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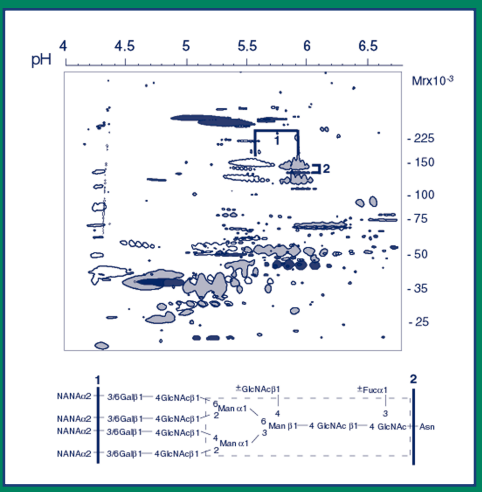
Posttranslational Modifications of Proteins

Tools for Functional Proteomics

Edited by

Christoph Kannicht



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Cover illustration: The figure shows the effect of a posttranslational modification (here N-glycosylation) on 2D-gel electrophoresis spot pattern. Figure supplied by Christoph Kannicht and Klemens Löster.

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Preface

Posttranslational Modifications of Proteins: Tools for Functional Proteomics is a compilation of detailed protocols needed to detect and analyze the most important co- and posttranslational modifications of proteins. Though, for reasons of simplicity not explicitly mentioned in the title, both kinds of modifications are covered, whether they occur during, or after, biosynthesis of the protein. My intention was to cover the most significant protein modifications, focusing on the fields of protein function, proteome research, and the characterization of pharmaceutical proteins.

The majority of all proteins undergo co- and/or posttranslational modifications. Knowledge of these modifications is extremely important, since they may alter physical and chemical properties, folding, conformation distribution, stability, activity, and, consequently, function of the proteins. Moreover, the modification itself can act as an added functional group. Examples of the biological effects of protein modifications include: phosphorylation for signal transduction, ubiquitination for proteolysis, attachment of fatty acids for membrane anchoring or association, glycosylation for protein half-life, targeting, cell–cell and cell–matrix interactions, and carboxylation in protein–ligand binding to name just a few. Full understanding of a specific protein structure–function relationship requires detailed information not only on its amino acid sequence, which is determined by the corresponding DNA sequence, but also on the presence and structure of protein modifications.

The individual chapters provide detailed step-by-step instructions for the analysis of the most important protein modifications, especially the assignment of disulfide bond sites in proteins (Chapter 1). Analysis of protein glycosylation is treated in great detail. Starting with the analysis of carbohydrate composition (Chapter 2), the methods for cleavage, labeling, separation, and sequence analysis of N-linked glycans are described (Chapters 3–5). Analysis of protein *O*-glycosylation in general and specific detection of *O*-linked *N*-acetyglucosamine residues follow (Chapters 6 and 7, respectively). Finally, Chapter 8 provides a method to analyze the oligosaccharides present at specific single glycosylation sites in a protein.

A method for analysis of glycosylphosphatidylinositols describes analysis of the carbohydrate and lipid portions as well (Chapter 10). Two more protocols on the analysis of lipid modifications, in particular S-acylation (Chapter 11) and ubiquitination (Chapter 12) follow.

Further protein modifications of interest and different nature complete the book: analysis of protein methylation, acetylation, phosphorylation, and sulfation

(in Chapters 13–15, respectively), and analysis of α-amidation, γ-glutamate, isoaspartate, and lysine hydroxylation in Chapters 16–19. Topics of more general interest are treated in the final two chapters. Chapter 20 describes the use of a heterologous expression system for the analysis of posttranslational modifications and Chapter 21 shows how to detect the influence of glycosylation on protein spot patterns in 2D gel electrophoresis.

Let me give special mention to two areas of research of high current interest: the fields of (1) proteomics and (2) the characterization of biological pharmaceuticals. (1) With respect to proteomics, research in the field of genomics has led to knowledge of the complete human DNA sequence. Measurement of the mRNA pool at a specific status of the cell, the "transcriptome," was found to not necessarily reflect the cells' actual protein expression pattern. In proteomics research, the description of expression levels of proteins related to a defined cell or tissue status will be incomplete without knowledge of the posttranslational modifications of those proteins.

In addition to possible changes in the activity or function of a protein, changes in its molecular weight or charge caused by protein modifications will influence the separation of proteins during 2D gel electrophoresis. Protein spot patterns generated by 2D electrophoresis will change because of altered protein expression or changes in the protein modifications. As an example, Chapter 21 describes detection and influence of sialylation and *N*-glycosylation on the protein spot pattern obtained by 2D gel electrophoresis.

(2) An additional important practical application of posttranslational modification analysis is to ensure the product quality of therapeutic pharmaceutical proteins. Recombinant proteins intended for therapeutic use in humans must be accorded particularly thorough investigation. Product quality depends on accurate posttranslational modification in the respective expression system during production, e.g., insect or several mammal cell lines. Note that different expression systems may vary in their ability to carry out posttranslational modifications and that changes in cell culture conditions also influence these modifications. Thus, posttranslational modifications of recombinant proteins have to be monitored during production and documented for registration. Directly related to this topic, Chapter 9 shows how to monitor glycosylation in order to ensure product consistency.

Growing knowledge of the biological roles of protein modifications, on the one hand, and the development and availability of sophisticated, sensitive analytical methods on the other hand, are already leading to increased interest in co- and posttranslational modifications of proteins. *Posttranslational Modifications of Proteins: Tools for Functional Proteomics* intends to serve as a practical guide for researchers working in the field of protein structure–function relationships in general, in the rapidly growing field of proteomics, as well as scientists in the pharmaceutical industries.

Christoph Kannicht

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1

Assignment of Disulfide Bonds in Proteins by Chemical Cleavage and Peptide Mapping by Mass Spectrometry

Jiang Wu and J. Throck Watson

1. Introduction

Among the twenty amino acids that comprise proteins, cysteine has unique properties. It may contribute to protein biological functions by using its sulfhydryl (-SH) group in the active site for enzyme catalysis such as in cysteine proteases, as the chelating site for metal ions, or as the active site of disulfide-reshuffling enzymes ***(1)***. The oxidation of sulfhydryl groups to form a disulfide bond is one of the most common posttranslational modifications in proteins. Disulfide bonds play an important role in folding/refolding processes and in maintaining the three-dimensional structure of proteins. The determination of disulfide-bond linkage is therefore an integral part of structural characterization of proteins ***(2)***.

Conventional methodology for determining disulfide linkage involves the cleavage of the protein backbone between half-cystinyl residues to obtain peptides that contain only one disulfide bond. Labor-intensive efforts are then pursued to isolate and sequence the various cystinyl peptides by Edman degradation or mass spectrometry, or both. The identified peptides are then related to specific segments of the protein. Although this approach has been well-described and used with much success ***(3,4)***, it is often inadequate for many challenging problems. A major constraint is the requirement for a proteolytic cleavage site between all cysteine residues, especially in cases where the cysteines reside close to one another in the sequence because the possibility of an intervening cleavage site becomes less likely ***(4)***. Proteins containing adjacent cysteines are usually refractory to conventional methodol-

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ogy. Furthermore, the conditions most frequently used for specific proteolytic digestion (pH ~8.0) promote disulfide scrambling ***(3,4)***. As a result, the disulfide linkage determined experimentally may not reflect the native structure of the protein.

Selective chemical modification and cleavage at cysteine residues in proteins can be utilized to great advantage in studies exploring the structure and function of the proteins. Jacobson ***(5)*** first showed that 2-nitro-5-thiocyanobenzoic acid (NTCB) specifically cyanylates protein sulfhydryl groups under mildly alkaline conditions, which leads to a base-catalyzed cleavage of the protein backbone at the N-terminal side of the S-cyano-cysteine residues to form an amino-terminal peptide and a series of 2-iminothiazolidine-4-carboxyl peptides (itz-peptides). A competitive reaction, β-elimination of HSCN from the S-cyanocysteine group, may also occur under the same conditions, depending primarily on the structure of amino acids on the N-terminal side of cyanylated cysteine (*see* **Note 1**). As an alternative, 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate (CDAP) was later proposed to be advantageous over the NTCB reagent for the specific cyanylation of sulfhydryl groups because the reaction is performed under acidic conditions (pH 3.0–5.0) to minimize sulfhydryl/disulfide exchange ***(6–8)***. Recently, extensive studies were carried out in our laboratory in an effort to optimize the cleavage of cyanylated cysteinyl proteins ***(9)***, which previously required long hours of incubation under mildly alkaline conditions. The cleavage reaction, which involves nucleophilic attack to the carbonyl carbon of the amide followed by a concerted cyclization, can be greatly accelerated in ammonia, giving rise to an α-amidated N-terminal peptide ***(9)***. **Figure 1** shows the general scheme of cyanylation and cleavage reactions that have been used in our laboratory.

The cyanylation and subsequent cleavage are specific to free cysteine residues, that is, the selective chemical cleavage at modified cysteinyl residues can be achieved in the presence of cystinyl residues ***(5)***, a feature that is particularly useful for identifying free cysteines and those involved in disulfide bonding. Utilizing the cyanylation chemistry, we describe in this chapter a new strategy for identifying sulfhydryls and disulfide bonds in proteins (including those containing adjacent cysteine residues) by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) ***(10–12)***.

2. Materials

Unless indicated, all the chemicals and proteins were obtained from Sigma and used without further purification.

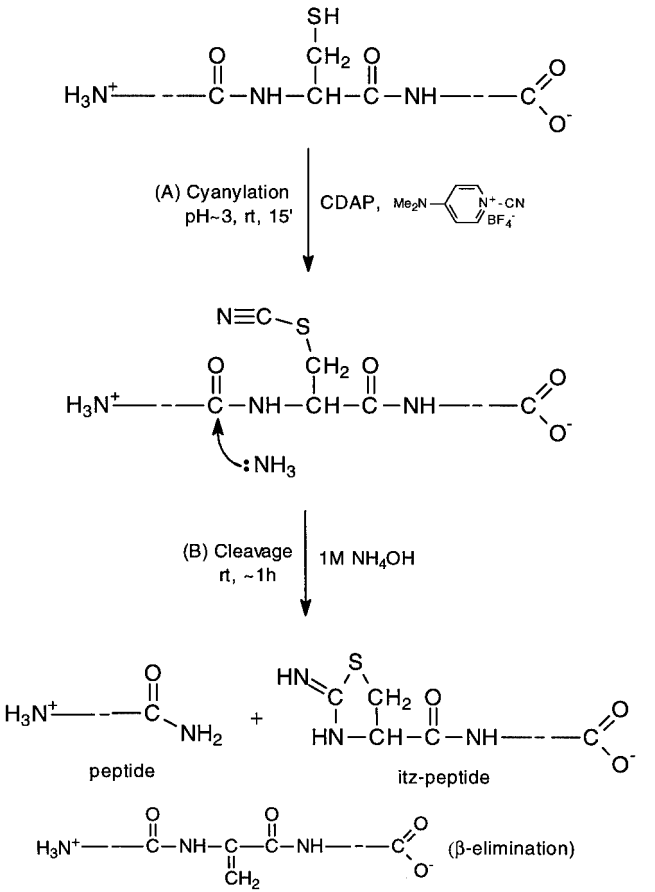


Fig. 1. Schematic illustration of **(A)** cyanylation and **(B)** cleavage reaction.

2.1. Peptides and Proteins

1. Ovalbumin.
2. Rabbit muscle creatine phosphokinase.
3. Bovine pancreatic Ribonuclease A, type III-A.
4. Recombinant long insulin-like growth factor I (LR^3IGF-1).
5. Bradykinin.
6. Bovine pancreatic insulin.
7. Horse skeletal myoglobin.

2.2. Solvents and Buffers

1. High-performance liquid chromatography (HPLC) solvent A: 0.1% TFA in water.

2. HPLC solvent B: 0.1%TFA in acetonitrile.
3. Aqueous acetonitrile (1/1, v/v) containing 0.1% TFA.
4. 0.1 *M* Citrate buffer, pH 3.0, containing 6 *M* guanidine-HCl.
5. 1 *M* NH_4OH aqueous solution.
6. 1 *M* NH_4OH solution containing 6 *M* guanidine-HCl.
7. 0.1 *M* 1-Cyano-4-dimethylamino-pyridinium tetrafluoroborate (CDAP) solution in 0.1 *M* citrate buffer, pH 3.0. Prepare fresh prior to use.
8. Saturated α-Cyano-4-hydroxycinnamic acid (α-CHCA) solution freshly prepared in aqueous CH_3CN (1/1, v/v) containing 0.1% TFA prior to use.
9. 0.1 *M tris*(2-Carboxyethyl)phosphine hydrochloride (TCEP) aqueous solution.
10. 0.1 *M* TCEP solution in 0.1 *M* citrate buffer, pH 3.0.

2.3. Instrumentation

2.3.1. MALDI-TOF MS

MALDI experiments were performed on a Voyager Elite time-of-flight (TOF) mass spectrometer (PerSeptive Biosystems Inc., Framingham, MA) equipped with a model VSL-337ND nitrogen laser (337-nm, 3-ns pulse length, Laser Science, Newton, MA) and a dual microchannel plate detector (Galileo, Sturbridge, MA). The accelerating voltage in the ion source was set to 25 kV. Data were acquired in the positive linear mode of operation. The spectra were collected just slightly above the laser threshold necessary for analyte ion production. Mass calibration was achieved by using external standards of bradykinin (m/z 1061.2), bovine pancreatic insulin (m/z 5734.5), and horse skeletal myoglobin (m/z 16,952).

2.3.2. Capillary LC/ESI-MS System

On-line LC/ESI-MS was performed on a Micromass Platform LC mass spectrometer equipped with an electrospray ionization source and a single quadrupole analyzer. The source and cone voltage were 3.26 kV, and 30 V respectively. The source temperature was set to 80°C. Separation of cleavage products was carried out on a Perkin-Elmer C_{18} capillary column at a flow rate of 7 μL/min with a linear gradient 5–65% B in 135 min, where A = 0.085% aqueous TFA and B = 0.085% TFA in CH_3CN.

2.3.3. HPLC

The separation of modified proteins was carried out using a reversed-phase Vydac C18 column (10-μm particle size, 300-Å pore, 4.6 × 250 mm) under a linear gradient elution controlled by a Waters 6000 system. The UV detection was at 215 nm. The mobile phases A and B were 0.1% TFA aqueous solution

and CH_3CN containing 0.1% TFA, respectively. The HPLC conditions should be optimized for individual proteins.

3. Methods

3.1. Identification of Free Cysteine Residues by Cyanylation/Chemical Cleavage/Mass Mapping

For proteins containing both sulfhydryls and disulfide bonds, the first step is to determine the number and location of sulfhydryl groups. The cyanylation and chemical cleavage of the denatured original protein under nonreducing conditions, and subsequent mass mapping of the cleavage products allow the number and locations of sulfhydryl groups to be deduced ***(10)***.

3.1.1. Experimental Procedure

The procedure used in our laboratory is as follows:

1. Dissolve 5 nmol of protein in 10 μL of 0.1 *M* citrate buffer, pH 3.0, containing 6 *M* guanidine-HCl.
2. Add a 10-fold molar excess (over equivalent cysteine content) of 0.1 *M* CDAP solution in 0.1 *M* citrate buffer, pH 3.0, to the protein solution. Incubate the mixture for 10–15 min at ambient temperature. The CDAP solution should be freshly prepared prior to use.
3. Remove immediately the excess reagent from the modified protein by HPLC (*see* **Note 2**). Collect HPLC fractions corresponding to the modified protein and remove solvent under reduced pressure (Speedvac concentrator).
4. Reconstitute a solution of the protein residue in 10 μL of 1 *M* NH_4OH containing 6 *M* guanidine-HCl, incubate the mixture for 1 h at ambient temperature, and remove the excess ammonia in the Speedvac.
5. Add 10 μL of 0.1 *M* TCEP aqueous solution. Incubate at 37°C for 30 min to promote the reduction of the disulfide-linked peptide fragments.
6. Dilute a 1-μL aliquot of the above solution with 100 μL of $CH_3CN:H_2O$ (1/1, v/v) containing 0.1% TFA (*see* **Note 3**). Mix equal volumes of the diluted protein sample and α-CHCA matrix solution on a stainless-steel sample plate, and allow to air-dry. The saturated α-CHCA solution should be freshly prepared in $CH_3CN:H_2O$ (1/1, v/v) containing 0.1% TFA.
7. Acquire MALDI spectra. Calculate the theoretical masses by adding to the MW of the parent peptide 25 Dalton for each site of cyanylation, adding 25 Dalton for peptide fragments containing the iminothiazolidine moiety, subtracting 34 Dalton for β-elimination.

If the protein is reduced prior to cyanylation and cleavage, the MALDI analysis will provide a fingerprint spectrum of the fragments resulting from

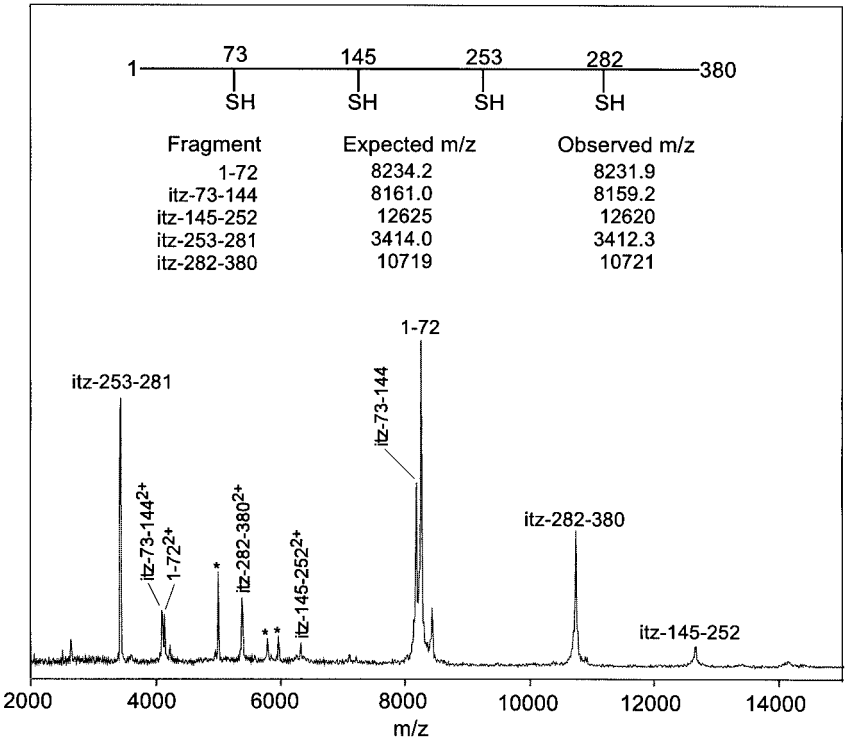


Fig. 2. MALDI spectrum of rabbit muscle creatine phosphokinase (MW. 42977), after cyanylation by CDAP and subsequent cleavage in 1 *M* NH_4OH solution. The peaks marked with asterisk are due to "carry-over" from an impurity in the sample. itz, iminothiazolidine derivative. Reproduced with permission from **ref. *9***.

cleavage at all cysteine sites. This procedure can be used to confirm the structure of a given protein and to examine the MALDI data obtained from nonreducing conditions. The procedure is as follows:

1. Add a 10-fold molar excess (over equivalent cysteine content) of the TCEP solution in 0.1 *M* citrate buffer, pH 3.0, to the protein solution. Incubate at 50°C for 15 min.
2. Add a 20-fold molar excess (over total equivalent cysteine content) of freshly prepared 0.1 *M* CDAP solution in the 0.1 *M* citrate buffer to the protein solution. Incubate the mixture for 10–15 min at ambient temperature.
3. Repeat **steps 3–7** as described previously.

3.1.2. Analysis of Creatine Phosphokinase

Figure 2 shows the MALDI spectrum of the cleavage products of rabbit muscle creatine phosphokinase, a protein containing 380 amino acids. The

cysteines reside at positions 73, 145, 253, and 282, respectively, among which Cys282 is in the active site. The primary structure of this protein was established from cDNA clones, which do not provide structural information regarding the cysteine status ***(13)***. The MALDI spectrum of the cleavage products showed peaks at m/z 3412.3, 8159.2, 8231.9, 10721, and 12620, corresponding to the fragments consisting of residues itz-253-281, itz-73-144, 1-72, itz-282-380, and itz-145-252, respectively. In addition, doubly charged fragments are also readily identified in the spectrum. The error of mass assignment is below 0.05%. It is clear that the cyanylation and cleavage must have taken place at each of the cysteine residues. Therefore, all four cysteine residues are in the reduced form and there is no disulfide-bonded cystine in rabbit muscle creatine phosphokinase.

3.1.3. Analysis of Ovalbumin

Another example illustrates application of the analytical strategy in the identification of sulfhydryl residues in chicken egg ovalbumin, which contains four free cysteine residues (at positions 11, 30, 367, and 382) and one disulfide bond (Cys73-Cys120). After cyanylation and cleavage, five fragments, ranging in size from 429.5–37684.7 Da, are expected due to cleavage at the four cyanylated cysteine residues. The calculated and observed mass values for these expected fragments are tabulated in **Fig. 3A**. We were able to identify by MALDI-MS only two cleavage products (**Fig. 3A**): itz-11-29 and itz-367-381. Other fragments may either have no response to MALDI-MS or be suppressed by the coexisting species (*see* **Note 4**). The observation of these two internal fragments is informative, however, indicating that Cys10, 30, 367, and 382 had undergone the cyanylation and cleavage.

In addition, two sets of partially cleaved fragments were observed, corresponding to the cyanylation of and subsequent β-elimination from an internal cysteine residue (**Fig. 3A**). **Figure 3C** illustrates the residues altered by the chemistry and represented by the fragments. The peak at m/z 3371.5 is due to the fragment 1-29 that contains a cyanylated (uncleaved) internal Cys11 (expected m/z 3371.8), while m/z 3311.1 is its corresponding β-elimination product (expected m/z 3312.8). The mass difference between the cyanylated peptide (+25 Dalton shift from parent peptide) and its β-elimination product (–34 Da shift from parent peptide) is 59 Dalton. Likewise, peaks at m/z 2126.6 and 2067.7 are due to the peptide chain itz-367-385, containing a cyanylated Cys382 and its β-elimination product, respectively (**Fig. 3C**). This analysis of the fragments resulting from partial cleavage provides evidence that all four sites (Cys11, 30, 367, and 382) were free cysteines that underwent cyanylation. In contrast, Cys73 and Cys120 are linked by a disulfide bond, and no cleavage occurs at these residues.

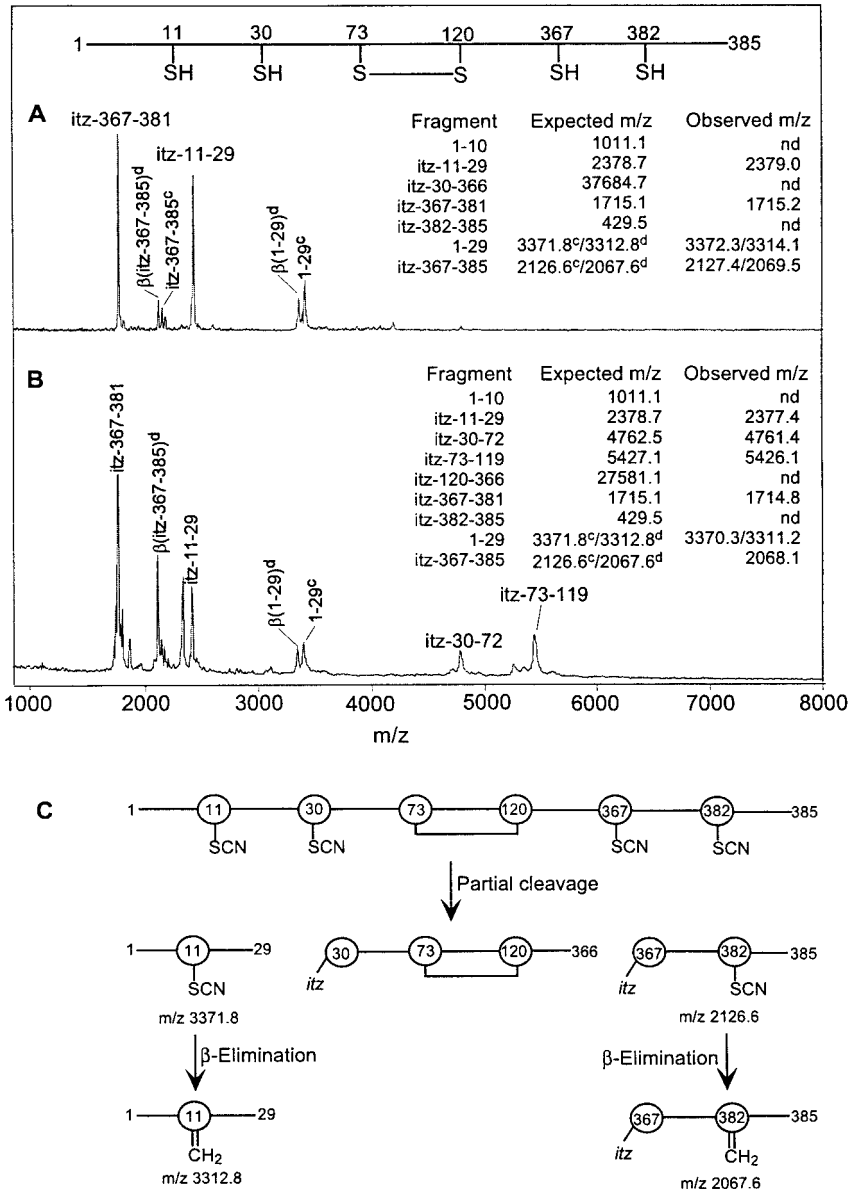


Fig. 3. MALDI spectra of ovalbumin (MW. 42699) after cleavage at **(A)** free cysteine sites and **(B)** total cysteine sites. Reproduced with permission from **ref. *9***. *c* and *d* represent cyanylated/uncleaved peptide and its β-elimination product, respectively. itz, iminothiazolidine derivative. **(C)** Schematic illustration of incomplete cleavage of cyanylated ovalbumin.

In comparison, the mass spectrum in **Fig. 3B** of the fragments from the cyanylation/cleavage of reduced ovalbumin gave two additional peaks at m/z 4761.4 and 5426.1, attributable to reduction of the disulfide bond (Cys73-Cys120), cyanylation, and cleavage to give fragments consisting of itz-30-72 and itz-73-119, respectively. The spectrum also shows evidence for the cyanylation/β-elimination pairs for partially cleaved fragments 1-29 and itz-367-385 as described previously. However, the fragments 1–10, itz-120-366, and itz-382-385 are still undetectable. Some of the peaks in **Fig. 3B** (e.g., m/z~2200) are not related to cleavage at any cysteine sites, they are apparently due to the impurities in the sample, or a side reaction, or both. Because our assignment relies only on the fragments arising from the specific cleavage at cysteine residues, the unexpected peaks do not seriously affect the data interpretation.

3.2. Assignment of Disulfide Linkage in Proteins Containing Multiple Cystines by Partial Reduction/Cyanylation/Chemical Cleavage/Mass Mapping

The foregoing descriptions and illustrations have shown that the cyanylation reagent does not react with nor modify an intact disulfide bond, but will modify the nascent cysteines generated by reduction of the disulfide bond. The analysis of a protein containing multiple disulfide bonds requires some means of reducing a particular disulfide bond and applying the cyanylation/cleavage methodology to corresponding pair of nascent cysteines ***(11)***. The technique of partial reduction developed by Gray ***(14,15)*** provides a convenient means of preparing a mixture of partially reduced isoforms of the parent protein. The general procedure is shown in **Fig. 4** for the simplest hypothetical peptide containing two cystines. This method has been applied to the characterization of disulfide structure in an unknown protein and in protein-folding intermediates ***(16,17)***.

In our experiments, the partial reduction procedure involves establishing a kinetically limited reduction reaction from which only a small portion of each disulfide bond (e.g., 10% each) is reduced while the majority of the protein remains intact ***(11)***. Experimental conditions, such as incubation time, temperature, and stoichiometry of reducing agents, can be controlled so that singly reduced isoforms of the protein are predominant products. The singly reduced isoforms of the original protein are most desirable as the cyanylation and cleavage can be applied in a straightforward manner as described above (*see* **Note 5**).

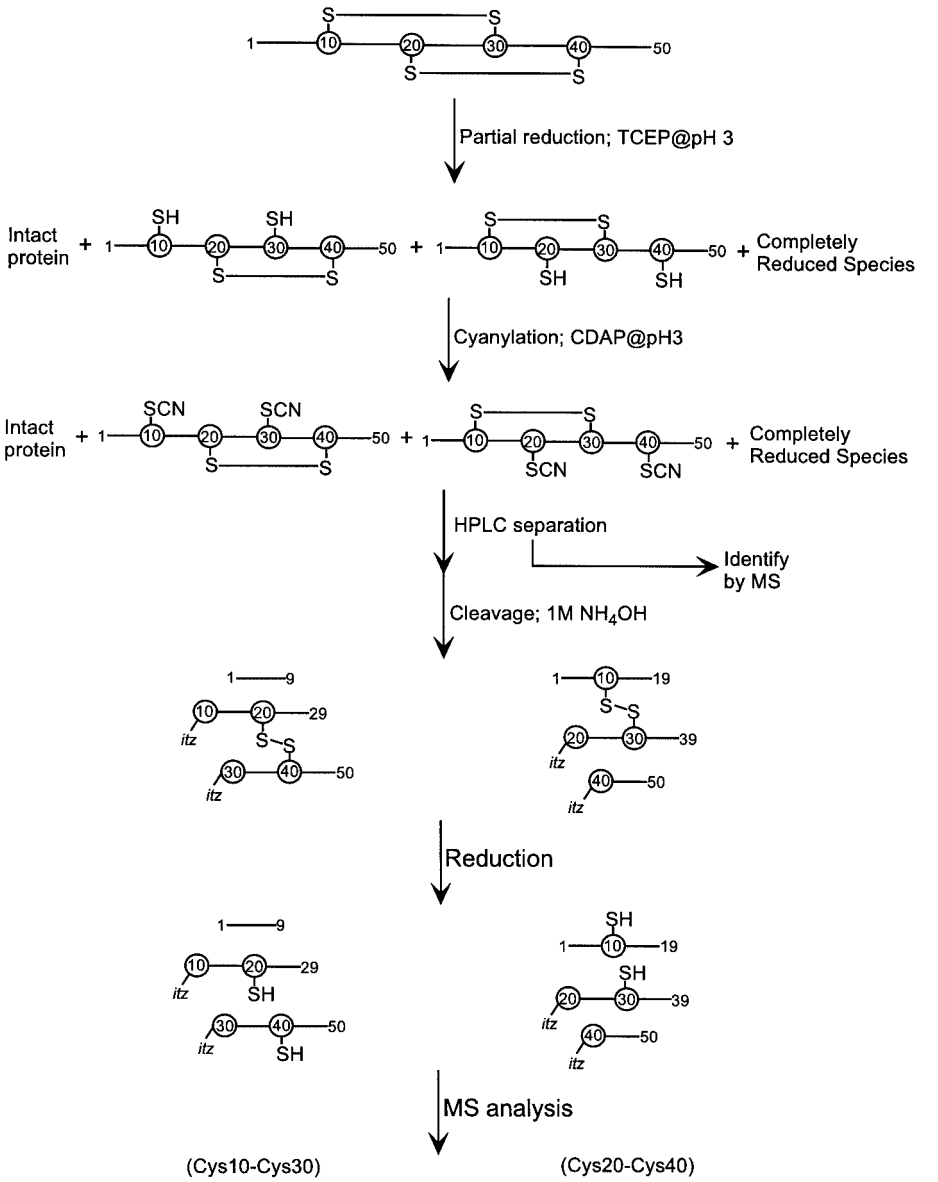


Fig. 4. Chemical overview of the methodology for locating disulfide bonds in proteins.

3.2.1. Experimental Procedure

The experimental conditions are summarized below:

1. Dissolve 10 nmol of protein sample in 10 μL of 0.1 *M* citrate buffer, pH 3.0, containing 6 *M* guanidine-HCl.
2. Add an equivalent of 0.1 *M* TCEP (in 0.1 *M* citrate buffer, pH 3.0) for the cystine content in the protein. Incubate at room temperature for 10–15 min (*see* **Note 6**). The TCEP solution was stored under N_2 at –20°C for weeks with little deterioration.
3. Add a 20-fold molar excess (over total cysteine content) of freshly prepared 0.1 *M* CDAP solution in 0.1 *M* citrate buffer, pH 3.0, to the protein solution (*see* **Note 7**). Cyanylation of the nascent sulfhydryl groups is achieved by incubation at ambient temperature for another 10–15 min.
4. Separate the partially reduced and cyanylated species by reversed-phase (RP)-HPLC with linear gradient elution (*see* **Subheading 2.3.3.** and **Note 8**). Collect the HPLC fractions manually.
5. Identify the HPLC fractions by mass analysis by MALDI (+52 Dalton for a singly reduced/cyanylated species; +104 Dalton for a doubly reduced/cyanylated species, etc.) (*see* **Note 9**). Those fractions corresponding to singly reduced/cyanylated species are dried in Speedvac.
6. Reconstitute a solution of the singly reduced/cyanylated protein residues in 2 μL of 1 *M* NH_4OH containing 6 *M* guanidine-HCl, then add another 5 μL of 1 *M* NH_4OH solution. Cleavage of peptide chains is carried out by reaction for 1 h at ambient temperature. The excess ammonia is removed in a Speedvac.
7. Add 5 μL of 0.1 *M* TCEP aqueous solution and incubate at 37°C for 30 min to release the truncated peptide chains still linked by the remaining disulfide bonds (*see* **Note 10**).
8. Dilute a 1-μL aliquot of the above solution to 100 μL with 1 : 1 (v/v) $CH_3CN : H_2O$ containing 0.1% TFA for analysis by MALDI.

3.2.2. Advantages of the Methodology

This methodology offers several potential advantages compared with the conventional methodologies:

1. The chemistry and data interpretation are simple, fast, and straightforward. Cleavage of the peptide chain takes place only at reduced and cyanylated cysteinyl sites, which, in principle, yields three fragments (and two β-elimination products if partial cleavage occurs) for each singly reduced/cyanylated protein isomer. The mass of each fragment is related to the position of the two cyanylated cysteinyl residues, which in turn can be used to deduce a particular disulfide-bond linkage. β-Elimination, an alternative to peptide-chain cleavage, provides mass spectral data corresponding to overlapped peptides and serves as a confirmation for the disulfide-bond assignment.

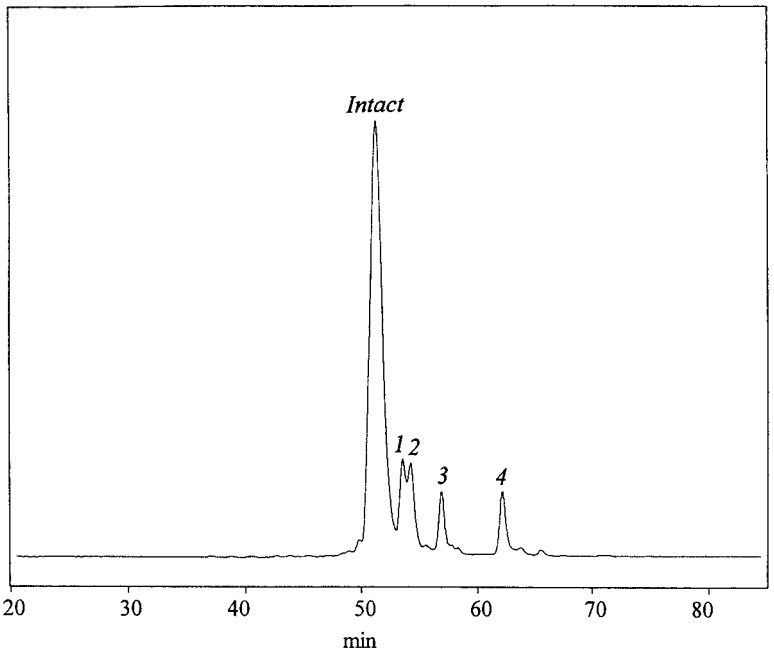


Fig. 5. HPLC separation of denatured ribonuclease A and its partially reduced/cyanylated isomers. The HPLC separation was carried out on a reversed-phase Vydac C18 column at a flow rate of 1.5 mL/min with a linear gradient 20–40% B in 90 min, where A = 0.1% TFA aqueous solution and B = 0.1% TFA in CH_3CN. The peak marked IP is intact ribonuclease A. Peaks 1–4 represent singly reduced/cyanylated ribonuclease A isomers, as identified by MALDI-TOF analysis. Reproduced with permission from **ref. *11***.

2. These experimental conditions minimize problems related to disulfide-bond scrambling because both partial reduction of disulfide bonds and cyanylation of nascent sulfhydryls are performed in an acidic medium.
3. The procedure can be used for locating disulfide bonds in proteins containing adjacent or closely spaced cysteines, for which conventional methodology fails (*see* **Subheading 3.3.**).

3.2.3. Synopsis of the Procedure Applied to Ribonuclease A

Ribonuclease A (Mr 13,683) contains 124 amino acids, among which eight cysteines are linked by four disulfide bonds (*see* **Scheme 1**). **Figure 5** shows the separation of residual intact ribonuclease A and its partially reduced/cyanylated isomers. The peaks marked 1–4, each representing ~10% of the intact protein, show +52 Dalton shift from the original protein, and corresponding to the reduction of a different disulfide bond and cyanylation of the corresponding two cysteines. Mass mapping of the truncated peptides allows one to deduce the disulfide pair that had undergone reduction, cyanylation, and cleavage.

Scheme 1. Disulfide bond linkage of Ribonuclease A.

Figure 6A–D are MALDI spectra of peptide mixtures resulting from cleavage of one of the four singly reduced/cyanylated isoforms of ribonuclease A corresponding to HPLC peaks 1–4 in **Fig. 5**, respectively. **Table 1** lists the calculated and observed m/z values for fragments resulting from the cleavage of the peptide chains at cysteine sites corresponding to reduction of different disulfide bonds (*see* **Note 11**).

In the **Fig. 6A**, three peaks at m/z 2705.3, 6548.5, and 4527.4 are assigned to fragments 1-25, itz-26-83, and itz-84-124, respectively (**Table 1**). From these data, one can deduce that reduction, cyanylation, and peptide-chain cleavage occur at Cys26 and Cys84. The overlapped peptide fragments resulting from β-elimination support the assignment. For instance, the MALDI peak at m/z 9176.7 corresponds to a partially cleaved peptide, β(1-83), resulting from peptide backbone cleavage at Cys84, but β-elimination at Cys26; thus, this peak corroborates information provided by the peaks representing residues 1-25 and itz-26-83. Likewise, the MALDI peak at m/z 10998.6 is another partially cleaved peptide, β(itz-26-124), with cleavage at Cys26 and β-elimination at Cys84; similarly, this peak corroborates information provided by the peak representing itz-26-83 and itz-84-124. Overall, a disulfide-bond linkage between Cys26-Cys84 can be unambiguously deduced.

The MALDI spectrum in **Fig. 6B**, corresponding to the cleavage products represented by HPLC peak 2, shows two main peaks at m/z 5907.7 and 7083.8, assigned to fragments itz-72-124 and 1-64, respectively. Another expected fragment itz-65-71 is missing (*see* **Note 12**). However, it is still possible to deduce the Cys65-Cys72 linkage from these two fragments because no other combination affords such masses. Two partially cleaved products at m/z 6617.9 and 7790.0, attributable to residues β(itz-65-124) and β(1-71), respectively, are particularly informative for the confirmation of the assignment in this case.

The HPLC peaks 1 and 2 in **Fig. 5** were not resolved completely. This is reflected in the MALDI spectra (**Fig. 6A**,**B**) of the cleavage products of these two fractions, each of which carries small fragments corresponding to the cleavage products of the other fraction. The unambiguous assignment of the respective disulfide bond pairs in the presence of another isomer is still possible because only a few fragments are produced, all of which are related (*see* **Note 13**).

With similar strategy, two other disulfide bond linkages, Cys40-Cys95 and Cys58-Cys110, can readily be recognized from **Fig. 6C**,**D**, respectively.

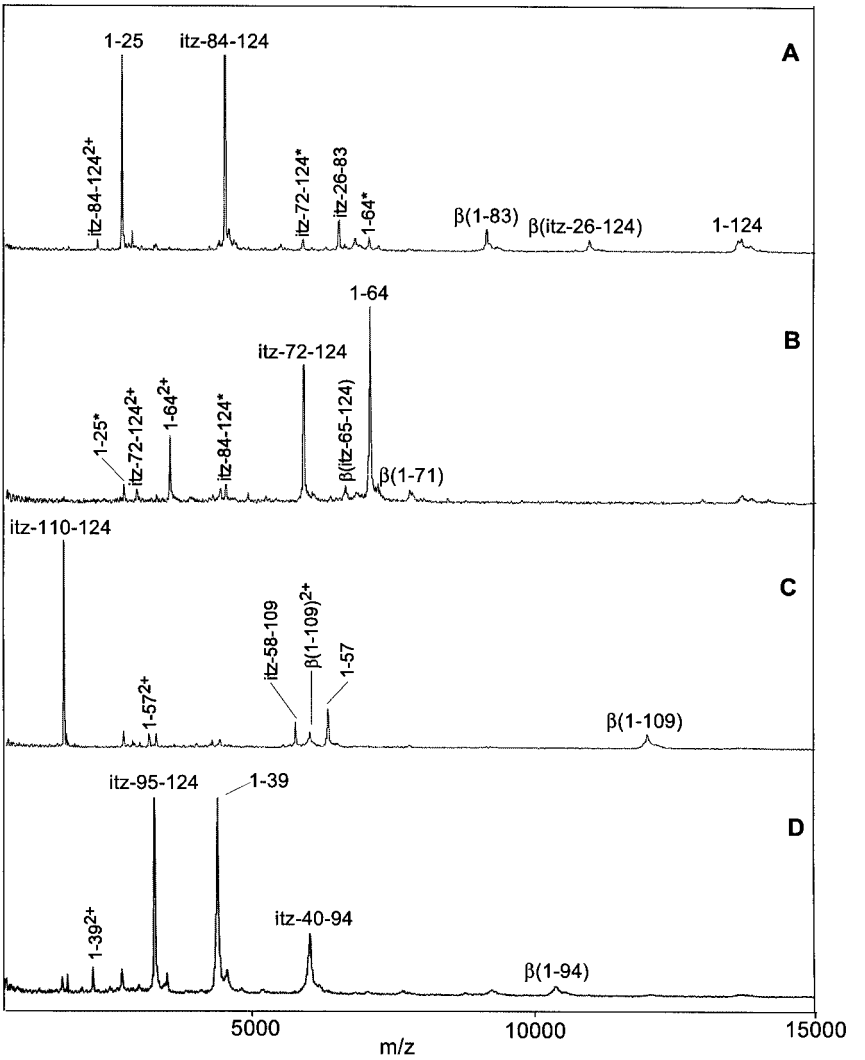


Fig. 6. The MALDI mass spectra of peptide mixtures resulting from the cleavage of the four singly reduced/cyanylated ribonuclease A isomers. Spectra **A–D** correspond to the HPLC peaks 1–4 in **Fig. 5**, respectively. The peaks with asterisk represent the "carry-over" from other HPLC fractions. *See also* **Table 1** for calculated and observed m/z values. Reproduced with permission from **ref. *11***.

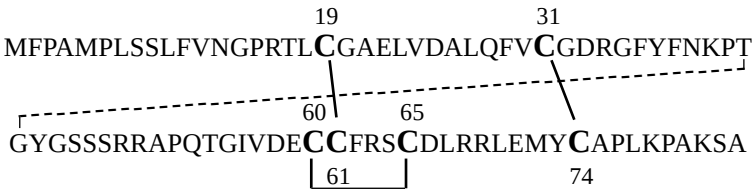
Table 1
Calculated and Observed m/z Values for Possible Fragments Resulting from the Cleavage Reaction of Ribonuclease A Chains at Sites of Designated Cysteine Pairs

Disulfide pair	Fragment	Calculated m/z	Observed m/z
Cys26-Cys84	1-25	2706.8	2705.3
	itz-26-83	6547.3	6548.5
	itz-84-124	4526.0	4527.4
	β(1-83)	9176.2	9176.7
	β(itz-26-124)	10995.3	10998.6
Cys65-Cys72	1-64	7083.9	7083.8
	itz-65-71	789.8	nd
	itz-72-124	5906.5	5907.7
	β(1-71)	7795.7	7790.0
	β(itz-65-124)	6618.3	6617.9
Cys58-Cys110	1-57	6353.1	6351.1
	itz-58-109	5767.4	5766.8
	itz-110-124	1659.8	1659.8
	β(1-109)	12042.4	12036.7
	β(itz-58-124)	7349.1	nd
Cys40-Cys95	1-39	4413.9	4414.4
	itz-40-94	6063.6	6061.2
	itz-95-124	3302.7	3303.7
	β(1-94)	10399.5	10430.5
	β(itz-40-124)	9288.3	9293.4

3.3. Assignment of Disulfide Bonds in Proteins Containing Adjacent Cysteines

Proteins containing adjacent cysteine residues represent a substantial challenge for disulfide characterization because the likelihood of achieving specific proteolytic cleavage between the adjacent cysteinyl residues is very low ***(3,4)***. Nevertheless, the structural problem presented by adjacent cysteines is amenable to the partial reduction/cyanylation/cleavage/mass mapping approach ***(12)***. The procedures used are the same as those described in **Subheading 3.2.**

An example of analysis of a protein containing adjacent cysteines is given with LR^3IGF-I, which consists of 83 amino acids and three disulfide bonds, two of which involve adjacent cysteines (*see* **Scheme 2**). LR^3IGF-I was subjected to partial reduction, and the nascent cysteines were cyanylated. **Figure 7** is the HPLC chromatogram resulting from the analysis of the reaction mixture. Only



Scheme 2. Amino acid sequence and disulfide bond linkage of LR^{3}IGF-I.

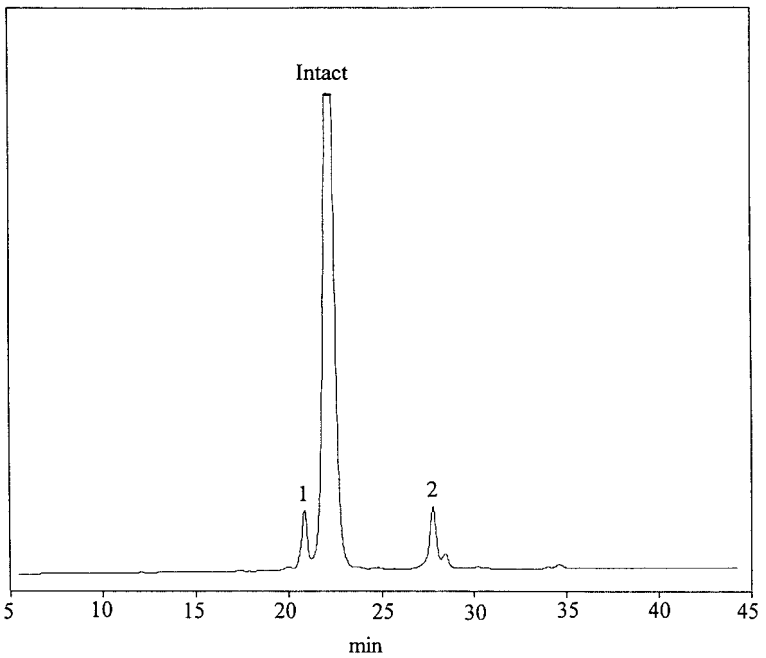


Fig. 7. HPLC chromatogram of denatured LR^{3}IGF-I and its partially reduced/cyanylated isomers. Separation was carried out on a reversed-phase Vydac C18 column at a flow rate of 1.0 mL/min with a linear gradient 30–50% B in 45 min, where A = 0.1% TFA aqueous solution and B = 0.1% TFA in CH_3CN. The peak marked IP is intact ribonuclease A. Peaks 1 and 2 represent singly reduced/cyanylated LR^{3}IGF-I isomers, as identified by electrospray (ESI). Reproduced with permission from **ref. *12***.

two of the expected three isoforms were produced in this case, because one of the disulfide bonds is unusually stable ***(18)***. Peaks 1 and 2 in **Fig. 7** represent the two singly reduced/cyanylated isoforms, according to a mass shift of 52 Da as determined by ESI-MS. (Note: the minor peaks following peak 2 in **Fig. 7** are identified to be doubly and completely reduced proteins, respectively.) Nevertheless, in dealing with a protein known to contain only ***n***-disulfide

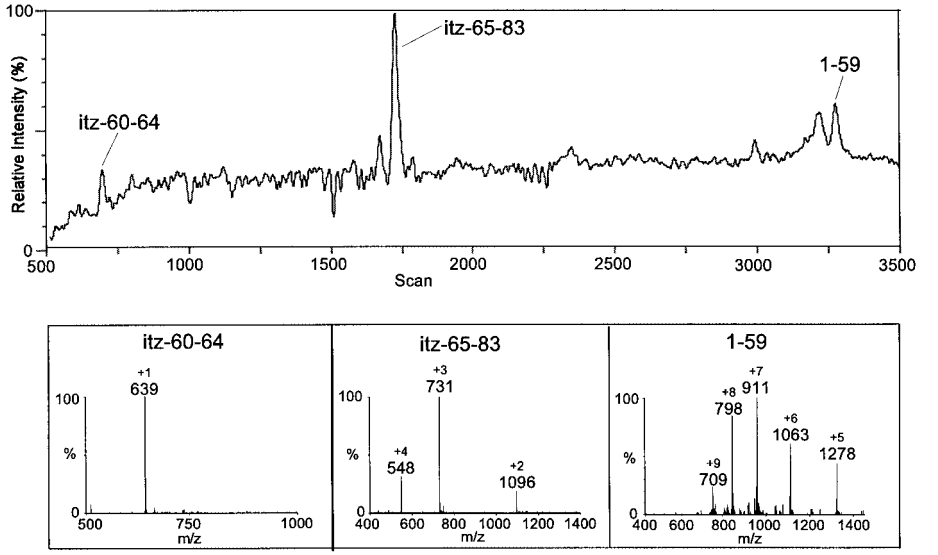


Fig. 8. Upper panel, RTIC from LC/ESI-MS of the peptide mixture resulting from cleavage of the cyanylated isoform represented by HPLC peak 1 in **Fig. 7**. Separation was carried out on a Perkin-Elmer C_{18} capillary column at a flow rate of 7 μL/min with a linear gradient of 5–65% B in 135 min, where A = 0.085% TFA aqueous solution and B = 0.085% TFA in CH_3CN. Lower panel, ESI mass spectra of the corresponding RTIC peaks. Reproduced with permission from **ref. *12***.

bonds, the determination of ***n-1*** disulfide pairs allows the remaining one to be determined by default.

The compound represented by HPLC peak 1 was subjected to the chemical reactions described in **Subheading 3.2.** The cleavage products were analyzed by capillary LC/ESI-MS to give the reconstructed total ion current (RTIC) chromatogram in **Fig. 8** (upper panel) (*see* **Note 14**). The corresponding ESI spectra of the LC peaks (lower panel of **Fig. 8**) give the deconvoluted masses, 638.0, 2189.7, and 6372.7 Da, which can be assigned to fragments itz-60-64, itz-65-83, and 1-59, respectively (*see* **Table 2** for comparison of the calculated and observed masses). From these data, one can deduce that Cys60 is linked to Cys65.

Likewise, the upper panel in **Fig. 9** represents the RTIC chromatogram of the cleavage products from the reduction/cyanylation of a different disulfide bond (HPLC peak 2 in **Fig. 7**); the ESI spectra corresponding to peaks in the RTIC are shown in the lower panel of **Fig. 9**. A similar analysis allows one to deduce that Cys19 is connected to Cys61. Overall, the disulfide-bond structure containing adjacent cysteines can be readily determined.

Table 2
Calculated and Observed m/z Values for the Fragments Resulting from the Cleavage Reaction of Singly Reduced and Cyanylated LR^{3}IGF-I Isoforms

Disulfide pair	Fragment	Calculated m/z	Observed m/z
Cys60-Cys65	1-59	6372.2	6373.7
	itz-60-64	638.7	639.0
	itz-65-83	2189.6	2190.7
Cys19-Cys61	1-18	1979.4	1978.8
	itz-19-60	4539.0	4541.0
	itz-61-83	2682.1	2684.9

4. Notes

1. β-Elimination presents as a major competitive side reaction under alkaline conditions in our experiments, although other minor side reactions such as reversibility of the cyanolysis were also reported. The cyanylated peptides showed marked variations in their tendency to undergo β-elimination. For most of the denatured peptides examined, the expected cleavage products are predominant, however, amino acids with rigid or bulky side chains, such as Pro and Tyr on the N-terminal side of cysteine, are more resistant to amide cleavage, giving β-elimination as a main product ***(9)***.
2. Although the cyanylation is generally specific to sulfhydryl groups, a large excess of CDAP (>50-fold over protein sulfhydryl groups) and excessive incubation time (>2 h) may result in minor modification of other amino acid side chains. It is recommended that the excess reagent be removed immediately after cyanylation to minimize side reactions. Ultrafiltration or use of a small protein desalting cartridge may be superior to a C18 column for reagent removal and purification of hydrophobic proteins.
3. Although MALDI is tolerant to salts and buffers, the high concentration of guanidine-HCl used in our procedure prevents a successful analysis because the protein-matrix mixtures do not co-deposit well on the probe tip. Upon dilution of sample, good-quality MALDI signals can be observed, presumably because a more favorable ratio of residual salts to matrix allows proper co-deposition on probe tip.
4. It is important to note that MALDI responses to different peptides vary significantly dependent on many factors, such as peptide size, amino acid composition, sample preparation, and so on. Usually medium-sized peptides (1000–10,000 Dalton) show higher sensitivity than low-mass (<600 Dalton) or high-mass (>20 kDa) peptides. In a peptide mixture, high-mass peptides are often suppressed, leading to weak or undetectable signals.
5. Singly reduced protein isomers are advantageous over other reduced protein isomers, because only a few fragments are obtained after cyanylation/cleavage, leading to the most simple mass spectrum. In principle, a singly reduced isomer

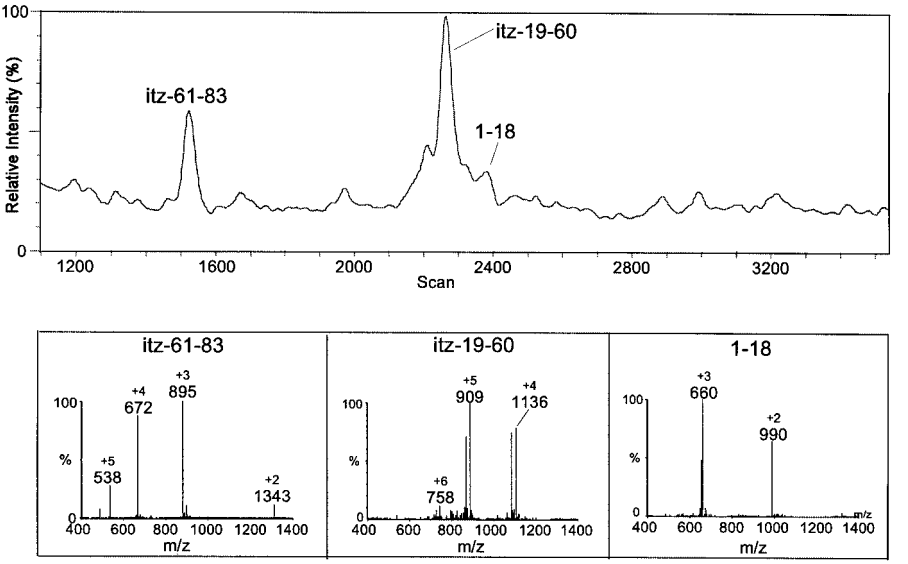


Fig. 9. Upper panel, RTIC from LC/ESI-MS) of the peptide mixture resulting from cleavage of the cyanylated isoform represented by HPLC peak 2 in **Fig. 7**. Separation was carried out under the same conditions as for **Fig. 8**. Lower panel, ESI mass spectra of the corresponding RTIC peaks. Reproduced with permission from **ref. *12***.

contains two nascent cysteine residues, which, after cyanylation and cleavage, only generate three cleavage products plus two possible β-elimination products. It is experimentally feasible to obtain singly reduced isomers as predominant products. Under the conditions where we assume a 10% reduction of each disulfide bond, the probabilities of reducing two and three disulfide bonds in the same protein molecule are only 10% × 10% = 1% and 10% × 10% × 10% = 0.1%, respectively. Our methodology requires that individual disulfide bonds be broken at comparable rates. To minimize structural diversity of disulfide bonds, proteins under study must be denatured by dissolution in 6 *M* guanidine so that difference in the accessibility of TCEP to the disulfide bonds is minimized.

6. Water-soluble TCEP has proved to be an excellent reducing agent for disulfide bonds ***(10,14,15)***. Reduction by TCEP can be carried out at pH 3.0 to suppress disulfide-bond scrambling. Furthermore, at pH 3.0, the reduction of disulfide bonds is kinetically controlled, which makes partial reduction possible. As the cystine content may not be known *a priori*, and the rate of disulfide reduction may vary, the extent of reduction should be monitored for individual proteins. From the limited number of proteins we have examined, it appears that an equivalent of TCEP for the cysteine content is a good initial stoichiometry. The extent of reduction may be readily adjusted by controlling the reaction time and temperature.

7. A fivefold molar excess of CDAP would drive the cyanylation reaction to completion. However, it should be noted that CDAP reacts instantly with residual TCEP. Therefore, a larger amount of CDAP is applied to ensure complete cyanylation.
8. The mechanism of HPLC separation is based on the hydrophobicity and conformational differences of different protein isomers ***(14,19,20)***. Opening a disulfide bond disrupts protein structure, exposes the protein's interior hydrophobic amino acids, and changes the protein's hydrophobicity to different extents. Therefore, different isoforms may exhibit different retention times on RP-HPLC columns. Some of the more hydrophobic proteins, especially after reduction and cyanylation, exhibited a long retention time or even irreversible retention on a C18 column in the water/acetonitrile mobile phase (data not shown). In such a case, a C4 column with water/1-propanol mobile phase may be utilized for faster elution ***(20)***. Most of the proteins can be eluted by 40% aqueous 1-propanol within a reasonable time.
9. To unambiguously identify intact proteins (~15,000 Dalton) and its singly reduced/cyanylated isomers (+52 Dalton mass shift), the resolution of the mass spectrometer should be better than 500. The accuracy of mass determination by MALDI can be improved by internal standard calibration. However, ESI-MS generally offers better resolution and mass accuracy, and may become the method of choice for the identification of cyanylated protein isomers.
10. Although the TCEP is recommended for the partial reduction step, other reducing agents such as DTT may be used at this stage to promote the complete reduction of remaining disulfide bonds.
11. For a protein with eight cysteine residues, there are as many as 105 isomers involving four disulfide bonds. It is preferable to list all the cleavage products for each of the possible isomers. In practice, a cursory analysis of the MALDI spectrum of the cleavage products can exclude many of the isomeric possibilities.
12. We have observed that the interval fragment from cleavage of a cyanylated peptide chain usually has the lowest abundance in the MALDI spectrum (e.g., m/z 6547.3, m/z 789.8, m/z 5767.4, and m/z 6063.6 in **Table 1**). A plausible explanation for this observation is that for the interval fragment, β-elimination is able to occur at either side, which significantly reduces the yield of the expected cleavage product.
13. In earlier work, before the HPLC conditions were optimized, peaks 1 and 2 were collected as a broad peak. The MALDI spectrum of the cleavage products from the mixture is an overlap of spectrum A and B in **Fig. 6**, allowing one to deduce two disulfide pairs simultaneously. However, the feasibility of such a mixture analysis depends on the components of the mixture, their cleavage pattern, and, to a large extent, on the MALDI behavior of the cleavage products. The explicit assignment can be problematic if key fragments are missing in the MALDI spectrum.
14. The fragments arising from the cleavage of cyanylated proteins are relatively simple, but the reaction mixture contains buffers and salts, which may affect

subsequent analysis by MS. MALDI is the method of choice for such a mixture because it is tolerant to salts and buffers. A complementary method for peptide mixture analysis is LC/ESI-MS, which affords comparable sensitivity, but better peptide coverage because of the reduced signal suppression.

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2

Carbohydrate Composition Analysis of Glycoproteins Using Highly Sensitive Fluorescence Detection Methods

George N. Saddic, Mary Beth Ebert, Shirish T. Dhume, and Kalyan R. Anumula

1. Introduction

Proteins with covalently bound sugars are known as glycoproteins and are widely distributed in nature, e.g., plants, animals, bacteria, and viruses. Hormones, enzymes, and toxins are examples of proteins that are both biologically active and glycosylated. In addition, a number of proteins on the cell's surface, in cytosol, and in nucleus are glycosylated. During the last few years, enormous advances have been made in the understanding of glycoproteins, specifically their structure and biochemistry. This increased understanding is primarily due to the advent of new tools for the study of complex carbohydrates. These new analytical methods have allowed for the reporting of a large number of well-characterized glycoproteins. The complete analysis of a glycoprotein provides information on the primary structure of the oligosaccharides as well as their variation at individual glycosylation sites. Such analysis requires a multipronged approach involving mapping and characterization of oligosaccharides, which is described in Chapter 9 of this book, and determination of carbohydrate composition.

Carbohydrate composition analysis of glycoproteins is similar to amino acid analysis of proteins. Just as an accurate amino acid composition is critical to protein-structure determination and identification by database searching, the accurate determination of the carbohydrate composition of a glycoprotein provides information as to the type and extent of glycosylation. In order

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to determine and understand the structure of a glycoprotein, the individual monosaccharides present must be identified and quantitated. This is critical since as a class, hexoses and hexosamines have the same molecular weights, and therefore, can not be determined by mass spectrometry.

Another aspect to carbohydrate analysis became apparent with the advent of glycoprotein biopharmaceuticals. With respect to biopharmaceuticals, there is a need to demonstrate consistency of glycosylation in production lots that are intended for human therapy. In addition, there is an increasing demand to provide a well-characterized product description for regulatory submissions. One aspect, which can be used to determine the consistency of glycoprotein production lots, is the amount of carbohydrate it contains (expressed in % carbohydrate). The other aspect is monitoring of the sialic acid content in glycoprotein drugs, which is important since sialic acid may be critical for biological function ***(1**,**2)***.

For the last decade, high performance anion exchange chromatography with pulsed amperometric detection method (HPAE-PAD) was used for determining carbohydrate composition ***(3**,**4)***. Today, newer highly sensitive and reproducible methods using reversed phase high-performance chromatography (RP-HPLC) with fluorescence detection are available and described in this report. The HPLC methods for carbohydrate composition, namely of monosaccharides and sialic acids, are based on pre-column derivatization with fluorescent tags.

Carbohydrate composition is determined by acid hydrolysis of a glycoprotein sample to release the individual monosaccharides. After hydrolysis, the monosaccharides (neutral and amino sugars) are derivatized with anthranilic acid (AA, 2-aminobenzoic acid), and then separated from each other and from excess reagent using RP-HPLC ***(5**,**6)***. The resulting peak areas are compared to those of concomitantly derivatized and analyzed monosaccharide standards to determine the amount of each monosaccharide in the sample.

For sialic acid determination, the sialic acids are initially released from the glycoprotein by mild acid hydrolysis followed by derivatization with o-phenylenediamine (OPD) to yield a fluorescent quinoxaline derivative. The derivative is separated from excess reagent by RP-HPLC for quantitation using fluorescence detection ***(6**,**7)***. The resulting peak areas are compared to those of concomitantly derivatized sialic acid standards to determine the amount of the sialic acids (*N*-acetyl and *N*-glycolylneuraminic acids).

The methods described here are based on the use of highly fluorescent tags. They offer the most sensitive approach to analyze glycoproteins at this time, and therefore, these methods are suitable for analyzing samples available in limited amounts.

2. Materials (*see* Notes 1 and 2)

2.1. Excipient Removal and Protein Concentration Determination

1. 2% (w/v) Ammonium bicarbonate.
2. Ethanol : ethyl acetate mixture 1 : 1 (v : v) with 0.5% acetic acid (*see* **Note 3**).
3. 5% (v/v) Acetic acid solution (used with basic glycoproteins only).
4. 50 m*M* Sodium hydroxide (used with acidic glycoproteins only). Store in a plastic container.
5. Glass tubes 13 × 100 mm with Teflon-lined screw caps (Reactor Vessels Oxford GlycoSciences, cat no. I-4022).
6. Vacuum centrifuge.

2.2. Monosaccharide Analysis

1. Neat trifluoroacetic acid (TFA).
2. 1% (w/v) Aqueous sodium acetate solution.
3. Polypropylene vials (1.6 mL) with O-ring seal screw caps (Fisher, 118448 or 2.5.15. National Scientific, BC16NA-BP) (*see* **Note 4**).
4. 4% (w/v) Sodium acetate (trihydrate)-2% (w/v) boric acid (granular) in methanol (*see* **Note 3**).
5. Anthranilic acid (AA) solution: Weigh approx 45 mg of anthranilic acid (2-amino benzoic acid) into a polypropylene vial. Add approx 30 mg of sodium cyanoborohydride. Dissolve the solids in 1.5 mL of the sodium acetate-boric acid-methanol solution. (*Note:* Sodium cyanoborohydride is a poison and tends to absorb moisture readily from the air, which may affect the derivatization reaction. Limit the exposure of this chemical to air when weighing.)
6. Monosaccharide Standard Solution (1.0 m*M*): Weigh exactly 43.0 mg each of glucosamine hydrochloride and galactosamine hydrochloride and 36.0 mg each of galactose, mannose, and glucose into a 200-mL volumetric flask. Add 32.8 mg of fucose into the same volumetric flask and bring to volume with Milli-Q water. Mix well and aliquot small volumes for storage. All the monosaccharides were from either Sigma or Pfansteihl Labs. Expiration: 1 yr at –20°C if used more than once (*see* **Note 5**). Monosaccharide Working Standard Solution: Dilute the 1.0 m*M* monosaccharide standard solution 1 : 100 with Milli-Q water (*see* **Note 6**).
7. Chromatographic solvents: *see* **Subheading 2.4.**
8. Temperature controlled oven and/or heating block

2.3. Sialic Acid Analysis

1. 0.5 *M* Sodium bisulfate ($NaHSO_4$).
2. 0.25 *M* $NaHSO_4$: dilute the 0.5 *M* sodium bisulfate solution with an equal volume of Milli-Q water.
3. OPD derivatization solution: prepare a 20 mg/mL OPD solution in 0.25 *M* sodium bisulfate.

4. 0.05% (v/v) Acetic acid-water solution for preparing the sialic acid stock standard.
5. 1.0 m*M* Sialic acid standard stock solution made in 0.05% acetic acid-water (*see* **Note 7**). Sialic acid working standard solution: dilute the sialic acid standard solution 1 : 100 to 500 with 0.25 *M* $NaHSO_4$ (*see* **Note 8**).
5. Chromatographic solvents: *see* **Subheading 2.4.** below.
6. Temperature controlled oven and/or heating block.

2.4. Chromatography System

1. HPLC with a fluorescence detector (highly sensitive fluorescence detectors such as Jasco FP 920, Waters 474, and HP 1100 were used in these studies.
2. Thermostatted column compartment.
3. C18 Column: Waters Symmetry (3.9 × 150 mm) for monosaccharide analysis.
4. C18 Column: Ultrasphere ODS (4.6 × 150 mm, 5 µm, Beckman, cat no. 235330) for sialic acid analysis.
5. Column prefilter (Upchurch Scientific, A-315) and 0.2 µm insert (Upchurch Scientific, A-101X).
6. The following solvents were used for the analysis of both monosaccharides and sialic acids: Solvent A is composed of 0.2% (v/v) 1-butylamine (Aldrich), 0.5% (v/v) phosphoric acid, and 1.0% (v/v) tetrahydrofuran (inhibited, Aldrich) in Milli-Q water (*see* **Notes 9** and **10**). Solvent B: Dilute solvent A with an equal volume of acetonitrile (HPLC grade) (*see* **Note 10**).

3. Methods

3.1. Removal of Formulation Ingredients and Protein Concentration Determination

Sugars (sucrose, mannitol), detergents, buffers, and so on, may also be present as excipients in the glycoprotein or biopharmaceutical samples. These excipients are likely to interfere in the analysis, and therefore, these substances must be removed prior to analysis of the sample. If the sample quantity is limited, a variety of desalting methods including dialysis and desalting using non-Sephadex resins, new types of centrifuge filters and mini-columns, and so on, could be used to remove the excipients. Samples can also be desalted by drying directly onto polyvinylidene difluoride (PVDF) membranes. In addition, glycoproteins that have been electroblotted onto a PVDF membrane following gel electrophoresis can be analyzed. This line of approach is useful when small amounts of the sample are available or several proteins exist as a mixture. Any gel technique may be used to perform this step. Once the band of interest has been identified (preferably on PVDF), it is excised and cut into small pieces. The pieces are placed in the bottom of the vial before proceeding with the hydrolysis step in **Subheading 4.3.1.** (*see* **Note 11**).

The following procedure is routinely used for removal of excipients from formulated biopharmaceuticals. If the amount of glycoprotein sample is limited, the procedure may be followed by reducing the reagents proportionally or by selecting another method for desalting as mentioned previously.

3.1.1. Removal of Formulation Ingredients (Excipients) (see **Note 12**)

1. Place about 1.0–2.0 mg of each sample into each of two or four separate glass tubes (13 × 100 mm) with Teflon-lined screw caps.
2. Add 0.5 mL of 2% ammonium bicarbonate solution to each and vortex briefly.
3. Add 2 mL of the ethyl acetate/alcohol mixture to each vessel. Cap the vials and mix on a Vortex for about 20 s.
4. Allow the samples to stand for at least 10 min after vortexing.
5. Centrifuge the samples for about 5 min at the maximum setting in a suitable centrifuge.
6. Remove the samples from the centrifuge without disturbing the pellet.
7. Decant the supernatant from the pellet into a suitable waste container.
8. Invert the vial on a clean tissue paper (Kimwipe) placed at the bottom of a test tube rack for a minute to allow the residual solvent to drain (*see* **Notes 13–15**).
9. Repeat **steps 2–8** for a total of 4 precipitations.
10. If the glycoprotein is basic: After the fourth extraction, invert the vial on a tissue paper (Kimwipe) and allow the protein pellet to dry to insure that all the organic solvent has been removed (approx 5 min). Add about 300 µL of 5% acetic acid solution to each vial and mix well. Approximate protein concentration should be 5 mg/mL. Skip to **step 14**.
11. If the glycoprotein is acidic: After draining the supernatant from the fourth extraction, cover the vials with several layers of parafilm and puncture the parafilm 5–7 times with a clean needle.
12. Dry the pellets in a vacuum centrifuge for 5–10 min (*see* **Note 16**).
13. Add about 300 µL of 50 m*M* sodium hydroxide to each vial and mix well. Approximate protein concentration should be 5 mg/mL.
14. Vortex the samples for about 30 s and allow them to stand for at least 48 h to clarify. Intermittent vortexing is recommended throughout the 48 h period (*see* **Note 17**).

3.1.2. Protein Concentration Determination

Each sample should be clear at this point (*see* **Note 18**). Pool the contents of the clarified samples if additional amount of protein is required (*see* **Note 19**).

1. Prepare a 10-fold dilution of the pooled material using Milli-Q water (acidic samples) or 5% acetic acid solution (basic samples). This diluted purified sample will be used in the monosaccharide analysis.

2. Read the absorbance of the diluted material at 280 nm against a blank, which has been diluted in similar manner to the sample (*see* **Note 20**).
3. Divide the observed absorbance by the extinction coefficient of that protein to obtain the protein concentration (*see* **Note 21**).

3.2. Analysis of Monosaccharides

This section describes a method for the determination of carbohydrate composition of glycoproteins ***(5**,**8)***. Carbohydrate composition is determined by acid hydrolysis of a glycoprotein sample to release monosaccharides. After hydrolysis, the monosaccharides are derivatized with anthranilic acid (a highly sensitive fluorescent tag) and separated from excess reagent using RP-HPLC. Monosaccharide standards are concomitantly derivatized with the samples and analyzed. The resulting peak areas are compared to determine the amount of each monosaccharide present.

3.2.1. Hydrolysis

1. Place 0.1 mL aliquots of the sample into three separately labeled sample vials or an excised and minced PDVF band into a vial for monosaccharide analysis. Place 0.1 mL of the appropriate blank into the fourth vial (*see* **Note 22**).
2. Add 0.3 mL of Milli-Q water and mix gently.
3. Add 0.1 mL of neat TFA to each vial using a positive-displacement pipet, cap tightly, seal with 4–6 layers of Teflon tape and vortex gently on a low setting.
4. Hydrolyze the samples by placing them in a temperature-controlled oven at 100 ± 2°C for 6 h ± 5 min (*see* **Note 23** and **Fig. 1**).
5. Remove the samples from the oven and allow them to cool to room temperature. Centrifuge these samples briefly to collect the solution at the bottom of the tube.
6. Remove the Teflon tape and open the caps half way. Dry the samples overnight without heat in a vacuum centrifuge.
7. Similarly, excised and minced PDVF bands containing glycoproteins and appropriate blanks are prepared for monosaccharide analysis. However, 0.3 mL glass crimp-top micro vials are used with a 75 µL hydrolysis volume ***(8)***.

3.2.2. Derivatization of Samples and Monosaccharide Standards

1. Dissolve the dried hydrolysis sample replicates in 100 µL each of 1% sodium acetate solution and vortex vigorously for at least 2 min at the highest possible setting (*see* **Note 24**). Allow the sample to sit at room temperature for at least 30 min, vortex intermittently (approx every 10 min) to ensure the pellet is completely dissolved (*see* **Note 25**).
2. Centrifuge the tubes briefly to spin the solution to the bottom of the tube and transfer 50 µL of each hydrolyzed sample to separately labeled 1.6 mL polypropylene vials (*see* **Note 26**). Appropriately label additional 1.6 mL polypropylene vials as the standards and aliquot 50 µL of the monosaccharide working solution into each vial.

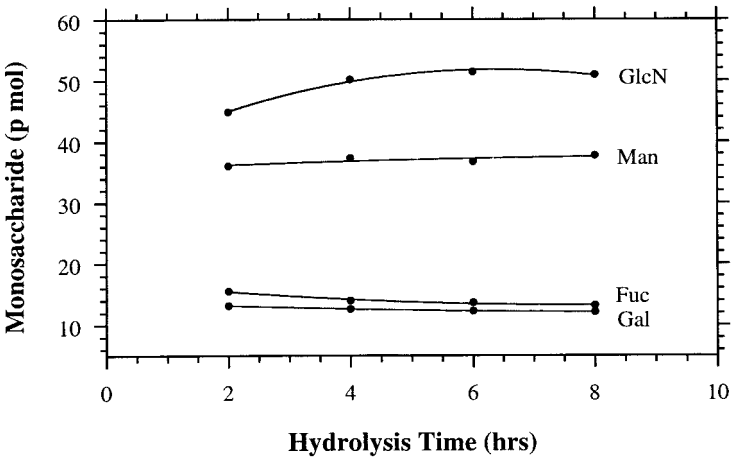

Fig. 1. Effect of hydrolysis time on the recovery of monosaccharides.

3. Add 100 µL of anthranilic acid reagent to each sample and monosaccharide standard, cap the vials tightly, vortex, and centrifuge briefly to collect the solution to the bottom of the tube.
4. Heat the tubes for 45 min at 80 ± 2°C in a thermal heating block (*see* **Note 27**).
5. After the incubation, remove all tubes from the heating block and allow them to cool to room temperature.
6. Add 850 µL of mobile phase A to each tube, cap, and vortex vigorously.
7. Centrifuge all tubes for 5 min at maximum speed in the centrifuge to obtain a solution that is free of particulates.
8. Transfer each solution to an autosampler vial (*see* **Note 28**). Install the vials on the HPLC system autosampler and analyze using appropriate injection volumes (typically 50 µL).

3.2.3. Chromatography

1. Equilibrate a Waters Symmetry column (C18, 3.9 × 150 mm) in 5% Solvent B at a flow rate of 1 mL/min.
2. Make duplicate injections of 50 µL from samples and standards.
3. Separate the monosaccharides as follows: 5% solvent B isocratic for 7 min followed by a linear gradient from 5 to 8% B over 18 min. This is followed by a 5 min wash using 100% B and an 8 minute equilibration at initial condition prior to next injection. The column temperature is maintained at 17°C (*see* **Note 29**). Total run time is 40 min with 28 min of data collection. The fluorescence detector settings are excitation λ = 360 nm and emission λ = 425 nm (*see* **Note 30**). *See* **Fig. 2** for representative standard and sample chromatograms (*see* **Note 31**). *See* **Fig. 3** for a chromatogram of fetuin using a PVDF membrane.

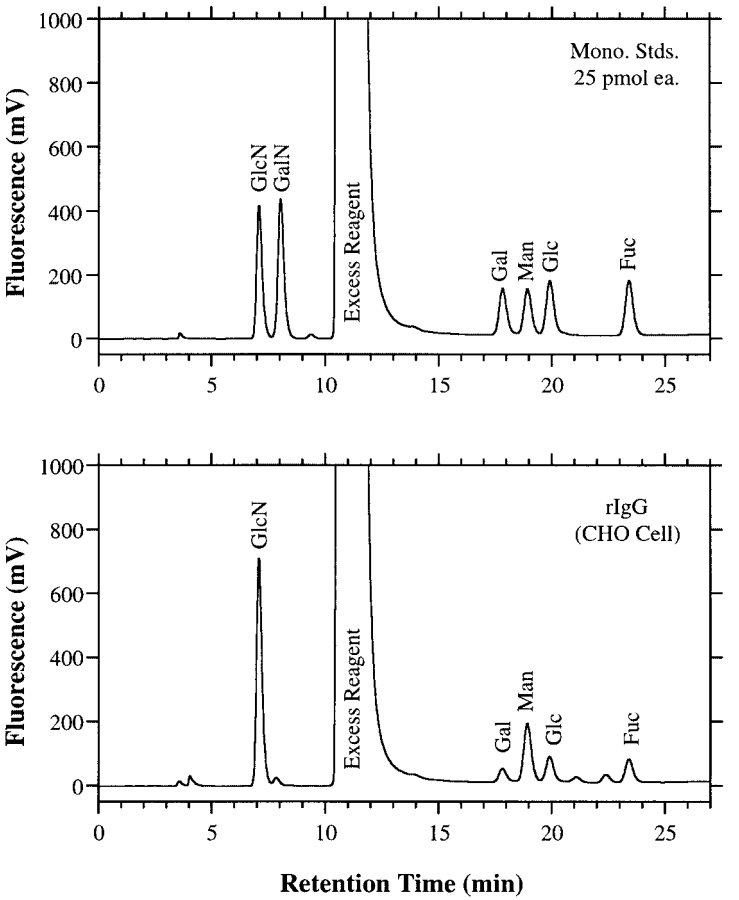

Fig. 2. Representative monosaccharide chromatograms obtained with a standard (top) and a rIgG produced in Chinese hamster ovary cells (bottom).

3.2.4. Calculations

1. Program the computer data system to calculate the peak area response for each sample (in pmol) from the average peak area response of the monosaccharide standard replicates (total six data sets, i.e., duplicate injections from three vials).
2. Calculate the amount of each monosaccharide in pmol per mg of protein taking into account dilution factors, sample volumes, and injection volumes used.
3. Calculate the moles of monosaccharide per mole of protein by the following formula:

$$\text{moles of each monosaccharide per mole of protein} = \frac{(\text{pmol/mg of protein} \times \text{MW})}{10^9}$$

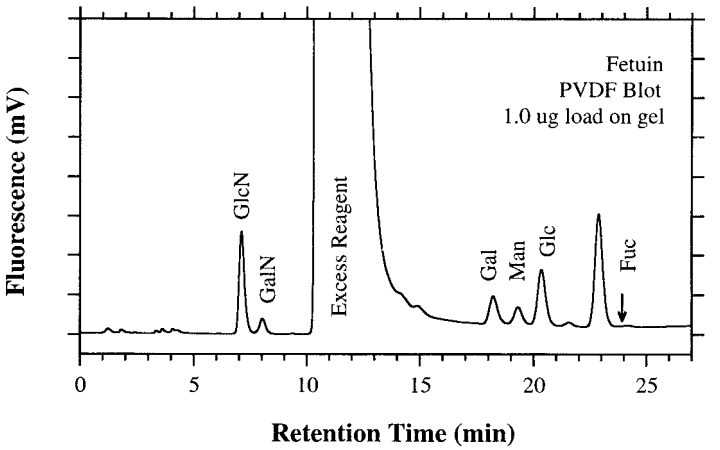

Fig. 3. An example of monosaccharides obtained from one of the three bands of fetuin electroblotted onto PVDF following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Glucose is a contaminant in this analysis ***(9)***.

where:
10^9 is the conversion factor from pmol to moles
MW is the molecular weight as determined from the amino acid sequence of the protein

4. Calculate the percent carbohydrate (w/w):
Percent carbohydrate (w/w) =

$$\frac{\text{all monosaccharides per mole of protein} \times \text{mol. wt. of that monosaccharide} \times 100}{\text{all monosaccharides per mole of protein} \times \text{mol. wt. of that monosaccharide} + \text{MW}}$$

Use the following molecular weights for the monosaccharides: Glucosamine; 221.2; Galactose; 180.2; Mannose; 180.2; Fucose; 164.2 and Sialic Acid; 309.3 (*see* **Note 32**).

3.2.5. Acceptance Criteria for Test Results

The relative standard deviation between six standard injections is less than 3.0% for each monosaccharide (usually 1.0%). The relative standard deviation between sample injections is less than 5.0% for each monosaccharide (usually 2.0%) (*see* **Note 33**).

3.3. Analysis of Sialic Acids

This section describes the method of quantitation of sialic acid (*N*-acetyl and *N*-glycolylneuraminic acids) in glycoproteins ***(6)***. The sialic acids are released

from the glycoproteins by mild acid hydrolysis followed by derivatization with OPD to yield a fluorescent quinoxaline derivative. The derivative is separated from excess reagent by RP-HPLC for quantitation using fluorescence detection.

3.3.1. Mild Acid Hydrolysis

1. Place 50 µL of the excipient free undiluted sample into labeled sample vials.
2. Prepare a vial with the appropriate blank (*see* **Note 34**).
3. Add 50 µL of 0.5 *M* sodium bisulfate to the blank and each sample, cap each vial tightly, and vortex each slowly for several seconds.
4. Hydrolyze the samples by placing them in a temperature controlled oven or heating block at 80 ± 2°C for 20 min.
5. Remove the vials from the heating block and allow them to cool to room temperature.

3.3.2. Derivatization of the Samples and Sialic Acid Standards

1. Aliquot 100 µL of the sialic acid working solution to labeled vials.
2. Add 100 µL of OPD solution to the hydrolyzed samples and standard vials. Cap the tubes tightly and vortex.
3. Heat the tubes for 40 min at 80 ± 2°C in a thermal heating block.
4. Remove all vials from the heating block and allow them to come to room temperature.
5. Add 800 µL of solvent A to each sample and standard. Cap the tubes and vortex them vigorously.
6. Centrifuge all the vials for 5 min at the maximum setting to obtain a particulate-free solution.
7. Transfer each solution to an autosampler vial (*see* **Note 26**). Install the vials on the autosampler and analyze.

3.3.3. Chromatography

1. Equilibrate a Beckman ODS column (4.6 × 150 mm, 5 µm) in 8–12% solvent B at a flow rate of 1 mL/min (*see* **Note 35**).
2. Inject 100 µL of each sample or standard in duplicate.
3. Separate the sialic acids as follows: isocratic at initial solvent B for 15 min followed by a 10-min wash at 95% solvent B and 10-min equilibration at initial conditions. The column temperature is maintained at 17°C (*see* **Note 27**). Total run time is 35 min with 20 min of data collection. The fluorescence detector settings are excitation λ = 230 nm and emission λ = 425 nm. *See* **Fig. 4** for representative standard and sample chromatograms (*see* **Note 36**).

3.3.4. Calculations

1. Calculate sialic acid amount (in pmol) per injection in samples using the average peak area response of the 100 pmol sialic acid standard.

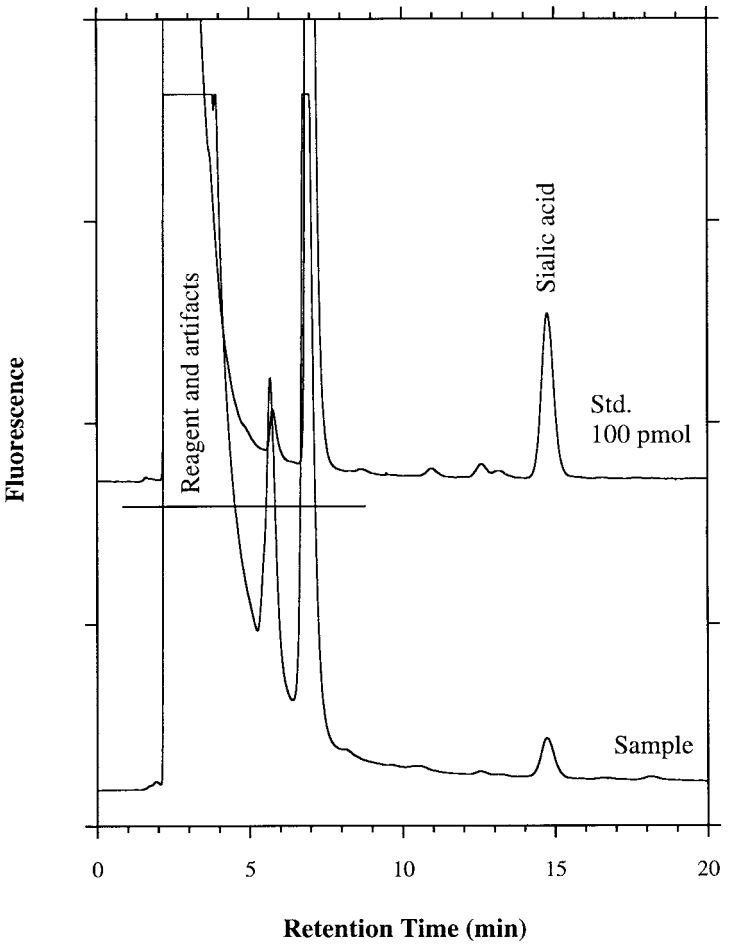

Fig. 4. Representative sialic acid (*N*-acetylneuraminic acid) chromatograms obtained with a standard (top) and a rIgG produced in Chinese hamster ovary cells (bottom). The rIgG typically contains about 2% (w/w) carbohydrate and less than 5% of the oligosaccharides are sialylated.

2. Calculate the quantity of sialic acid (in pmol) per mg of protein taking into account dilution factors, sample volumes, and injection volumes used.
3. The moles of sialic acid per mole of protein is determined by the following formula:

$$\text{moles of sialic acid per mole of protein} = \frac{(\text{pmol/mg of protein} \times \text{MW})}{10^9}$$

where:
10^9 is the conversion factor from pmol to moles
MW is the molecular weight of the protein as determined from the amino acid sequence.

3.3.5. Acceptance Criteria for Test Results

The relative standard deviation for six sialic acid standard injections is less than 3.0% as determined by peak area. The relative standard deviation between sample injections is less than 5.0% for sialic acid as determined by peak areas (usually 2.0%) (*see* **Note 37**).

4. Notes

1. CAUTION: Cyanoborohydride, trifluoroacetic acid, 1-butylamine, THF, acetonitrile, and OPD are toxic and/or flammable. Avoid contact with skin or inhalation. Wear gloves when preparing all solutions. When acetonitrile, phosphoric acid, THF, TFA, 1-butylamine, and cyanoborohydride are not in use they should be stored in an appropriate place. Dispose these chemicals appropriately.
2. The manufacturer's part numbers for the equipment and reagents are meant as a guide. Equivalent substitutions may be made.
3. Expiration: 6 mo at room temperature.
4. This type of vial and cap are critical and should not be substituted.
5. An unthawed stock standard stored at –20°C is accurate for 5 yr.
6. The working standard contains 10 nmol of each monosaccharide/mL.
7. Aliquot the solution into 500 µL portions. Expiration: 1 yr at –15 to –25°C.
8. Typical working standard contains 2–10 nmol/mL of sialic acid.
9. Make sure the butylamine has completely dissolved before proceeding to the next step. For convenience, use graduated glass pipet to add the THF.
10. Expiration: 1 mo at room temperature if containers are sealed.
11. Make sure to prepare simultaneously a blank piece of PVDF as a control.
12. If the glycoprotein is basic then follow the procedure using acetic acid as the diluent. If the glycoprotein is acidic then follow the procedure using sodium hydroxide as the diluent.
13. It is important to mix the sample vigorously to disperse the pellet before proceeding to the next step.
14. The solution should become cloudy. If the protein settles at the bottom then intermittent vortexing may be appropriate during this time.
15. Be careful: the pellet may slide out of the inverted vial. Before proceeding, it is helpful to gently tap the opening of the inverted vial on a clean tissue paper (Kimwipe) to remove any residual solvent remaining in the treads of the vial.
16. Do not over-dry. The pellet may not go into solution.
17. Most proteins will clarify in less time but the actual time may vary depending on the protein.

18. Additional dilution and/or clarification time may be necessary if the samples are not completely clear. Inspect the bottom of the tube for undissolved protein.
19. It is recommended that the pooled samples be allowed to stand overnight, however this is not a necessity.
20. This diluted blank would be either a 1 : 10 dilution of the 50 m*M* sodium hydroxide solution in water or the 5% acetic acid solution depending upon which diluent was used in **Subheading 3.2.**
21. Multiply the calculated protein concentration by the dilution factor (i.e., 10×). It is best if the protein concentration is between 3–5 mg/mL after correcting for the dilutions (e.g., rIgGs with approx 2% (w/w) carbohydrate). Lower concentrations may make the accurate quantitation of residual sialic acid difficult for proteins with very low sialic acid content (e.g., rIgGs). Higher concentrations may produce off-scale peaks for glucosamine and galactosamine, which would require an additional dilution prior to analysis. The recommended ranges are dependent on the degree of glycosylation of the protein.
22. For the blank, use either the 5% acetic acid solution or a 10-fold dilution of the 50 m*M* sodium hydroxide solution prepared in **Subheading 3.3.2.**
23. It may be necessary to determine the optimum hydrolysis time through the use of a time-course study over 4–8 h.
24. It is crucial to completely dissolve the pellet before proceeding. It is recommended that the end of a pipet tip be used to physically crush/scrape the pellet from the bottom of the tube. Rinse the tip with the sample's sodium acetate diluent several times to ensure that any residual pellet is cleared from the tip.
25. If a flat-top Vortex mixer is available, samples can be bound together by a rubberband or placed in a small box and placed on the vortex for 15 min.
26. Transfer only the supernatant and none of the pellet.
27. Cover the top of the heating block with insulating material (e.g., foam, paper towels, and/or wool pads, etc.) to maintain the temperature.
28. Some samples may contain particulates at the bottom of the tube. These particulates should not be transferred and injected into the HPLC. It is best to use amber autosampler vials to minimize light exposure to the derivatized samples.
29. The actual temperature is not critical, however, the column should be maintained at a constant temperature in order to obtain reproducible retention times. A column cooler (Cool Pocket, Keystone Scientific) can be used for this purpose.
30. For less sensitive fluorescence detectors, an excitation of 230 nm may be used.
31. Sample chromatograms may have some additional peaks but the monosaccharides peaks should be resolved from any artifact peaks. It is common to observe varying levels of glucose in the samples and blank. Due to the abundance of glucose-containing polymers (e.g., lint) in the environment, complete elimination is nearly impossible. Glucose levels, therefore, cannot be accurately determined.
32. The value for the number of moles of sialic acid is determined by the procedure described in the sialic acid method.
33. This value may be greater with very low amount of a particular monosaccharide in the glycoprotein. The tailing factor for each of the peaks in the standard

chromatogram must be less than 1.2. The resolution of the mannose in the standard chromatogram peak must be greater than 1.2.

34. The blank would either be a similarly diluted blank of the 50 m*M* sodium hydroxide solution or the 5% acetic acid solution, depending on which solution was used in **Subheading 3.2.**
35. It is usually necessary to optimize the chromatography on a particular lot of columns by adjusting the initial percentage of solvent B to between 8 and 12%. Sialic acid peak must separate from the artifacts.
36. Sialic acid should elute within 15 min.
37. This value may be greater with very low abundance of sialic acid in the glycoprotein. The tailing factor for the sialic acid peaks in the standard chromatogram must be less than 1.4. The standard theoretical plate count should be greater than 4000.

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3

Enzymatical Hydrolysis of N-Glycans from Glycoproteins and Fluorescent Labeling by 2-Aminobenzamide (2-AB)

Rolf Nuck

1. Introduction

When performing a structural analysis of N-glycans, a number of aspects should be considered. N-Glycans may be hydrolyzed from purified glycoproteins, serum glycoprotein mixtures, or delipidated membrane fractions by chemical hydrolysis using hydrazine or enzymatical hydrolysis using PNGase F.

Chemical deglycosylation may be an economical alternative to produce N-and O-glycans in a preparative scale ***(1)***, but it is less suitable for analytical purposes. By chemical hydrazinolysis the protein core is destroyed completely and all acyl groups are cleaved from neuraminic acid residues as well as from N-acetylhexosamine residues. Therefore, re-N-acetylation is necessary prior to the carbohydrate analysis procedure ***(2,3)***.

If not only a structure analysis of N-glycans is intended but also a sequencing of the protein core, an analysis of the different types of neuraminic acids or an elucidation of the carbohydrate structures in distinct glycosylation sites has to be performed; in addition, enzymatical deglycosylation using PNGase F is the most suitable way to hydrolyze N-glycans from the protein backbone.

Enzymatical hydrolysis may be performed from the intact glycoprotein or from glycopeptide fractions as obtained by prior proteolytic digestion of the glycoprotein by different proteases of sequencing grade such as trypsin or proteases from *Pseudomonas fragi* (Asp-N), *Staphylococcus aureus* V8 (Glu-C), and *Lysobacter enzymogenes* (Lys-C). Because in many cases complete enzymatical deglycosylation of the intact glycoprotein requires the use of detergents and enzyme concentrations of up to 100 mU/mL of PNGaseF ***(4)***, a

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prior proteolytic digestion ***(5)*** is advantageous, minimizing the concentration of the enzyme required to 2–10 mU/mL, and does not necessitate a purification of the N-glycans from detergent ***(6)***.

Note that some glycoproteins of plant and insect origin may be resistant to PNGaseF from *Flavobacterium meningosepticum*, if they contain a fucose residue, α1-3 linked to the core N-acetylgucosamine, which inhibits a deglycosylation by PNGase F. In this case, the use of PNGase A from almonds ***(7)*** is recommendable.

Note that for enzymatical hydrolysis of glycoproteins and/or glycopeptides for an analytical purpose, a mixture of Endoglycosidase F and PNGaseF, which is offered by some companies, should not be used. Because Endoglycosidase F is hydrolyzing the oligosaccharides in the chitobiose core between the two N-acetlyglucosamine residues, but PNGaseF is hydrolyzing the intact N-glycans directly from asparagine, a mixture of both enzymes is producing two oligosaccharides chains differing in size by an N-acetylglucosamine residue from a defined homogeneous asparagine linked glycan ***(8)***.

Moreover some critical aspects should be taken into account with respect to the analysis of N-glycans. Oligosaccharides in small amounts (picomol to nanomol range) are difficult to detect, because they do not contain a chromophore. A sensitive detection of sugars without prior derivatization may be performed by high-performance anion-exchange chromatography combined with pulsed amperometric detection (HPAEC-PAD). However, in most cases this separation method is not sufficient to obtain homogeneous N-glycans. A multidimensional separation of complex oligosaccharide mixtures has been proven to be superior to come to homogeneous N-glycans, consisting of high-performance liquid chromatography (HPLC) on anion exchange (Mono Q, GlykoSep C), hydrophilic interaction (APS 2-Hypersil), and hydrophobic interaction columns (ODS-Hypersil) ***(6,9)***. However, the multidimensional separation methods described require a sensitive labeling of the carbohydrate chains for their detection, such as reductive amination using 2-aminobenzamide ***(9)*** or radioactive labeling using sodium borotritide ***(3)***.

To perform an optimized labeling reaction, a purification of the N-glycans from detergent, proteine/petides, and salt is essential. If the N-glycans to be analyzed are exclusive of neutral character such as in the case of high-mannose oligosaccharides, N-glycans may be very effectively purified by passing them through mixed bed resins. However it should be kept in mind that most N-glycans are negatively charged, containing sialic acid, phosphate, or sulfate residues. In principal, in those cases gel-permeation chromatography using Bio-Gel P-6 or Sephadex G-25 is usually applied for desalting. However this approach has been found to cause substantial losses of material if only small amounts of N-glycans have to be analyzed.

Keeping in mind the considerations mentioned previously, a protocol was developed that allows an economical, short, and simple procedure for the enzymic hydrolysis and the fluorescent labeling of N-glycans, which may be applied in each laboratory and which does not require expensive special instrumentation.

2. Materials

2.1. Trypsin Digestion of Glycoproteins

1. Trypsin, modified, sequencing grade (Boehringer Mannheim).
2. 1% (w/v) Acetic acid.
3. Deglycosylation buffer: 100 m*M N*-Methyl-2,2-iminodiethanol-trifluoroacetate, pH 7.5., 1.15 mL (1,19 g) *N*-Methyl-2,2-iminodiethanol (Merck-Schuchardt, Hohenbrunn) are dissolved in 100 mL aqua bidest. and adjusted to pH 7.5 using 4 *N* anhydrous trifluoroacetic acid (TFA) (sequencing grade, Sigma, Deisenhofen).

2.2. Enzymatical Deglycosylation with PNGase F

1. PNGaseF from the culture medium of *Flavobacterium meningosepticum* or the recombinant enzyme expressed in *Escherichia coli* (Glyko, Inc., Novato, CA or Boehringer Mannheim).
2. Deglycosylation buffer (*see* **Subheading 2.1., item 3**).
3. 1 *N* Acetic acid and 1% (v/v) acetic acid.
4. 50% Aqueous ethanol.
5. Centricon YM-10 microconcentrator tubes (Millipore, Bedford).
6. Vacuum centrifuge.
7. Temperable centrifuge.
8. Affi-Gel Blue Gel 50–100 mesh (wet), 150–300 μm (Bio-Rad, Hercules, CA).
9. Dowex 50 W-X12 (H^+)-cation exchanger (Bio-Rad Laboratories, Richmond).
10. Pasteur pipets with cotton.
11. Acilit pH-indicator-strips (Merck, Darmstadt).
12. Temperable shaker.
13. Glass centrifugation tubes (10 mL).

2.3. Fluorescent Labeling of N-Glycans with 2-Aminobenzamide

1. Signal™ 2-AB Glycan labeling kit (Glyko, Inc., Novato, CA).
2. Screw-capped microvials (500 μL or less) with conical bottom.
3. Whatman 3 MM paper (3 × 10 cm).
4. Heating oven.
5. Chromatography chamber (5 × 7 × 14.5 cm).
6. Solvent for paper chromatography: n-Butanol/ethanol/distilled water (4/1/1, each by vol).
7. Clothes pegs (clothespins) and cord.

8. Fume cupboard.
9. Millex-HV (4 mm) filter, 0.45 µm (Nihon Millipore, Yonezawa, JP).
10. Luer-Lok Syringes (3 mL, Plastipak, Becton Dickinson, Franklin Lakes, NJ).

3. Methods

3.1. Trypsin Digestion of Glycoproteins

1. Dissolve 400 µg of glycoprotein to be analyzed, in 400 µL of 100 m*M* volatile deglycosylation buffer.
2. Dissolve 100 µg of trypsin in 100 µL 1% acetic acid immediately prior to use.
3. Add 8 µL (8 µg) of trypsin in 1% acetic acid (ratio of trypsin/protein 1/50 [w/w]) to the dissolved glycoprotein (**Subheading 2.1., step 1**).
4. Incubate the solution for 4 h at 37°C.
5. Add again 16 µL (16 µg) of trypsin and incubate the solution overnight.
6. Inactivate the enzyme by incubation at 95°C in a water bath and cool the digest for 30 min on ice.
7. If sequencing of the peptides should be performed, evaporate the obtained glycopeptide mixture to dryness in a vacuum centrifuge, dissolve it in 500 µL of water, and apply it to reversed-phase (RP) HPLC ***(5)***.
8. If deglycosylation of the complete glycoprotein only is performed, apply the tryptic digest directly to enzymatic deglycosylation.

3.2. Enzymatical Deglycosylation with PNGase F

1. Dissolve 50 mU PNGaseF (50–250 U of commercially available enzyme, *see* **Note 1**) in 2 mL of volatile deglycosylation buffer and apply it to dialysis by threefold ultrafiltration (*see* **Note 2**) in a Centricon microconcentrator tube by centrifugation at 5000*g* for 90 min at 4°C. Dilute the concentrated enzyme in the upper layer with volatile deglcosylation buffer to 50 mU/mL.
2. Let the trypsinized glycoprotein come to room temperature (**Subheading 3.1., step 6**).
3. Take an aliquot corresponding to about 0.1–0.5 nmol of glycans for monoaccharide analysis (Analysis 1) ***(11)***.
4. Add 50 µL (2.5 mU) of PNGaseF to the proteolyzed glycoprotein solution (about 400 µL) and incubate for 2 h at 37°C in a temperable shaker.
5. Add another 50 µL of PNGaseF and continue incubating overnight at a final enzyme concentration of 10 mU/mL.
6. Remove glycerol from the filter of the Centricon YM-10 microcencentrator tubes by the addition of 2 mL distilled water to the top and centrifugation at room temperature for 30 min at 5000g. Discard the flow-through.
7. Fill up the digest from **Subheading 3.2., step 5** with water to 2 mL, put it onto the top of the purified microconcentrator tubes, and apply it to ultrafiltration by centrifugation at 5,000*g* at 4°C for 2 h. Collect the filtrate, containing the N-glycans, and separate the remaining amount of hydrolyzed N-glycans, peptides, and salt in the top of the concentrator tubes from protein (trypsin and

PNGaseF) by twice adding 2 mL distilled water to the top of the tubes and centrifuging as described earlier.

8. Collect the combined filtrates containing the N-glycans in 10-mL glass tubes and evaporate them to dryness in a vacuum centrifuge tempered to 30°C at 0.05 mbar.
9. Remove main part of volatile buffer by twice adding of 1 mL 50% (v/v) aqueous ethanol and evaporation in a vacuum centrifuge.
10. Remove residual volatile buffer by twice adding of 1 mL methanol and evaporation to dryness.
11. Redissolve the dried sample in 0.5 mL distilled water, adjust the pH to 3.0–3.5 with 1 *N* acetic acid, and improve the pH by dipping an aliquot of 0.5–1 µL to Acilit pH-indicator strips (*see* **Note 3**).
12. Prepare cation-exchange/affinity-columns by filling 0.5 mL Affi-Blue Gel into a cotton stoppered Pasteur pipet. Wash the column with 3 mL distilled water and allow the gel to settle down completely. Add 0.5 mL of Dowex 50 WX-H^+ to the top of the columns and wash the resulting cation-exchange/affinity column with the fivefold column volume (5 mL) of distilled water.
13. Apply the acidified digest from **step 11** to the cation-exchange/affinity column (*see* above) and wash the column with 5 mL (fivefold bed volume) of 1% acetic acid.
14. Collect the column eluate to a 10-mL centrifugation tube and evaporate it to dryness using a vacuum centrifuge at 30°C (*see* **Note 4**).
15. Redissolve the dried sample in 500 µL of distilled water.
16. Take an aliquot corresponding to about 0.1–0.5 nmol of glycan for monoaccharide analysis (Analysis 2) (*see* **Note 5**).
17. Transfer residual sample to a 500 µL conical screw-capped microvial and evaporate the sample in a vacuum centrifuge to dryness. Remove acid and residual volatile buffer by two times redissolving the samples in 200 µL of 50% (v/v) aqueous ethanol and then in 200 µL of methanol followed by evaporation in vacuo as described earlier.
18. Apply the desalted and completely dried sample to fluorescence labeling.

3.3. Fluorescent Labeling of N-Glycans with 2-Aminobenzamide

1. Concentrate the released N-glycans (5–10 nmol) carefully from 10 µL of water to the bottom of a screw-capped microvial by evaporation at 30°C in a vacuum centrifuge (*see* **Note 6**).
2. Let the commercially available Signal™ 2-AB glycan labeling kit, which should be held at 4°C, come to room temperature to thaw dimethylsulfoxide and acetic acid. Prepare the 2-AB labeling reagent as recommended by the manufacturer. The labeling kit contains two batches of kit reagents, which are sufficient to label up to 9 samples of N-glycans each. Each sample can contain up to 50 nmol glycans.
3. Label the sample with 10 µL of the reducing amination reagent, containing 2-AB (250 µg) and sodium cyanoborohydride (500 µg), dissolved in dimethylsulfoxide/acetic acid (7/4, by vol) for 2 h at 65°C in an heating oven in the dark.

4. Allow the reaction mixture to come to room temperature, and spot the samples in two separate portions of 5 µL onto a paper strip each (3 cm × 8 cm).
5. Dry the paper strips upright in a fume cupboard overnight (*see* **Note 7**).
6. Separate the labeled glycans from unreacted 2-AB by paper chromatography for about 1 h using n-butanol/ethanol/water (4/1/1, by vol) as chromatography solvent.
7. Dry the papers for 2 h in a fume cupboard.
8. Improve the positions of N-glycans near to the origin and of unreacted dye, which moves to about 6–8 cm from the origin under an UV-lamp operating at 254 nm or 366 nm (*see* **Note 8**).
9. Label the positons of the N-glycans using a pencil and cut out the paper where the N-glycans are located.
10. Put the cut out and folded papers at the top of prewashed Luer-locked syringes equipped with 0.45 mm HV-filters. Put the syringes in 10-mL centrifugation glass tubes and dissolve the N-glycans by incubating the papers with 1 mL of distilled water for 5 min, so that they are completely wet. Centrifuge the tubes together with the syringes for 5 min at 750*g*. Complete the elution by incubating twice with 1 mL water followed by centrifugation.
11. Combine the filtrates of each sample containing the labeled oligosaccharides and evaporate to dryness in a vacuum centrifuge.
12. Redissolve samples in 1 mL of water and keep at –20°C in the dark until further use (*see* **Note 9**).

4. Notes

1. PNGase F is commercially available as solution in 20–50 m*M* sodium phosphate buffer, 12.5–50 m*M* EDTA, 50% glycerol, pH 7.2 and pH 7.5 or as a lyophilisate without glycerol, which when reconstituted as recommended by the manufacturer, results in a concentration of 100 m*M* sodium phosphate buffer, 25 m*M* EDTA, pH 7.2. Note that, 1 U is defined as the enzyme activity that hydrolyzes 1 nmol of dabsyl fibrin glycopeptide or 0.2 nmol dansyl fetuin glycopeptide within 1 min at 37°C at pH 7.8, if the enzyme used is obtained from Boehringer Mannheim. In contrast, if the enzyme from Glyko is applied, 1 U is defined as the enzyme activity that hydrolyzes 1 nmol of dansyl fetuin glycopeptide within 1 min at 37°C at pH 7.5. Note that 50 mU, which is used in this chapter, corresponds to 50 U of the enzyme from Glyko and to 250 U of the enzyme from Boehringer. Keep in mind that the recombinant enzyme, expressed in *E. coli*, is thought to have an optimal enzyme activity at pH 7.5. In contrast, the pH optimum of the enzyme purified from the culture medium of *Flavobacterium meningosepticum* is reported to be 8.6, also the enzyme is as least 80% active over the range 7.5–9.5.
2. PNGaseF preparations should not contain glycerol—which may be present as stabilizing reagent in some commercially obtainable preparations—because this can interfere with subsequent fluorescence labeling-reaction efficiencies. The

enzyme is therefore dialyzed to a volatile buffer, which may be removed in vacuo without the necessity of also desalting the solution by gel filtration.

3. At pH 3.0–3.5 most peptides are cationic and are removed by passing through a cation-exchange resin (capacity: 2.1 meq/mL resin bed). Uncharged peptides may be removed by passing through Affi-Gel Blue (capacity: 11.7 mg albumin per mL gel).
4. By passing of the N-glycan mixture through cation-exchange-affinity columns, all salts are converted to their corresponding acids. The presence and enrichment of phosphoric acid during the following evaporation has to be avoided by prior dialysis of the commercially obtained enzyme solution to volatile deglycosylation buffer (*see* **Subheading 3.2., step 1**). It is very important not to increase the temperature above 30°C, because acetic acid and traces of TFA that are in the flow-through may hydrolyze fucose and sialic acids residues from the N-glycan at higher temperatures.
5. From the comparison of the monosaccharide analyses after PNGaseF digestion, before and after the passage through a cation-exchange-affinity column, it can be concluded to a succcessful deglycosyltion reaction as well as to the presence of O-glycans, which do not pass the column.
6. To avoid an uncomplete derivatization of glycans, a careful concentration of the sample to the bottom of the microvial, as well as a double volume of 10 µL labeling reagent is essentially neccessary. By MALDI-TOF mass spectrometry, an incomplete derivatization may be recognized by the occurence of additional peaks showing a lowered mass of m/e minus 120 Dalton.
7. Careful drying of the papers overnight is essential to remove dimethyl sulfoxide (DMSO) and acetic acid from the paper strips, which would cause an unefficient separation of excess label from N-glycans during paper chromatography.
8. Note, that low molecular oligosaccharides from mono- to penta-saccharides may also migrate from the origin near to the position of unreacted dye (6–8 cm from origin).
9. Working under illumination by fluorescent light may cause a decrease of fluorescence in 2-AB labeled glycans even at 4°C. Therefore, if possible, all steps during the purification procedure should be performed at daylight and the labeled oligosaccharides are stored at –20°C in the dark.

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4

Separation of N-Glycans by HPLC

Martin Gohlke

1. Introduction

Most glycoproteins carry a very heterogeneous mixture of oligosaccharides and even a single glycosylation site of a pure glycoprotein is often heterogeneously glycosylated. The structural diversity of oligosaccharides arises from linkage variants, from differences in the size and number of charges of glycans, and from differences in the monosaccharide composition of glycans. Fortunately, the biosynthetic pathway is subject to certain restrictions, so that structural diversity is limited and amenable to laborator investigation. Different approaches have been developed to the structural characterization of oligosaccharides, including nuclear magnetic resonance (NMR), mass-spectrometry, linkage analysis by gas chromatography-mass spectrometry (GC-MS), sequence analysis using specific exoglycosidases and others, but a crucial part of these strategies is the separation of the glycan mixture into homogeneous glycan fractions. In this chapter some high-performance liquid chromatography (HPLC)-techniques are described for the isolation of oligosaccharides, in particular N-linked glycans.

The complex heterogeneity of N-glycans demands refined systems for the separation of oligosaccharide mixtures, both for "mapping" analysis and for further structural characterization ***(1**,**2)***. Normal-phase columns with primary, secondary, or quaternary amines, as well as reversed-phase (RP) columns have been used for the separation of glycoprotein-derived oligosaccharides ***(3–5)***. In a highly organic phase, the neutral glycans interact with the stationary phase via hydrogen bonding and the sugars are eluted by increasing the polarity of the eluents (hydrophilic interaction chromatography). Normal-phase HPLC gives a high resolution and a detailed profile of fluorescence-labeled N-glycans

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from glycoproteins ***(6)***. In addition to hydrophilic interaction chromatography, the basic properties of amine-bonded resins can be used for anion-exchange chromatography for the separation of negatively charged oligosaccharides. High-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has proved a useful tool for the separation of sialylated glycans, as well as uncharged oligosaccharides. Since the introduction of HPAEC-PAD in 1988 by Hardy and Townsend ***(7)*** for the separation of N-linked oligosaccharides, this method has become standard for the separation of N-glycans; it produces a high resolution and allows the sensitive detection of underivatized oligosaccharides. Depending on the chromatographic conditions applied, negatively charged glycans or neutral oligosaccharides can be separated to provide a "fingerprint" map for the glycans of a given protein. Sugars are separated using strong basic eluents (pH 13.0). Under these conditions the oligosaccharides are present as oxyanions and can bind to the amino groups of the stationary phase of the column. The selectivity of the chromatographic behavior may be determined by the particular hydroxyl groups of the oligosaccharides that become deprotonated and undergo interaction with the quaternary ammonium groups of the column resin. The separation depends on charge, molecular size, sugar composition, and linkage of the monosaccharides, but prediction of the elution order is largely empirical. At this high base concentration, an epimerization of the reducing GlcNAc to ManNAc has been observed. Thus a single oligosaccharide species may elute as two peaks differing only in the monosaccharide at the reducing end ***(8,9)***. Further characterization of the oligosaccharides requires neutralization and removal of eluent salts. Automated systems for on-line desalting have been introduced ***(10)***. While HPAEC is usually coupled with PAD, the combination of HPAEC and fluorescence detection of labeled glycans has been reported recently ***(11)***.

Despite rapid progress in the development of separation techniques in the last decade and availability of high-resolution methods, a complete separation of all structures (even after release of the sialic acid residues) present in a mixture is rarely achieved in a single chromatographic step. Thus, rechromatography using a complementary HPLC method is often needed. It is important to note that for both one- and multidimensional HPLC-based methods, the use of retention times is not sufficient for the structural identification of sugars.

In addition to the problems presented by the structural diversity of the N-glycans, sensitive on-line detection is difficult because oligosaccharides do not contain a chromophore. The requirement for sensitive detection of carbohydrates has led to the development of two approaches, based on electrochemical detection and fluorimetric detection. Underivatized glycans can be monitored by pulsed amperometric detection (PAD) at low picomole concentrations, but this method is nonspecific for carbohydrates ***(12)***. Pulsed

amperometric detection is usually combined with HPAEC to provide a sensitive and selective detection system for carbohydrates and other oxidizable species. In principle, a repeating waveform potential is applied in a flow-through cell. The standard waveform is a triple potential waveform, which has worked well in our hands (the triple-pulse voltage sequence is given in **Subheading 3.2.**). The potential E1 is used for oxidation of the oligosaccharide; this generates the signal that is detected for the analyte. The subsequent two steps at potentials of more positive or negative potential are necessary for cleaning the gold electrode. Detection is based on the measurement of a current that is proportional to the oxidation rate of the analyte, which in turn depends on various factors. Changes in the cell, like a decrease in the reaction area of the gold electrode, therefore impair the response.

The requirement for highly sensitive detection in carbohydrate analysis has promoted increased interest in the fluorescence labeling of glycans. Generally derivatization of the oligosaccharides with a fluorescent label is nonselective and allows detection of glycans in subpicomolar concentrations in their correct molar proportions. Different labels such as 2-aminopyridine (2-AP) ***(13)***, 2-aminobenzoic acid (2-AA) ***(14)***, 2-aminobenzamide (2-AB) ***(15)***, and others have been introduced for specific detection at very high sensitivity. The chromatographic behavior of 2-AP ***(16,17)***, 2-AA ***(18)*** and 2-AB-tagged oligosaccharides ***(6,19)*** has been extensively investigated.

In this chapter techniques for separating sialylated and/or neutral oligosaccharides using anion-exchange-HPLC, normal-phase-HPLC, and RP-HPLC are introduced.

2. Materials

All salts used for the preparation of the eluents should be of analytical quality.

1. Double-distilled water was used for HPLC. Double-distilled water and aqueous buffers should be filtered through a 0.45 μm membrane (Millipore, Durapore Membrane Filter, 0.45 μm HV, Bedford, USA). NaOH (50%) is available from J. T. Baker (Deventer, Netherlands).
2. A common HPLC-system capable of delivering accurate gradients at flow rates of 0.5–1.5 mL/min is needed. A low dead volume of the HPLC-system and a decreased injection volume increase the resolution. The loop size depends on the amount of the sample and the separation mode. For analytical purposes a 10–20 μL loop, and for preparative separation a 50–100 μL loop, is adequate. For the detection of 2-AB-labeled glycans a fluorescence detector is required (excitation wavelength: 330 nm, excitation wavelength: 420 nm).
3. For HPAEC, a DIONEX system, e.g., DX500 is preferred, equipped with a pulsed electrochemical detector (e.g., DIONEX ED 40), and a Helium degassing

system. If a different HPLC-system is used, it must be capable of resisting the very basic conditions (pH 13.0) and must consist of titan or peek parts.
4. Samples should be stored at –20°C. Prior to injection, samples should be sonicated for 3 min to ensure a homogeneous solution.

2.1. Charge Profiling of N-Glycans (Low Resolution)

1. Mono Q HR 5/5 (Pharmacia, Uppsala, Sweden).
2. 0.6 *M* NH_4OAc, pH 7.0.
3. GlycoSepC (4.6 × 100 mm, Glyko, Novato, CA).
4. 20% Acetonitrile, 80% H_2O, (v/v) by volume.
5. 20% Acetonitrile, 30% H_2O, 50% 0.5 *M* NH_4OAc, pH 4.5, (v/v) by volume.

2.2. Charge Profiling of N-Glycans (High Resolution)

1. Carbopak PA-100 and guard PA-100 (Dionex).
2. Eluent 1: 0.1 *M* NaOH (5.75 mL 50% NaOH/1 L).
3. Eluent 2: 0.1 *M* NaOH, 0.6 *M* NaOAc (5.75 mL 50% NaOH/1 L with 49.2 g/1 L NaOAc anhydrous, crystalline).

2.3. Separation of Neutral N-Glycans

2.3.1. HPAEC-PAD

1. Column, Carbopak PA-100 and guard PA-100.
2. Eluent 1: 0.1 *M* NaOH (5.75 mL 50% NaOH/1 L).
3. Eluent 2: 0.1 *M* NaOH, 0.6 *M* NaOAc (5.75 mL 50% NaOH/1 L with 49.2 g/1 L NaOAc anhydrous, crystalline).
4. Eluent 3: 0.1 *M* NaOH, 120 m*M* NaOAc (5.75 mL 50% NaOH/1 L with 9.84 g/1 L NaOAc anhydrous, crystalline).
5. Eluent 4: 0.2 *M* NaOH (11.5 mL 50% NaOH/1 L).

2.3.2. Separation of Neutral N-Glycans Using Two-Dimensional-HPLC

2.3.2.1. Separation of Neutral N-Glycans Using NH_2-HPLC

1. Column: APS 2-Hypersil column (4 × 250 mm, 3 µm, Knauer [Berlin, Germany] or Bishoff [Leonberg, Germany]) (*see* **Note 1**).
2. Eluent 1: Acetonitrile.
3. Eluent 2: 15 m*M* NaH_2PO_3 / Na_2HPO_3, pH 5.2.

2.3.2.2. Separation of Neutral N-Glycans Using RP-18 HPLC

1. Column: ODS-Hypersil column (4 × 250 mm, 3 µm, Knauer [Berlin, Germany]).
2. Eluent 1: H_2O.
3. Eluent 2: Acetonitrile.

3. Methods

In "charge profiling," negatively charged N-linked oligosaccharides are separated by various HPLC-methods to provide a characteristic elution pattern.

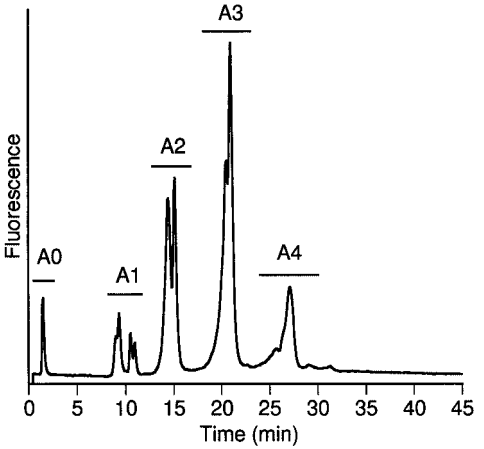

Fig. 1. Charge profiling on a Mono Q-column (low resolution). 2-AB labeled N-linked oligosaccharides from α1 acid glycoprotein were separated according to the number of negative charges using a Mono Q-column. The elution positions of neutral, mono-, bi-, tri-, and tetracharged glycans are marked A0, A1, A2, A3, and A4, respectively.

Depending on the columns used and the chromatographic conditions applied, sialylated glycans can be separated at low resolution or at high resolution.

3.1. Charge Profiling of N-Glycans (Low Resolution)

Oligosaccharides can be separated strictly according to their number of negative charges. Under appropriate chromatographic conditions, other structural features of the oligosaccharides do not significantly influence the elution profile (*see* **Note 2**). Charge profiling at a low resolution is often used as a preparative method to fractionate oligosaccharides according to their charge state, prior to subjecting the glycans to further structural characterization. Two methods using a strong anion exchanger (Mono Q-column) or a weak anion exchanger (GlycoSepC-column), are described (*see* **Note 3**). Both columns can be used for the separation of 2-AB-labeled glycans with fluorimetric detection (*see* **Notes 4–6**). Mono Q was also used for the separation of underivatized glycans detected by PAD ***(20)***. However the post-column addition of 0.5 *M* NaOH is required for high sensitivity.

3.1.1. Charge Profiling on a Mono Q-Column ***(Fig. 1)***

Column: Mono Q HR 5/5 (Pharmacia, Uppsala, Sweden).
Eluent 1: H_2O.
Eluent 2: 0.6 M NH_4OAc, pH 7.0.
Flow: 1 mL/min.

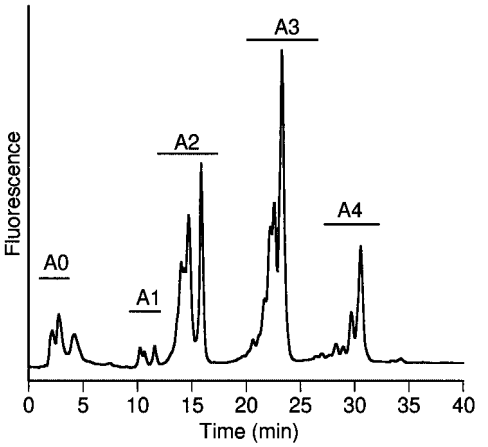

Fig. 2. Charge profiling on a GlycoSepC-column (low resolution). 2-AB labeled N-linked oligosaccharides from α1 acid glycoprotein were separated according to the number of negative charges using a GlycoSepC-column. The elution positions of neutral, mono-, bi-, tri-, and tetracharged glycans are marked A0, A1, A2, A3, and A4, respectively.

1. Wash the column by either running the complete gradient or using a shortened gradient (in 15 min from 0 to 100% eluent 2, wash the column 10 min with 100% eluent 2, equilibrate the column 20 min in eluent 1) (*see* **Note 2**).
2. Inject the sample in water while the sample loop is filled with eluent 1 and elute the analyte with the following gradient. For detection of 2-AB-labeled glycans use flourimetric detection (excitation 330 nm, excitation: 420 nm).
3. Store the column in 80% H_2O and 20% ethanol.

3.1.2. Charge Profiling on a GlycoSepC-Column ***(Fig. 2)***

The GlycoSepC-column can be used for multiple applications in glycan analysis including the separation of charged glycans, neutral glycans, and a mixture of both, depending on the eluents and gradients applied ***(21)***. This column has been described for the separation of 2-AB oligosaccharides, but might be generally applicable for fluorescent-tagged oligosaccharides.

Column: GlycoSepC (4.6 × 100 mm).
Eluent 1: 20% acetonitrile, 80% H_2O.
Eluent 2: 20% acetonitrile, 30% H_2O, 50% 0.5 *M* NH_4OAc, pH 4.5.
Flow: 0.5 mL/min.

1. Wash the column by either running the complete gradient or using a shortened gradient (in 15 min from 0 to 100% eluent 2, wash the column 10 min with 100% eluent 2, equilibrate the column 20 min in eluent 1).

Time	%1	%2	Event
0	100	0	Injection
5	100	0	
45	75	25	
47	0	100	
57	0	100	
59	100	0	
80	100	0	Restart

2. Inject the sample in a small volume of water while the sample loop is filled with eluent 1 or inject the sample in 20% acetonitrile, 80% H_2O. Elute the analyte with the gradient. For detection of 2-AB-labeled glycans use fluorimetric detection (excitation: 330 nm, excitation: 420 nm).
3. Store the column in 80% H_2O and 20% acetonitrile.

3.2. Charge Profiling of N-Glycans (High Resolution) (Fig. 3)

A separation technology providing a high resolution is required to give a detailed profile of the overall glycosylation of a protein, including neutral and sialylated glycans. HPAEC-PAD has proved to be a high-resoluting system for mapping sialylated N-glycans ***(22)***. Recently a high resolution and extremely sensitive method for the separation of 2-aminobenzoic acid-labeled sialylated N-glycans using an NH_2-bonded column was published ***(18)***.

Column: Carbopak PA-100 and guard PA-100.
Eluent 1: 0.1 *M* NaOH (5.75 mL 50% NaOH/1 L).
Eluent 2: 0.1 *M* NaOH, 0.6 M NaOAc (5.75 mL 50% NaOH/1 L + 49.2 g/1 L NaOAc anhydrous, crystalline).

1. Filter 1 L double-destilled water for each eluent through a 0.45 µm membrane (Millipore, Durapore Membrane Filter, 0.45 µm HV) and sparge with helium for 15 min (*see* **Note 7**).
2. For preparation of eluent 1 add 5.75 mL of 50% NaOH (Baker, Deventer, Netherlands) to the 1 L double-distilled water and mixing by gently shaking the flask (*see* **Notes 8** and **9**).
3. Dissolve 49.2 g NaOAc (anhydrous, crystalline) in 1 L double-distilled water and gently shake. After 5 min of sparging with helium, add 5.75 mL of 50% NaOH and mix gently (eluent 2). Continue sparging for 5 min for both eluents.
4. Wash the column by either running the complete gradient or using a shortened gradient (in 15 min from 0 to 100% eluent 2, wash the column 10 min with 100% eluent 2, equilibrate the column 25 min in eluent 1, *see* **Note 10**).
5. Inject the sample in water and elute the analyte with the gradient (*see* **Notes 11–13**).

Time	%1	%2	Event
0	100	0	Injection
2	100	0	
45	25	75	
50	0	100	
60	0	100	
61	100	0	
81	100	0	Restart

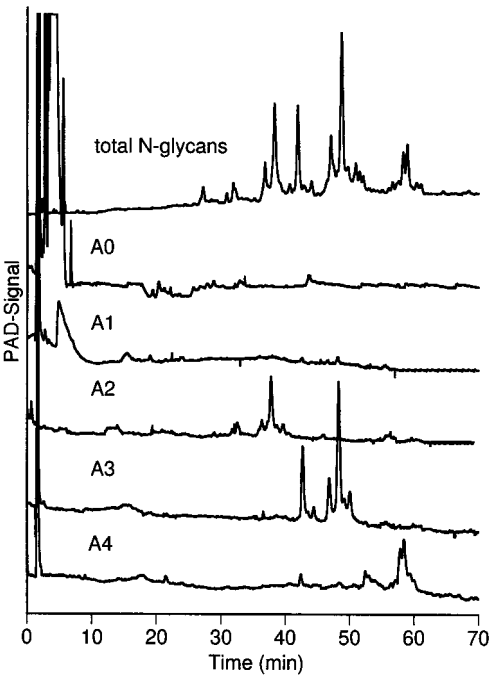

Fig. 3. Charge profiling of N-glycans from α1 acid glycoprotein on HPAEC-PAD (high resolution). Underivatized N-linked oligosaccharides from α1 acid glycoprotein were separated using the gradient for sialylated N-glycans on HPAEC-PAD. The elution pattern of the total moiety is shown in the upper panel. The traces AO, A1, A2, A3, and A4 show the rechromatography of corresponding glycan fraction obtained by Mono Q-HPLC and defines the elution times for neutral, mono-, bi-, tri-, and tetrasialylated N-glycans. Since α1 acid glycoprotein carries only traces of neutral and monosialylated glycans, no signal was detected for these fractions. Neutral N-glycans elute at about 15–25 min and monosialylated at about 25–35 min.

Time	%1	%2	Event
0	100	0	Injection
1	100	0	
2	100	0	
82	60	40	
85	0	100	
93	0	100	
94	100	0	
120	100	0	Restart

The detector settings for PAD-detection are (*see* **Note 14**):

Integration:	0.20–0.40 s
E1 = 0.05 V	0.00–0.40 s
E2 = 0.75 V	0.41–0.60 s
E3 = –0.15 V	0.61–1.00 s

3.3. Separation of Neutral N-Glycans

In this section two different methods are described: (1) for the separation of underivatized sugars using HPAEC-PAD, and (2) for the separation of glycans fluorescence-labeled with 2-aminobenzamide, using two-dimensional NH_2-HPLC and RP-HPLC.

*3.3.1. HPAEC-PAD **(Fig. 4)***

HPAEC-PAD is a high-resolution, sensitive method for the separation of native oligosaccharides. The relationship between N-glycan structures and their retention times is summarized in **Table 4**. In general, larger structures of the same charge elute later but separation is greatly influenced by structural features like branching, type of linkage, and monosaccharide composition. The gradient is useful for separation of neutral oligosaccharides but not for sialylated N-glycans ***(20)***. The main difference between the gradients for separation of neutral or sialylated sugar chains is the higher salt concentration needed for the elution of negatively charged structures. A gradient build-up from a low-salt concentration is favored for the separation of neutral oligosaccharides because even subtle differences in the glycan structure influence the retention time. However, as mentioned in the introduction, a complete separation of all structures present in a mixture is rarely achieved in a single chromatographic step.

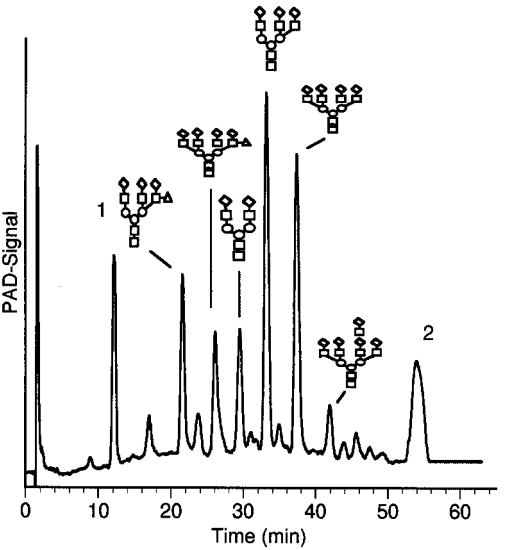

Fig. 4. Separation of desialylated N-glycans from α1 acid glycoprotein using HPAEC-PAD. Underivatized N-linked oligosaccharides from α1 acid glycoprotein were separated using the gradient for neutral N-glycans on HPAEC-PAD. Peak 1 and 2 are internal standards used for calibration of the chromatographic system. The potential structures of the oligosaccharides are given in symbols. For structural details, *see* **ref. *22***. Glycan key: ○, mannose, □ = N-acetylglucosamine, ◇ = galactose, △ = fucose.

1. Wash the column by either running the complete gradient or using a shortened gradient (in 15 min from 0–100% eluent 2, wash the column 10 min with 100% eluent 2, elute 5 min with eluent 4, and equilibrate the column 20 min in eluent 1).
2. Inject the sample in water and elute the analyte with the gradient.

Column:	Carbopak PA-100 and guard PA-100.
Eluent 1:	0.1 *M* NaOH (5.75 mL 50% NaOH/1 L).
Eluent 2:	0.1 *M* NaOH, 0.6 *M* NaOAc (5.75 mL 50% NaOH/1 L + 49.2 g/1 L NaOAc anhydrous, crystalline).
Eluent 3:	0.1 *M* NaOH, 120 mM NaOAc (5.75 mL 50% NaOH/1 L + 9.84 g/1 L NaOAc anhydrous, crystalline).
Eluent 4:	0.2 *M* NaOH (11.5 mL 50% NaOH/1 L).

The detector settings for PAD-detection are:

Integration:	0.20–0.40 s
E1 = 0.05 V	0.00–0.40 s
E2 = 0.75 V	0.41–0.60 s
E3 = –0.15 V	0.61–1.00 s

Table 4
Relationship of N-Linked Glycan Structure and Retention Time on HPAEC-PAD and NH2-HPLC

Structural characteristic	Change in retention time on NH_2-HPLC	Change in retention time on HPAEC-PAD
Increasing antennarity, bi-, tri- and tetraantennary glycans	Increasing retention times (about 5 min per antenna)	Increasing retention times (about 4 min per antenna)
Fucose α1-6 linked to the core	Increasing retention times (about 2 min)	Decreasing retention times (about 3 min)
Fucose linked to the antennae (Fucα1-3GlcNAc)	Increasing retention times (about 2 min)	Decreasing retention times (about 12 min)
Galβ1-4GlcNAc (type II chain)/ Galβ1-3GlcNAc (type I chain)	Slightly different	Galβ1-3GlcNAc elutes later (about 6 min)
Addition of an *N*-acetyl lactosamin-repeat	Increasing retention times, (about 4 min)	Increasing retention times (about 4 min)
24-Branched triantennary/ 26-branched triantennary glycans	24-Branched triantennary elutes earlier (about 2 min)	24-Branched triantennary elutes earlier (about 4 min)
Reduction or labeling with 2-AB	Based on 2-AB-labeling	Decreased retention time
Addition of an bysecting GlcNAc	Not measured	Increase of retention time
Glycans with a complete core/glycan with only one GlcNAc in the core	Decreased retention time	Increase of retention time (about 2 min)

The influence of the carbohydrate structure on the retention time given in this table was empirically determined (*see* **ref. *24*** for HPAEC-PAD). Changes in the retention times depend on the gradients used. The time values in brackets correspond to differences in the retention time estimated in our laboratory applying the methods introduced in this chapter.

3.3.2. Separation of Neutral N-Glycans Using Two-Dimensional-HPLC

Two and three-dimensional techniques have been developed either for sugar mapping ***(16,17)***, e.g., in combination with exoglycosidase digestion, or for fractionation to obtain homogeneous glycan fractions for further analysis ***(19,23)***. Multidimensional HPLC-techniques have been preferentially applied for the separation of fluorescence-labeled oligosaccharides.

These techniques often use amine-bonded columns, because these columns can perform as both hydrophilic interaction media and as an anion-exchange

Time	%1	%2	%3	%4	Event
0	100	0	0	0	Injection
1	100	0	0	0	
2	100	0	0	0	
72	50	0	50	0	
82	0	0	100	0	
87	0	100	0	0	
97	0	100	0	0	
98	0	0	0	100	
103	0	0	0	100	
104	100	0	0	0	
125	100	0	0	0	Restart

phase. RP-HPLC can be applied as a complementary system with a different chromatographic characteristic. Different combinations of amine-bonded columns, working either in the hydrophilic mode or used as an anion exchanger, and RP-HPLC have been reported for the separation of oligosaccharides. In this section, a complimentary technique for 2-AB-labeled glycans using NH_2-bonded HPLC in the first chromatographic step and RP-18-HPLC in the second chromatographic dimension is described (*see* **Note 15**).

3.3.2.1. Separation of Neutral N-Glycans Using NH_2-HPLC (Fig. 5)

1. Wash the column by either running the complete gradient or using a shortened gradient (in 15 min from 0–100% eluent 2, wash the column 10 min with 100% eluent 2, equilibrate the column 20 min in eluent 1).
2. Inject the sample in a small volume of water while the sample loop is filled with acetonitrile (*see* **Notes 16** and **17**). Elute the analyte with the following gradient. For detection of 2-AB-labeled glycans use flourimetric detection (excitation: 330 nm, excitation: 420 nm).

   Column: APS 2-Hypersil column (4 × 250 mm, 3 µm, Knauer or Bishoff) (*see* **Note 11**).
   Eluent 1: Acetonitrile.
   Eluent 2: 15 mM NaH_2PO_3/Na_2HPO_3, pH 5.2.
   Flow: 1.5 mL/min.

3. Store the column in acetonitrile.

3.3.2.2. Separation of Neutral N-Glycans Using RP-18 HPLC (Fig. 6)

1. Wash the column by either running the complete gradient or using a shortened gradient (in 15 min from 0–100% eluent 2, wash the column 10 min with 100% eluent 2, equilibrate the column 20 min in eluent 1).

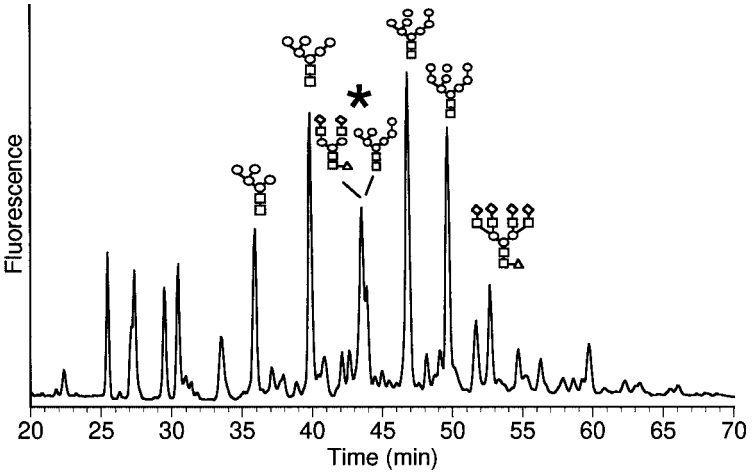

Fig. 5. Separation of desialylated 2-AB-labeled N-glycans. N-linked glycans released from K562-cells were desialylated and fluorescently labeled with 2-AB. Neutral oligosaccharides were separated mainly according to their size on NH_2-HPLC. Fractions were subjected to mass determination and the potential structures of the main fractions are given in symbols. The glycan fraction marked with an asterisk was rechromatographed using RP-18-HPLC (**Fig. 6**). Glycan key: ○ = mannose, □ = N-acetylglucosamine, ◇ = galactose, △ = fucose.

Time	%1	%2	Event
0	100	0	Injection
10	80	20	
80	50	50	
82	30	70	
92	30	70	
95	100	0	
115	100	0	Restart

2. Inject the sample in water while the sample loop is filled with eluent 1 (*see* **Notes 18** and **19**). Elute the analyte with the gradient. For detection of 2-AB-labeled glycans use flourimetric detection (excitation: 330 nm, excitation: 420 nm) (*see* **Note 20**).

   Column: ODS-Hypersil column (4 × 250 mm, 3 µm, Knauer).
   Eluent 1: H_2O.
   Eluent 2: Aacetonitrile.
   Flow: 1 mL/min.

3. Store the column in acetonitrile.

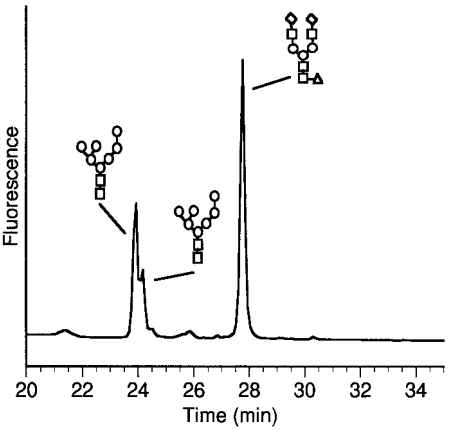

Fig. 6. Rechromatography of N-glycans using RP18-HPLC. The resulted glycan fraction from NH_2-HPLC (**Fig. 5**, fraction marked with an asterisk) was rechromatographed on RP-18-HPLC. Mass spectrometry revealed the presence of a biantennatry and two isomers of high mannose-sugars (Man7). Glycan key: ○ = mannose, □ = N-acetylglucosamine, ◇ = galactose, △ = fucose.

4. Notes

1. Columns filled with 3 µm instead of 5 µm material give a slightly enhanced resolution. Back pressure can be high (about 300 bar), but this is not a problem for conventional HPLC-systems.
2. It is essential that the sample is free of salts, since anions present in the sample behave as eluents.
3. Adjust the limit for maximum back pressure to 50 bar.
4. A proper reequilibration of the column is important for reproducible results.
5. The quantification of the neutral sugars can be difficult since they elute in the void volume or only slightly retarded close to the signal of residual 2-AB-label. The ratio of the neutral and charged oligosaccharides can be calculated after rechromatography of the resulting fractions. We use amino-phase HPLC for rechromatography of glycan fractions after digestion with neuraminidase (described in **Subheading 4.2.**). If no high-mannose or hybrid-type structures are present, a monosaccharide-composition analysis is helpful for quantification of all resulting fractions (A0-A4).
6. Both columns, Mono Q and GlycoSepC, are eluted with NH_4OAc, which can be removed in a vacuum centrifuge. For glycan fractions containing higher salt concentrations, the total removal of salts can be difficult even after repeated dissolving and evaporation of the samples. Alternatively, glycan fractions can be desalted by gel-chromatography on a Sephadex G-25 superfine column (0.5 × 20 cm, Pharmacia, Uppsala, Sweden) at a flow rate of 0.5 mL/min.

Time	%1	%2	Event
0	100	0	Injection
6	100	0	
46	80	20	
47	0	100	
57	0	100	
58	100	0	
80	100	0	Restart

7. The careful preparation of the eluents is extremely important for HPAEC-PAD to ensure reproducibility of retention times and elution profiles.
8. NaOH containing eluents must be prepared so as to minimize the content of Na_2CO_3. Do not use NaOH pellets, since these are coated with a Na_2CO_3 film. We recommend 50% NaOH solution (J. T. Baker, Deventer, Netherlands).
9. Store the 50% NaOH-solution under argon to minimize absorption of CO_2 from the air.
10. Reequilibration of the system influences the retention time. Thus if several analyses are performed, use the same intervals for injection or use an autosampler with titan or peek equipment.
11. Avoid high salt concentrations in the sample, or the analyte may elute earlier or with poor resolution. Anions present in the sample act as eluents.
12. Generally, detergents, Tris, and hydroxylated compounds should be avoided (e.g., glycerol, other alcohols).
13. Samples should be dissolved in water prior to injection, no organic solvent (e.g., acetonitrile) is allowed.
14. The gold electrode of the detector can be cleaned by rubbing. The reference electrode should be regenerated in KCl-solution depending on the time used.
15. The eluent salts are usually no obstacle for mass determination by MALDI-TOF-MS. DHB (2,5-dihydroxybenzoic acid) is a suitable matrix, which is relatively tolerant of salt contamination.
16. Injection of more than 30 µL of the sample might result in doubling of the peaks.
17. Samples should be free of salt.
18. Salts present as contamination of the analyte do not affect the separation in this system.
19. The injection volume is not critical (10–100 µL are adequate).
20. Volatile mobile phases can be easily removed in a vacuum centrifuge. Thus eluents used in this system are compatible with mass spectrometry and other techniques for analyzing the resulting glycan fractions.

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5

Enzymatic Sequence Analysis of N-Glycans

Christoph Kannicht and Anke Flechner

1. Introduction

Enzymatic sequencing of oligosaccharides gives structural information on sequence of monosaccharides and type of linkage within the oligosaccharide chain. This data can be obtained by stepwise enzymatic digestion of a single, isolated oligosaccharide using different mixtures of specific exoglycosidases. N-glycans have to be fractionated from mixtures prior to sequence analysis.

In order to keep the method simple, we describe the use of a commercially available N-linked sequencing enzyme set, which is easy to handle and gives fast results. The enzyme set contains five exoglycosidases and the enzyme reaction buffer. These enzymes cleave the glycan stepwise from the nonreducing terminal linkage-releasing monosaccharides. They are highly specific for monosaccharide type, anomeric configuration, linkage, and branching. The digestion will stop at the monosaccharide, which cannot be cleaved off with the enzymes available in the respective enzyme mixture. Enzymatic digestion results in a mixture of truncated forms of the analyzed N-glycan.

For evaluation of the assay, the size of the fragments has to be determined. From this data and the known specificity of the exoglycosidases, type, order, and linkage of monosaccharide within the N-glycan chain can be deduced.

Size of oligosaccharide fragments can be determined by gel-permeation chromatography with internal standard ***(1)***, a special gel-electropheresis system ***(2)*** or mass-spectrometry ***(3)***. Here we describe a method using MALDI-TOF mass spectrometry for size determination of the exoglycosidase digestion products. Using this method, oligosaccharides without fluorescence label can be measured as well. Moreover, you do not need special instrumentation used solely for oligosaccharide analysis (*see* **Note 1**).

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Table 1
Monosaccharides and Possible Linkage Commonly Found in Mammalian Glycoproteins

Monosaccharide	Anomer	Bound to C
N-Acetyl neuraminic acid	α	3, 6, 8
N-Glycolyl neuraminic acid	α	3, 6
D-Galactose	α	3
	β	3, 4, 6
N-Acetyl D-glucosamine	β	2, 3, 4, 6
N-Acetyl D-galactosamine	α	3
	β	4
D-Mannose	α	2, 3, 6
	β	4
L-Fucose	α	2, 3, 4, 6
D-Xylose	β	2

Occurrence and action of the glycosyltransferases involved in synthesis of N-glycans is limited and only a number of monosaccharides are accepted as substrates in N-glycan-synthesis (*see* **Table 1**). As a result, the number of possible oligosaccharide chains found in mammalian cells is limited. Moreover, N-glycans share a common core structure and sugar chains bound to the core follow some structural rules. N-linked glycans are divided into three subgroups: high-mannose type, complex type, and hybrid type ***(4,5)***. For this reason, it is possible to perform sequence analysis of most N-glycans from mammalian proteins using only few exoglycosidases.

2. Materials

2.1. Desialylation

1. Thermomixer (Eppendorf, Hamburg, Germany).
2. Sialidase from *Arthrobacter ureafaciens* (Glyko, Inc., Novato, CA).
3. Sialidase incubation buffer: 50 m*M* sodium acetate, pH 5.5.
4. Mixed bed column for desalting of oligosacharides: add one after the other 0.5–1 mL 50% aqueous suspensions of anion exchange resin (Dowex 3-X4A, 400 mesh, OH^- form), and cation exchange resin (Dowex AG 50 W-X12, 200–400 mesh, H^+ form) into a small column (5 mL) (Bio-Rad, or equivalent).
5. Centrifugal evaporator.

2.2. Enzymatic Sequencing

1. Centrifugal evaporator.
2. N-Linked sequencing enzyme set (No. 50300, Glyko) or single exoglycosidases β-Galactosidase III (*S. pneumoniae*), β-N-Acetylhexosaminidase III (Jack bean), α-Mannosidase II (Jack bean), α-Mannosidase VI (*X. manihotis*).
3. Fivefold enzyme-reaction buffer (No. 50360, No. 50300, Glyko).
4. Thermomixer (Eppendorf, Hamburg, Germany).
5. Mixed-bed column for sample purification: add one after the other 0.5 mL 50% aqueous suspensions of protein-binding resin (Mimetic Blue AX6LSA), anion exchange resin (Dowex AG3-OH^- form), cation-exchange resin (Dowex AG50-H^+ form), and anion-exchange resin (Dowex AG1-OH^- form) into a 5 mL plastic column.
6. Pyridine (for safety instructions and disposal please refer to the Safety Data Sheet of the manufacturer).

2.3. Mass Determination

1. MALDI-TOF Mass Spectrometer equipped with a 337-nm nitrogen laser, e.g., Biflex (Bruker, Germany).
2. Matrix solution: saturated solution of 2,5-Dihydroxybenzoic acid (DHB) in 60% ethanol.
3. Calibration standard for mass spectrometry. For example dextran hydrolyzate (Glucose α1-6)n (Glucose Homopolymer Standard, Glyko).

3. Methods

3.1. Desialylation

This section describes the enzymatic release of sialic acids from oligosaccharides in order to obtain desialylated neutral N-glycans for use in positive ion mass spectrometry or enzymatic sequencing as described in **Subheading 3.2.** (*see* **Note 2**). We recommend use of sialidase from *Arthrobacter ureafaciens* for its ability to split off α2-3, -6 and -8 linked sialic acids ***(6)***.

In many cases, desialylation has already been performed prior to separation of N-glycans by high-performance liquid chromatography (HPLC). Neutral oligosaccharides obtained from chromatogaphic fractionation can be subjected to mass spectrometry or enzymatic sequencing without further sialidase treatment.

N-glycan mixtures obtained from enzymatic or chemical cleavage of oligosaccharides from glycoproteins or glycopeptides can be subjected to MALDI-TOF mass spectrometry using negative ion mode for measurement of

sialylated glycans ***(7)*** or have to be desialylated prior to positive ion MALDI-TOF mass spectrometry (*see* **Subheading 3.3.** and **Note 3**).

1. Dissolve 0.1 U sialidase from *Arthrobacter ureafaciens* in 50–100 µL incubation buffer (*see* **Note 4**).
2. Dissolve salt-free oligosaccharides in 50–100 µL sialidase solution.
3. Check pH of incubation buffer.
4. Incubate overnight at 37°C under gentle shaking.
5. Load the sample onto the mixed bed column and elute the sample with fivefold bed volume water.
6. Dry the sample by centrifugal evaporation.

3.2. Enzymatic Sequencing of N-Glycans

The principle of enzymatic sequencing is to stepwise release the terminal monosaccharides until digestion stops at a fragment that none of the enzymes available in the respective sample can cleave. The sequence can then be deduced from: (1) substrate specificity of enzymes present in the reaction mixture, and (2) size of N-glycan fragments resulting from enzymatic cleavage. The specificity of endoglycosidases applied for sequencing is known and the fragment sizes can be determined by MALDI-TOF mass spectrometry (*see* **Subheading 3.3.**).

Note that the enzymatic sequencing method described here is not applicable to sialylated N-glycans. Sialylated N-glycans have to be desialylated first as described in **Subheading 3.1.**

Enzymatic sequencing of N-glycans can be performed exclusively with single, fractionated oligosaccharides. N-glycans from mixtures therefore have to be fractionated first, for example by aminophase- and/or reversed phase (RP)-chromatography ***(7)***. Depending on the chromatographic methods employed, oligosaccharides have to be desialylated prior to chromatography. Fractionated samples may contain organic eluent like acetonitrile, which will have to be removed before exoglycosidase treatment.

1. Transfer the neutral N-glycan sample to a 1 mL Eppendorf tube (*see* **Note 5**).
2. Remove possible organic solvents and dry the sample in a centrifugal evaporator.
3. Dissolve the sample in water to a final volume of 32 µL.
4. Divide the N-glycan sample into 4 equal volumes and transfer aliquots to 0.5 mL Eppendorf tubes.
5. Follow the reaction scheme in **Table 2**.
6. Incubate at 37°C for 12–16 h. Then add 1 µL Mannosidase VI to tube 4 and incubate at 37°C for 30 min (*see* **Note 6**).
7. Stop the reaction by addition of 10 µL pyridine (*see* **Note 7**).

Table 2
Pipet Scheme for Preparation of the Exoglycosidase Reaction Mixtures

	Tube number			
	1	2	3	4
Reagent [μL]				
Oligosaccharide Solution	8	8	8	8
H_2O	8	6	4	2
5X Reaction buffer	4	4	4	4
β-Galactosidase	–	2	2	2
β-N-Acetylhexosaminidase	–	–	2	2
α-Mannosidase II	–	–	–	2
Total volume	20	20	20	20

8. Pool the content of all four reaction vials over the mixed-bed column, and elute the sample with 3×1 mL water.
9. Dry the sample by centrifugal evaporation.

3.3. Mass Determination

In principle, relative masses of sialylated and desialylated neutral N-glycans can be determined by MALDI-TOF mass spectrometry. Sialylated N-glycans can be analyzed using negative ion mode ***(7)***. For measurement of desialylated neutral N-glycans, positive ion mode is applied. Concentration of N-glycans needed for MALDI-TOF mass spectrometry depend on purity of sample, residual salt concentration, and instrument and typically range from 2–20 pmol/μL oligosaccharide (*see* **Note 8**).

Please note that N-glycans may be labeled with fluorophores like 2-aminobenzamide (2-AB) ***(8)***. 2-AB labeled N-glycans can be subjected to MALDI-TOF mass spectrometry without any problems, but other fluorophores may not (*see* **Note 9**).

3.3.1. Sample Preparation and Measurement

1. Freshly prepare 2,5-dihydroxybenzoic acid matrix solution.
2. Dissolve N-glycan sample from enzymatic sequencing with water to a final concentration of typically 2–50 pmol/μL of each digestion product.
3. Mix equal amounts (v/v) of aqueous solution of standard glycans or sample and matrix solution.
4. Place 0.5–1 μL of the sample/matrix mixture on the target and let the sample dry at room temperature (*see* **Note 10**).

Table 3
Theoretical Monoisotopic Masses of Neutral N-Glycans of the Complex- and Hybrid-Type

Structure (core + monosaccharides)			Calculated mass	
GlcNAc	Gal	Fuc	$[M+H]^+$	$[M+Na]^+$
Biantennary				
2			1316.5	1338.5
2	2		1640.6	1662.6
2		1	1462.5	1484.5
2	2	1	1786.6	1808.6
Triantennary				
3			1519.6	1541.6
3	3		2005.7	2027.7
3		1	1665.6	1687.6
3	3	1	2151.8	2173.8
Tetraantennary				
4			1722.6	1744.6
4	4		2370.9	2392.9
4		1	1868.7	1890.7
4	4	1	2516.9	2538.9
Bisecting GlcNAc, e.g., biantennary, 1 bis. GlcNAc			1843.7	1865.7
Hybrid type, e.g., 1 GlcNAc, 2 Man			1437.5	1459.5

5. Perform measurements. Refer to the appropriate parameter setting recommended by the manufacturer of your instrument (*see* **Note 11**).
6. Calibrate mass spectrometer using an oligosaccharide standard sample.
7. For mass determination of digestion products from enzymatic sequencing use positive ion mode and the reflector if available.

3.3.2. Interpretation of Results

The calculated masses $[M+H]^+$ and $[M+Na]^+$ of some widely found complex N-glycans are summarized in **Table 3**. Neutral N-glycans may form $[M+H]^+$-, $[M+Na]^+$-, or $[M+K]^+$-ions, depending on the extent of salts in the sample. Mainly Na^+-adducts and small amounts of K^+-adducts of N-glycans are found using MALDI-TOF mass spectrometry. Corresponding data for high-mannose type N-glycans is given in **Table 4**. **Tables 3** and **4** show calculated masses for some N-glycans commonly found in glycoproteins. For calculation of further theoretical masses of N-glycans the use of the GlycanMass software available

Table 4
Calculated Monoisotopic Masses $[M+H]^+$ and $[M+Na]^+$ of High Mannose-Type N-Glycans

Core + no. of mannoses	$[M+H]^+$	$[M+Na]^+$
2	1234.4	1256.4
3	1396.5	1418.5
4	1558.5	1580.5
5	1720.6	1742.6
6	1882.6	1904.6

Table 5
Mass Differences Caused by Addition or Loss of Monosaccharides of N-Glycans. Calculated Single Monoisotopic Mass Values

Monosaccharide		Calculated mass difference
Hexose	e.g., Galactose, Mannose, Glucose	162.05
N-Acetylhexosamine	e.g., N-Acetylglucosamine, N-Acetylgalactosamine	203.08
Deoxyhexose	e.g., Fucose	146.06
Pentose	e.g., Xylose	132.04
Sialic acid	e.g., N-Acetylneuraminic acid	291.10

on the Expert Protein Analysis System (ExPASy) proteomics server from the Swiss Institute of Bioinformatics (SIB) website is very helpful ***(9)***: <www.expasy.ch> Alterations of molecular weight of N-glycans due to labeling with fluorophores such as 2-aminobenzamide (2-AB; calculated mass difference + 122.1) have to be taken into account for interpretation of mass spectrometry data as well (*see* **Table 5**).

Mass spectrometry of a sample obtained by enzymatic sequencing of a single N-glycan will give a number of different masses. Calculated mass differences caused by cleavage of monosaccharides are summarized in **Table 5**. As the aliquot in tube 1 of the sequencing assay is not treated with exoglycosidases (*see* **Subheading 3.2.**), the highest relative mass determined by mass spectrometry corresponds to the complete N-glycan structure. The lower masses found in the mass spectrum result from the action of β-galactosidase III, β-N-acetylhexosaminidase III (*see* **Note 12**), α-mannosidase II, mannosidase VI, or combinations of these enzymes (*see* **Table 2**). Substrate specificity of the exoglycosidases is given in **Table 6**.

Table 6
Specifity of Exoglycosidases Used for N-Glycan Sequencing

Enzyme name	Specificity
Sialidase (*Arthrobacter ureafaciens*)	(α2-3,6,8)-linked N-acetylneuraminic acid
β-Galactosidase III (*S. pneumoniae*)	(β1-4)-linked galactose
β-N-Acetylhexosaminidase III (Jack bean)	(β1–2,3,4,6)-linked N-acetylglucosamine
α-Mannosidase II (Jack bean)	(α1-2,3,6,)-linked mannose
α-Mannosidase VI (*X. manihotis*)	(α1-6)-linked mannose

1. Check the mass spectrum of standard glycans for $[M+H]^+$-, $[M+Na]^+$-, or $[M+K]^+$-ions.
2. Consider mass difference caused by possible fluorophore label of the N-glycan.
3. Compare the highest mass found in the mass spectrum of the sample obtained from enzymatic sequencing with corresponding calculated masses listed in **Tables 3** and **4**.
4. Try to classify the N-glycan as complex, hybrid, or high-mannose structure (*see* **Note 13**).
5. Calculate mass differences between digestion products found in the mass spectrum.
6. Compare measured mass differences with values given in **Table 5**. For example mass differences of 162, 324, or 486 indicate loss of one, two, or three hexoses, respectively.
7. Refer to **Table 6** and check specificity of exoglycosidases. For example, if you identified your sample as complex type N-glycan, mass difference of 324 indicates loss of two (β1-4)-linked galactoses.
8. Deduce suggested N-glycan structure from relative mass of uncleaved N-glycan, mass differences of cleavage products found in mass spectrum, enzyme mixtures used for sequencing, and specificity of applied enzymes (*see* **Note 14**).

4. Notes

1. For electrophoretic separation N-glycans have to be labeled with ANTS fluophore to introduce negative charges. ANTS labeled N-glycans can be analyzed using a special FACE™ electrophoresis system.
2. The manufacturers protocol of the enzymatic sequencing kit suggests adding siladase to the reaction tubes. If you follow this protocol, sialylated oligosaccharides can be subjected to enzymatic sequencing as well.

3. We recommend performing mass determination of the complete mixture of oligosaccharides before chromatographic fractionation. Perform positive ion spectrum for neutral oligosaccharides or negative ion spectrum for sialylted or charged oligosaccharides ***(7)***.
4. Alternatively, 0.01 U sialidase from Newcastle disease virus can be used instead of 0.1 U sialidase from *Arthrobacter ureafaciens*.
5. The manufacturer of the enzyme-digestion kit recommends to add 50–100 pmol N-glycan to every reaction vial. Following the recommendation you will need about 200–500 pmol oligosachaccharide for enzymatic sequencing. Concentration of samples needed for mass determination by MALDI-TOF is typically 10–100 pmol oligosaccharide.
6. Do not add mannosidase VI to mannosidase II as it will inhibit mannosidase II.
7. Complete inactivation of exoglycosidases is important to avoid further cleavage of stop-point fragments. For this reason mix the stop solution thoroughly with the sample and transfer aliquots from each reaction tubes separately to the mixed-bed column.
8. In general, measurement of sialylated N-glycans in negative ion mode is less sensitive compared to measurement of neutral N-glycans using positive ion mode.
9. The fluorophore 8-aminonaphtalene-1,3,6-trisulphonate (ANTS) used for labeling oligosaccharides for analysis by the FACE system contains sulfonic acid groups. ANTS imparts negative charge to drive the electrophoretic separation. These may cause problems for measurement of ANTS labeled N-glycans. However, we did not try these derivatives for use in mass spectrometry so far.
10. The target can be stored at room temperature in the dark for several days.
11. Appropriate parameter setting of mass spectrometer depends on the instrument used for measurements. Please refer to recommendation of the manufacturer of the instrument and use these settings as starting point. We use a Bruker Biflex instrument. Parameter settings in positive ion mode with reflector for measurements of neutral glycans are, for example: (a) high voltage IS/1 19 kV, IS/2 12.8 kV, Refl. 20 kV, Lens 7 kV; (b) cut-off mass 1200, deflection HV on; (c) detector neutrals refl. 1.55 kV, lin. 1.6 kV. A typical parameter set for measurement of glycans in negative ion mode with reflector is, for example: (a) high voltage IS/1 19 kV, IS/2 12.8 kV to 13.3 kV, Refl. 20 kV, Lens 6.8 to 7 kV; (b) cut-off mass 400, deflection HV on; (c) detector neutrals refl. 1.55 kV, lin. 1.6 kV.
12. β-N-Acetylhexosaminidase III from Jack bean will not fully cleave bisecting N-Acetylglucosamin at the concentrations given in the reaction scheme.
13. A single fucose residue attached to the core structure of the N-glycan causes mass shift of all fragments of about 146.1 compared to the unfucosylated structures (*see* **Table 5**). If you want to remove the fucose you may use commercial available fucosidase (e.g., Glyco Fucosidase I, Part #80080), which cleaves Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc core structure. Oligosaccharides from plant glycoproteins may contain Fucα1-3 linked to the core structure, which will not be cleaved by this fucosidase.

14. Several modifications in glycosylations have been published that are not covered by the aforementioned N-glycan structures. In case you have no clear results with the exoglycosidase assay, further experiments with other exo- or endoglycosidases will be necessary.

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6

Immunological Detection of O-GlcNAc

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1. Introduction

N-acetylglucosamine O-glycosidically linked (O-GlcNAc) to serine or threonine residues of proteins has been shown to be ubiquitous among eukaryotic proteins of the nucleus, cytoskeleton, cytoplasm, and has also been detected on cytosolic tails of membrane proteins ***(1,2)***. The exact function of this carbohydrate modification is not yet known. Studies from several groups point at potential roles in: (1) signaling as an alternative to phosphorylation, (2) protein–protein interactions, and (3) protection against protein degradation.

The assumption that O-GlcNAc might be involved in signaling was initially based on the observation that O-GlcNAc is turned over much faster than the proteins to which it is bound. Later it was found that the addition and removal of O-GlcNAc is controlled by cytosolic O-GlcNAc transferases and O-GlcNAc hydrolases in an analogous manner to the control of phosphorylation by kinases and phosphatases ***(3–5)***. Furthermore, the same serine or threonine residues of some proteins exist either in phosphorylated or O-GlcNAc modified form ***(6)***. Additional evidence that O-GlcNAc might be involved in signaling came from the observation that O-GlcNAc levels respond reciprocally to inhibition or activation of certain kinases or phosphatases by pharmacological manipulations ***(7)***. That O-GlcNAc may mediate or modulate molecular interactions is suggested because several transcription factors have been shown to have higher affinity to DNA and to activate more efficiently transcription in the glycosylated compared to the nonglycosylated form ***(8)***. As to the possible protective role against protein degradation, it has been shown by Kudlow and coworkers ***(9)*** that the nonglycosylated form of the transcription factor

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Sp1 is rapidly degraded by proteasomes. In another study it was shown that inactivation of the eukaryotic initiation factor 2 (eIF2) is prevented by an associated O-GlcNAc modified 67 kDa protein. In response to specific cellular signals, this protein is rapidly deglycosylated and subsequently degraded ***(10)***. In addition, PEST sequences, which target proteins for degradation, sometimes contain O-GlcNAc, supporting a function of O-GlcNAc in protein stabilization, since phosphorylated PEST sequences seem to represent a signal for protein degradation ***(11,12)***.

Taken together, these observations suggest that one and the same protein may be endowed with different binding capacities or functional activities in either the phosphorylated, O-GlcNAc modified or nonmodified form.

All O-GlcNAc modified proteins identified so far are phosphoproteins. If O-GlcNAcylation represents an alternative to phosphorylation, it is most likely that many, if not most phosphoproteins, are also O-GlcNAc modified. If one takes the fairly small number of identified O-GlcNAc modified proteins into consideration, it seems that to date only a minority of O-GlcNAc modified proteins is known ***(1)***.

One reason for the slow process of identification of O-GlcNAc modified proteins is probably that the methods applied for the detection of O-GlcNAc are time-consuming, require fairly large amounts of proteins, and are not easily available to every lab. A rapid screening method is therefore desirable for the detection of O-GlcNAc on proteins. We describe here immunological detection methods, which, although not as accurate as the methods reported by Greis and Hart in the previous volume of this series ***(13)***, have some advantages in time, cost, and techniqual expenditure. The analytical methods described by Greis and Hart ***(13)*** include capillary liquid chromatography-electron spray mass spectrometry (LC-ESMS) +/– enzymatic galactosylation and chemical β-elimination. They allow also the determination of peptide sequences to which O-GlcNAc is attached. Although the immunological detection methods described here have some drawbacks, as discussed later, they have been proven to be suitable to detect O-GlcNAc modifications under different conditions. Three monoclonal antibodies (MAbs) recognizing O-GlcNAc modified proteins are compared here with respect to their reactivity in enzyme-linked immunosorbent assay (ELISA), Western blot, and immunohistochemistry.

The HGAC 85 antibody belongs to a group of antibodies raised against streptococcal group A carbohydrate and is a mouse MAb of the IgG3 subtype ***(14,15)***. It was shown to recognize GlcNAc residues of the cell-wall polysaccharide composed of a polyrhamnose backbone and β1-3 linked GlcNAc side chains ***(16)***. In immunocytological studies, the antibody stained the nuclear periphery of mammalian cells and, although less intensive, the cytoplasma. The staining pattern of a mutant cell line, which expresses N-glycosidically linked

glycans with terminal GlcNAc residues on many glycans, was indistinguishable from that of wild-type cells, indicating that the antibody is specific for the O-linked GlcNAc. That the antibody recognizes the O-GlcNAc modification of eukaryotic glycoproteins is further supported by the observation that the antibody stains eukaroytic cells and that in ELISA it reacts with a human erythrocyte cytosolic fraction. Its reactivity is completely abolished either in the presence of 22 m*M* free GlcNAc or after galactosylation using UDP-galactose and galactosyltransferase ***(17)***. Similar binding characteristics were observed when the covalent conjugate of GlcNAc coupled to bovine serum albumin (BSA) was used as substrate: only free GlcNAc, but not glucose, rhamnose, or N-acetylgalactosamine competitively inhibited binding of the antibody to GlcNAc-BSA at concentrations of about 10 m*M* ***(18)***.

MUD 50 is a monoclonal IgG antibody raised in mice against urea extracted sheath proteins from the cellular slime mold *Dictyostelium discoideum* ***(19)***. The antibody was shown to recognize an O-linked carbohydrate epitope on several glycoproteins of this species. It has a strong requirement for the sugar moiety as it does not show any reactivity with a glycosylation-defective mutant ***(20)***. Using solid-phase sequencing, gas chromatography, high pH anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD), and LC-ESMS the carbohydrate moiety was identified as GlcNAc linked O-glycosidically to a PTVTPT repeat of *Dictyostelium* cell-surface glycoprotein ***(21,22)***. Further analysis of acceptor sequences for O-GlcNAc led to the interesting conclusion that the O-GlcNAc bearing sequences from *Dictyostelium* are similar or identical to the $PX_{0\text{-}4}S/T$ sequences and KSP sequences shown to be glycosylated by mammalian O-GlcNAc transferases, i.e., sequences that contain proline in position –1 or in close C-terminal proximity to the serine or threonine residue ***(23,24)***. That the antibody recognizes mammalian O-GlcNAc-modified peptide sequences is further supported by comparable results obtained in ELISA with cellular fractions of neurons from mouse brain using either the MUD 50 antibody or the more tedious galactosyltransferase assay ***(7)***.

The RL 2 antibody is the only antibody of the three tested that was raised against mammalian O-GlcNAc-modified glycoproteins. A nuclear pore complex-lamina fraction isolated from rat liver nuclear envelopes was used for the immunization of mice ***(25)***. Several MAbs were obtained, of which the RL 2 had the strongest dependence on the carbohydrate modification. This was shown by the almost complete loss of antibody reactivity in Western blots after removal of O-GlcNAc by hexosaminidase treatment and by the failure to immunoprecipitate O-GlcNAc modified glycoproteins after galactosylation. It was found, however, that the amount of immunoprecipitated proteins was reduced only to 55% in the presence of 1 *M* GlcNAc. In the presence of 1 *M* GalNAc, 92% of proteins were immunoprecipitated compared to

immunoprecipitation in the absence of monosaccharides, indicating that the O-GlcNAc residue is involved in antibody binding, but in contrast to MUD 50 and HGAC 85 antibodies, the RL 2 has a significant requirement for a peptide epitope ***(25)***. After pronase digestion of O-GlcNAc-modified proteins RL 2 reactivity is lost, although this may also be due to lower avidity of peptides possibly modified with only a single O-GlcNAc residue compared to multiply O-GlcNAc-modified intact proteins ***(24)***. Nevertheless, the antibody seems to react not only with eight different proteins of the nuclear-pore complex but also with several other O-GlcNAc-modified proteins ***(9,25)***.

We describe here the strengths and weaknesses of these three antibodies in ELISA, Western blot, and immunohisto/cytochemical analyses.

Note added in proof: Another O-GlcNAc specific MAb was recently described by Comer et al. ***(25a)***. This antibody (CTD 110.6) is available from Babco (Richmond, CA).

2. Materials

2.1 Antibodies

The three O-GlcNAc specific antibodies used were obtained from the following sources: Hybridoma cells of the HGAC 85 antibody were kindly donated by Dr. N. Greenspan (Case Western University, Cleveland, OH). The antibody was purified from hybridoma supernatant by affinity chromatography on GlcNAc-Agarose (Sigma cat. no. A 2278, Deisenhofen, Germany). After elution with 220 m*M* N-acetylglucosamine, the antibody was dialyzed against phosphate-buffered saline (PBS) and kept at a concentration of 1 mg/mL in glycerol/ PBS (1 : 1) at –20°C. After aliquots were taken out for experiments, the antibody was stored again at –20°C.

The MUD 50 antibody used was an ascites preparation kindly provided by Dr. M. Slade and Dr. K. Emslie, School of Biological Sciences, Macquarie University NSW, Australia. It was used without further purification at dilutions as indicated in the Methods section and was stored in PBS at –20°C. After aliquots were taken out for experiments, the antibody was stored again at –20°C or kept for up to 4 wk at 4°C.

The RL 2 antibody is commercially available from Affinity Bioreagents Inc., Golden, CO 80401, USA (cat. no. MA1-072). The original description of the antibody is given in Snow et. al ***(23)***. It was kept at –20°C in glycerol/PBS (1 : 1). After aliquots were taken out for experiments, the antibody was stored again at –20°C.

2.2. ELISA

1. GlcNAc modified BSA (GlcNAc-BSA): bovine p-aminophenyl-N-acetyl-β-D-glucosaminide albumin (Sigma cat. no. A 1034; Deisenhofen, Germany).

2. Peroxidase (POD) conjugated goat anti-mouse antibodies (Jackson Immunoresearch, Dianova cat. no. 115 035 068; Hamburg, Germany).
3. ABTS solution: 0.5 mL 2,2′-azino-di-3-ethylbenzthiazolinesulfonate (ABTS) stock solution (2 g ABTS in 100 mL demineralized water (aqua dem.); Novartis, Mannheim, Germany), 9.5 mL acetate buffer (0.1 *M* sodium acetate and 0.05 *M* sodium dihydrogenphosphate adjusted to pH 4.2 with glacial acetic acid) and 3 µL hydrogen peroxide. The solution should be freshly prepared directly before use.

2.3. Western Blots

1. Ponceau reagent: 0.2% Ponceau S, 3% glacial acetic acid.
2. Peroxidase (POD) conjugated goat anti-mouse antibodies (Jackson Immunoresearch, cat. no. 115 035 068; Dianova, Hamburg, Germany).

2.4. Immunohistochemistry

1. Paraformaldehyde (PFA) solution: 4.0 g paraformaldehyde is suspended in 25 mL aqua dem. and hold at 60°C under stirring. Then 0.6 *M* NaOH is added until the solution turns clear. The mixture is then filtered, cooled to room temperature, and filled up to 100 mL with phosphate buffer (0.33 g NaH_2PO_4 ($\times H_2O$); 2.24 g Na_2HPO_4 ($\times 2\ H_2O$).
2. Cy-3 (Cyanin-3™) and DTAF (dichlorotriazinyl-amino-fluoresceine)-labeled secondary goat anti-mouse antibodies (Jackson Immunoresearch, cat. no. 115 015 146 and cat. no. 115 165 146; Dianova, Hamburg, Germany).
3. Peroxidase (POD) conjugated goat anti-mouse antibodies (Jackson Immunoresearch, cat. no. 115 035 068; Dianova, Hamburg, Germany).
4. Mowiol embedding solution: 12.0 g glycerol (Merck cat. no. 4095; Darmstadt, Germany), 4.8 g Mowiol 4-88 (cat. no. 17951, Polysciences, Eppelheim, Germany) and 12 mL aqua dem. are mixed in a 50 mL Falcon tube and held for 2 h at room temperature under occasional shaking. Then 24 mL 0.2 *M* Tris, pH 8.5, are added, incubated for 10 min at 50°C in a water bath (shaken occasionally), and centrifuged at about 1000*g*. The supernatant is stored in 2-mL aliquots in glass tubes at –20°C for up to 12 mo. The solution is stable for 1 mo at room temperature.
5. Diaminobenzidine (DAB) solution: Sigma Fast DAB Tablet Set D 4168 (Sigma, Deisenhofen, Germany).
6. Silver enhancement solution: Reagents are mixed in the following order: 200 µL aqua dem., 100 µL 0.1 *M* NH_4NO_3, 100 µL 0.047 *M* $AgNO_3$, 90 µL 0.12 *M* silicotungstic acid (Sigma cat. no. T-2786), 7.5 µL formalin, and 500 µL 0.47 *M* Na_2CO_3. The reagent is stable for about 20 min.

3. Methods

3.1. Enzyme-Linked Immunosorbent Assay (ELISA)

1. Coat Nunc Maxisorp 96-well flat-bottom plates overnight at 4°C with GlcNAc-BSA in 0.1 *M* $NaHCO_3$ at concentrations ranging from 100–500 ng per well.

2. Wash coated plates three times with PBS and then block by incubation with 400 µL 1% BSA/0.1 *M* $NaHCO_3$ for 1 h at room temperature.
3. Add the primary antibody in appropriate dilutions (*see* **Figs. 1** and **2**) in 50 µL 1% BSA/PBS per well and either incubate at 4°C for 16 h or at room temperature for 2–3 h (*see* **Note 1**).
4. Perform three washing steps with PBS, then incubate wells with 50 µL POD conjugated anti-mouse antibodies (dilution 1:4000) in 1% BSA/PBS for 1 h at room temperature and wash as described above (**step 2**).
5. For the development incubate each well with 50 µL ABTS solution for up to 30 min at room temperature and terminate the reaction by adding 50 µL 0.6% aqueous SDS solution per well.
6. Determine antibody reactivity by measuring the optical density of the reaction products of the POD conjugated to the secondary antibodies at 405 nm wavelength (OD 405) in a Titertec ELISA reader from ICN (Meckenheim, Germany). **Figure 1** shows that all three O-GlcNAc specific antibodies react with GlcNAc-BSA (*see* **Note 2**). For the detection of other O-GlcNAc modified proteins in ELISA, *see* **Notes 3** and **4**.

To obtain further evidence of the importance of the O-GlcNAc modification of proteins for the antibody reactivities, competition experiments with free N-acetylglucosamine (GlcNAc) and for control, N-acetylgalactosamine (GalNAc) might be performed.

1. Coat wells with 250 ng GlcNAc-BSA and block as described above (**step 2**).
2. Incubate diluted antibodies (1:10 for HGAC 85; 1:100 for MUD 50 and RL 2) with monosaccharides (10 m*M*, 50 m*M*, and 1 *M* for HGAC 85, MUD 50, and RL 2, respectively) for 30 min at room temperature.
3. Add the respective monosaccharide solutions (50 µL/well in PBS at concentrations of 10 m*M*, 50 m*M*, and 1 *M* as above) to all wells except the first.
4. Finally add the preincubated antibody solutions (100 µL) to the first well and generate a twofold dilution series. All further steps were performed as described above.

Figure 2 shows results of the competition experiments (*see* **Note 5**).

3.2. Western-Blot Analysis

Determine protein concentration using the D_cProtein Assay Kit from Biorad (Munich, Germany). Use 10% polyacrylamide gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli ***(28)***. Load each lane with approx 25 µg protein (*see* **Note 6**). Perform Western Blot analysis according to the method of Towbin et al. ***(29)***.

1. Control success of the electrophoretical transfer of proteins to nitrocellulose sheets by semi-dry blotting by visualizing the proteins with Ponceau reagent.

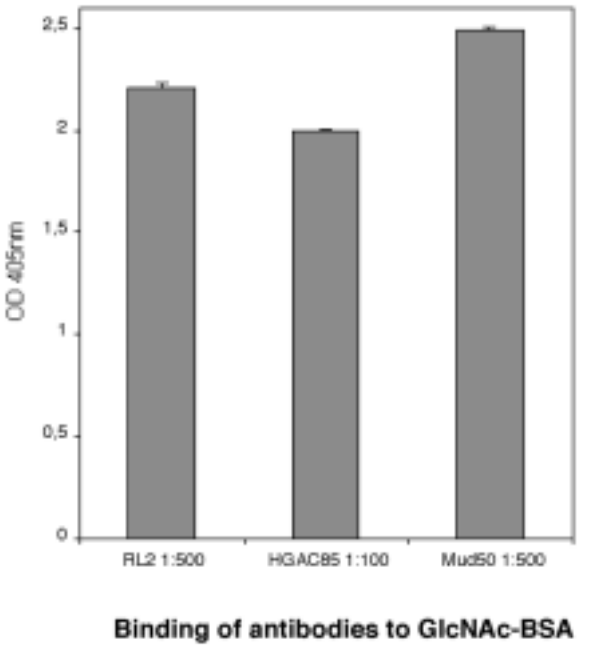
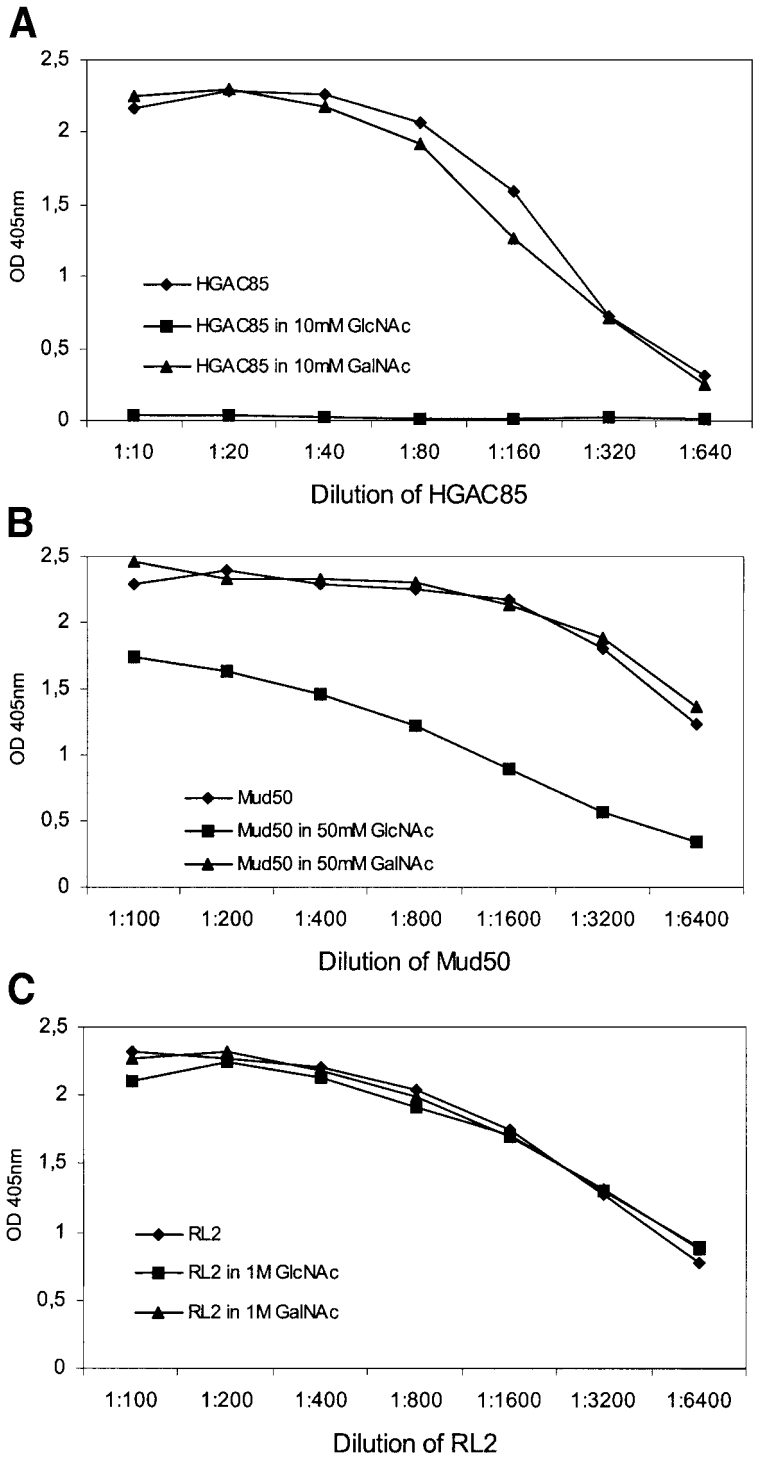


Fig. 1. Binding of the O-GlcNAc specific monoclonal antibodies MUD 50, HGAC 85, and RL 2 to GlcNAc-BSA as determined by ELISA. Microtiter wells were coated with 250 ng GlcNAc-BSA/well. After incubation with the antibodies at the indicated dilutions and secondary anti-mouse antibodies, the optical density at 405 nm (OD 405) of reaction products with POD-conjugated secondary antibodies was measured. Mean values ± standard deviations of one representative out of three experiments carried out in quadruplicate are shown.

2. Destain blot with water.
3. Keep blot either for 1 h at 70°C in PBS or for 5 min in boiling PBS (*see* **Note 7**).
4. Block unspecific binding sites by adding 2% BSA and 0.5% Triton X-100 in PBS for 1 h at room temperature.
5. Incubate blots with the appropriate dilution of the respective antibody overnight at 4°C (1 : 500 for MUD 50 and 1 : 1000 for RL 2) or for 1 h at room temperature (only for RL 2).
6. Perform five washing steps with 0.5% Triton X-100 in PBS for 5 min.
7. Use POD conjugated anti-mouse antibodies in a 1 : 2000 dilution in 1% BSA/PBS as secondary antibody. Incubate for 1 h at room temperature.
8. Repeat five washing steps as described described above (*see* **step 6**).
9. Detect peroxidase activity by enhanced chemoluminescence using the Super Signal West Pico Kit from Pierce (KMF, St. Augustin, Germany) according to the description of the manufacturer.


A
2,5
2
1,5
1
0,5
0
OD 405nm
HGAC85
HGAC85 in 10mM GlcNAc
HGAC85 in 10mM GalNAc
1:10
1:20
1:40
1:80
1:160
1:320
1:640
Dilution of HGAC85
B
2,5
2
1,5
1
0,5
0
OD 405nm
Mud50
Mud50 in 50mM GlcNAc
Mud50 in 50mM GalNAc
1:100
1:200
1:400
1:800
1:1600
1:3200
1:6400
Dilution of Mud50
C
2,5
2
1,5
1
0,5
0
OD 405nm
RL2
RL2 in 1M GlcNAc
RL2 in 1M GalNAc
1:100
1:200
1:400
1:800
1:1600
1:3200
1:6400
Dilution of RL2

In **Fig. 3**, the reactivity of the RL 2 and MUD 50 antibodies with O-GlcNAc modified proteins from human brain is shown (*see* **Notes 8** and **9**).

3.3. Immunohistochemistry

1. Freshly prepared mouse brain must be snap frozen in liquid nitrogen for 5 min.
2. Warm frozen brain up to –20°C, cut into 10 µm sections and mount onto slides.
3. Air-dry sections at room temperature for 2 h and store at –80°C.
4. Before incubations with antibodies air-dry sections again for some minutes.
5. Fix sections in methanol at –20°C for 10 min followed by a 30 s treatment with acetone (*see* **Note 10**).
6. Dry again at room temperature.
7. Incubate for 15 min with PBS.
8. Incubate sections with 50 µL of the primary antibody diluted in 1% BSA/PBS at 4°C overnight in a moist atmosphere to prevent from drying (dilutions: 1:10 or 1:20 for HGAC 85; 1:25 for MUD 50 and 1:100 or 1:250 for RL 2).
9. Wash slides three times for 5 min with PBS.
10. Incubate with 50 µL of POD conjugated anti-mouse antibodies (diluted 1:600 in 1% BSA/PBS) for 1 h at room temperature in a moist atmosphere.
11. Wash again three times with PBS and once with aqua dem.
12. Develop with DAB solution. Pipet 100–200 µL onto each section and incubate for approx 15 min until brown color develops.
13. Incubate sections with 100–200 µL silver enhancement solution for 5–15 min at room temperature to amplify the color reaction. Terminate the reaction by washing three times with aqua dem. as soon as the brown DAB staining turns black, but before the control experiment in the absence of first antibody shows unspecific silver deposition ***(27)***.
14. Dehydrate sections with 70%, 90% and twice with 100% ethanol (v/v) for 5 min each. Wash sections in xylol and dry before embedding in one drop of Entellan (Merck, Darmstadt, Germany) per coverslip (**Notes 11** and **12**).

Figure 4 shows MUD 50 reactivity with sections from mouse brain (*see* **Note 13**).

Fig. 2. *(previous page)* Binding of the O-GlcNAc specific monoclonal antibodies HGAC 85 **(A)**, MUD 50 **(B)**, and RL 2 **(C)** to GlcNAc-BSA in the presence of N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc). Microtiter wells were coated with 250 ng GlcNAc-BSA/well. After preincubation of the antibody with or without the carbohydrates for 1 h at room temparature (or at 4°C for the HGAC 85; *see* **Note 1**) a twofold dilution series of the antibodies was performed in the absence or presence of the carbohydrates at the indicated concentrations. The optical density at 405 nm (OD 405) of reaction products of POD-conjugated secondary antibodies was measured.

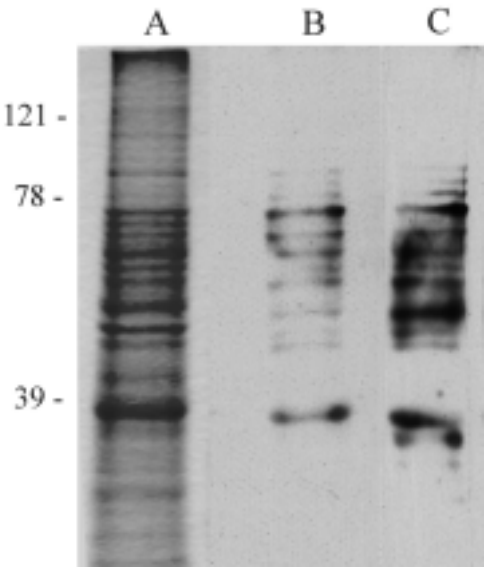


Fig. 3. Western blot of detergent-soluble homogenates from human brain using RL 2 (B) or MUD 50 (C) antibodies. Coomassie blue staining is shown in (A). Note the striking similarity of protein patterns stained by RL 2 and MUD 50.

4. Notes

1. Both methods gave the same results except for the HGAC 85 antibody, which requires low temperatures (4°C) for binding ***(18)***.
2. Results shown for the HGAC 85 confirm earlier results ***(18)***. In this paper it was also shown that yet another antibody raised against streptococcal group A carbohydrate, HGAC 39, has similar binding characteristics to GlcNAc-BSA. We also used this antibody in some of the experiments with similar results. In order to enhance the sensitivity of the HGAC 85, we also used this antibody after coupling to digoxigenin according to the supplier's recommendation (Novartis, Mannheim, Germany) and detection with POD-conjugated anti-digoxigenin antibodies. Although there was an increase in sensitivity, the specificity of the antibody appeared to have been altered since competition with free GlcNAc could no longer be observed (*see also* **Notes 5** and **13**).
3. When O-GlcNAc modified proteins from other sources (e.g., tissue or cell fractions) were used the protein amounts for coating ranged from 10–20 µg per well. Some protein mixtures were coated according to a modified protocol particularly useful for the effective coating of proteins from solutions containing high amounts of detergents ***(7**,**30**,**31)***. Nunc Immunosorb 96 well plates were pretreated with a 0.5% aqueous glutaraldehyde solution overnight, washed twice with PBS, then incubated with the protein solutions to be analyzed for 1 h at room temperature. After removal of the protein solutions the remaining reactive

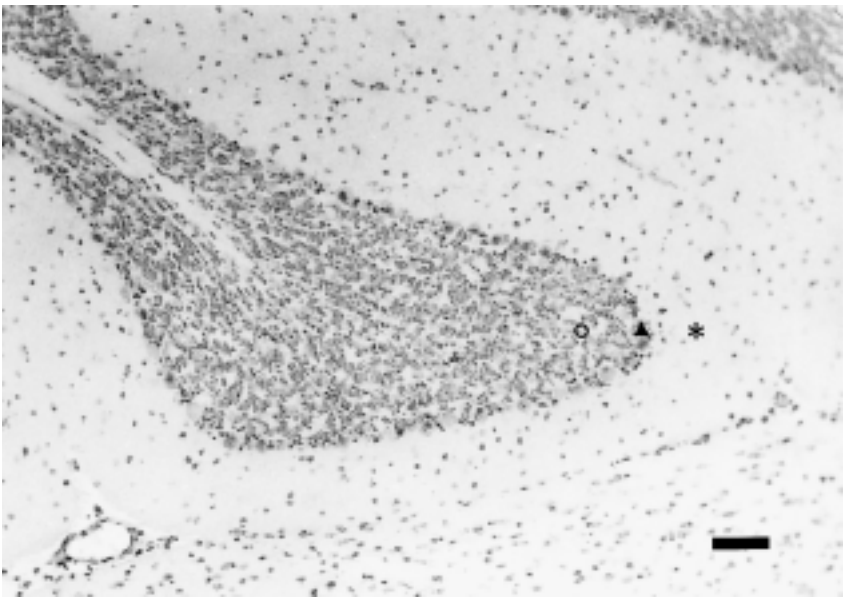


Fig. 4. Detection of O-GlcNAc in cerebellum of 30-d-old mice using the MUD 50 antibody. The DAB- silver reaction product of the POD conjugated to the secondary antibody shows a marked staining of nuclei and probably also cytoplasm of cells in the granule-cell layer (○) and the Purkinje cells (▲) next to the granule-cell layer. The molecular layer (*) is poor of cells but shows presumably like the others nuclear and/or cytoplasmic staining of cells. Similar immunostaining is observed with RL 2 and HGAC 85 supporting that the antibodies recognize at least similar epitopes (*see also* **Note 13**). Control sections without first antibody showed no staining. The bar represents 80 μm.

aldehyde groups were blocked with 150 m*M* Tris-buffered saline (TBS, pH 7.2). Wells were blocked with 400 μL of a 1% BSA/PBS solution for 30 min. Then 4 μL 100% formic acid were added, thoroughly mixed with the BSA/PBS solution and incubated for 4 h at room temperature. This treatment serves as an additional denaturation step to release possibly masked epitopes. Wells were washed three times with PBS and the procedure was then continued as described in **Subheading 3.1.** with the respective primary and secondary antibodies.

4. All three antibodies react also with mammalian proteins in ELISA (***7***,***17***,***32*** and our own unpublished observations with RL 2). They do not recognize terminal GlcNAc on N-linked glycans as they did not show any reactivity with ovalbumin, which is known to contain large amounts of hybrid-type glycans with GlcNAc at the nonreducing end (unpublished observations). In case of the MUD 50 antibody, the values obtained by ELISA were shown to be comparable to the

values obtained by the radioactive labeling method using β-galactosyltransferase and radiolabeled UDP-galactose ***(7)***.

5. Results for the HGAC 85 are comparable to those published by Greenspan et al. ***(18)***. The reactivity of this antibody shows the strongest dependence on the carbohydrate portion in comparison to MUD 50 and RL 2. MUD 50 is about 50% inhibitable by 50 m*M* GlcNAc but not by 50 m*M* GalNAc. Higher concentrations of O-GlcNAc do not result in higher inhibition. The reactivity of the RL 2 with GlcNAc-BSA can not be inhibited in the presence of GlcNAc at a concentration even as high as 1 *M*. Concerning this point, *see* **Subheading 1.** and **Note 8**.
6. Since we were interested in the detection of an altered expression of O-GlcNAc on proteins in specific subcellular fractions we prepared a detergent-soluble and a detergent-insoluble cytoskeletal fraction. The preparation of these fractions is described in Griffith and Schmitz ***(7)***. Other mammalian cell homogenates or cellular fractions were shown to react with the antibodies in Western blots (**9**, **25**, and C. Mäss, unpublished results with MUD 50; but *see also* **Note 9** concerning HGAC 85).
7. As described by Snow et al. ***(25)*** an additional denaturation step was carried out by either keeping the blot for 1 h at 70°C in PBS or for 5 min in boiling PBS. The heat treatment is recommended as a necessary step to obtain reproducibly high levels of labeling of antigen bands on immunoblots with the MAbs used.
8. Although RL 2 and MUD 50 were raised against O-GlcNAc-modified proteins isolated from different species they are specific and suitable for the rapid detection of the O-GlcNAc modification of proteins in Western blots. With respect to the similarity of the protein acceptor sequences for the O-GlcNAc modification in *D. discoideum* and in mammals, it is possible that both antibodies recognize not only the O-GlcNAc modification but also a certain amino acid sequence so that it cannot be excluded that some proteins bearing the O-GlcNAc modification at different sites are not detected. As discussed by Hart et al. ***(33)*** it also cannot be excluded that some O-GlcNAc epitopes are cryptic and therefore not detectable. They observed that some sites are available for the galactosyltransferase only after proteins have been proteolytically digested. Snow et al. ***(25)*** have shown that galactosylation of O-GlcNAc modified proteins with UDP-galactose/galactosyltransferase abolishes RL2 reactivity in Western blots, confirming the specificity of the antibody.
9. The antibodies HGAC 85 and HGAC 39 (*see* **Note 2**) give, at least in our hands, varying staining of proteins in Western blots but seem to stain the same protein bands as MUD 50 and RL 2.
10. The fixation of the sections can also be carried out for 15 min at room temperature with a 4% PFA solution. Fixation is terminated by incubation with 50 m*M* NH_4Cl for 10 min at room temperature and slides are washed twice with PBS.
11. In case of the detection of antibody reactivity by indirect immunofluorescence, sections were fixed with PFA and treated with primary antibodies as described in **Subheading 3.3.** They were then incubated with Cy-3 labeled secondary antibodies (diluted 1 : 200 in 1% BSA/PBS) or DTAF-labeled secondary antibod-

ies (diluted 1:25 in 1% BSA/PBS) for 1 h at room temperature, washed three times with aqua dem., dehydrated in ethanol, dried, and embedded in Mowiol.

12. Make sure that all solutions used are clear. Filter buffers and spin down antibody solutions to avoid particles sticking to the sections.
13. HGAC 85 and RL 2 gave similar staining patterns as MUD 50. Staining by HGAC 85 and MUD 50 could be almost completely inhibited in the presence of 10 m*M* and 100 m*M* GlcNAc, respectively, whereas staining by RL 2 was only partially inhibitable by 0.5 *M* GlcNAc (not shown). Similar results for the inhibition of HGAC 85 by GlcNAc have been obtained by Turner et al. ***(17)***. Digoxigenation of HGAC 85 led to a greater insensitivity of the antibody towards competition by GlcNAc (not shown;, *see also* **Note 2**). Immunocytological detection of neurons from mouse brain in culture or of N2a neuroblastoma cells was also carried out using PFA fixation and the indirect immunofluorescence technique (*see* **Subheadings 3.3.** and **Notes 10** and **11**).

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7

Analysis of O-Glycosylation

Juan J. Calvete and Libia Sanz

1. Introduction

Secreted as well as membrane-associated eukaryotic proteins are most commonly glycosylated. Saccharides are attached to proteins mainly through N- and O-glycosydic bonds or as part of the glycosylphosphatidyinositol-membrane anchor. In contrast to N-glycosylation, which involves the co-translational transfer in the endoplasmic reticulum (ER) of the glycan portion of $Glc_3Man_9GlcNAc_2$-PP-dolichol to suitable Asn residues on nascent polypeptides, O-glycosylation begins with the addition of a single monosaccharide. Contrary to N-glycosylation, which involves an asparagine residue in the sequon Asn-Xaa-Thr/Ser (Xaa can be any amino acid except Pro, and it is rarely Cys), no particular sequence motif has been described for O-glycosylation. This may reflect the fact that: (1) the specificity of the UDP-GalNAc : polypeptide N-acetylgalactosaminyltransferase is presently unknown; and (2) seems to be modulated by sequence context, secondary structure, and surface accessibility ***(1)***. An internet server, accessible at http://www.cbs.dtu.dk/netOglyc/cbsnetOglyc.html, produces neural network predictions of mucin-type GalNAc O-glycosylation sites in mammalian proteins based on 299 known and verified mucin-type O-glycosylation sites. The sequence context of glycosylated threonines was found to differ from that of serine, and the sites were found to cluster. Nonclustered sites had a sequence context different from that of clustered sites, and charged residues were disfavored at position –1 and +3.

In animal cells, O-glycosylation is normally initiated in the Golgi apparatus by enzymatic transfer of a N-acetylgalactosamine (GalNAc) residue to the hydroxyl side chain of a serine or a threonine residue. This reaction is catalysed by GalNAc transferase (GalNAc-T, EC 2.4.1.41) using UDP-GalNAc as the sugar donor. O-glycosylation of hydroxylysine and hydroxyproline (in collagen

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Table 1
The Ser/Thr O-Linked Core Sequences of Animal Glycoproteins

1	Galβ1,3-GalNAcol
2	GlcNAcβ1,6 | GalNAcol | Galβ1,3
3	GlcNAcβ1,3-GalNAcol
4	GlcNAcβ1,6 | GalNAcol | GlcNAcβ1,3
5	GalNAcα1,3-GalNAcol
6	GlcNAcβ1,6-GalNAcol
7	GalNAcα1,6-GalNAcol
8	Galα1,3-GalNAcol

and in plant proteins) and O-linked GlcNAc (often on cytoplasmic and nuclear proteins as a reversible regulatory modification) have also been characterized. In addition, O-linked fucose occurs together with O-linked glucose on specific sequons in the EGF domains of several proteins ***(2)***.

In yeast, unlike in animal cells, O-glycosylation begins in the endoplasmic reticulum by addition of a single mannose from from Man-P-dolichol to selected Ser/Thr residues; once transported to the Golgi, sugar transferases add one or more α1,2-linked mannoses that may be capped with one or more α1,3-linked mannose residues ***(3,4)***. In animal cells, however, stepwise enzymatic elongation by specific transferases yields several core structures, which can be further elongated or modified by sialylation, sulphation, acetylation, fucosylation, and polylactosamine extension. The eight core structures identified to date ***(2,5)*** are shown in **Table 1**.

The diversity of O-linked oligosaccharides linked to eukaryotic glycoprotein is comparable to that described for N-linked glycans, and site microheterogeneity and variable site occupancy are both common phenomena. The consequence of this is that many isolated glycoproteins are actually a set of glycoforms exhibiting small differences in their physicochemical properties. Thus, in the practice, glycosylation analysis is performed on a mixture of glycoforms.

Most analytical strategies involve several stages that seek to address questions like “is the protein glycosylated?”; “does it contain N- and/or O-glycans?”; “which amino acid residues are glycosylated?”; “which are the structures of the sugar chains?” The methods outlined below are simple protocols that can be carried out in many noncarbohydrate specialized biochemical laboratories.

1.1. Occurrence and Extent of O-Glycosylation

Diverse strategies may aid in determining the occurrence of O-glycosylation in a protein. As a first approach monosaccharide compositional analysis provides a good estimate of the presence of N- and/or O-glycosylation sites. Thus, GalNAc is present in all O-linked oligosaccharides, while this aminosugar is uncommon in N-linked glycans. On the other hand, all N-linked carbohydrate chains contain mannose residues, but it is not present in most O-linked oligosaccharide chains. Thus, detection of GalNAc and absence of Man strongly indicate that the protein may be O-glycosylated. The situation is normally not so simple and a given glycoprotein may exhibit both N- and O-glycosylation sites.

2. Materials

2.1. Determination of Aminosugars Using an Amino Acid Analyzer

1. D-(+/–) glucosamine hydrochloride (2-amino-2-deoxy-D-glucopyranoside) and D-(+/–). galactosamine (2-amino-2-deoxy-D-galactopyranoside), crystalline, minimum 99%.
2. Amino acid standard solution (Sigma) for protein hydrolysates.
3. L-norleucine (D-α-amino *n*-caproic acid).
4. Hydrochloric acid (HCl) 37% (12 *M*) for analysis.
5. Deionized water (MilliQ [18 MΩ] or equivalent).
6. Glass ampoules or Pasteur pipets.
7. Blowlamp.
8. Vacuum pump.
9. Thermobloc (temperature range up to 130°C).
10. Amino acid analyzer with postcolumn (ninhidrin) derivatization.

2.2. Determination of Dansyl Hydrazone Derivatives of Neutral Sugars by Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

1. 2 *N* Hydrochloric acid (HCl, for analysis).
2. Standard neutral sugars (maltose, lactose, galactose, glucose, mannose, fucose, xylose; minimum 99%).
3. Dowex 50W X4 (200–400 mesh, H^+ form) resin.

4. 2 *N* NaOH.
5. Deionized water (MilliQ [18 MΩ] or equivalent).
6. 10% (v/v) Trichloroacetic acid (TCA).
7. 5% Solution of dansyl hydrazine (dansyl-(5-dimethylaminonaphtalene-1-sulphonyl)-hydrazine) in acetonitrile.
8. Rotary evaporator (SpeedVac).
9. Water bath.
10. C18 Sep-Pak cartridges (Waters Ass.).
11. 5, 18, and 20% (v/v) acetonitrile in water.
12. HPLC equipment.
13. Reversed-phase C18 analytical column (25 × 0.4 cm, 5 μm particle size, 100 Å pore size).

2.3. Molecular Mass Determination of Intact Glycoproteins

1. 0.1% TFA.
2. Acetonitrile.
3. Matrix solution: 22 mg of 3,5-dimethoxy-4-hydroxycinnamic acid in 1 mL of 40:60 (v/v) acetonitrile: 0.1% TFA in water.
4. MALDI mass spectrometer.

2.4. Release of O-Linked Oligosaccharides

2.4.1. Mass Spectrometric Analysis of O-Deglycosylated Protein

1. 0.1 *M* NaOH.
2. Thermobloc (temperature range up to 60°C).
3. Gel-filtration column (Bio-Gel P10 or P30 [Bio-Rad]; Superdex HR10/30 [Pharmacia] or equivalent).
4. Lyophilisator.
5. Deionized water (MilliQ [18 MΩ] or equivalent).
6. MALDI mass spectrometer.

2.4.2. Recovery and Initial Characterization of Released Oligosaccharides

1. 0.1 *M* NaOH.
2. 1 *M* $NaBH_4$.
3. Thermobloc (temperature range up to 60°C).
4. 4 *M* Acetic acid.
5. Rotary evaporator (SpeedVac).
6. Methanol:acetic acid (99:1, v/v).
7. Sephadex G25 Superfine Fast-Desalting HR 10/10 column.
8. FPLC system.
9. Matrix solution: mixture of 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid, each at a concentration of 10 mg/mL in 30% (v/v) acetonitrile/water.

2.5. Fractionation of Oligosaccharides: Anion-Exchange Chromatography

1. FPLC system.
2. MonoQ HR 5/5 column (Pharmacia).
3. Deionized water (MilliQ [18 MΩ] or equivalent).
4. 0.5 *M* NaCl.

2.6. Fractionation of Oligosaccharides: Amino-Bonded Phase Chromatography

1. Rotary evaporator (SpeedVac).
2. 85% (v/v) Acetonitrile: 15% 5–15 m*M* potassium phosphate, pH 7.0.
3. 5–15 m*M* Potassium phosphate, pH 7.0.
4. Lichrosorb-NH2 (Merck) 25 × 0.4 cm, 5 μm particle size.
5. HPLC system.

2.7. Location of O-Glycosylation Sites

1. 100 m*M* Ammonium bicarbonate, pH 8.3.
2. Proteolytic enzymes (TPCK-trypsin, α-chymotrypsin, endoproteinase Lys-C or endoproteinase Asp-N).
3. HPLC system.
4. (25 × 0.4 cm) C18 (5 μm particle size, 100 Å pore size) column.
5. 0.1% (v/v) TFA in water.
6. 0.1% (v/v) TFA in acetonitrile.
7. Amino acid analyzer with postcolumn (ninhidrin) derivatization.
8. N-terminal sequencer.
9. MALDI mass spectrometer.

3. Methods

3.1. Determination of Aminosugars Using an Amino Acid Analyzer

Aminosugars (GlcNAc and GalNAc) can be easily identify and quantitate using an automated amino acid analyzer with postcolumn (ninhydrin) derivatization ***(6)***.

1. Make a stock solution of glycoprotein in water or 0.1% TFA.
2. Put aliquots of 0.5–1 nmol glycoprotein in two glass ampoules (*see* **Note 1**).
3. Add HCl to final concentrations of 6 *M* (for total protein hydrolysis) and 4 *M* (for aminosugar analysis).
4. To avoid decomposition of hydroxy amino acids, the ampoules should be evacuated to below 50 microns for 1–2 min.
5. Seal the ampoules under vacuum using a blowlamp.
6. Hydrolyze samples in a thermobloc at 110°C for 24 h (for protein hydrolysis) and 4 h (for aminosugar analysis).

7. Open the ampoules, add a known amount (i.e., 5–10 nmol) of norleucine as internal standard, and remove HCl in a rotary evaporator (SpeedVac).
8. Run the samples in an automated amino acid analyzer calibrated with a mixture of standard amino acids containing known amounts of galactosamine and glucosamine (*see* **Note 2**).

3.2. Determination of Dansyl Hydrazone Derivatives of Neutral Sugars by RP-HPLC

The method outlined below for labeling of the reducing end of sugars with a fluorescent tag followed by HPLC separation and quantitation of the resulting dansyl hydrazones of neutral sugars has been employed in the author's laboratory for compositional analysis of human platelet $\alpha_{IIb}\beta_3$ integrin subunits ***(8,9)***. It is simple, reproducible, and does not need a dedicated carbohydrate analyzer.

1. The optimal conditions for the release of neutral sugars may vary from glycoprotein to glycoprotein. Therefore optimal conditions should be determined in a case-by-case manner. However, hydrolysis with 2 *N* HCl for 2 h at 110°C in sealed tubes or ampoules (*see* **steps 1–6** of **Subheading 3.1.**) are good starting conditions.
2. Treat a mixture of neutral sugars (10 nmol of each maltose, lactose, galactose, glucose, mannose, fucose, and xylose) in a separate tube the same way as the glycoprotein (*see* **Note 3**).
3. After acid hydrolysis, dry standards and samples in their hydrolysis tubes under reduced pressure, at 40–45°C, using a rotavapor or SpeedVac evaporator.
4. Dissolve dried samples in 0.5 mL of deionized water (MilliQ) and add 10 nmol of an internal standard (maltose or lactose) to each hydrolysate.
5. Prepare 0.5 mL columns of Dowex 50W X4 (200–400 mesh, H^+ form) (Fluka) using a Pasteur pipet containing a piece of glass-wool as filter. Wash the resin exhaustively with 2 *N* NaOH, water, 2 *N* HCl, and water.
6. Apply the samples and wash the columns with 5 mL water. Neutral sugars are not retained in the Dowex columns and thus will be recovered in the flow-through fraction.
7. Dry the samples in a rotavapor or SpeedVac evaporator and dissolve the residues containing the neutral sugars in 100 µL water.
8. Add 10 µL of TCA 10% and 50 µL of a 5% solution of dansyl hydrazine (dansyl-(5-dimethylaminonaphtalene-1-sulphonyl)-hydrazine) (Sigma) in acetonitrile.
9. Cap the tubes and incubate for 20 min at 65°C in a water bath in the dark.
10. Place the reaction mixtures in an ice-cold water bath and dilute with 2 mL water.
11. Activate a Sep-Pak C18 cartridge (Waters Associated) by rinsing with 3 mL acetonitrile followed by 5 mL water.
12. Load slowly the derivatized samples (**step 10**) into the activated Sep-Pak cartridge and rinsed it slowly with 5 mL of 5% (v/v) acetonitrile (*see* **Note 4**).
13. Recover the dansyl hydrazones by elution with 6 mL of 20% (v/v) acetonitrile (*see* **Notes 5** and **6**).

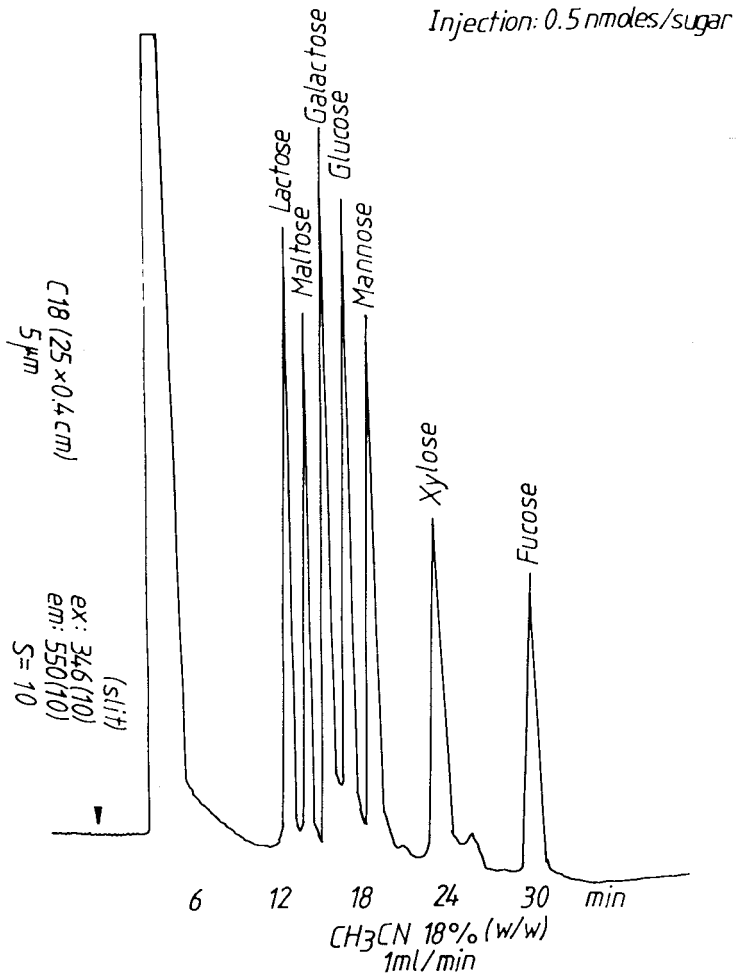

Fig. 1. Separation of the dansyl hydrazones of standard sugars by reverse-phase HPLC using a 5 µm C18 analytical column (25 × 0.4 cm) and isocratic elution with 18% (w/w) acetonitrile, at room temperature and a flow-rate of 1 mL/min 0.5 nmol of each sugar were injected. Fluorescence detection was done in a Perkin-Elmer MPF 3 spectrofluorimeter equipped with a 15 µL flow cell. Excitation wavelength: 346 nm (slit 10); emission wavelength: 550 nm (slit 10); sensitivity: 10.

14. Dry the dansyl hydrazone fraction in a SpeedVac.
15. Dissolve in 0.3 mL of 18% (v/v) acetonitrile and store in the dark and at 4°C until use (*see* **Note 7**).
16. Inject aliquots of 25–100 µL of the dansyl hydrazone solution into an analytical (25 × 0.4 cm) HPLC C18 reversed-phase column (100 Å pore size, 5 µm particle size) eluted at a flow rate of 1 mL/min isocratically with 18% (w/w) acetonitrile at room temperature (around 22°C) (**Fig. 1**) (*see* **Note 8**).

17. Wash the column with 100% acetonitrile until baseline indicates no further elution of interfering material, and equilibrate with 18% (w/w) acetonitrile for subsequent analyses.

3.3. Molecular Mass Determination of Intact Glycoproteins

Mass spectrometric analysis of the glycoprotein before and after release of O-glycans provide an estimate of the relative amount of total O-linked carbohydrate. The molecular mass of intact glycoproteins may be accurately determined by matrix-assisted laser-desorption ionization (MALDI) mass spectrometry:

1. Dissolve the glycoprotein (approx 10–50 pmol/μL) in 0.1% TFA. Add acetonitrile in 10% steps if necessary for solubilization.
2. Prepare the matrix solution: dissolve 22 mg of 3,5-dimethoxy-4 hydroxycinnamic acid in a 1 mL of 40 : 60 (v/v) acetonitrile: 0.1% TFA in water.
3. Mix equal volumes (0.5–1 μL) of protein and matrix solution onto the stainless-steel MALDI target and dry at room temperature.
4. Set an acceleration voltage of 30 kV and operate in the linear mode.

3.4. Release of O-Linked Oligosaccharides

3.4.1. Mass Spectrometric Analysis of O-Deglycosylated Protein

Although the enzyme O-glycosidase from *Streptococcus pneumoniae* (endo-α-N-acetyl-galactosaminidase, EC 3.2.1.97 or 3.2.1.110) can be used for cleavage of O-linked oligosaccharides from glycoproteins, it has a narrow substrate specificity, releasing only unsubstituted galactose β-3-N-acetylgalactosamine. Thus, a mixture of exoglycosidases (sialidase, α-fucosidase, α-N-acetylgalactosaminidase) is often required to be used with or prior to digestion with this enzyme. For mass spectrometric analysis of deglycosylated glycoproteins, O-linked oligosaccharides can be optimally released by mild alkali-catalyzed β-elimination:

1. Dissolve the glycoprotein (approx 10 mg/mL) in 0.05–0.1 *M* NaOH.
2. Incubate for 16–24 h at 40–50°C.
3. Desalt on a gel-filtration column (approx 30 × 0.5 cm) Bio-Gel P10 or P30 (BioRad) or Superdex HR10/30 (Pharmacia).
4. Dialyze against deionized water and lyophilize.
5. Determine the molecular mass by MALDI mass spectrometry as in **Subheading 3.3.**

3.4.2. Recovery and Initial Characterization of Released Oligosaccharides

1. For release the O-linked glycans by β-elimination dissolve the glycoprotein as in **step 1** of **Subheading 3.4.1.** However, to prevent breakdown of the oligosaccharide chains, 0.5–1 *M* $NaBH_4$ must be included in the reaction mixture. The

released oligosaccharides are recovered as alditols. Because of the inclusion of the borohydride, release of N-glycans may also occur.
2. Incubate the reaction mixture for 16–24 h at 40–50°C.
3. Cool on ice and neutralize to pH 6.0 with 4 *M* acetic acid.
4. Remove boric acid by repetitive co-evaporation (using a rotavapor) with methanol containing 1% acetic acid.
5. Resuspend in 1 mL deionized water and desalt on a Sephadex G25 Superfine Fast-Desalting HR 10/10 column using an FPLC system (Pharmacia). Monitor the elution of oligosaccharides at 206 nm.
6. The degree of compositional heterogeneity and the relative abundance of oligosaccharides in the pool of released glycans may be conveniently analysed by mass spectrometry. Underivatized oligosaccharides can be analyzed by MALDI-MS using a matrix such as a mixture of 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid, each at a concentration of 10 mg/mL in 30% acetonitrile/water with a detection limit of about 100 fmol ***(7)***. Alternatively, determination of the molecular masses of reduced and permethylated ***(7)*** oligosaccharides (~10 pmol/μL) can be determined by MALDI mass spectrometry (MS) (using 10 mg/mL DHB in 10% ethanol as matrix) or by electrospray ionisation (ESI) mass spectrometry. For ESI-MS, the reduced and permethylated oligosaccharides are dissolved in methanol saturated with NaCl at a concentration of ~10 pmol/μL. In this case, oligosaccharides are detected as the natrium adducts (M + 23 Da) (*see* **Note 9**).

3.5. Fractionation of Oligosaccharides: Anion-Exchange Chromatography

Released oligosaccharide chains can be fractionated according to their charge (i.e., neutral, monosialylated, disialylated) by anion-exchange chromatography on a MonoQ HR 5/5 column using a Pharmacia FPLC system.

1. Dissolve the oligosaccharide pool in 0.5 mL of deionized (MilliQ) water.
2. Apply the sample and run the column at 1 mL/min with a mixture of water (solvent A) and 0.5 *M* NaCl (solvent B). Separation of different mixtures may require optimization of the elution conditions. Tentatively starting conditions could be: isocratic (100% A) for 5 min followed by a linear gradient from 100% A–100% B for 30–45 min. Oligosaccharides are detected at 206 nm.

3.6. Fractionation of Oligosaccharides: Amino-Bonded Phase Chromatography

Oligosaccharide fractions isolated by anion-exchange chromatography might be further subfractionated by means of amino-bonded HPLC. Oligosaccharides are eluted in order of increasing molecular weight.

1. Dry the MonoQ fractions (isolated as described in **Subheading 3.5.**) containing 10–200 μg oligosaccharide using a SpeedVac.

2. Dissolve each fraction in 0.5 mL of 85% (v/v) acetonitrile containing 5–15 m*M* potassium phosphate, pH 7.0 (solvent A).
3. Apply to a Lichrosorb-NH2 (0.4 × 25 cm, 5 μm particle size) Merck HPLC column eluting at 1 mL/min with linear gradients of solvent A and 5–15 m*M* potassium phosphate pH 7.0 (solvent B). Monitor oligosaccharide elution by absorbance at 206 nm.

3.7. Location of O-Glycosylation Sites

The simple strategy for determination of the position of O-glycosylated residues along the amino acid sequence of a glycoprotein includes fragmentation of the protein, separation of the resulting peptides, and structural characterization of the isolated fragments by combination of N-terminal sequencing and carbohydrate analysis (*see* **Subheading 4.**). This approach requires that the primary structure of the protein is known.

1. Degrade the glycoprotein (1–2 mg/mL in 100 m*M* ammonium bicarbonate, pH 8.3 [*see* **Notes 10** and **11**]) with the chosen proteolytic enzyme (usually TPCK-trypsin, α-chymotrypsin, endoproteinase Lys-C, or endoproteinase Asp-N) at an enzyme : substrate ratio of 1 : 100 (w/w) overnight at 37°C.
2. Separate peptides by reversed-phase HPLC (*see* **Note 12**).
3. Determine the presence of galactosamine in the HPLC-separated fractions by amino acid analysis as in **Subheading 3.1.**
4. Determine the N-terminal sequence of those peptides containing galactosamine. A blank in the sequence may indicate the existence at that position of a glycosylated residue (*see* **Note 13**).
5. Determine the molecular mass of galactosamine-containing peptides. The difference between the experimentally determined mass and that calculated from the amino acid sequence indicates the size of the glycan.

3.8. Sequence Analysis

Sequencing of O-linked oligosaccharides involves two separate steps: identification of the monosaccharide units followed by the linkages between units in the sequence. Carbohydrate compositional analysis of glycans or glycopeptides isolated as in **Subheadings 3.4.2.** or **3.7.** involve the same protocols as described in **Subheadings 3.1.** and **3.2.** Several techniques are available for the determination of the anomericity of the glycosidic linkages, specific linkages between monosaccharides, and branch configuration, including: (1) methylation analysis in combination with diverse mass spectrometric techniques (fast-atom-bombardment mass spectrometry; collision-induced dissociation using tandem [electrospray] mass spectrometry; postsource decay by MALDI mass spectrometry); (2) enzymatic analysis using exo- and endoglycosidases; and (3) ^{1}H-NMR. However, a comprehensive review of these techniques, which are also used for structural characterization of N-linked oligosaccharides, is beyond the aim of this chapter.

4. Notes

1. Ampoules can be easily made from Pasteur pipets using a small blowlamp. Due to their manufacturing process, Pasteur pipets from local suppliers are free from amino acids, and need not to be pretreated.
2. Although glycoproteins contain N-acetylgalactosamine and N-acetylglucosamine, these residues are hydrolyzed to their respective deacetilated aminosugars. The amount of galactosamine and glucosamine added to the amino acid standard (Sigma) should be roughly the same as the amount of any amino acid present in the mixture.
3. Mixtures of standard sugar may be adequately prepared by carefully weighting of desiccated pure sugars.
4. Since the recovery of eluting sugars is affected by the flow-rate through the Sep-Pak cartridge, loading, and elution should not exceed about 2 mL/min.
5. This sample clean-up procedure removes both high- and low-polarity material, consisting primarily of dansyl sulphonic acid and excess of dansyl hydrazine, reducing thereby the presence of junk peaks in the chromatogram. Recovery of dansyl hydrazones of neutral sugars is usually greater than 90%.
6. The Sep-Pak cartridge can be reused following regeneration and activation with 3 mL of acetonitrile followed by 5 mL water.
7. The derivatized sugar fraction is 85–100% stable if stored 2 d at 4°C or 2–3 wk at –20°C in the dark.
8. Detection of dansylhydrazones can be done with a spectrophotometer monitoring at 240 nm or with a fluorimeter recording the emission at 550 nm after excitation at 346 nm. The detection limit is of the order of 25–50 pmol, although for accurate compositional analysis, 100–500 pmol are recommended.
9. A description of derivatization (permethylation or peracetylation) and fragmentation behavior of oligosaccharides is far beyond the scope of this chapter. The methods and analytical techniques are the same as for structural elucidation of N-linked carbohydrates described elsewhere in this book and in excellent previous book chapters and reviews ***(10,11)***.
10. The composition and pH of the digestion buffer may vary. Ammonium bicarbonate buffer is a convenient medium for digestion with many enzymes, including the serine proteinases listed. It is a volatile buffer and therefore easy to remove by lyophilization. Other suitable buffers in the pH range 7.5–8.5, with or without 50–150 m*M* NaCl, are based on Tris-HCl, phosphate, N-ethylmorpholine-HCl, and so on. If necessary for solubilization of the glycoprotein, guanidine hydrochloride up to a final concentration of 2 *M* may be added. Most serine proteinases retain 40–100% proteolytic activity under these conditions.
11. If the polypeptide chain is strongly crosslinked by disulphide bonds, the protein must be reduced and alkylated before cleavage. To this end, dissolve the protein (5–10 mg/mL) in a buffer pH 8.0–9.0 containing 6 *M* guanidine-hydrochloride, add 2-mercaptoethanol (5% [v/v] final concentration and incubate for 2 min in a boiling water bath. Thereafter, let cool at room temperature and add a 2 molar fold excess of alkylating reagent (i.e., iodoacetamide or 4-vynilpyridine) over

reducing agent. Let react for 30 min, and dialyze extensively against digestion buffer (*see* **Note 10**).

12. Many different chromatographic conditions can be employed for reversed-phase peptide mapping. Most common conditions employ a C18 25 × 0.4 cm (5 μm particle size, 100 Å pore size) column eluted at 1 mL/min with a linear gradient of 0.1% TFA in water (solution A) and acetonitrile (solution B). For detection of peptides, monitor the eluate at 206–220 nm.
13. If the sequence of the glycosylated fragment is not completely determined by N-terminal sequencing, it might be necessary to subfraction the glycopeptide by digestion with another enzyme followed by separation of the secondary peptides and sequence analysis.

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8

Characterization of Site-Specific Glycosylation

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1. Introduction

With the aid of polymerase chain reaction (PCR) even minuscule amounts of DNA or RNA can be amplified. Thus, the sequence of practically any encoded protein can be obtained. However, posttranslational modifications have to be studied on the protein level. Even if a consensus sequence has been identified for a post-translational modification, the presence of such a sequence motif only indicates the possibility, not the certainty that the modification actually occurs. Proteins can be glycosylated on certain amino acid side-chains, and these modifications are designated as N- and O-glycosylation. N-glycosylated species are modified at Asn residues. There is a consensus sequence for N-glycosylation: AsnXxxSer/Thr/Cys, where Xxx can be any amino acid except proline. N-linked oligosaccharides share a common core structure of $GlcNAc_2Man_3$. In addition, an enzyme, peptide N-glycosidase F (PNGase F), removes unaltered most of the common N-linked carbohydrates from proteins while hydrolyzing the originally glycosylated Asn residue to Asp. O-glycosylation occurs at Ser or Thr-residues, usually in sequence-stretches rich in hydroxy amino acids, but there has been no consensus sequence determined for this modification. In addition, O-glycosylation lacks a common core structure: mammalian proteins have been reported bearing O-linked N-acetylgalactosamine ***(1)***, fucose ***(2)***, glucose ***(2,3)***, and corresponding elongated structures, as well as N-acetylglucosamine ***(4)***. Chemical methods are used to liberate these oligosaccharides because no enzyme has been discovered that would cleave all the different O-linked carbohydrates. Characterization of both types of glycosylation is complicated by the fact that the same amino acids within a population of protein molecules may be derivatized with an array of different carbohydrate structures, or remain unmodified. This site-specific heterogeneity may

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vary by species, tissue ***(5,6)***, and may be affected by physiologycal changes ***(7)***, and so on.

Glycosylation of a given protein has usually been characterized from its enzymatically or chemically released carbohydrate pool, using a wide variety of methods, such as high pH anion-exchange chromatography ***(8)*** or fluorophore-assisted carbohydrate electrophoresis (FACE) ***(9)***, sequential exoglycosidase digestions ***(10,11)***, mass spectrometry ***(12–16)***, and nuclear magnetic resonance (NMR) ***(16)***. For addressing site-specific carbohydrate heterogeneity, mass spectrometry has become the method of choice. Although matrix-assisted laser desorption ionization (MALDI) mass spectrometry of collected high-performance liquid chromatography (HPLC)-fractions has been used successfully for this purpose ***(17,18)***, RP-HPLC directly coupled with electrospray ionization mass spectrometry (LC/ESIMS) offers better resolution ***(6,19–27)***. Using a mass spectrometer as on-line detector not only assures the analysis of every component eluting (mass mapping), but at the same time diagnostic carbohydrate ions can be generated by collisional activation in the ion-source that permit the selective detection of glycopeptides ***(21)***. These characteristic nonreducing end oxonium ions at m/z 204, 274, and 292, 366, and 657 indicate the presence of N-acetylhexosamine, neuraminic (sialic) acid, hexosyl-N-acetylhexosamine, and sialyl-hexosyl-N-acetylhexosamine, respectively (*see* **Note 1**). During LC/ESIMS experiments fragmentation can be induced and these diagnostic ions can be monitored (Selective Ion Monitoring; SIM) in alternate scans, while every second scan will yield molecular-weight information of the intact species. Glycopeptide-containing fractions should exhibit components with molecular weights that do not match any predicted proteolytic peptide, and these species would be further analyzed. Prior to LC/ESIMS analysis N-linked carbohydrates can be eliminated by PNGase F leaving only the Ser- Thr-glycosylated species ***(22)***. However, for the characterization of O-linked sites, other methods are frequently required, such as tandem mass spectrometry, additional proteolytic and/or glycosidase cleavages, Edman sequencing, or the combination thereof, depending on the actual carbohydrate structure ***(24,28–30)***. Thus, this chapter will focus on the characterization of N-glycosylated proteins, where a more general protocol can be followed because of the shared features of these modifications. From a known amino acid sequence, potential N-glycosylation sites can be identified, and a proteolytic enzyme can be selected that would separate these sites on individual peptides. Then the digestion mixture can be analyzed by LC/ESIMS. Glycopeptide-containing fractions will be identified by SIM, and from the molecular weights measured for the glycopeptides the site-specific heterogeneity may be addressed ***(6,19,23–26)***. Studying recombinant proteins may help in overcoming some of the hurdles, for example, developing new approaches and techniques, and/or

working out optimal conditions for the characterization of the native species available in much lower quantities.

2. Materials

2.1. Reduction and Alkylation of Glycoprotein

1. 1 nmol purified glycoprotein, little salt, *no* detergent (*see* **Note 2**). In our example, thrombin activated recombinant human Factor VIII (rhFVIIIa) was separated into its four polypeptides by RP-HPLC on a semipreparative C4 column ***(25)***.
2. 6 *M* guanidine hydrochloride, 200 m*M* NH_4HCO_3 buffer (pH ~8.0).
3. Freshly prepared DTT solution in water: 250 n*M*/μL.
4. Iodoacetic acid sodium salt solution in H_2O: 550 n*M*/μL. Prepare just before use.
5. Microdialyzer, dialysis membrane of the appropriate molecular weight (MW) cut-off.
6. 20 m*M* NH_4HCO_3 buffer, pH ~7.4.

2.2. Tryptic Digestion (see *Note 3*)

1. Side-chain protected porcine trypsin (Promega).
2. 1 m*M* TFA in H_2O.

2.3. LC/ESIMS Analysis of the Tryptic Digest

1. HPLC system with a microbore C18 column (1.0 × 150 mm).
2. UV detector, variable wavelength.
3. HPLC solvents: 0.1% TFA in water, 0.08% TFA in acetonitrile (*see* **Note 4**).
4. Make-up solvent: 2-methoxyethanol/isopropanol 1 : 1 mixture.
5. Additional pump providing stable flow at 50 μL/min flow rate, for the make-up flow.
6. Mixing tee, splitting tee, fused silica capillaries for plumbing, teflon sleeves.
7. Mass spectrometer equipped with an electrospray source and with hardware and software permitting selective ion monitoring and data acquisition with alternating scans at high and low cone-voltage.

In our example the digests were injected onto a microbore (C18, 1.0 × 150 mm, Vydac) reversed-phase column fitted to an Applied BioSystems 140B HPLC system. Fused silica capillaries 285 μm OD/75 μm ID were used for plumbing (LC Packings). The UV-detector (Applied Biosystems 759A) was equipped with an U-Z View flow cell (LC Packings). Capillaries were connected wherever it was necessary with LC Packings teflon sleeves. Make-up solvent was added after UV-detection, and to accomplish this a separate syringe pump (Isco μLC-500) delivered a 2-methoxyethanol/isopropanol 1 : 1 mixture to a 3.1 μL dead volume PEEK mixing tee (Upchurch Scientific) at a flow rate of 50 μL/min. After the mixing tee a zero dead volume tee (Upchurch Scientific) was included to split the column effluent at a rate of 1 : 4 (*see* **Note 5**). The split ratio was adjusted by cutting the outlet capillary to the right size while

measuring the flow rates. Mass spectrometric detection was performed on a Micromass AutoSpec SE mass analyzer equipped with an electrospray source (*see* **Note 6**).

3. Methods

3.1. Reduction and Alkylation of Glycoprotein

1. Dissolve 1 nmol of the glycoprotein in 100 µL of a 6 *M* guanidine hydrochloride/200 m*M* NH_4HCO_3 pH ~8.0 solution.
2. Add 2 µL DTT in H_2O 250 n*M*/µL. Incubate the mixture at 60°C for 1h.
3. Add 2 µL sodium iodoacetate in H_2O: 550 n*M*/µL. Incubate the mixture at room temperature, for 2 h, in the dark.
4. Dialyze the mixture against 20 m*M* NH_4HCO_3 buffer overnight to remove the reagent excess.
5. Concentrate the sample by vacuum centrifugation to approx 100 µL.

In our example approx 1 nmol of the rhFVIIIa proteins was derivatized with 1000-fold excess of vinylpyridine in 6 *M* guanidine hydrochloride, 200 m*M* NH_4HCO_3 solution, at 20°C, for 3.5 h, in the dark. The only purpose of this derivatization step is the identification of free sulfhydryls in the protein; therefore for glycosylation studies it could have been eliminated. Incubation with 2000-fold excess with dithiothreitol (DTT) followed, at 60°C, for 1 h, then new sulfhydryls were derivatized with 4400-fold excess of iodoacetic acid sodium salt at 20°C, for 2 h, in the dark. Finally the reagent excess was eliminated by dialysis against 20–50 m*M* NH_4HCO_3 solution ***(25)***.

3.2. Tryptic Digestion

1. Side-chain protected trypsin dissolved in 1 m*M* TFA solution (0.1–1 µg/µL) is added to the glycoprotein solution. The amount of trypsin should be approx 4% w/w.
2. Incubation for 8 h, at 37°C (*see* **Note 7**).
3. Stop the digestion by boiling the mixture for 5 min (*see* **Note 8**).

The reduced and alkylated rhFVIIIa proteins required longer incubation time: 16–24 h.

3.3. LC/ESIMS Analysis of the Tryptic Digest

1. Prepare solvents.
2. Set up the microbore HPLC system with a make-up solvent added after the UV-detector and the flow-splitter. Measure flow rates. Equilibrate the column in 5% solvent B.
3. Set up an LC/ESIMS method of alternating high and low cone-voltage scans. In high cone-voltage mode monitor ions at m/z 204 and 292. Usually an approx

1 Dalton mass window is monitored around these m/z values. In most cases these two values are sufficiently informative, because all N-linked oligosaccharides contain at least two N-acetylhexosamines, while the presence or absence of neuraminic (sialic) acid will provide some indication of what class of N-linked structure is present. In low cone-voltage scans monitor the mass range from m/z 350–2000 (*see* **Note 9**).

4. Inject approx 100 pmol (approx 10 µL) of the tryptic digest. Start mass spectrometry data acquisition.
5. Keep the flow isocratic (5% B) for 5 min, then start a linear gradient increasing the percentage of solvent B to 50% over 90 min (*see* **Note 10**). Collect fractions at the splitting tee, if further analysis is planned.

Approximately 100 pmol of a tryptic digest of a reduced and alkylated rhFVIIIa protein (73 kDa or B-domain ***[25]***) was injected onto a microbore (C18, 1.0 × 150 mm, Vydac) reversed-phase column that was equilibrated in 5% solvent B, at a flow rate of 50 µL/min. The gradient was started 5 min after the injection, the amount of solvent B was linearly increased to 40 or 50% over 80 min. Data shown here were collected while applying a make-up solvent after UV-detection, at a flow rate of 50 µL/min. Two identical LC/ESIMS analyses were performed using an AutoSpec SE mass spectrometer as the detector. The source voltage was 4000 V, the needle voltage was 7800 V. During the first analysis, fragmentation was induced in the ion source by increasing the sample-cone voltage to 313 V above the source potential and the skimmer voltage to 325 V. During the second analysis the sampling cone and skimmer voltages were kept at a lower value, 196 and 235 V, respectively. Under these lower-voltage conditions, no carbohydrate fragmentation was observed. For normal scans the magnet was scanned to monitor the spectra from m/z 2000 to 350, while for the high cone-voltage scans the lower mass limit was set at m/z 200 (*see* **Note 11**). Mass resolution was approx 1500 (*see* **Note 6**).

3.4. Data Interpretation

3.4.1. Glycopeptide Identification

1. Using Protein Prospector (www.prospector.ucsf.edu/) MS-digest (or other similar programs) print out the list of expected tryptic peptides. Do not forget to select the appropriate modification for the Cys-residues (*see* **Note 12**).
2. Print out the total ion current (TIC) and SIM chromatograms. The TIC chromatogram will show a UV-like elution profile, while the SIM-peaks will indicate the glycopeptides.
3. Print out the full-scan electrospray spectra of glycopeptide-containing TIC peaks, i.e., combine the scans, beginning where the SIM trace starts to rise to the edge of the SIM peak.

Table 1
Some Common Components of N- and O-Linked Oligosaccharides

Sugar	M_r	In chain: nominal mass	Exact mass	Average mass
Pentose	150	132	132.0423	132.1161
Arabinose, Xylose	$C_5H_{10}O_5$	$C_5H_8O_4$		
DeoxyHex	164	146	146.0579	146.1430
Fucose	$C_6H_{12}O_5$	$C_6H_{10}O_4$		
Hexose	180	162	162.0528	162.1424
	$C_6H_{12}O_6$	$C_6H_{10}O_5$		
Hexuronic acid	194	176	176.0321	176.1259
HexA	$C_6H_{10}O_7$	$C_6H_8O_6$		
HexNAc	221	203	203.0794	203.1950
	$C_8H_{15}NO_6$	$C_8H_{13}NO_5$		
NeuAc/SA	309	291	291.0954	291.2579
N-acetyl-neuraminic acid/ sialic acid	$C_{11}H_{19}NO_9$	$C_{11}H_{17}NO_8$		
NeuGc	325	307	307.0903	307.2573
N-glycolyl-neuraminic acid	$C_{11}H_{19}NO_{10}$	$C_{11}H_{17}NO_9$		
H_2O		+18	18.0106	18.0153
Phosphate		+80	79.9663	79.9799
Sulphate		+80	79.9568	80.0642

4. Determine the MH^+ values of the coeluting digest components: based on charge state determination (high resolution) or based on the presence of a set of differently charged ions (low resolution) (*see* **Note 13**, and *see* detailed explanation below).
5. Compare these MH^+ values (monoisotopic for high resolution and average for low resolution) to the MS-digest list. Identify unmodified peptides.
6. Identify glycopeptide ion-series from mass differences that reflect carbohydrate heterogeneity (*see* **Notes 13–15**, **Tables 1** and **2**, and *see* below).
7. Identify the modified peptide (*see* below; **Table 3**).
8. Determine/confirm the carbohydrate structure by sequential exoglycosidase digestions/LC/ESIMS analyses of the collected glycoprotein fractions (**Table 4**, and *see* below).

LC/ESIMS analysis of the 73 kDa protein of rhFVIIIa is presented here as an example for addressing site-specific glycosylation by SIM and mass mapping. **Figure 1** shows the extracted ion chromatograms (the intensity changes of given m/z value ions during the analysis) of diagnostic carbohydrate fragments

Table 2
Carbohydrate Heterogeneity in Mass Differences

	Mass difference for ions			
Saccharide structure	2+	3+	4+	5+
Fucose	73	48.7	36.5	29.2
Hexose	81	54	40.5	32.4
N-Acetylhexosamine	101.5	67.7	50.8	40.6
Neuraminic acid	145.5	97	72.8	58.2
HexHexNAc	182.5	121.7	91.3	73
SAHexHexNAc	328	218.7	164	131.2

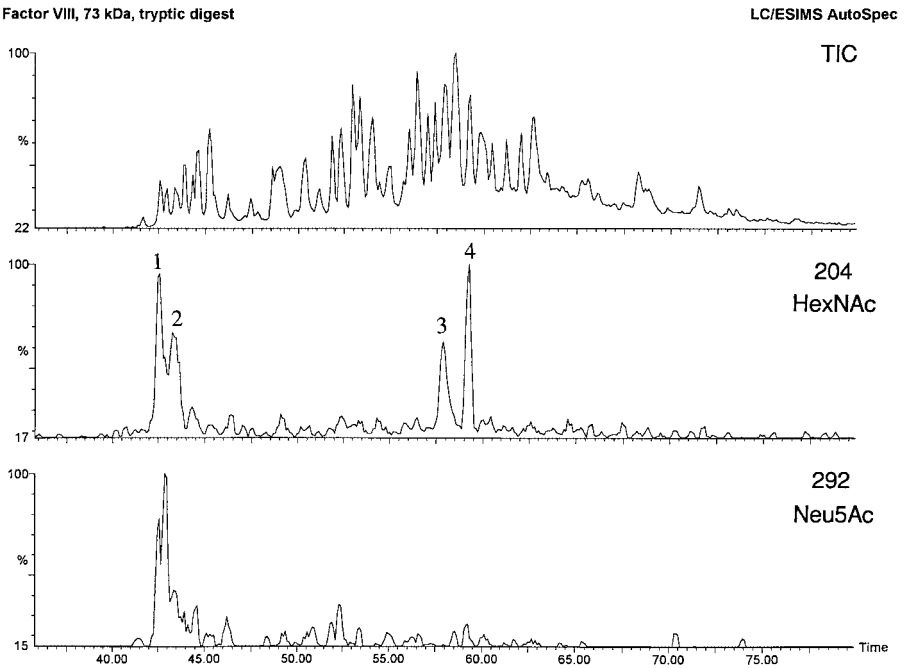

Fig. 1. Total ion chromatogram and selected ion chromatograms of diagnostic carbohydrate ions, m/z 292 (Neu5Ac) and 204 (HexNAc), acquired during high cone voltage LC/ESIMS analysis of a tryptic digest of the 73 kDa protein of rhFVIIIa. Reprinted with permission from **ref. *25***. Copyright (1997) American Chemical Society.

Table 3
Mass Addition to Peptides: Some Common N-Linked Oligosaccharides

Glycan	Composition	Mass added to peptide	
		Monoisotopic	Average
Oligomannose structures			
Man_3 core	$Man_3GlcNAc_2$	892.32	892.82
Man_3 core fucosylated	$Man_3GlcNAc_2Fuc$	1038.37	1038.96
Man_5	$Man_5GlcNAc_2$	1216.42	1217.10
Man_6	$Man_6GlcNAc_2$	1378.48	1379.24
Man_7	$Man_7GlcNAc_2$	1540.53	1541.39
Man_8	$Man_8GlcNAc_2$	1702.58	1703.53
Man_9	$Man_9GlcNAc_2$	1864.63	1865.67
Bisecting hybrid	$Man_5GlcNAc_4$	1622.58	1623.49
Bi-antennary structures			
Asialo	$Gal_2Man_3GlcNAc_4$	1622.58	1623.49
Asialo, fucosylated	$Gal_2Man_3GlcNAc_4Fuc$	1768.64	1769.64
Bisecting asialo fucosylated	$Gal_2Man_3GlcNAc_5Fuc$	1971.72	1972.83
Bisecting asialo	$Gal_2Man_3GlcNAc_5$	1825.66	1826.69
Asialo agalacto	$Man_3GlcNAc_4$	1298.48	1299.21
Asialo agalacto fucosylated	$Man_3GlcNAc_4Fuc$	1444.54	1445.35
Bisecting asialo agalacto fucosylated	$Man_3GlcNAc_5Fuc$	1647.61	1648.55
Monosialo	$SAGal_2Man_3GlcNAc_4$	1913.68	1914.75
Monosialo fucosylated	$SAGal_2Man_3GlcNAc_4$ Fuc	2059.74	2060.89
Disialo	$SA_2Gal_2Man_3GlcNAc_4$	2204.77	2206.01
Disialo fucosylated	$SA_2Gal_2Man_3GlcNAc_4$ Fuc	2350.83	2352.15
Tri-antennary structures			
Asialo	$Gal_3Man_3GlcNAc_5$	1987.71	1988.83
Asialo fucosylated	$Gal_3Man_3GlcNAc_5Fuc$	2133.77	2134.97
Asialo agalacto	$Man_3GlcNAc_5$	1501.56	1502.40
Asialo agalacto fucosylated	$Man_3GlcNAc_5Fuc$	1647.61	1648.55
Monosialo	$SAGal_3Man_3GlcNAc_5$	2278.81	2280.09
Monosialo fucosylated	$SAGal_3Man_3GlcNAc_5$ Fuc	2424.87	2426.23
Disialo	$SA_2Gal_3Man_3GlcNAc_5$	2569.90	2571.35
Disialo fucosylated	$SA_2Gal_3Man_3GlcNAc_5$ Fuc	2715.96	2717.49
Trisialo	$SA_3Gal_3Man_3GlcNAc_5$	2861.00	2862.60
Trisialo fucosylated	$SA_3Gal_3Man_3GlcNAc_5$ Fuc	3007.06	3008.75
Tetrasialo	$SA_4Gal_3Man_3GlcNAc_5$	3152.10	3153.86
Tetrasialo fucosylated	$SA_4Gal_3Man_3GlcNAc_5$ Fuc	3298.15	3300.00

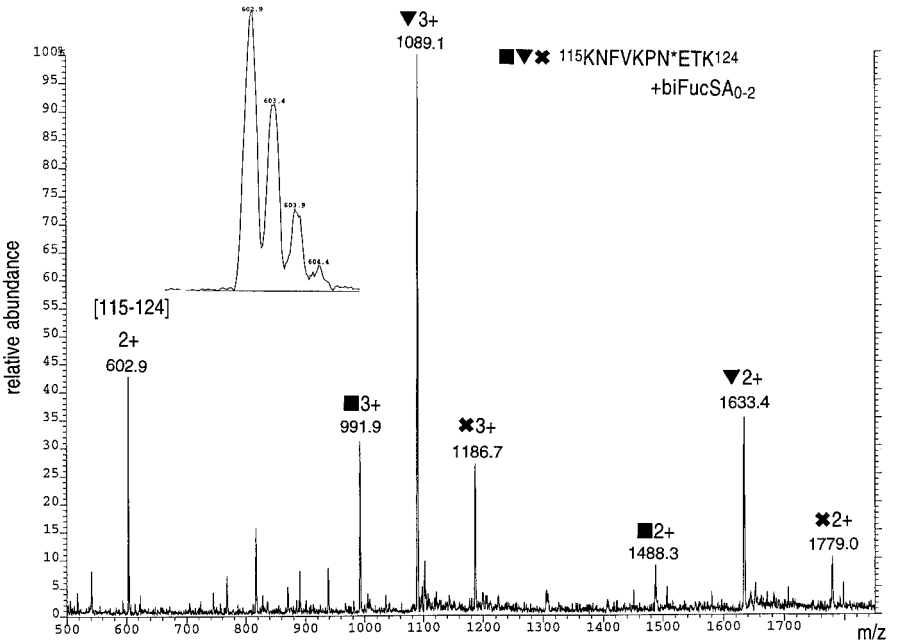

Fig. 2. Electrospray mass spectrum of the first partially resolved glycopeptide-containing peak from the tryptic digest of the 73 kDa protein of rhFVIIIa (**Fig. 1)**. These data were acquired at normal cone voltage. Insert illustrates the resolution during this LC/ESIMS experiment. Reprinted with permission from **ref. *25***. Copyright (1997) American Chemical Society.

at m/z 204 and 292 and the TIC chromatogram during the high cone voltage LC/ESIMS analysis of a tryptic digest of this protein. There are four consensus sequences in the 73 kDa protein, and from the SIM data *alone* the presence of four glycopeptide-containing fractions can be concluded. The first two partially resolved peaks exhibited the diagnostic ion for neuraminic acid, thus those glycopeptides should be modified by complex or hybrid oligosaccharides. From the lack of neuraminic acid oligomannose structures may be suspected for the other fractions. The next step is the analysis of the full-scan data of the chromatographic peaks producing the diagnostic carbohydrate fragments. The AutoSpec SE mass spectrometer afforded a high enough mass resolution to allow the charge state and the monoisotopic mass to be determined for each ion (*see* **Note 13**). **Figure 2** shows the electrospray spectrum of the first partially resolved glycopeptide-containing fraction. The peaks are labeled with their monoisotopic masses and charge states. Whenever a molecule was represented by ions of different charge states, usually the more highly resolved ions were used for the molecular-weight determination, if they were sufficiently abundant.

Table 4
Recommendations for Exoglycosidase Digestion

Enzymes (in sequencing order)	Enzyme source	Linkage specificity	Concentration (U/mL)	Digestion buffer[a]	Digestion time (h)
1. Neuraminidase	Newcastle Disease Virus	NeuAcα2-3,8R NeuGcα2-3,8R	0.02–0.05	A	3–7
2. β-Galactosidase	*Streptococcus pneumoniae*	Galβ1-4GlcNAc Galβ1-4GalNAc	0.03–0.07	A	3–7
3. β-GlcNAse	Chicken liver	GlcNAcβ1-3,4R	1.0	A	3–7
4. α-Mannosidase	Jack bean	Manα1-2,3,6Man	3.5	A+25 m*M* $ZnCl_2$	3–6
5. β-Mannosidase	*Helix pomatia*	Manβ1-4GlcNAc	2.0–4.0	A+25 m*M* $ZnCl_2$	3–6
6. β-GlcNAse	Chicken liver	GlcNAcβ1-3,4R	2.0	A	3–7
7. α-Fucosidase	Bovine epididymis	Fucα1-6(>2,3,4)R	0.2	A	3–6

[a]Buffer A: 30 m*M* sodium acetate, pH 5.0
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From these values the protonated molecular mass of each component was calculated: $n \times (MH_n)^{n+} - (n - 1)1.0$ (1.0 is the approximate mass of a proton). The MH^+ value was selected over the molecular mass because the software for protein digestion, MS-digest, lists this value. MH^+ values calculated for the fraction in **Fig. 2** were 1204.9, 2974.1, 3264.4, and 3556.0 Dalton, from ions at m/z 602.9, 991.9, 1089.1, and 1186.7, respectively. First the predicted tryptic peptides were identified: m/z 1204.9 corresponds to peptide ^{115}Lys-Lys^{124} (MH^+_{cal} at m/z 1204.7). Surprisingly, this sequence contains a consensus sequence, obviously unmodified. The three higher masses did not match predicted tryptic products and differed by approx 291 Dalton. Different glycoforms of the same glycosylation site usually elute very close together, generally the larger the oligosaccharide, the shorter the retention time (slightly). Heterogeneity of the carbohydrate structure is reflected in closely related masses (*see* **Note 14**; **Tables 1** and **2**). Thus, a series of ions differing in mass by 291 Dalton indicate different numbers of neuraminic acids in the oligosaccharides. Similarly, an ion series with 162 Dalton differences would indicate the presence of an array of oligomannose structures. A mass difference of 365 Dalton shows the presence of an additional HexHexNAc unit, that may indicate another antenna or an addition to the repetitive N-acetyllactosamine chain, and a 146 Dalton mass difference indicates fucosylation (*see* **Note 15**). The modified peptides can be identified readily if the structure of the oligosaccharides, separated from the protein, have been studied prior to the LC/ESIMS analysis, which is frequently the case for recombinant protein pharmaceuticals. The masses of these structures can be calculated (*see* **Table 3**) and subtracted from the molecular weights determined. If the correct oligosaccharide has been considered, the result of this subtraction will be the MH^+ value of a predicted tryptic peptide containing a potential glycosylation site. For example, it had been reported that the major component of the N-linked carbohydrate pool of rhFVIIIa was a monosialo biantennary core-fucosylated oligosaccharide ***(9)***. The addition of this carbohydrate would increase the peptide molecular mass by 2059.7 Dalton (monoisotopic; **Table 3**). Thus, this number was subtracted from the masses above and the results were compared to the mass table of the predicted tryptic peptides. 3264.4 – 2059.7 = 1204.7, is indeed a tryptic peptide with a consensus site: ^{115}Lys-Asn^{121}-Lys^{124} and interestingly the same peptide that had previously been identified unmodified in this fraction. The glycoforms represent this sequence bearing the aforementioned complex carbohydrate with zero, one, and two neuraminic acids. Thus, this consensus site occurs in 4 different forms (*see* **Note 16**).

The full scan mass spectrum of fraction 2 was analyzed as described earlier. Its glycopeptide-component was identified as ^{116}Asn-Asn^{121}-Lys^{124} bearing the same oligosaccharides as reported for fraction 1 (data not shown).

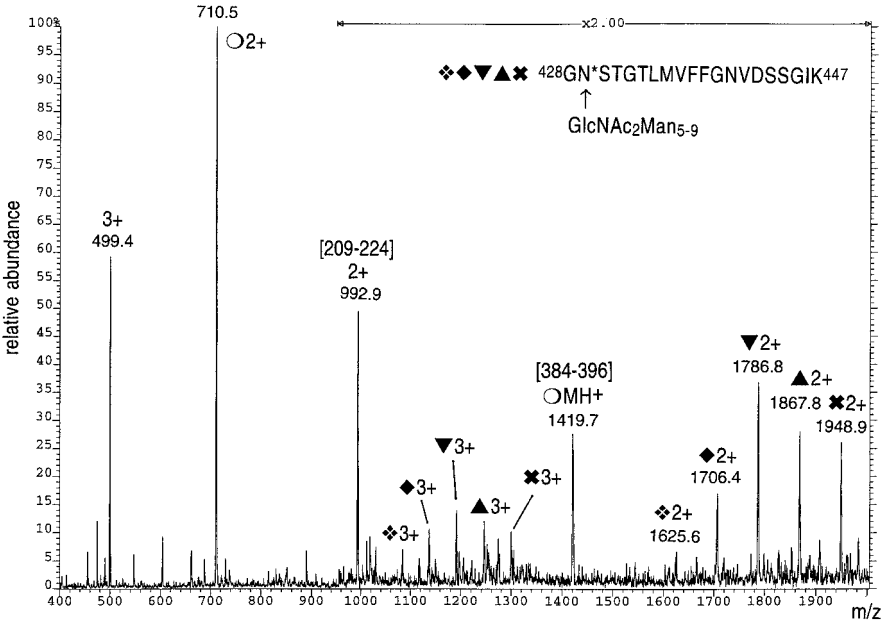

Fig. 3. Electrospray mass spectrum of the third glycopeptide-containing peak from the tryptic digest of the 73 kDa protein of rhFVIIIa (**Fig. 1**). These data were acquired at normal cone voltage. Reprinted with permission from **ref. *25***. Copyright (1997) American Chemical Society.

Figure 3 shows the electrospray mass spectrum of fraction 3. Let us focus only on the glycopeptides. A series of doubly charged ions was detected in this fraction that differed by approx 81 Dalton (the corresponding triply charged ions are present too, and labeled accordingly). When multiplied by 2, this number yields the residue weight of a hexose unit (**Tables 1** and **2**), which together with the lack of sialic acids seems to suggest the presence of an oligomannose-modified peptide. To identify the modified sequence, the predicted molecular masses of the unmodified tryptic peptides with the potential glycosylation sites ("consensus" peptides): 1204.7 [115–124], 1186 [116–124], 2666.3/2619.3 [309–331] with S-β-4-ethylpyridyl (PE) or carboxymethyl (CM) Cys, 2031.0 [428–447] and 2386.0/2291.9 [475–494] with PE or CM-Cys, respectively, were subtracted from the MH^+ values determined for the glycopeptides: 3247.0, 3410.2, 3571.3, 3733.9, and 3896.5 Dalton. The resulting mass differences revealed the potential additional masses of the carbohydrates. This number must be bigger than 892 Dalton (core structure). First, let us suppose that the protein was modified with one of the known oligosaccharides (*see* **Table 3**; **Note 17**). Results of the subtractions were compared with masses corresponding to asialo

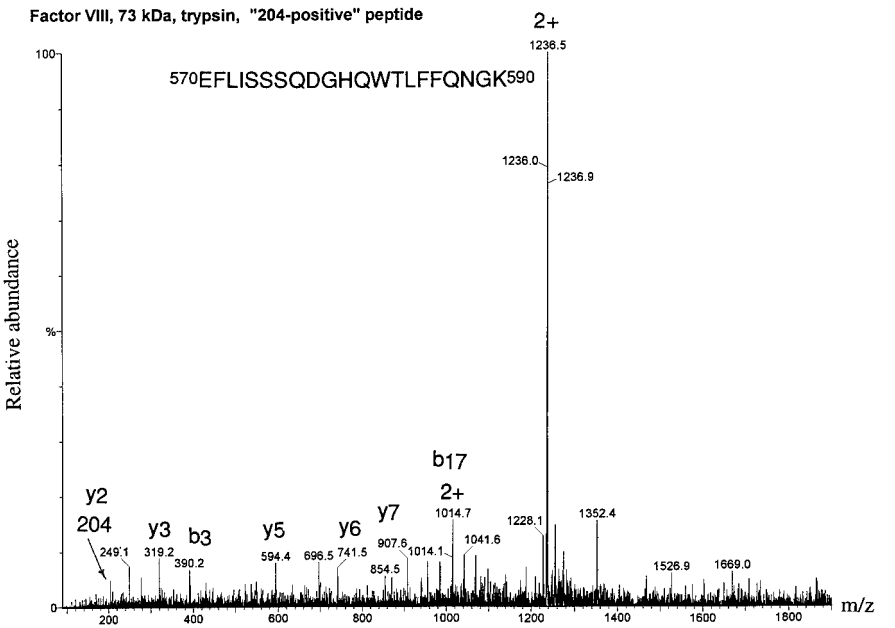

Fig. 4. Electrospray mass spectrum of the last 204-positive peak from the tryptic digest of the 73 kDa protein of rhFVIIIa (peak 4 in **Fig. 1**). These data were acquired at high cone voltage. Peptide fragments are labeled according to Biemann ***(31)***. All peptides with Gly-Lys C-terminus may yield a y_2 ion at m/z 204.

carbohydrate structures commonly described for mammalian glycoproteins (**Table 3**). From this analysis the presence of ^{428}Gly-Lys^{447} bearing Man_5-Man_9 oligomannose structures was established. Indeed, FACE-analysis identified these carbohydrates present in the carbohydrate pool ***(9)***.

The electrospray mass spectrum of chromatographic peak 4 (**Fig. 4**) exhibits no obvious glycopeptide ion series, only a doubly charged ion at m/z 1236.0. MH^+ calculated from this ion yields 2471.0, in good agreement with the predicted mass for peptide Glu^{570}-Lys^{590}. It is apparent that the ion at m/z 204 in this fraction did not come from carbohydrates. Under conditions that induce in-source carbohydrate fragmentation some peptides may fragment as well. Peptide Glu^{570}-Lys^{590} yielded a series of fragments (**Fig. 4**) ***(31)***. Fragment y_2 formed via peptide bond cleavage at the N-terminus of the penultimate Gly-residue, with the charge retained on the newly formed dipeptide is at m/z 204. All peptides with GlyLys C-terminus may yield this ion, and thus are potential false carbohydrate-positives.

LC/ESIMS analysis combined with SIM of diagnostic carbohydrate ions is of only limited utility for highly glycosylated proteins. Thrombin digestion

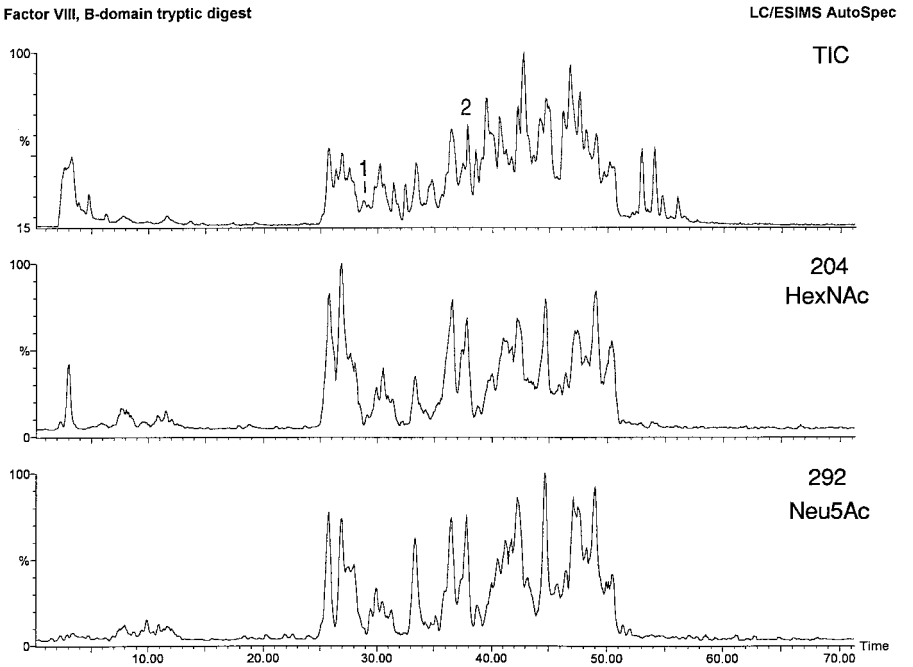

Fig. 5. Total ion chromatogram and selected ion chromatograms of diagnostic carbohydrate ions, m/z 292 (Neu5Ac) and 204 (HexNAc), acquired during high cone voltage LC/ESIMS analysis of a tryptic digest of the B-domain of rhFVIIIa. Reprinted with permission from **ref. *25***. Copyright (1997) American Chemical Society. Electrospray spectra of peaks labeled 1 and 2 are shown in **Figs. 6** and **7**.

of Factor VIII yields also the B-domain, a 909 amino acid protein featuring 20 consensus sequences for N-glycosylation. This protein also has been reported O-glycosylated. The comparison of SIM and TIC chromatograms of its tryptic digest rather reveals which fractions do *not* contain glycopeptides (**Fig. 5**). Unfortunately, glycopeptides that display strong SIM signals, frequently produce ions of low abundance in the full-scan spectra, especially in comparison to the signals of coeluting peptides. This may be partly because of their substoichiometric quantities due to the carbohydrate heterogeneity. The electrospray spectrum of a carbohydrate-positive fraction (**Fig. 6**; peak 1 in **Fig. 5**) showed a series of weak doubly charged signals at m/z 1676.3, 1773.3, and 1894.9. MH^+ values were calculated as 5026.9, 5317.9, and 5682.7, respectively. Subtracting the molecular masses of the predicted "consensus" tryptic peptides, peptide 513–520 was identified, doubly glycosylated at Asn-515 and -519 with two biantennary core-fucosylated oligosaccharides and two sialic acids, and a bi- and a triantennary core-fucosylated oligosaccharide

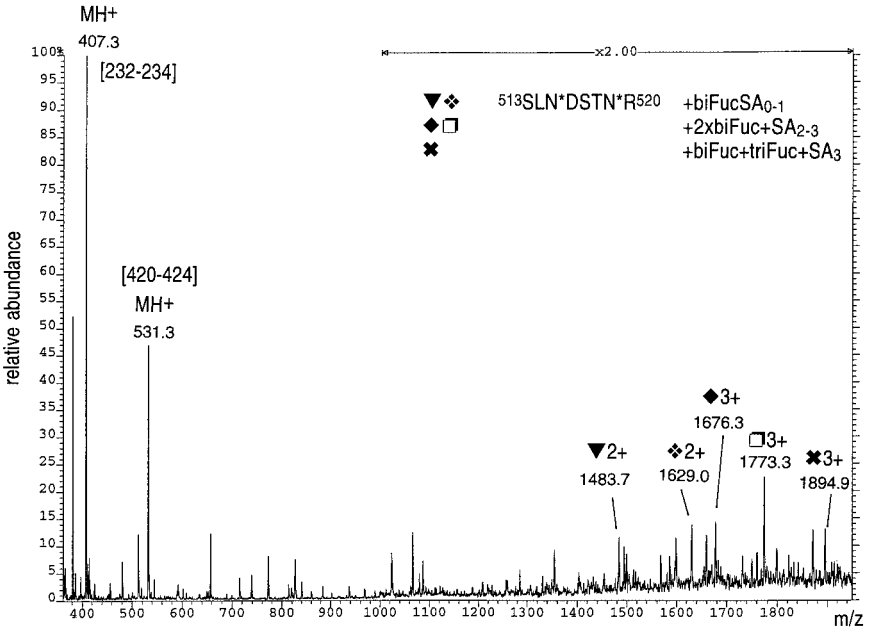

Fig. 6. Electrospray mass spectrum of the fraction labeled 1 in **Fig. 5**. These data were acquired at normal cone voltage. For simplicity only the discussed glycopeptide ions are labeled in the high mass region with their monoisotopic mass, and charge state. The peptide is modified with bi- and tri-antennary core-fucosylated oligosaccharides. From these data in cannot be determined wich structures are sialylated.

and three sialic acids, respectively. From these data, it cannot be determined which site bears the triantennary structure, or the location of the sialic acids. A tryptic digest of the B-domain was analyzed by LC/ESIMS after PNGase digestion ***(25)***. PNGase removes the N-linked carbohydrates while transforming the modified Asn into Asp, increasing the MH^+ value for the unmodified peptide by 1 Dalton per N-linked site. Peptide 513–520 gave a double peak showing both 1 and 2 Dalton molecular-weight increases. The glycoforms described earlier contained both sites modified. Careful inspection of the full-scan data revealed the presence of two previously overlooked doubly charged ions at m/z 1483.7 and 1629.0, glycoforms with one monosialo and disialo biantennary core-fucosylated oligosaccharide, respectively (**Fig. 6**). **Figure 7** illustrates the complexity of the digest mixture (peak 2 in **Fig. 5**). Abundant ion series representing different glycoforms were identified from mass differences reflecting the carbohydrate heterogeneity as discussed previously. Because the high mass resolution allowed the charge determination of the peaks, even minor glycoforms represented by a single ion could be identified quite convincingly.

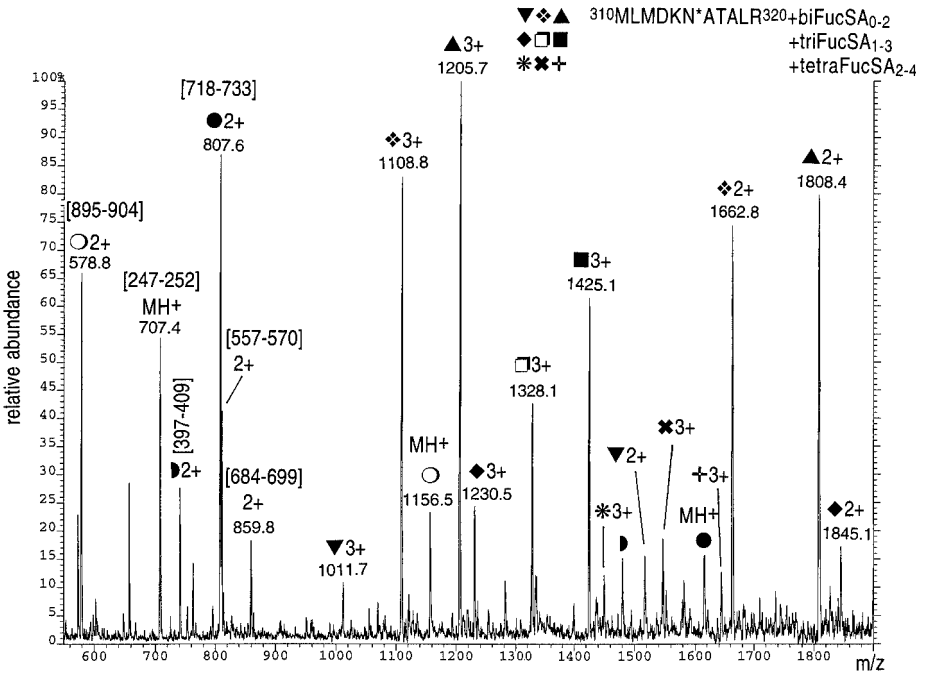

Fig. 7. Electrospray mass spectrum of the fraction labeled 2 in **Fig. 5**. These data were acquired at normal cone voltage. Reprinted with permission from **ref. *25***. Copyright (1997) American Chemical Society.

Obviously, these experiments determined only the masses of the carbohydrate(s) at any given site, and yielded some information on the carbohydrate heterogeneity. The more accurate the mass measurement, the more precisely the composition of the oligosaccharide could be determined. However, the identity of these residues and their linkage and stereochemistry cannot be deduced from these data. For rhFVIIIa the enzymatically freed carbohydrate pool was analyzed by FACE-analysis ***(9)***, the results of which were combined with LC/ESIMS results to draw conclusions about the carbohydrate structure at any given site. However, glycopeptide-containing fractions can be collected on-line at the splitting tee and subjected to exoglycosidase digestions and consequent mass spectrometric analyses that may reveal information about the identity and linkage of the saccharide units. **Table 4** shows the specificity and optimal digestion conditions of some exoglycosidases recommended for these analyses. **Figure 8** illustrates the results that can be expected from these experiments with a glycopeptide as isolated from a bovine fetuin tryptic digest (panel A), then treated with neuraminidase (panel B) and β-galactosidase (panel

C) ***(23)***. Panel A shows the glycopeptide bearing disialo biantennary and di-, tri- and tetrasialo-triantennary oligosaccharides. As expected neuraminidase digestion yielded only two components, when all the sialic acids were removed, a biantennary and triantennary glycoform (panel B). N-linked carbohydrates of bovine fetuin have been studied extensively. The Gal-units are mostly in β(1,4)GlcNAc linkages, but it has been reported that the tetrasialo triantennary structure also contains Galβ(1,3)GlcNAc linkages (~20%)***(23,32,33)***. The β-galactosidase (from *Streptococcus pneunomiae*) used for the second digestion step was expected to cleave only the Galβ(1,4)GlcNAc linkages, yielding three components: biantennary glycoform-2 × 162 Dalton, triantennary glycoform-3×162 and –2×162 Dalton (~20%). The carbohydrates were cleaved as expected. However, one additional component was detected due to some exopeptidase activity exhibited by the β-galactosidase preparation (**Fig. 8**, panel C).

3.4.2. Complete Analysis of LC/ESIMS Data

1. Consider TIC peaks as chromatographic fractions and print out their corresponding full-scan mass spectra.
2. Determine the MH^+ values for every signal more abundant than approx 5% of the base peak. Identify the peptides by comparing these values to the MS-digest mass list (*see* **Note 18**).
3. Search more thoroughly for missing peptides using selected ion extraction: use a ~2 Dalton window around the m/z value of interest or less depending on the mass accuracy.
4. Prepare a summary of your data, including sequence coverage, peptides still missing, relatively abundant MH^+ values unaccounted for. In this process consider missed alkylation of Cys residues, trypsin-autolysis (peptides of MH^+ at m/z 842.5, 1045.6, and 2211.1 are the most frequently observed), chymotryptic cleavages, and other contaminating proteins. One of the most frequent side-reactions, the oxidation of Met-residues, is included in MS-digest.

Discussing the complete interpretation of LC/ESIMS data for the tryptic digest of the 73 kDa protein of rhFVIIIa goes beyond this chapter. However, only two glycosylation sites were characterized earlier, and two other potential glycosylation sites, Asn-330 and -483, were unaccounted for when only the carbohydrate-positive peaks were analyzed. Data processing software allows creation of the ion chromatogram of any given mass (ion extraction), thus the LC/ESIMS data can be searched for any predicted peptides. Doubly, triply charged, and so on, masses of peptides are also calculated for this search. If the relative abundance of ions at this m/z value was changing during the LC/ESIMS analysis, ion extraction will produce some “chromatographic” peaks. Then full-scan data of these chromatographic regions should be reviewed. If the full-scan data show ions of other charge states of the same species, its pres-

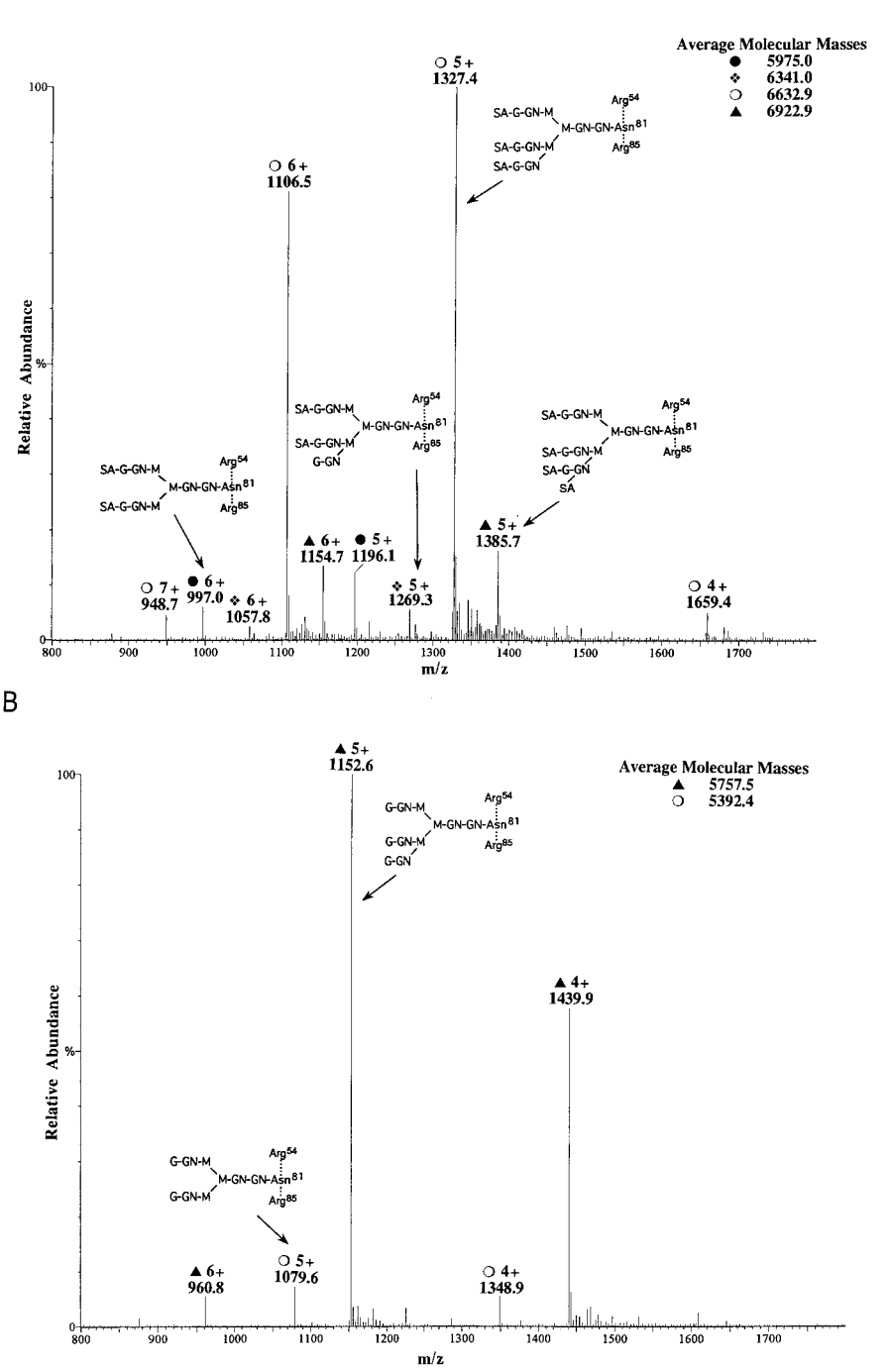
A
Average Molecular Masses
5975.0
6341.0
6632.9
6922.9
○ 5+ 1327.4
○ 6+ 1106.5
▲ 6+ 1154.7
● 5+ 1196.1
❖ 5+ 1269.3
▲ 5+ 1385.7
○ 7+ 948.7
● 6+ 997.0
❖ 6+ 1057.8
○ 4+ 1659.4
Relative Abundance
m/z
B
Average Molecular Masses
▲ 5757.5
○ 5392.4
▲ 5+ 1152.6
▲ 4+ 1439.9
▲ 6+ 960.8
○ 5+ 1079.6
○ 4+ 1348.9
Relative Abundance
m/z

Fig. 8.

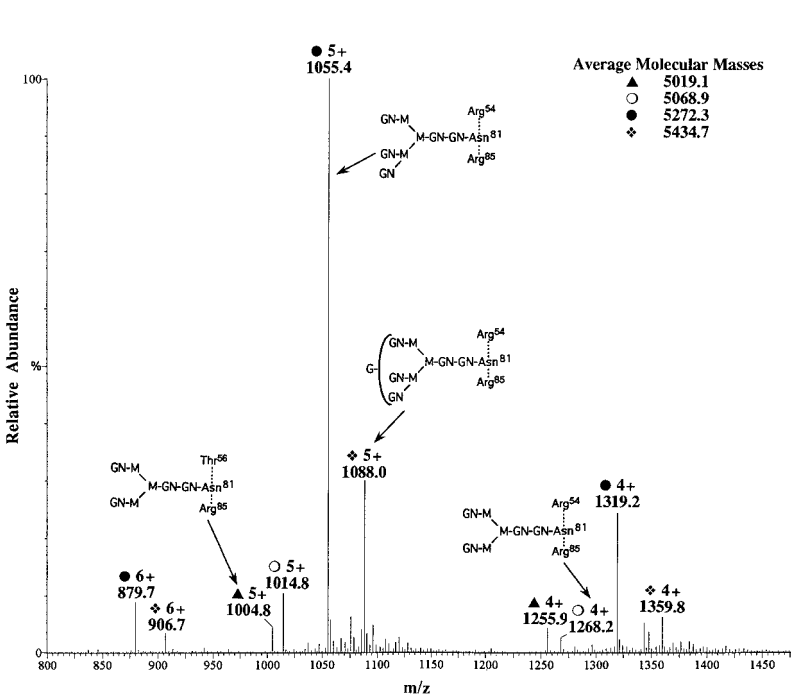

Fig. 8. *(continued)* Electrospray mass spectrum of bovine fetuin tryptic glycopeptide ^{54}Arg-Asn^{81}-Arg^{85}, as isolated from a digest **(A)**; after neuraminidase digestion **(B)**, and after β-galactosidase (from *Streptococcus pneunomiae*, *see* **Table 4**) digestion **(C)**. The N-linked carbohydrate at this site contains approx 20% Galβ1-3GlcNAc, and the enzyme is specific for Galβ1-4GlcNAc linkage. Reprinted with permission from **ref. *23***.

ence is confirmed. If the extracted ion is the only signal from a peptide, it should show an abundance comparable with some other components identified unambiguosly (*see* **Note 18**). Based on these criteria "consensus" peptides Val^{309}-Lys^{331} and Met^{475}-Lys^{494} were both observed unmodified: giving MH^+ values 2665.9/2619.1 and 2386.3/2292.4 with PE- and CM-Cys, respectively. For additional confirmation, the CM-Cys peptides eluted slightly earlier than the PE-Cys peptides, showing similar retention time differences as other Cys-containing peptides in the digest.

4. Notes

1. The numbers listed here are ions diagnostic of common oligosaccharide structures shared by a wide variety of species. However, any structure that yields a characteristic fragment of relatively unique m/z value can be monitored. For

example, m/z 803 was monitored when tracing sialyl Lewis^x antigen on α_1-acid glycoprotein ***(27)***. The mass of any B-type oxonium ion can be calculated by adding together the residue weights of its components + 1 (H) (**Table 1**).

2. Since the different glycoforms are present at substoichiometric quantities, and the glycopeptide-containing fractions may have to be recollected, further digested, and reanalyzed, if there is enough sample available reduce, alkylate, and digest at least 1 nmole glycoprotein.

   When preparing samples for mass spectrometry experiments one has to avoid involatile salts, or they must be removed thoroughly by dialysis or chromatography. Salts interfere with mass spectrometric detection and they may clog the capillaries.

   Detergents may form micelles and thus may remain in the sample even after dialysis. Due to their exceptional mass spectrometric response and evenly spaced ion series, several detergents can be used for calibration, but they will completely suppress every other signal.
3. The proteolytic enzyme used for digestion should separate the consensus sequences into individual peptides and must show some cleavage specificity so that the molecular masses for the cleavage products can be predicted. Trypsin, endoproteases Lys-C and Asp-N are considered the most reliable enzymes in this regard. However, if digestion periods are too long and too much enzyme is employed, any of these may cleave at nonspecific sites, and/or undergo autolysis.
4. Using a water/acetonitrile/TFA mobile phase for the HPLC separation provides the best chromatographic resolution, however TFA adversely affects mass spectrometric sensitivity. Addition of the make-up solvent, 2-methoxyethanol:isopropanol 1:1 mixture, significantly (5–10-fold) increases the detection sensitivity for hydrophilic components eluting at high water concentrations.

   Another HPLC mobile phase: solvent A: 0.1% formic acid in water, solvent B: 0.08% formic acid in ethanol/propanol (5:2), yields comparable chromatographic resolution while displaying improved ESIMS detection sensitivity without any make-up solvent addition. In this solvent system glycoforms may elute separated, and in reverse order, the most sialylated featuring the longest retention time. In addition, hydrophobic peptides and glycopeptides may give wide, tailing peaks.
5. The splitting ratio depends on the optimal flow rate for the electrospray source of the mass spectrometer used as detector.
6. Other mass spectrometers equipped with an electrospray source also may be coupled with HPLC. Magnetic analyzers or orthogonal-acceleration-time-of flight instruments afford higher resolution and mass accuracy than the quadrupole mass spectrometers. Iontraps exhibit higher resolution and mass accuracy only in zoom-in mode for selected digest components.
7. Unfortunately, the optimal digestion period depends on the individual protein. For example, bovine fetuin is fully digested by trypsin (~1% w/w) in an hour, while other proteins require longer incubation times and even the addition of

denaturing agents, such as urea or organic solvents. Trypsin tolerates both. However, long incubation with urea may result in carbamoylation of the ε-amino groups of Lys residues as well as the newly formed N-termini. Similarly, longer incubation times or larger amounts of trypsin may lead to chymotryptic and other nonspecific cleavages and to enzyme autolysis, despite the side-chain protection.

8. Acidification of the mixture to stop the digestion may lead to loss of sialic acid.
9. Even the best-quality solvents used for the LC/ESIMS experiments produce abundant series of low mass ions. Thus, the low mass region of the mass spectra will be affected by this background. In most cases no significant background ions were observed at m/z values corresponding to diagnostic carbohydrate fragments.
10. This linear, approx 0.5%/min gradient, started at 5% B provides a good chromatographic separation for most of the digests. If there are concerns about the loss of hydrophilic peptides, the column should be equilibrated at 2% B. Similarly, when more hydrophobic peptides are present the final organic content of the eluant could be increased to higher level.
11. Many electrospray instruments permit alternate acquisition of high and low cone-voltage scans in a single LC/ESIMS analysis, although the AutoSpec did not. They also permit monitoring a series of diagnostic masses, hence the name selective ion monitoring, usually at higher sensitivity, rather than scanning the entire mass range.

    The voltage settings required for in-source fragmentation depend on the electrospray source and the mass spectrometer, as do all the other tuning parameters.
12. Either the sequence of the protein can be imported from a word processing program, or for Protein Prospector MS-digest its database accession number is sufficient information.
13. Remember, that mass spectrometers separate ions according to their mass-to-charge ratios, i.e., m/z values. Thus, stable isotope pacing can be used for charge-state determination. The isotope peaks of a singly charged ion are separated by approx 1 Dalton, while this mass difference is reduced to 0.5 Dalton for doubly charged ions: $MH^{2+}/2$ is measured, and so on. The monoisotopic ion is the lowest mass member of this cluster, containing only ^{12}C, ^{1}H, ^{14}N, ^{16}O, ^{32}S, and so forth, atoms. Monoisotopic masses are different from values calculated from the chemical atomic weights that are a weighted average for all the isotopes. Average masses are measured when the isotope peaks are not separated.
14. **Table 1** shows the elemental composition and mass of some oligosaccharide units frequently encountered in glycoproteins.
15. For low-resolution mass spectrometers these carbohydrate unit differences (**Table 2**) can aid the charge-state determination. For example, complex structures almost always exhibit heterogeneity in the number of neuraminic (sialic) acids or the antennae. Thus, one can look for mass differences that would separate differently sialylated structures at different charge states. For example, a 97 Dalton mass difference would indicate triply charged ions, while the differently sialylated

quadruply charged ions will be separated by approx 73 Dalton. A similar approach can be used to trace other carbohydrate heterogeneities. **Table 2** below lists multiply charged mass differences corresponding to different carbohydrate structures.

16. In general, mass spectrometry is not a quantitative method unless careful measurements are made with known quantities of internal standards. However, approximate relative amounts of different glycoforms may be estimated from the relative abundances, taking all the glycopeptide ions into consideration.
17. Unusual carbohydrate compositions can be dealt with using a simple computer program that generates the oligosaccharide compositions of any given mass. A Macintosh program, Gretta Carbos, written by Wade Hines is available by writing to Professor B.W. Gibson: gibson@socrates.ucsf.edu. However, elucidating the structure of unusual oligosaccharides is beyond this chapter, although the steps described here may serve as starting point for that research.
18. Deciding whether or not a peptide is really present sometimes requires judgement to be made by the analyst. If the signals are of low abundance, independent proof or repeated analysis is desirable, especially with mass spectrometers giving low-resolution, lower-mass accuracy data.

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9

Monitoring Glycosylation of Therapeutic Glycoproteins for Consistency Using Highly Fluorescent Anthranilic Acid

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1. Introduction

Understanding the structure and function of proteins is a major area of interest and significance in biochemistry. Many proteins frequently reported in scientific journals are glycosylated. The oligosaccharide chains attached to the proteins may elicit important functional or structural properties ***(1,2)***. The characterization of oligosaccharide chains in addition to its protein part is important in order to fully understand the structure and function of glycoproteins. There are cases where either no apparent function is obvious or not yet identified for the carbohydrate part. However, characterization of carbohydrate units is necessary to define the therapeutic glycoprotein structure for regulatory purposes. This may include studying the extent of glycosylation in terms of its monosaccharide composition (including sialic acids and percent carbohydrate) and the nature of oligosaccharide chains.

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) had been the method of choice for carbohydrate analysis ***(3)***. This method does not require derivatization but requires specialized dedicated equipment. However, it has many disadvantages, e.g., high noise, unstable baseline, high pH, high salt, and is not very user-friendly, to name a few. An additional problem with HPAEC-PAD is the interference of amino acids, peptides, and other oxidizable compounds in the carbohydrate analysis ***(4)***. High salt present in the eluents precludes the use of HPAEC-PAD in recent techniques such as liquid chromatography-mass spectrometry

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(LC-MS) and liquid chromatography-nuclear magnetic resonance (LC-NMR). Often, resolution of oligosaccharides is a problem with HPAEC-PAD in cases where a high level of carbohydrate microheterogeneity exists. Recently, a number of methods based on fluorescent labeling of carbohydrates to address some of these problems have been introduced (for a short review, *see* **ref. *5***).

Oligosaccharide analysis with fluorescent detection provides a great improvement over HPAEC-PAD in terms of sensitivity (~100–200×) and resolution ***(6)***. In addition, it does not require a dedicated instrument. Oligosaccharide mapping using anthranilic acid (AA, 2-aminobenzoic acid) derivatization is specific for reducing monosaccharides and oligosaccharides, thus precluding the need to purify glycans from peptides or proteins prior to chromatography ***(6)***. It is applicable to all types of glycans as long as a reducing end is available for derivatization. N-linked oligosaccharides can be conveniently released with the enzyme PNGase (A or F), and both N- and O-linked oligosaccharides can be released using hydrazinolysis ***(7)***. We report two chromatographic methods for high-resolution analysis of glycans released from glycoproteins ***(6)***. The mapping/profiling method offers a high-resolution map in which the degree of sialylation, desialylated complex, and high mannose type of oligosaccharides can be identified in a single run. The second method is mainly used for further characterization of oligosaccharides following desialylation. In this method, branch and linkage isomers are easily separated, and the method can be used for determining the sequence of sugars following treatment with an array of glycosidases.

Oligosaccharide mapping/profiling following derivatization with AA affords very high-sensitivity and high-resolution technology for studying glycoproteins. In fact, to date, the highest resolution of sialylated as well as neutral oligosaccharides has been achieved by oligosaccharide mapping with AA technology. For example, in contrast to AA analysis, high-mannose and asialo-complex glycans from Chinese hamster ovary (CHO) cell derived rIgG coeluted in HPAEC-PAD analysis, which may lead to erroneous conclusions. Such HPAEC-PAD profiles may be interpreted as a “normal” distribution of glycans in a rIgG and the presence of high-mannose structures can go undetected. However, in the AA map the high mannose and asialo-complex oligosaccharides are easily discernible since they elute as separate sharp peaks ***(6)***. In addition, the fluorescent-mapping method can be used for oligosaccharide analysis of the glycoproteins run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels preferably after electroblotting onto polyvinylidene difluoride (PVDF) membranes in a manner similar to monosaccharide analysis ***(8)***.

The oligosaccharide methods described here are compatible with physical techniques (e.g., MS and NMR), since the AA mapping method uses only volatile organic solvents. The oligosaccharide fractions can be isolated simply

by evaporation for further analysis, e.g., MALDI-TOF ***(6)***. The AA derivatized oligosaccharides can be easily analyzed by MALDI-TOF in either mode for neutral and acidic glycans using 2,5-dihydroxybenzoic acid (DHB) as a matrix. MALDI-TOF has also been used for more detailed structural studies in conjunction with exo-glycosidase treatments without the need to remove buffers or salts.

The fluorescent AA tag was reported to be at least twice as intense as its amide derivative, 2-aminobenzamide (AB) ***(6)***. In fact, among all fluorescent tags tested to date ***(9)***, AA is the most sensitive tag of choice for oligosaccharide analysis. In addition, the AA derivatized oligosaccharides are stable for over 3 yr at –20°C. The reagents and solvents used in the method have a "shelf-life" of several months. The mapping solvents (A and B), washing solution (95% acetonitrile in water), eluting solution (80% water in acetonitrile), all stored at room temparature, and AA reagent, stored at –20°C, for more 5 mo gave oligosaccharide maps that were comparable to those obtained with freshly made reagents and solvents.

Monitoring of production lots or in-process samples for glycosylation using the AA technology is done by comparison of their profiles with a well-defined reference standard. In order to pass the test, all peaks in the sample are expected to co-elute with the corresponding peaks in the reference standard within a set of chromatographic runs. Acceptance criteria are set such that high confidence in the results is assured. For example, ±0.25 min standard deviation for retention times is used for peaks in the experimental sample compared to corresponding peaks in the reference standard. Additionally, relative standard deviation (RSD) for percent area of peaks is set at less than 15% for the main peaks. Main peaks are defined as peaks with relative percent area greater than 10% of total area of all the glycan peaks. All main peaks should have resolution greater than 1.0 and tailing less than 1.4. In addition to retention time, resolution and tailing are important parameters for monitoring system performance and are used in all the analysis. Often, additional peaks are encountered in the map and they require further characterization, for example, by using mass spectrometry, co-elution, and/or exo-glycosidase studies. However, these may not necessarily present a problem for the release of clinical materials if the amounts are below a previously set criteria.

For detailed characterization of CHO cell-derived IgGs by high-performance liquid chromatography (HPLC), commercially available sialyl- and asialo-complex glycans with core fucose and oligosaccharides from well-defined human IgG can be used as standards. For IgGs that contain high-mannose structures in addition to complex glycans, RNase B glycans can be used as a reference, either alone or in combination with the standards suggested earlier. Some oligosaccharide standards are not easily available, such as the

monogalactosyl complex glycan, and oligosaccharides with defined sialic acid linkages (2–3 vs 2–6), etc. In rIgGs, there are two isomers resulting in the presence of a galactose on either arm of the biantennary oligosaccharide chain. Interestingly, these isomers are easily separated by normal-phase HPLC method described earlier ***(6)***. Thus, each can be isolated, characterized by NMR or mass spectrometry for subsequent use as a glycan standard. For sequence and linkage determinations, specific exo-glycosidases are now available that can distinguish various linkages in an oligosaccharide chain. NMR and mass spectrometry offer a definite proof of structure but require a level of expertise that may not be available in most biopharmaceutical companies or biotechnology laboratories.

The AA oligosaccharide mapping method has also been in use in other laboratories, and tested for most validation parameters. It passed rigid acceptance criteria set for accuracy, precision, reproducibility, robustness, ruggedness, and so on. In addition, it has been rigorously tested in different laboratories by different analysts for monitoring consistency of carbohydrates in various glycoprotein drug lots as part of regulatory submissions. Oligosaccharide mapping with AA tag is routinely used in conjunction with monosaccharide composition analysis ***(10–12)*** for comparison of various production lots intended for human use.

Furthermore, the technology described here is simple in terms of derivatization of saccharides with fluorescent AA and analysis by HPLC. Therefore, any biochemist with an access to HPLC system with a fluorescence detector can successfully analyze the glycoprotein of interest.

2. Materials

2.1. Preparation of the Oligosaccharides Using rIgG as an Example

1. Fetuin, bovine (Sigma).
2. Polypropylene vials (1.6 mL) with O-ring seals (Fisher, cat. no. 118448).
3. 1:100 Diluted ammonium hydroxide (0.15 *M*, prepare fresh for each use).
4. 10% (w/v) SDS stock solution.
5. Diluted ammonium hydroxide-0.5% SDS-1% 2-mercaptoethanol solution for PNGase F digestion (prepare fresh for each use from respective stock solutions).
6. Heating Block.
7. 5% (w/v) Nonidet P-40.
8. Peptide N-glycosidase F (Glyko).

2.2. Derivatization and Purification

1. Acetic acid, glacial.
2. 4% (w/v) Sodium acetate trihydrate and 2% (w/v) boric acid in methanol.

3. Anthranilic acid reagent: 30 mg/mL anthranilic acid (Aldrich) and approx 20 mg/mL sodium cyanoborohydride in acetate-borate-methanol solution. Although it is stable for a week in the dark at ambient temperature, it is recommended that this reagent be prepared fresh. NOTE: Sodium cyanoborohydride is a poison and tends to absorb moisture readily from the air, which may affect the derivatization reaction. Limit the exposure of this chemical to air when weighing.
4. Heating block.
5. 3- or 5-mL Plastic syringe with luer lock.
6. Nylon Acrodisc syring filter, 0.45 μm (Gelman, cat. no. 4438).
7. 95% (v/v) Acetonitrile in water.
8. 20% (v/v) Acetonitrile in water.

2.3. Chromatography

1. HPLC with fluorescence detector (highly sensitive fluorescence detectors such as Jasco FP 920, Waters 474, and HP 1100 were used in these studies).
2. Thermostatted column compartment.
3. Polymeric-amine bonded HPLC column, 0.46 × 25 cm (Astec, cat. no. 56403 or Asahipak, NH2P-50 4E, Phenomenex, cat. no. CHO-2628) for mapping.
4. TSK Amide-80 column, 0.46 × 25 cm (Toso Haas, cat. no. 13071) for neutral oligosaccharide analysis.
5. Column prefilter with column prefilter insert (0.2 μm).
6. Autosampler vials (amber).
7. Eluent A = 2% (v/v) acetic acid and 1% (v/v) tetrahydrofuran (inhibited) in acetonitrile (all HPLC grade).
8. Eluent B = 5% (v/v) acetic acid, 1% (v/v) tetrahydrofuran (inhibited) and 3% (v/v) triethylamine (all HPLC grade) in Milli-Q filtered water.
9. Eluent C = 0.2% (v/v) acetic acid in acetonitrile (all HPLC grade): for neutral oligosaccharide chromatography.
10. Eluent D = 0.2% (v/v) acetic acid and 0.2% (v/v) triethylamine (all HPLC grade) in Milli-Q water: for neutral oligosaccharide chromatography.

3. Methods

3.1. Preparation of the Oligosaccharides Using rIgG as an Example

1. Dilute the rIgG experimental, reference standard and fetuin to about 5 mg/mL in Milli-Q water.
2. Appropriately label one 1.6 mL polypropylene freeze vial for each blank, sample, and fetuin.
3. Place 5 μL of the diluted samples and fetuin into the corresponding labeled vials.
4. Prepare a blank by placing 5 μL of Milli-Q water into the corresponding labeled vial.
5. Add 30 μL of the ammonium hydroxide-SDS-2-mercaptoethanol solution to each vial. Cap the vials and mix them on a Vortex mixer (*see* **Note 1**).
6. Heat the vials in a heating block set at 100°C for 2–3 min to denature the proteins. Remove the vials and allow them to cool to room temperature.

7. Add 5 µL of 5% Nonidet P-40 to each vial and mix the vials using a Vortex mixer.
8. Add 2 µL of the PNGase F to each vial and gently mix by aspiration in and out of the pipet tip.
9. Place the vials in a heating block that is set at 37°C for about 18 h (overnight digestion is sufficient for most glycoproteins).

3.2. Derivatization and Purification

1. Allow the blank, samples, and fetuin to cool to room temperature. Briefly centrifuge the vials at the maximum setting in a microcentrifuge to collect the solutions at the bottom of the tubes (*see* **Note 2**).
2. Add 2 µL of glacial acetic acid to each vial and mix.
3. Add 100 µL of the anthranilic acid reagent to each vial. Cap the vials tightly and mix.
4. Heat the vials for 1 h in a heating block that is set at 80°C. Allow the vials to come to room temperature before proceeding.
5. Cut one 3- or 5-mL plastic syringe with luer lock for each sample at 2-mL mark to use as a funnel for Nylon syringe filters.
6. Plug the nozzle lightly with glass wool in order to prevent the formation of air bubbles during filtration.
7. Prime the Nylon syringe filters with about 2 mL of 95% acetonitrile-water using an uncut syringe.
8. Attach cut syringe to the filter and rinse the filters with additional 2 × 1 mL of 95% acetonitrile using gravity flow.
9. Dilute the oligosaccharide reaction mixture with 1.0 mL of 95% acetonitrile and vortex.
10. Transfer the diluted reaction mixture into the precut syringe and allow it to flow through the Nylon filter. The oligosaccharides will bind to the Nylon filter membrane.
11. Wash the bound oligosaccharides only two times with 1.0 mL of 95% acetonitrile. Discard the flow-through and washes (*see* **Notes 3** and **4**).
12. Appropriately label one autosampler vial for each sample.
13. Elute the bound oligosaccharides with 2 × 0.5 mL of 20% acetonitrile into the appropriately labeled autosampler vial and mix. Inject an aliquot of 50–200 µL for analysis.
14. Chromatographic runs are serially arranged as follows: blank, fetuin, reference standard, and experimental samples.

3.3. Chromatography

1. For mapping, freshly prepare eluents A and B.
2. For mapping use the gradient program as follows (Astec or Asahipak column at 50°C, flow rate of 1.0 mL/min and detection at λ_{ex} = 360 nm and λ_{em} = 425 nm):
   0 min, B = 30% (A = 70%)
   2 min, B = 30%
   82 min, B = 95% (linear increase)

97 min, B = 95% (Wash)
97.1 min, B = 30%
112.1 min, B = 30% (Equilibration) (*see* **Note 5**).

3. Prepare eluents C and D for neutral oligosaccharide chromatography.
4. For neutral oligosaccharide chromatography run the gradient program (Amide column at 25°C, flow rate of 1.0 mL/min and detection at λ_{ex} = 360 nm and λ_{em} = 425 nm) (*see* **Note 6**):

0 min, D = 28% (C = 72%)
20 min, D = 28%
80 min, D = 45% (linear increase)
80.1 min, D = 95%
90.1 min, D = 95% (Wash)
90.2 min, D = 28%
105.2 min, D = 28% (Equilibration)

3.4. Acceptance Criteria for Results

Suitability of the HPLC system for the analysis is deemed acceptable when the oligosaccharide map of bovine serum fetuin compares favorably to the representative chromatogram provided in **Fig. 1.** Fetuin glycan maps are routinely used as system check for good manufacturing practice (GMP) purposes. In the event of abnormal fetuin glycan map, the chromatographic runs are aborted for further investigation prior to lot analysis.

Test results are deemed acceptable when the oligosaccharide map of the experimental sample compares favorably to the reference standard. The retention times of the main peaks should have a standard deviation (SD) of less than 0.25 min and RSD of less than 15% for relative percent area in comparison to corresponding peaks in the reference standard. Main peaks are defined as peaks with relative areas more than 10% of total.

4. Notes

1. Dilute ammonium hydroxide can be used for PNGase F release of oligosaccharides from glycoproteins, and the yields from this procedure are similar to buffer systems provided by the suppliers precluding the need to prepare buffers. The profile of fetuin glycans released by PNGase F using dilute ammonium hydroxide is comparable to the hydrazine-released glycans (**Fig. 1**). However, the hydrazine released oligosaccharide mixture contains both N- and O-linked glycans, whereas the PNGase released oligosaccharides contain only the N-linked chains.
2. PNGase F reaction mixture and/or exo-glycosidase reaction mixture does not require additional purification prior to labeling. The Nylon purification step used for the removal of excess AA is sufficient to cleanup the samples (i.e., removal of SDS, Nonidet P-40, buffers, etc.) prior to chromatography.
3. Nylon purification is a crucial step that may cause loss of glycans due to excessive washing. Generally washing the filter with bound oligosaccharides with 95%

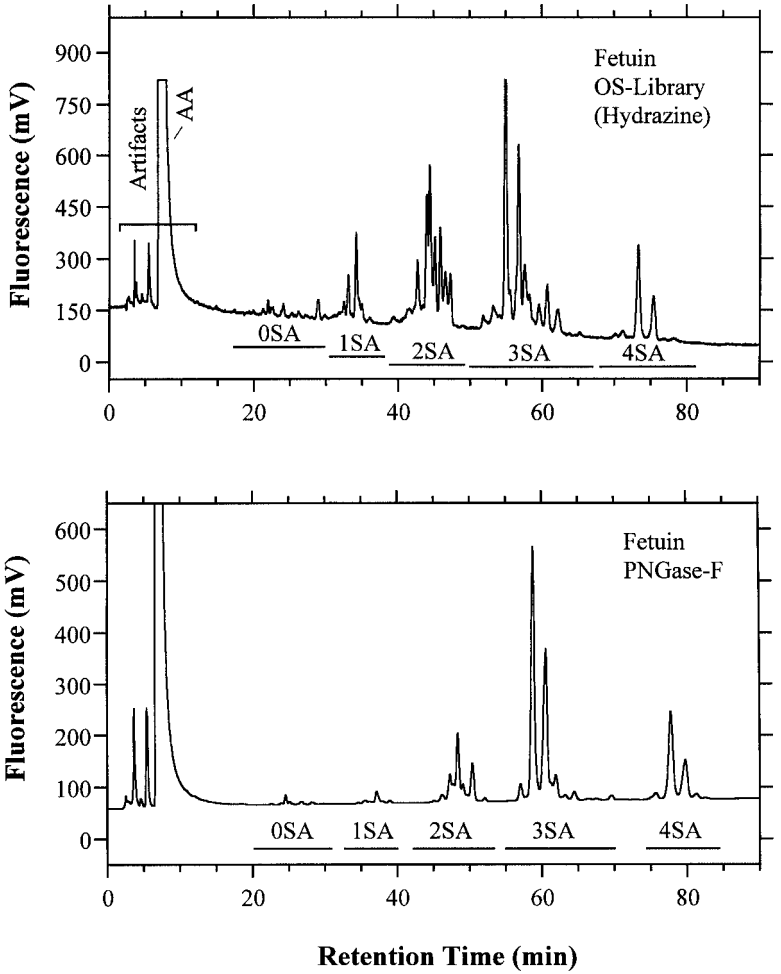

Fig. 1. Comparison of fetuin oligosaccharide maps: oligosaccharides released using hydrazine (top) and PNGase F (bottom). Bars indicate the regions where oligosaccharides with 0–4 sialic acids (SA) elute (*see* **Note 7**).

acetonitrile should be limited to two as suggested in the method, and elution with 0.5 mL × 2 is sufficient for most of the glycoproteins tested.

4. Oligosaccharides with less than four monosaccharide units are lost on the Nylon filter, and therefore, require direct analysis, i.e., without purification. This may be of importance to O-linked oligosaccharides where short-chain oligosaccharides are likely to be encountered. AA labeling has been successfully applied to O-linked oligosaccharides released from bovine submaxillary mucin (BSM) using hydrazine (**Fig. 6**). The O-linked glycan profile with and without purification is

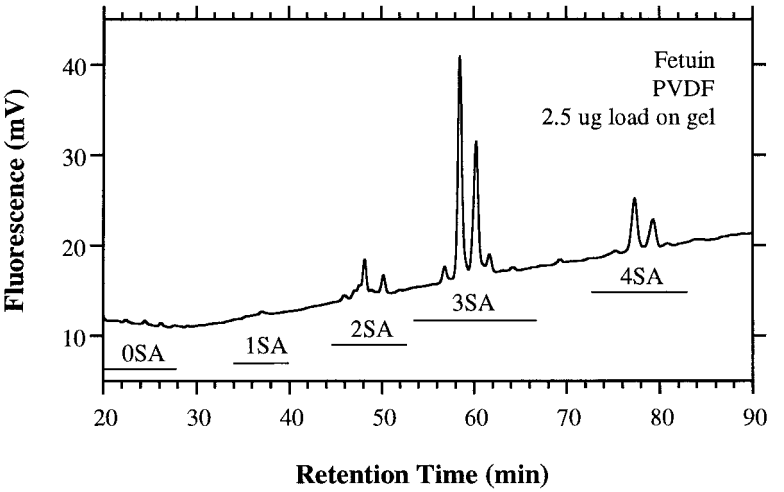

Fig. 2. Oligosaccharide map obtained with 2.5 μg of fetuin run on SDS-PAGE and electroblotted onto PVDF membrane. One of the three bands was used for PNGase F digestion (*see* **Note 8**).

similar in the case of BSM sugar chains, albeit the losses (>30%) encountered upon purification.

5. **Figure 3** shows the separation of neutral oligosaccharides on an amide column. The top two chromatograms are the maps of two different rIgG molecules produced in CHO cells. Although these two rIgGs have the same constant region of heavy chains, they differ in their oligosaccharide profile. Compared to rIgG-2, rIgG-4 has diminished amount of 2ACFG1 (biantennary complex with fucose and one galactose residue) and practically no 2ACF (biantennary complex with fucose) (*see* **Fig. 4** for structures). However, it has measurable amounts of high-mannose oligosaccharide structures, Man5-Man8, similar to the glycan chains from RNase B released by PNGase F (RNase B-NG). In addition, the two isomers of 2ACFG1 are easily separated in this chromatography, which in turn are easily differentiated from the Man6 glycan chain. Such resolution of oligosaccharide structures could not be obtained by HPAEC-PAD analysis.
6. For less sensitive detectors, the excitation wavelength can be changed to 230 nm.
7. The oligosaccharide profile obtained by the AA fluorescent-labeling procedure is similar to the ones obtained using HPAEC-PAD except for higher resolution, which has been discussed in detail earlier ***(6)***. The fetuin oligosaccharide profiles obtained with oligosaccharide released by hydrazine and PNGase F are shown in **Fig. 1**.
8. The oligosaccharide mapping technology can be applied to glycoproteins blotted onto PVDF membrane following SDS-PAGE or directly from gels after electroelution. Commercial fetuin typically resolves into three bands of equal

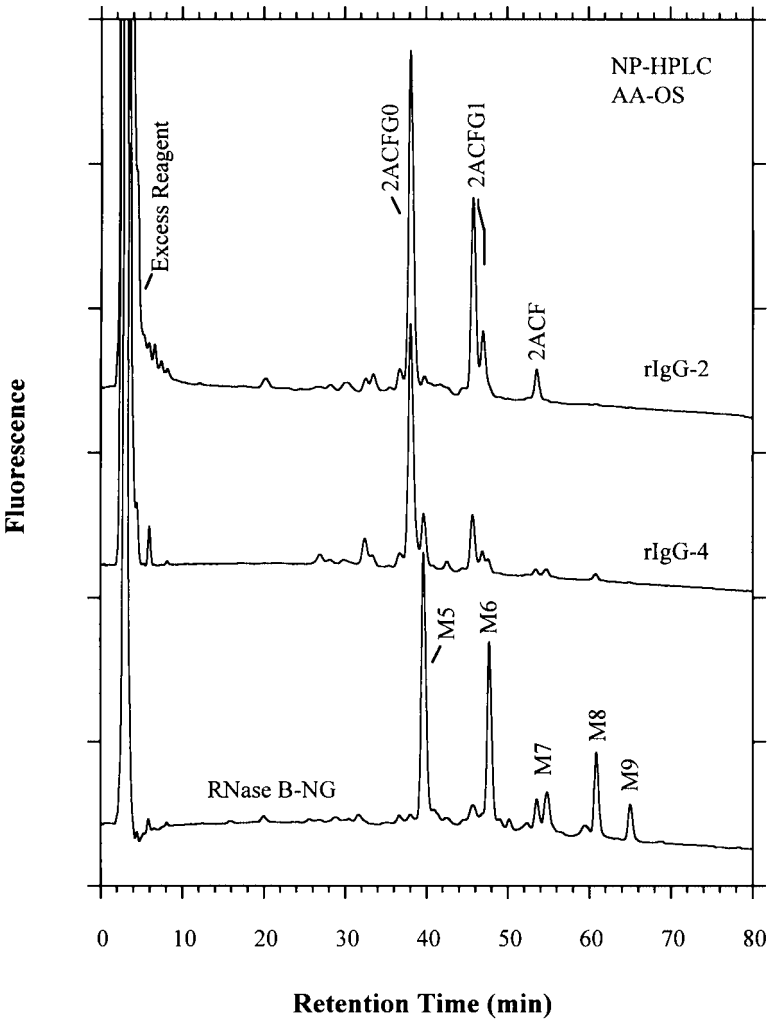

Fig. 3. Separation of neutral oligosaccharides on a TSK amide-80 column. Neutral oligosaccharides from two different rIgGs (top two chromatograms), in comparison with RNase B high mannose oligosaccharides.

intensity upon SDS-PAGE. One of the bands from a 2.5 μg load was excised, minced into small pieces, and digested with PNGase F as in the case of a sample in solution. The oligosaccharide map/profile obtained from a fetuin band is shown in **Fig. 2**. Characteristic profile of the fetuin oligosaccharides is clearly seen even with this level (<1.0 ug) of glycoprotein.

9. **Figure 5** shows the oligosaccharide maps of rIgG treated with and without exo-glycosidases demonstrating the sequential loss of monosaccharide units from

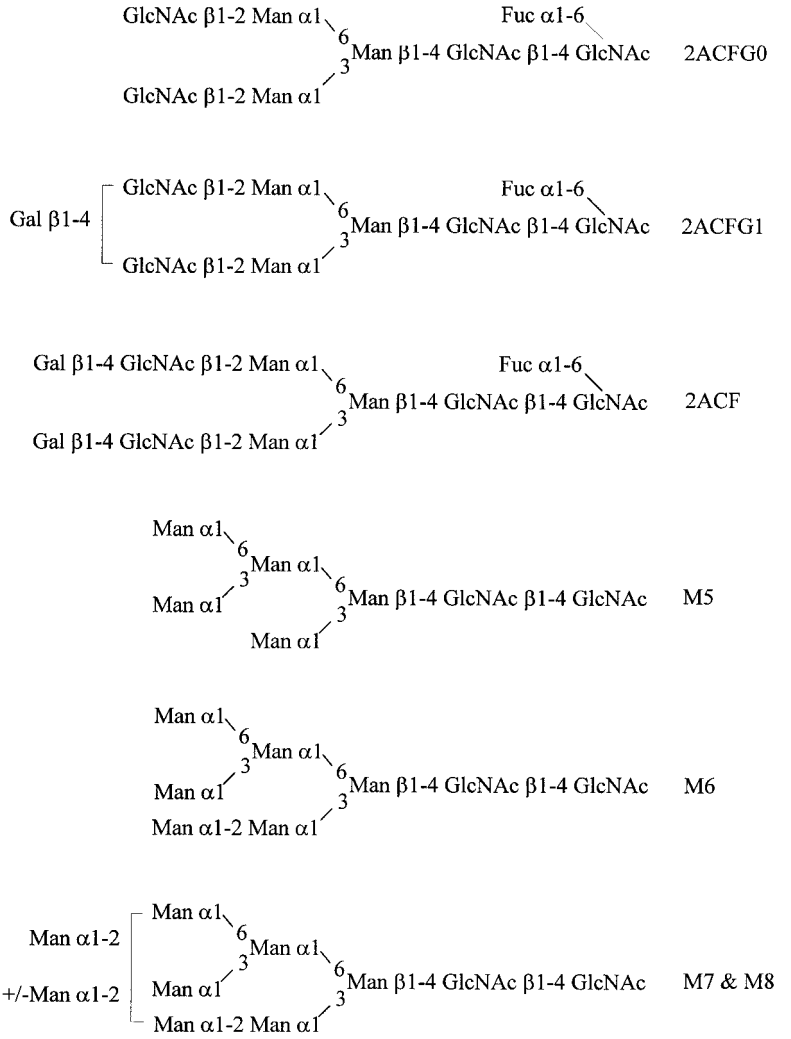

Fig. 4. Oligosaccharide structures for the peaks in **Figs. 3** and **5**.

the nonreducing end and their comparison with the standards. Co-elution of the rIgG peaks with commercial standards confirm the structures identified in the map. Maps were obtained without any additional purification to remove the digestion buffers.

10. The oligosaccharide mapping method has been validated for several IgG proteins. It has been shown to be accurate via NMR, mass spectrometry, co-elution (with glycan standards), and exo-glycosidase experiments. Precision of the method has also been established. Ruggedness and robustness with respect to

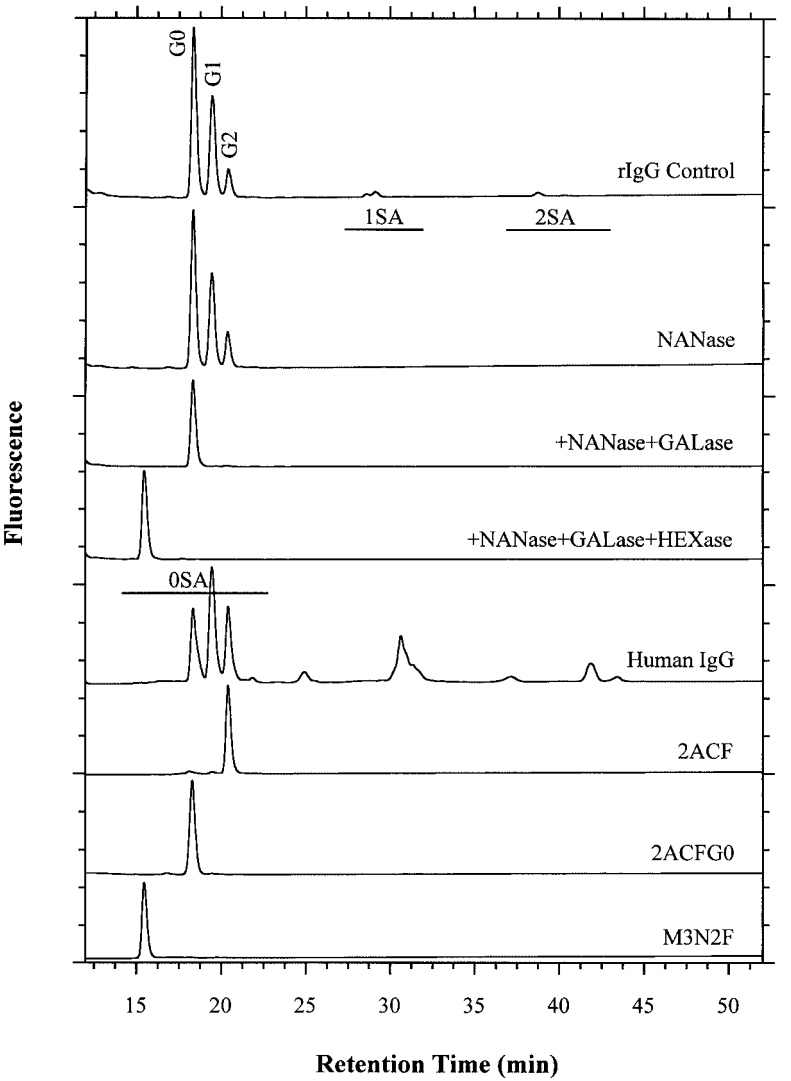

Fig. 5. Effect of exoglycosidase treatments on a CHO cell-derived rIgG oligosaccharides. The oligosaccharide standards (hIgG, 2ACF (G2), 2ACFG1 (G1), 2ACFG0 (G0), and M3N2F (fucosylated tri-mannose core structure) were purchased from Oxford GlycoSciences, and the exoglycosidases were from Glyko. The oligosaccharides following enzyme digestions were analyzed by the mapping method (*see* **Note 9**).

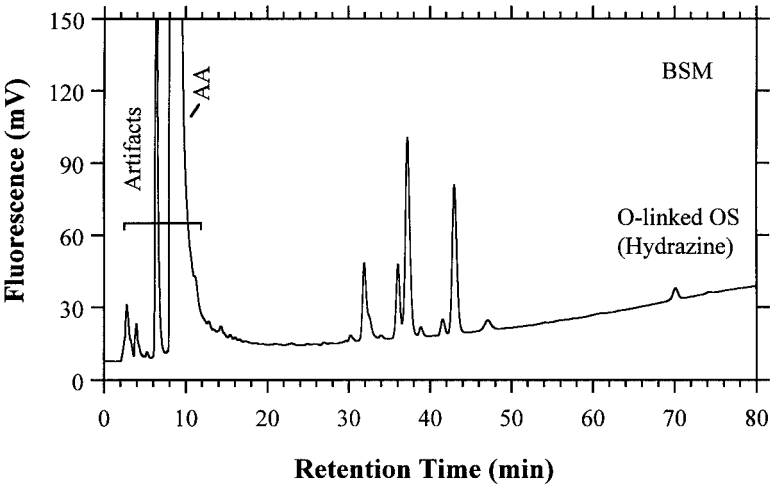

Fig. 6. Profile of O-linked oligosaccharides from bovine submaxillary mucin released by hydrazine. The oligosaccharides were analyzed using the mapping method.

Table 1
Analyst to Analyst Reproducibility with Oligosaccharide Mapping Method

Peaks	Analyst 1	Analyst 2	Average
Gal0	49.93[a]	51.39	50.66
	(0.08)[b]	(1.38)	(2.04)
Gal1	35.32	34.88	35.10
	(0.10)	(0.69)	(0.88)
Gal2	10.18	9.24	9.71
	(0.35)	(3.29)	(6.85)
SA1	3.09	3.02	3.05
	(0.92)	(3.99)	(1.74)
SA2	1.50	1.49	1.49
	(0.47)	(2.38)	(0.47)

[a]Average of relative percent peak area from two replicates.
[b]Percent relative standard deviation (*see* **Note 10**).

variations in HPLC systems, analysts, wavelengths, gradient slope, solvent A and B composition, temperature, and so on, has been demonstrated. Results of a reproducibility experiment is summarized in **Table 1**. Relative area %RSDs are less than 15% for all peaks and pass acceptance criteria.

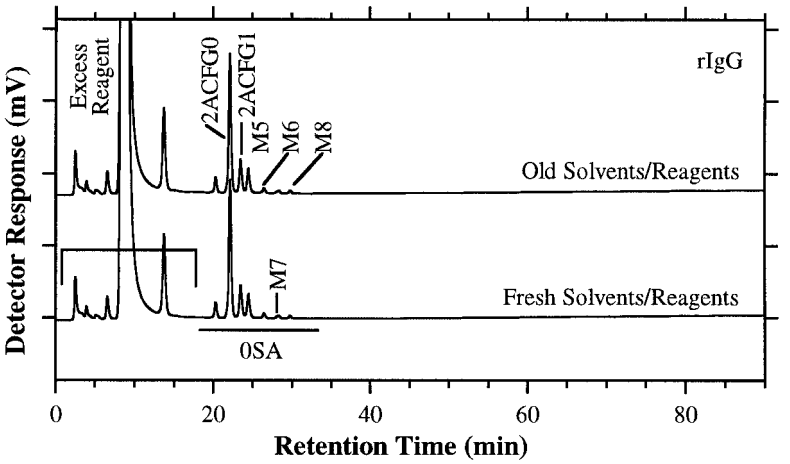

Fig. 7. Comparison of oligosaccharide map obtained using more than 5-mo-old solvents and reagents with a map obtained using "fresh" solvents and reagents (*see* **Note 11**).

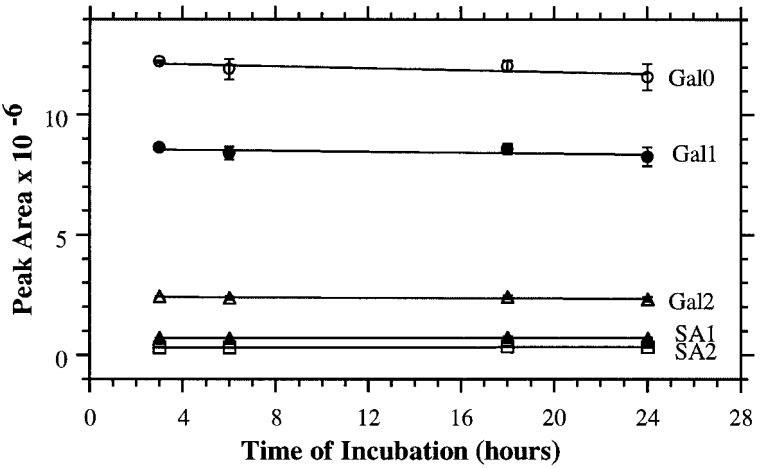

Fig. 8. Time course for release of oligosaccharides from a rIgG with PNGase F enzyme. Plots are obtained by averaging peak area values from two replicates at each time point. Error bars represent standard deviation of replicates (*see* **Note 12**).

11. Several experiments were also carried out to test the "shelf-life" of reagents and solvents used in the oligosaccharide-mapping method. The compare maps in **Fig. 7** are a testimony to the ruggedness of the method. All reagents and solvents were more than 5 mo old. Except for the AA reagent, which was stored at –20°C, the other solvents and reagents were at room temparature. Oligosaccharide

profiles obtained with these reagents/solvents were comparable to ones obtained with "fresh" solvents/reagents.

12. For most glycoproteins, 3 h of incubation with PNGase F suffices for complete oligosaccharide release (**Fig. 8**). However, a time course experiment may need to be done to confirm this in individual cases. Additionally, no degradation (diminution of peak area) of any oligosaccharide species is observed at 24 h of incubation (**Fig. 8**) or even at 48 h (not shown).

Acknowledgment

We express our gratitude to Ms. Ping Du for providing information on glycoproteins from PVDF membranes.

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10

Metabolic Labeling and Structural Analysis of Glycosylphosphatidylinositols from Parasitic Protozoa

Peter Gerold and Ralph T. Schwarz

1. Introduction

Glycosylphosphatidylinositols (GPIs) were first identified as membrane anchors of the variant-surface glycoprotein of the parasitic protozoan parasite *Trypanosoma b. brucei* ***(1,2)***. Since then, GPIs have been described as protein- and glycoconjugate-membrane anchors, and as free GPIs on the surface of a variety of organisms ranging from ancient eucaryotes (e.g., flagellates) to mammalian cells ***(3–10)***. This family of glycolipids exhibits the conserved structural motif: mannoseα1-4glucosamineα1-6*myo*inositol-1-PO_4-lipid. GPI-biosynthesis consists of a sequence of single molecule transfers from activated donors (e.g., nucleotide sugars and dolichol-phosphate-mannose) onto inositol-phosphate containing lipids of various structures (**Fig. 1**) ***(11–17)***. A prerequisite for the function of GPIs as membrane anchors of proteins is the presence of an ethanolamine-phosphate linked to the terminal mannose of the highly conserved trimannosyl core-glycan. The preassembled GPI-anchor precursor is posttranslationally transfered to a variety of membrane proteins in the lumen of the endoplamatic reticulum in a transamidase-like reaction ***(18)***. The transamidase complex leads to the replacement of the c-terminal hydrophobic GPI-attachment sequence by the preformed GPI-anchor precursor (**Fig. 1**). Some of the genes involved in the GPI-biosynthesis have been cloned from yeast and mammalian cells ***(19,20)***.

Besides the function as membrane anchors of proteins or glycoconjugates additional functions of GPIs have been described during the last few years involving signaling and intracellular transport ***(21–23)***. In particular, GPIs of

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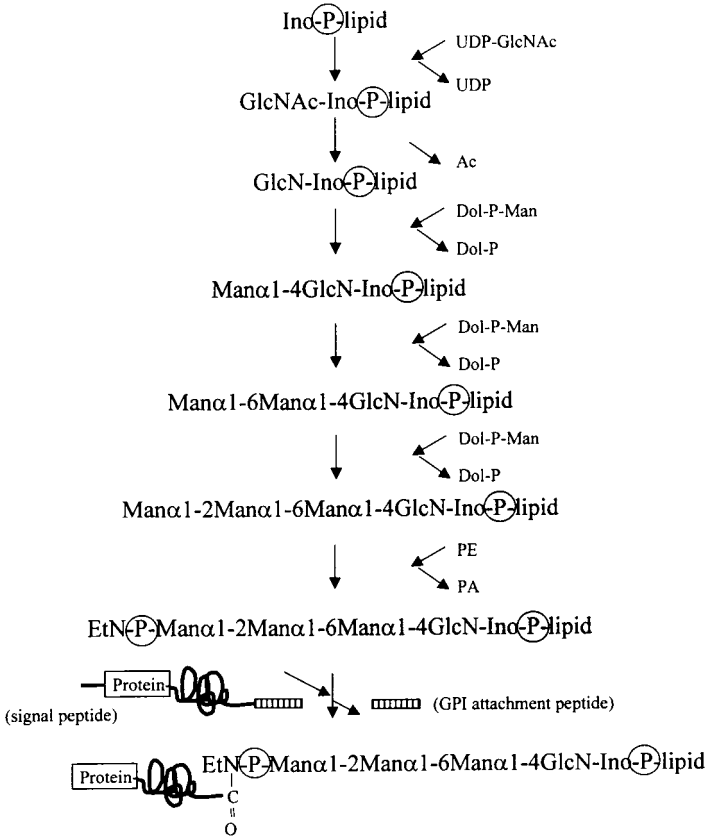

Fig. 1. Schematic representation of the general features of GPI-biosynthesis. The shadowed area represent the structrual features indicative for a glycosylphosphatidylinositol glycoconjugate. UDP-GlcNAc, uridine-diphosphate-*N*-acetyl-glucosamine; Ac, acetyl-residue; Dol-P-Man, dolichol-phosphate-mannose; PE, phosphatidylethanolamine; PA, phosphatidic acid.

parasitic protozoa have been described as parasite-derived factors affecting host-cell signaling and immunity ***(24–26)***. GPIs of the human malaria parasite *Plasmodium falciparum* have been described as a novel type of toxin involved in the development of severe malaria pathology ***(27–30)***.

In this chapter, we will describe protocols to identify and analyze GPI-biosynthesis intermediates and GPI-anchor precursors in the malaria parasite *P. falciparum*, although they are identical for the analysis of GPIs of other eucaryotes apart from cell culture and labeling medium conditions.

The protocols described for identification and characterization depend upon metabolic labeling techniques using radioactive GPI-precursor molecules,

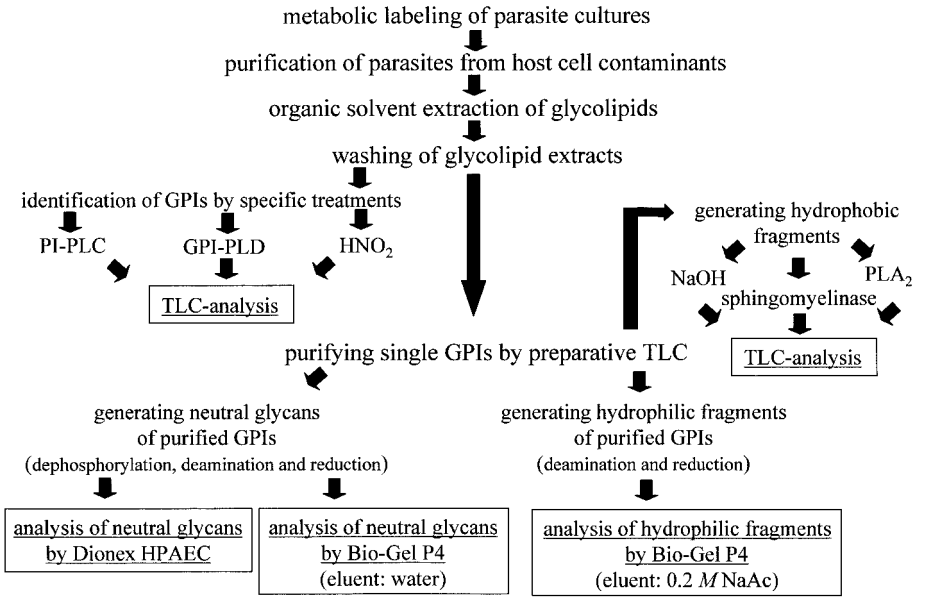

Fig. 2. Schematic representation of the protocol to identify and analyze nonprotein-bound glycosylphosphatidylinositol. PI-PLC, phosphatidylinositol-specific phospholipase C; GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D, HNO_2, nitrous acid deamination and reduction; NaOH, alkaline saponification; PLA_2, phospholipase A_2.

organic solvent extraction procedures, the presence of nonacetylated glucosamine, and the use of GPI-specific phospholipases (**Fig. 2**). GPIs were identified by their sensitivity towards nitrous acid deamination (HNO_2), and specific enzymatic treatments with phosphatidylinositol-specific phospholipase C (PI-PLC) and glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) using thin-layer chromatography (TLC) analysis. Structural characterization can be achieved by analyzing hydrophilic fragments and neutral core-glycans. Hydrophilic fragments are generated by nitrous acid deamination. This procedure leads to the cleavage of the linkage between the inositol and the nonacetylated glucosamine present in all GPIs. Anhydromannose generated at the reducing terminus of the GPI-glycan will be reduced by sodium borohydride and converted to anhydromannitol. This reaction prevents unspecific destruction of the glycan. After purification, the hydrophilic fragments can be analysed by size-exclusion chromatography (Bio-Gel P4). Neutral core-glycans are prepared by dephosphorylation, deamination, and reduction. The released core-glycans are intensively desalted prior the analysis by high-pH-anion-exchange chromatography (Dionex) along with an internal

standard of β-glucan oligomers. Exoglycosidase treatments are used to confirm the predicted structures of the GPI-glycans. Hydrophobic fragments of GPIs metabolically labeled with fatty acids were generated by specific treatments like alkaline saponification, phospholipase A_2 (PLA_2) and sphingomyelinase treatment and were investigated by TLC-analysis in comparison to standard lipids.

2. Materials

2.1. Metabolic Labeling, Extraction and Analysis of P. falciparum Glycolipids

1. Deinonized and filtered water.
2. Culture medium: RPMI-1640 medium (GIBCO-BRL) supplemented with gluthation, neomycin, Albumax I, and $NaHCO_3$.
3. Labeling medium: RPMI-1640 medium without glucose (Abimed, Muttenz Schweiz) supplemented with fructose, gluthation, neomycin, Albumax I, and $NaHCO_3$.
4. Radioactivity was purchased from Amersham, NEN-DuPont and Hartmann Analytic.
5. Save-lock or screw-top Eppendorf tubes.
6. Analytical-grade chloroform and methanol mixed in the ratio 1:1 (v/v)
7. Analytical-grade chloroform, methanol, and water mixed in the ratio 10:10:3 (by vol) or 4:4:1 (by vol).
8. Butan-1-ol shaken with an equal volume of water in a clean bottle.
9. Access to a liquid-scintillation counter.
10. Glass-backed silica gel 60 and silica 60 HPTLC plates (Merck), and a TLC-chamber (Desaga).
11. Bio-Imager plates (Tritum, Fuji Film) and film cassettes.
12. Access to a TLC-scanner (e.g., Berthold TLC Linear Analyzer).

2.2. Enzymatic Characterization of Glycolipids as GPIs

1. *Bacillus cereus* phosphatidylinositol-specific phospholipase C (Boehringer Mannheim, Oxford Glycosystems). Store enzyme stock at 4°C.
2. Phosphatidylinositol-specific phospholipase C incubation buffer: 0.1% Triton X-100, 50 m*M* Tris-HCl, pH 7.4.
3. Glycosylphosphatidylinositol-specific phospholipase D incubation buffer: 0.1% Triton X-100, 50 m*M* Tris-HCl, pH 7.4, 2 m*M* $CaCl_2$.
4. Access to a Speed-Vac concentrator.

2.3. Chemical Characterization of Glycolipids as GPIs

1. 0.2 *M* Sodium acetate, pH 4.0, with (for glycolipids) or without (for GPI-anchored proteins) 0.1% sodium dodecyl sulfate (SDS). Made by titrating sodium acetate solution (0.2 *M* final) to pH 4.0 with glacial acetic acid. Stable at room temperature for several month.

2. 1 *M* Sodium nitrite. Prepare just before use.
3. Access to a water-bath sonicator.

2.4. Generating Hydrophilic Fragments by Deamination and Reduction

1. 0.8 *M* Boric acid. Stable at room temperature for several months.
2. 2 *M* Sodium hydroxide.
3. 1 *M* Sodium borohydride. $NaBH_4$ is dissolved in 0.1 *M* NaOH before use.
4. 5 % and 50 % glacial acetic acid.
5. Toluene: analytical-grade (Merck).
6. Dextran from *Leuconostoc spp.* (Serva).
7. Access to a Bio-Gel P4 system, using 0.2 *M* ammonium acetate as eluent.
8. 96-Well microtiter plates.
9. H_2SO_4/0.2% orcinol.

2.5. Generating Neutral Core Glycans by Dephosphorylation, Deamination, and Reduction

1. 48% Aqueous HF (Sigma).
2. Dowex AG50-X12, 200-400 mesh (Bio-Rad) converted to the H^+ form by washing 5× with 10 vol 1 *M* HCL and 5–7× with 10 vol water. Store with an equal volume of water at 4°C.
3. Chelex 100 (Na^+) (Bio-Rad). Store with an equal volume of water at 4°C.
4. Dowex AG3-X4, 200–400 mesh (Bio-Rad) converted to the OH^- form by washing with 5 × 10 vol 1 *M* NaOH and 5–7× 10 vol water. Store with an equal volume of water at 4°C.
5. QAE-Sephadex-A25 (Pharmacia). Swollen in water and washed with 5 × 10 vol water. Store with an equal volume of water at 4°C.
6. Access to a Dionex HPAEC system (Dionex Corp., CA).

2.6. Sequencing of Neutral Glycans by Exoglycosidase Treatment

1. Jack bean α-mannosidase (JBAM) (Oxford Glycosystems). Store enzyme stock at –20°C.
2. *Aspergillus saitoi* α-mannosidase (ASAM) (Oxford Glycosystems). Store enzyme stock at –20°C.

2.7. Bio-Gel P4 Analysis of Neutral Glycans

1. Access to a Bio-Gel P4 system, using water as eluent.

2.8. Dionex HPAEC Analysis of Neutral Glycans

1. 0.2 μm HPLC-filter (Schleicher and Schüll).
2. 0.1 *M* NaOH (carbonate-free).
3. 0.1 *M* NaOH (carbonate free)/0.25 *M* sodium acetate.

2.9. Identification of Hydrophobic Fragments of GPIs

1. Phospholipase A_2 from bee venom (Boehringer Mannheim). Store enzyme stock at 4°C.
2. Reactivials (Pierce).
3. *Staphylococcus aureus* Sphingomyelinase (Sigma).
4. 40 µL 1 *M* Tris-acetate pH 7.6/20 µL 0.4 *M* $MgCl_2$/ 300 µL diethylether.

2.10. Characterization of Hydrophobic Fragments of Parasite GPIs

1. Soybean lyso-phosphatidylinositol (Sigma), bovine-brain phosphatidylinositol (Sigma), egg yolk phosphatidic acid (Sigma), and bovine brain ceramide (Sigma) dissolved in chloroform/methanol (1:1, by vol) at a concentration of 1 µg/µL. Store at –20°C.
2. Analtical grade chloroform/methanol/water mixed in the ration 25:75:5 (by vol).

3. Methods

3.1. Metabolic Labeling, Extraction, and Analysis of P. falciparum Glycolipids

1. Remove medium from cultures of intraerythrocytic stages of the malaria parasite *P. falciparum* by washing two times with phosphate-buffered saline (PBS) (*see* **Note 1**).
2. Resuspend the parasites (5×10^8 parasite infected erythrocytes) in glucose-deficient RPMI-1640 medium containing 20 m*M* fructose, 0.5% Albumax I, and 100–200 µCi 5 mL^{-1} of the tritiated presursors glucosamine, mannose, ethanolamine, or fatty acids (*see* **Note 2**) and incubate the parasite cultures for 3–4 h with the labeling medium (*see* **Note 3**) at 37°C under reduced (5%) oxygen pressure.
3. Release the parasites from their host cells by saponin lysis (*see* **Note 4**) and wash two times with PBS. Transfer the washed parasites to an Eppendorf tube.
4. Extract the glycolipids from the parasite pellet by addition of 7 vol chloroform/methanol (1:1, by vol) to 1 vol cells (*see* **Note 5**) and incubate at room temperature for 30 min. Pellet the insoluble material by centrifugation (5 min, 10,000*g*) and remove the supernatant to a fresh tube. Add 500 µL chloroform/methanol/water (10:10:3, by vol) to the pellet and sonicate for 5 min. Spin down the insoluble material (5 min, 10,000*g*), and combine the first and second extract.
5. Dry the combined extracts under a stream of nitrogen and resuspend the extracted glycolipids in water-saturated butan-1-ol. Wash the organic phase with butan-1-ol saturated water to remove hydrophilic contaminants (*see* **Note 6**).
6. Count an aliquot of the washed glycolipid extract in a liquid scintillation counter (*see* **Note 7**).
7. Dry about 20,000 cpm of glycolipid extracts labeled with tritiated glucosamine or mannose and about 100,000 cpm of glycolipid extracts labeled with tritiated ethanolamine or fatty acids (*see* **Note 8**) to the bottom of Eppendorf tubes (*see*

Note 9) and resupend in 10 µL chloroform/methanol (1:1, by vol). One µL at a time of the glycolipid extracts are spotted as a band 0.5 cm wide and 2 cm from the bottom onto silica 60 plates. Develop the plate in organic solvents (e.g., chloroform/methanol/water [4:4:1, by vol] or [10:10:3, by vol]) up to a line 2 cm from the top of the plate (*see* **Note 10**). Allow the plate to dry in a fume hood. Place it in a film cassette against a Bio-Imager Tritium plate and leave it for 10–14 d prior developing.

3.2. Enzymatic Characterization of Glycolipids as GPIs

1. About 20,000 cpm of glycolipid extracts labeled with tritiated glucosamine or mannose are dried to the bottoms of Eppendorf tubes and are resuspended in 5 µL 1% Triton X-100 by mixing and sonication. Subsequently, 45 µL Tris-HCl, pH 7.4 (final concentration 50 m*M*), is added. Incubate the samples with 0.5 U PI-PLC for 16 h at 37°C. For GPI-PLD (rabbit serum; *see* **Note 11**) add 2 m*M* $CaCl_2$ (final concentration) to the PI-PLC buffer and incubate with 10% rabbit serum (final concentration) for 16 h at 37°C. Terminate the incubation by heating at 100°C for 5 min (*see* **Note 12**).
2. Add 50 µL butan-1-ol saturated water and 100 µL water-saturated butan-1-ol to do the butanol/water-phase partition (*see* **Note 6**). Pool the butanol phases in an Eppendorf tube and dry them in a Speed-Vac concentrator.
3. Analyze the organic phases on silica 60 TLC-plates (*see* **Subheading 3.1., step 7**).

3.3. Chemical Characterization of Glycolipids as GPIs

1. About 20,000 cpm of glycolipid extracts labeled with tritiated glucosamine or mannose are dried to the bottom of an Eppendorf tube and are resuspended in 50 µL 0.2 *M* NaAc pH 4.0/0.1% SDS by mixing and sonication.
2. Add 50 µL freshly prepared 1 *M* $NaNO_2$ and incubate for 3 h at 37°C.
3. Add a further 25 µL 0.2 *M* NaAc, pH 4.0, and 25 µL freshly prepared 1 *M* $NaNO_2$ and incubate for further 16 h at room temperature.
4. Add 100 µL water-saturated butan-1-ol to do the butanol/water-phase partition (*see* **Note 6**). Pool the organic phases in an Eppendorf tube and dry them in a Speed-Vac concentrator.
5. Analyze the organic phases on silica 60 TLC-plates (*see* **Subheading 3.1., step 7**).

3.4. Generating Hydrophilic Fragments by Deamination and Reduction

1. Single glycolipids are purified by scraping out the relevant areas from the TLC plates. Transfer the silica in a glass tube. GPIs are eluted from the silica by adding 5 vol of chloroform/methanol/water (10:10:3, by vol), sonicating for 5 min, and incubating for 30 min at room temperature. Spin down the samples at 2800*g* for 5 min, and remove the supernatant in a clean tube. Repeat the elution twice and combine the supernatants. The combined extracts are dried in Eppendorf tubes (*see* **Note 9**) and the residual silica is remove by butanol/water-

phase partition (*see* **Note 13**). Resuspend in 100 µL butan-1-ol and count an aliquot in the liquid-scintillation counter. Dry about 2000 cpm of the organic phases in a Speed-Vac concentrator.

2. Hydrophilic fragments of TLC purified [^{3}H]-mannose or [^{3}H]-glucosamine labeled GPIs were generated by nitrous acid deamination (*see* **Subheading 3.3., steps 1** and **2**).
3. Add 16 µL 0.8 *M* boric acid followed by quickly 16 µL 2 *M* NaOH (*see* **Note 14**) and 30 µL 1 *M* $NaBH_4$. Incubate for 3 h at room temperature without closing the lid of the Eppendorf tubes.
4. Destroy the excess of $NaBH_4$ by adding 5 µL 50% glacial acetic acid until effervescence ceases.
5. Dry down the samples and flash-evaporate 2× with 100 µL methanol, 2 × 100 µL methanol/5% acetic acid, and 1× toluene.
6. Resuspend the samples in 100 µL water, add 20 mg β-glucan oligomers (*see* **Note 15**) as internal standards and spin down the sample in a microfuge at maximum speed for 1 min. Analyze the material by size-exclusion chromatography on 140 × 1 cm Bio-Gel P4 (mesh > 400) columns using 0.2 *M* ammonium acetate as solvent (*see* **Note 16**). Collect fractions of about 1 mL. Take 25 µL aliquots from each fraction and transfer them in a microtiter plate. Visualize the elution of the glycans by adding 100 µL conc. H_2SO_4 /0.2% orcinol (caution: highly corrosive) and heating at 100°C for 10 min. The maxima can be determined by eye. Determine the radioactivity in the samples by liquid-scintillation counting. Elution of the radioactivity is given in "glucose units" (GU).
7. Look up the GU values in **Table 1** to see if the chromatographic properties of the hydrophilic fragments(s) correspond to known structures.

3.5. Generating Neutral Core Glycans by Dephosphorylation, Deamination, and Reduction

1. 3000 cpm of single TLC-purified GPIs (*see* **Subheading 3.4., step 1**) are dried in an Eppendorf tube. Add 50 µL 48% ice-cold HF and incubate for more than 60 h at 0°C (*see* **Note 17**). Caution: highly corrosive.
2. Dry the HF under a stream of nitrogen (*see* **Note 18**).
3. Deaminate and reduce the sample as described (*see* **Subheading 3.4., steps 3** and **4**).
4. Apply the sample to a column of 0.4 mL Dowex AG50-X12 and eluate with 3 vol of water. Dry in a speed-vac concentrator.
5. Remove volatile contaminants as described (*see* **Subheading 3.4., step 5**) and dry the sample.
6. Redissolve in 100 µL water, pass through a column of 0.1 mL Chelex 100 (Na^+), over 0.3 mL Dowex AG50-X12 (H^+), over 0.3 mL Dowex AG3-X4 (OH^-), over 0.1 mL QAE-Sephadex-A25 (OH^-), and elute with 3 vol water.
7. Dry the eluate and redissolve in 100 µL water. Store the neutral glycans at –20°C. Analyze the products by Bio-Gel P4 (*see* **Subheading 3.7.**) or Dionex HPAEC (*see* **Subheading 3.8.**).

3.6. Sequencing of Neutral Glycans by Exoglycosidase Treatment

1. Dissolve purified GPI neutral glycans (5,000 cpm) in enzyme containing buffers (*see* **Note 19**) and treat for 16 h at 37°C.
2. Inactivate the enzymes by heating to 100°C for 5 min and desalt by passage through a column of 0.2 mL Dowex AG50-X12 (H^+) over 0.2 mL Dowex AG3-X4 (OH^-). Elute with 1 mL water.
3. For JBAM use 0.5 U enzyme in the buffer delivered by the supplier (0.1 *M* sodium acetate, pH 5.0).
4. For ASAM use 10 μU enzyme in the buffer delivered by the supplier (0.1 *M* sodium acetate, pH 5.0).

3.7. Bio-Gel P4 Analysis of Neutral Glycans

1. Dry the desalted samples (3000 cpm) and redisolve in 50 μL water.
2. Mix the core-glycans with 20 mg β-glucan oligomers as internal standards and spin down the sample in a microfuge at maximum speed for 1 min.
3. Apply each sample to a Bio-Gel P4 column (1 × 140 cm) and analyze as described (*see* **Subheading 3.4., step 6**).
4. Look up the GU-values in **Table 1** to see if the chromatographic properties of the hydrophilic fragment(s) correspond to known structures.

3.8. Dionex HPEAC Analysis of Neutral Glycans

1. Dissolve the desalted neutral core glycans (1500 cpm) (*see* **Subheading 3.5.**) in 100 μL water and filter through a 0.2 μm HPLC-syringe filter. Dry the filtered sample and resuspend it in 15 μL water.
2. Add 5 μL β-glucan oligomer standards (2 μg), which will be detected by pulsed ampheriometric detection (*see* **Note 15**).
3. The elution program for Dionex HPAEC analysis using a Carbopak™ PA1 (4 × 250 mm) and the corresponding guard column: 100% buffer A (0.1 *M* NaOH), 0% buffer B (0.1 *M* NaOH, 0.25 *M* sodium acetate) up to 6 min after injection, then increase of buffer B to 30% at 36 min, at a flow rate of 1 mL/min. Wash the column for 20 min at 100% buffer B and reequilibrate for 15 min at 100% buffer A before starting the next run. Elution of the radioactivity is given in "Dionex units" (DU).
4. Look up the DU-values in **Table 1** to see if the chromatographic properties of the hydrophilic fragment(s) correspond to known structures.

3.9. Identification of Hydrophobic Fragments of GPIs (see *Note 20*)

1. About 20,000 cpm of glycolipid extracts labeled with tritiated glucosamine or mannose are dried to the bottom of Eppendorf tubes. Add 100 μL methanol and 100 μL 0.2 *M* NaOH. Mix and incubate the tubes at 37°C for 2 h. Dry the samples in a Speed-Vac concentrator and do a butan-1-ol/water-phase partition (*see* **Note 6**).

Table 1
Chromatographic Properties of some GPI Neutral Glycans

Structure	DU values	GU values
AHM	0.9	1.7
Manα1-4AHM	1.1	2.3
Manα1–6Manα1–4AHM	2.1	3.2
Manα1–2Manα1–6Manα1–4AHM	2.5	4.2
Manα1–2Manα1–2Manα1–6Manα1–4AHM	3.0	5.2
Manα1–2Manα1–6Man(Galα1-3)α1–4AHM	3.6	5.2
Manα1–2Manα1–6Man(Galα1-3Galα1-6)α1–4AHM	3.8	6.1
Manα1–2Manα1–6Man(GalNAcβ1-4)α1–4AHM	3.0	6.5
Manα1–2Manα1–6Man(GalNAcβ1-4Glcα1-6)α1–4AHM	3.0	7.5
Manα1–2Manα1–6Man(GalNAcβ1-4Galβ1-3)α1–4AHM	3.0	7.5
Manα1–2Manα1–6Man(GlcNAcβ1-4)α1–4AHM	3.3	6.2
Manα1–2Manα1–6Man(GlcNAcβ1-4Galβ1-6)1–4AHM	3.8	7.2

DU, Dionex units; GU, glucose units; Man, mannose; Gal, galactose; GalNAc, *N*-acetyl-galactosamine; GlcNAc, *N*-acetyl-glucosamine; Glc, glucose; AHM, anhydromannitol.

2. About 20,000 cpm of glycolipid extracts labeled with tritiated glucosamine or mannose are dried to the bottom of Eppendorf tubes and are resuspended in 5 µL 1% Triton X-100 by mixing for 5 min. Subsequently, 45 µL Tris-HCl, pH 7.4 (final concentration 50 m*M*) and 2 m*M* $CaCl_2$ (final concentration) is added. Incubate the samples with 50 U PLA_2 for 16 h at 37°C. Terminate the incubation by heating at 100°C for 5 min. Apply the samples to a butan-1-ol/water-phase partition (*see* **Note 6**).
3. About 20,000 cpm of glycolipid extracts labeled with tritiated glucosamine or mannose, and 10 µL Triton X-100, are dried to the bottom of Reacti-vials (Pierce). Redissolve the sample in 40 µL 1 *M* Tris-acetate, pH 7.6, 20 µL 0.4 *M* $MgCl_2$, 300 µL diethylether, and 20 µL chloroform in the presence of 1 U sphingomyelinase (*Staphylococcus aureus*). Samples are incubated for 16 h at 37°C under constant stirring. Subsequently the organic solvent is evaporated under a stream of nitrogen. Apply the samples to butan-1-ol/water-phase partition (*see* **Note 6**).
4. Analyze the organic phases on TLC compared to untreated controls.

3.10. Characterization of Hydrophobic Fragments of Parasite GPIs

1. The hydrophobic fragment is released from 10,000 cpm of TLC purified (*see* **Subheading 3.4., step 1**) fatty acid labeled GPI by PI-PLC or GPI-PLD (*see* **Subheading 3.2.**). The organic phase is analyzed on TLC using chloroform/methanol/

water (4:4:1, by vol). Use 10 µg phosphatidylinosiol, lyso-phosphatidylinosiol, and phosphatidic acid as standards, which can be visualized by exposing the TLC plate to iodine vapor in a TLC-chamber.

2. The hydrophobic fragment is released from 10,000 cpm of TLC purified (*see* **Subheading 3.4., step 1**) fatty acid-labeled GPI by sphingomyelinase (*see* **Subheading 3.9., step 3**). The organic phase is analyzed on HPTLC-plates using chloroform/methanol (9:1, by vol). Use 10 µg ceramide as a standard, which can be visualized by exposing the TLC plate to iodine vapor in a TLC-chamber.
3. The ester-linked fatty acids are released from 10,000 cpm of TLC purified (*see* **Subheading 3.4., step 1**) fatty acid-labeled GPI by alkaline treatment (*see* **Subheading 3.9., step 1**). Released fatty acids can be analyzed on reversed-phase (RP-18) HPTLC plates (Merck) using chloroform/methanol/water (25:75:5, by vol) as solvent system (*see* **Note 21**).

4. Notes

1. Only minor amounts of *N*-glycans have been described for proteins *P. falciparum*. Dolichol-cycle intermediates (except dolichol-phosphate-mannose), the lipid-linked precursors for protein N-glycosylation, have not been demonstrated in asexual stages of *P. falciparum*. Therefore, they do not interfere with the synthesis of GPIs by this parasite. For efficient labeling of GPIs in other organisms, exhibiting substantial *N*-glycosylation, it is necessary to preincubate the cells with 1–10 µg/mL, tunicamycin. Having this antibiotic present in the medium will inhibit incorporation of radioactive sugars (glucosamine and mannose) into dolichol-cycle intermediates.
2. Labeling with radioactive ethanolamine will lead to a massive incorporation of labeled ethanolamine into phospholipids, especially phosphatidylethanolamine, whereas the labeling efficiency of GPI-anchor precursors is relatively low. Metabolic labeling with fatty acids is more efficient if they are coupled to defatted bovine serum albumin (BSA) V (Sigma) prior to the addition to the labeling medium. Dry 500 µCi fatty acids in Eppendorf tubes. Resuspend them in 10 µL ethanol and add 484 µL water and 6 µL defatted BSA V (100 mg/mL). Mix for 1 h at room temperature immediately before use. In addition, for efficient fatty acid-labeling reduce the amount of Albumax I or serum present in the labeling medium to 1/10 of the original amount present in the culture medium.
3. To get maximum incorporation of radioactive precursors it will be necessary to establish the time for steady-state labeling for each specific cell type. Usually labeling periods of 1–4 h give efficient incorporation of radioactive precursors when investigating parasitic protozoa.
4. Saponin-lysis ***(31)*** is a *Plasmodium*-specific treatment that releases intraerythrocytic parasites from their host cells by solubilizing cholesterol from erythrocyte membranes. Parasite membranes are low in cholesterol and therefore are not effected by this treatment.
5. The extraction protocol described will extract phospholipids, neutral lipids, dolichol-cycle intermediates, sphingolipids, and GPIs efficiently. For some

systems a sequential extraction using chloroform/methanol (2:1, by vol) prior to chloroform/methanol/water (10:10:3, by vol) has the advantage to separate phospholipids, neutral lipids, some sphingolipids, and some dolichol-cycle intermediates (found in the chloroform/methanol phase) from the ethanolamine-phosphate-containing GPIs, more hydrophilic sphingolipids, and dolichol-cycle intemediates (found in the chloroform/methanol/water phase).

6. For butanol/water-phase partition mix water and butan-1-ol (1:1) and wait until the two phases are clearly separated. Resuspend glycolipids in 1 vol of water-saturated butan-1-ol (upper phase) and mix with 1 vol butan-1-ol saturated water (lower phase) by intensive mixing. Separate the two phases by centrifugation at 13,000*g* for 2 min. Remove the lower, aqueous phase into a new Eppendorf tube and add 1 vol of fresh butan-1-ol saturated water to the remaining organic phase. Mix both phases and spin for phase separation. Remove the lower, aqueous phase and combine both aqueous phases. Add 1 vol of fresh water-saturated butan-1-ol to the combined aqueous phases. Mix both phases and spin for phase separation. Remove the upper, organic phase and combine both organic phases. Add 1 vol of fresh butan-1-ol saturated water to the remaining organic phase and mix. Separate the two phases by centrifugation and remove the lower, aqueous phase. The organic phase will be almost completely free of aqueous soluble contaminants and salt.
7. To determine the incorporation of radioactive precursors into glycolipids count an aliquot of 1/50 of the sample.
8. Labeling with radioactive ethanolamine or fatty acids will lead to a massive incorporation of radioactivity into phospholipids, whereas GPIs are usually underrepresented. Therefore, to get detectable signals of labeled GPIs it is necessary to use large aliqouts of the glycolipid extracts for TLC analysis.
9. To reduce losses of material at the wall of tubes it is necessary to dry the samples in steps of one-third of the previous sample volume using an appropriate solvent.
10. To perform reproducible TLC analyses, it is important to mix the solvent systems very carefully. Fill the solvent into a TLC-chamber and wait for gas-phase saturation in the chamber before you run the TLC.
11. As GPI-PLD is not commercially available, rabbit serum is used as a source for GPI-PLD. The enzyme activity present in serum varies. Therefore, a new batch should be tested prior to use. GPI-PLD is an unstable enzyme. It is recommended to store the serum in small aliquots at –80°C. After thawing the serum can be stored at +4°C for up to 1 wk.
12. PI-PLC will only cleave GPIs having an unsubstituted C-2 atom at the inositol ring. Fatty acids present at this position will hinder the formation of the cyclic phosphate at the inositol ring, which is an obligate intermediate in the cleavage of GPIs by PI-PLC. GPI-PLD is not affected by the presence of a substitution at C-2 atom of the inositol ring
13. The glycolipids will adsorb to the residual silica. Therefore, do not dry the sample completely. Redisolve the purified glycolipids in 200 µL water-saturated

butan-1-ol by sonication. Add 200 µL butan-1-ol saturated water, mix the two phases, and separate the phases by centrifugation. Remove the silica from the bottom of the tube together with the aqueous phase.

14. The amount of 2 *M* NaOH will vary. Titrate the NaOH to give pH 10.0–11.0. If you have overtitrated the sample, add acetic acid until a pH of 10.0–11.0 is reached. Be careful as pH-values above pH >11.0 lead to fragmentation of the glycans.
15. The β-glucan oligomers (Glc1 – Glc20) are prepared by partial hydrolysis of 100 mg of dextran (Sigma) in 1 mL 0.1 *M* HCl, 2 h, 100°C. The acid is removed by flash-evaporating with MeOH (5×) and by passage through a column of 1 mL of Serdolit MB3 (Serva). Elute with 3 mL water and filtrate through a 0.2-µm filter. The resulting set of β-glucan oligomers are stored at –20°C.
16. The hydrophilic fragments generated by nitrous acid deamination contain charged groups like ethanolamine-phosphate, mannose-phosphate, or sialic acid. These groups interfere with the Bio-Gel P4 size-exclusion chromatography matrix, which will result in an increase in the effective size of the hydrophilic fragment. Using 0.2 *M* ammonium acetate as eluent minimizes the effects of charged groups on the elution behavior and reduces losses of material on the Bio-Gel P4 column. 0.2 *M* ammonium carbonate is preferable if the Bio-Gel P4 chromatography will be used for preparative purposes. This eluent can easily be removed by serveral rounds of lyophilisation.
17. The incubation time of 60–65 h will lead to cleavage of the phosphodiester linkages found in GPIs. Ethanolamine-phosphate linked to the mannosyl-coreglycan is completely cleaved by 48% HF within 36 h at 0°C, whereas the inositol-phosphate linkage will only be cleaved after longer incubation periods.
18. Using a stream of nitrogen is an easy and convenient way to remove the HF. This method will not lead to destruction of the neutral glycans of most GPI structures. However, few substitutions of GPIs might be destroyed using this conditions. Therefore, it might be useful to neutralize the HF by adding frozen lithiumhydroxide and sodium hydrogencarbonate ***(32)***.
19. Besides the two α-mannosidases described, configuration, linkage-type, and attachment-side of substitutions attached to the conserved tri-mannosyl coreglycan can be investigated by using various exo-glycosidases ***(32)***.
20. The characterization of the hydrophobic fragment of GPIs make use of different specific chemical and enzymatic treatments. Alkaline saponification cleaves ester-linked fatty acids from glycerol-based GPIs. PLA_2 specifically releases the C-2 fatty acid ester-linked to the glycerol. Sphingomyelinase releases ceramide-based hydrophobic fragments from GPIs.
21. Radioactivity detection on RP-18 TLC-plates by a scanner or Bio-Imager is poor. Therefore, use more radioactivity than for the analysis of glycolipids on silica-60 plates.

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11

Analysis of S-Acylation of Proteins

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1. Introduction

Palmitoylation or S-acylation is the posttranslational attachment of fatty acids to cysteine residues and is common among integral and peripheral membrane proteins. Palmitoylated proteins have been found in every eukaryotic cell type examined (yeast, insect, and vertebrate cells), as well as in viruses grown in these cells. The exact functions of protein palmitoylation are not well understood. Intrinsically hydrophilic proteins, especially signaling molecules, are anchored by long-chain fatty acids to the cytoplasmic face of the plasma membrane. Palmitoylation may also promote targeting to membrane subdomains enriched in glycosphingolipids and cholesterol or affect protein–protein interactions (*see* **refs. *1–5*** for recent reviews).

Subheading 3.1. describes our standard protocol for metabolic labeling of palmitoylated proteins and also the procedures to prove a covalent and ester-type linkage of the fatty acids. Palmitic acid is a major fatty acid of cellular lipids. Thus, the vast majority (>99.5%) of the radioactivity is incorporated into lipids and only a tiny amount remains for the labeling of proteins. A protein must be fairly abundant in the cell type analyzed to detect palmitoylation. If DNA-clones of the potentially acylated protein are available, expression of the protein in vertebrate cells is often successful to increase its amount. **Subheading 3.1.** also describes our protocol for transient expression of recombinant proteins with the Vaccinia virus/T7-RNA-polymerase system.

Palmitate is usually found as the predominant fatty acid in S-acylated proteins, but other fatty acid species (myristic, stearic, oleic, and arachidonic acid) are often minor and sometimes even main components. In accordance, many S-acylated proteins can be labeled with more than one fatty acid and the palmitoyl-transferase shows no strict preference for palmitate in vitro. The

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functional significance of protein acylation with more than one fatty acid is not known ***(6,7)***. **Subheading 3.2.** describes a simple method to analyze the fatty acid content of S-acylated proteins.

Palmitoylation is unique among hydrophobic modifications because the fatty acids may be subject to cycles of de- and reacylation. The turnover of the fatty acids is often enhanced upon treatment of cells with physiologically active substances. It is supposed that reversible palmitoylation plays a role for the function of these proteins by controlling their membrane-binding and/or their protein-protein interactions ***(8)***. Two palmitoyl-thioesterases have recently been purified and their cDNAs have been cloned ***(9,10)***. However, for reason that are not known, turnover of fatty acids does not occur on every palmitoylated protein. **Subheading 3.3.** describes two methods to analyze dynamic palmitoylation for a given protein.

Integral membrane proteins are palmitoylated at cysteine residues located at the boundary between the transmembrane segment and the cytoplasmic tail. Peripheral membrane proteins are often acylated at an N-terminal MGCXXS motif, which possibly provides a dual signal for amide-myristoylation as well as S-palmitoylation. However, comparison of the amino acids in the vicinity of all known palmitoylated cysteine residues reveals no obvious consensus signal for palmitoylation ***(11,12)***. Thus, palmitoylation of a protein can not be predicted from its amino acid sequence and the palmitoylation site(s) have to be determined experimentally for each protein. This is usually done by site-specific mutagenesis of cysteine residues and subsequent expression of wild-type and mutant proteins in vertabrate cells. The most popular mutagenesis approach is the polymerase chain reaction (PCR) overlap extension method, which we describe in **Subheading 3.4.** Once a nonpalmitoylated mutant has been created, it can be used for funcional studies to determine the role of the fatty acids in the life-cycle of the protein.

The enzymology of protein-palmitoylation is poorly understood. Two putative palmitoyl-transferases (PAT) have recently been purified ***(13,14)***. However, their molecular identity and their relevance for the palmitoylation of proteins in vivo remain unresolved. Furthermore, enzymatically activities extracted from microsomal membranes have been characterized, but from neither of them PAT could be purified to homogeneity ***(15–20)***. On the other hand, some proteins are palmitoylated in vitro in the absence of an enzyme source when incubated with palmitoyl-Coenzyme A (Pal-CoA), which serves as an acyldonor in the palmitoylation reaction ***(21)***. This autocatalytic or nonenzymatic palmitoylation shows all the characteristics of authentic palmitoylation. In **Subheading 3.5.** we describe protocols to study cell-free palmitoylation of proteins.

2. Materials

2.1. Detection of Palmitoylated Proteins

1. Fetal calf serum (FCS).
2. Tissue-culture medium without FCS.
3. Recombinant vaccinia virus vTF7-3 diluted to 200 µL with medium without FCS (*see* **Note 1**).
4. CO_2 incubator.
5. Lipofectin solution: 10 µL of Lipofectin (Life Technologies) with 90 µL medium without FCS (*see* **Note 2**).
6. Vector DNA solution: Dilute 1–3 µg of pTM1 vector DNA with medium without FCS to a final volume of 100 µL (*see* **Note 2**).
7. Tritiated fatty acids, [9, 10-^{3}H (N)]-palmitic acid, and [9, 10-^{3}H (N)]-myristic acid, both at a specific activity of 30–60 Ci/mmol, are available from Amersham (Arlington Heights, IL), NEN-DuPont (Boston, MA), or ARC (St. Louis, MO). [9, 10-^{3}H]-stearic acid, 10–30 Ci/mmol, is delivered by ARC only.
8. Phosphate-buffered saline (PBS): 0.14 *M* NaCl, 27 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4, pH 7.2.
9. RIPA-buffer: 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% deoxycholate. 0.15 *M* NaCl, 20 m*M* Tris-HCl, 10 m*M* ethylenediaminetetraacetic acid (EDTA), 10 m*M* Jodacetamide, 1 m*M* PMSF, pH 7.2.
10. Protein A Sepharose CL-4B is available from Sigma (St. Louis, MO). Wash the beads three times with PBS. The packed beads are then resuspended in an equal volume of PBS and stored at 4°C.
11. SDS-PAGE sample buffer (nonreducing, 4X concentrated): 0.1 *M* Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.005% (w/v) bromophenole blue.
12. Gel-fixing solution: 20% methanol, 10% glacial acetic acid.
13. Scintillators for fluorography are available from Amersham (Amplify) or DuPont (Enlightening, En^{3}Hance). En^{3}Hance is also available as spray for fluorography of thin-layer plates.
14. 1 *M* Sodium salicylate, adjusted to pH 7.4.
15. Whatman 3MM filter paper.
16. Kodak X-OMAT AR film (Rochester, NY).
17. Phosphate-buffer (10 m*M*, pH 7.4; supplemented with 0.1% SDS).
18. Chloroform/methanol (2/1, by vol).
19. SDS-PAGE sample buffer (reducing, 4X concentrated): 0.1 *M* Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.005% (w/v) bromophenole blue, 10% mercaptoethanol.
20. 1 *M* Hydroxylamine, pH 7.0 and pH 10.0, respectively (*see* **Note 3**).
21. 1 *M* Tris, adjusted to pH 7.0 and pH 10.0, respectively.
22. Dimethylsulfoxide (DMSO).
23. Mercaptoethanol.

2.2. Analysis of Protein-Bound Fatty Acids

1. Tritiated fatty acids, [9, 10-^{3}H (N)]-palmitic acid, and [9, 10-^{3}H (N)]-myristic acid, both at a specific activity of 30–60 Ci/mmol, are available from Amersham (Arlington Heights, IL), NEN-DuPont (Boston, MA) or ARC (St. Louis, MO). [9, 10-^{3}H]-stearic acid, 10–30 Ci/mmol, is delivered by ARC only.
2. Scintillators for fluorography are available from Amersham (Amplify) or DuPont (Enlightening, En^{3}Hance). En^{3}Hance is also available as spray for fluorography of thin-layer plates.
3. 1 *M* Sodium salicylate, adjusted to pH 7.4.
4. DMSO.
5. 6 *N* HCl.
6. Glass ampoules for hydrolysis (e.g., Wheaton micro product V Vial, 3.0 mL with solid screw cap) are available from Aldrich (Milwaukee, WI).
7. Hexane.
8. HPTLC RP 18 thin-layer plates and hydroxylamine are available from Merck (Darmstadt, Germany).
9. Reference ^{3}H-fatty acids (^{3}H-myristate, ^{3}H-palmitate and ^{3}H-stearate).
10. TLC-solvent: acetonitrile/glacial acetic acid (1/1, by vol).
11. Kodak X-OMAT AR film (Rochester, NY).

2.3. Determination of a Possible Turnover of the Protein-Bound Fatty Acids

1. Tritiated [9, 10-^{3}H (N)]-palmitic acid at a specific activity of 30–60 Ci/mmol, Amersham (Arlington Heights, IL), or NEN-DuPont (Boston, MA) or ARC (St. Louis, MO).
2. Tissue-culture medium containing 0.1% fatty acid free bovine serum albumin (BSA).
3. Palmitic acid stock solution: 100 m*M* palmitic acid in ethanol.
4. Cycloheximide stock solution: 50 mg/mL in ethanol.

2.4. PCR-Based Mutagenesis of DNA Sequences

1. GeneAmp PCR Reagents Kit (Perkin-Elmer) containing Taq-Polymerase, PCR buffer, and dNTPs.
2. HPLC-purified synthetic oligonucleotides (primers).
3. JETsorb kit (Genomed, Germany).
4. Plasmid containing the gene of interest.

2.5. Cell-Free Palmitoylation of Proteins

1. Acyl-CoA Synthase (from pseudomonas fragi, 20 U in 2.8 mg of protein; Boehringer Mannheim, Germany) stock solution: 10 mg/mL in phosphate-buffer.
2. Coenzyme-A stock solution: 10 m*M* in aqua dest.

3. ^{3}H-palmitic acid (125 µCi, 50–60 Ci/mmol, dissolved in 25 µL ethanol).
4. Phosphate-buffer 20 m*M*, pH 7.4.
5. ATP stock solution: 100 m*M* in phosphate-buffer.
6. 100 m*M* $MgSO_4$ solution.
7. Stop solution: acetonitrile/1 *M* phosphoric acid (9/1 by vol).
8. Toluene.
9. TNE-buffer: 20 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 5 m*M* EDTA.
10. TLC-plates, Silica 60 (Merck, Darmstadt, Germany).
11. TLC solvent: butanol/acetic acid/aqua dest. (8/3/3, by vol).
12. PalCoA standard.
13. TNE-buffer with 2% Triton X-100.
14. Hydroxylamine.
15. PD-10 column.
16. TNE-buffer containing 0.1% Triton X-100.
17. Haemoglobin solution: 1% in TNE-buffer.
18. TNE-buffer with 1 m*M* DTT.
19. Chloroform/methanol, 1 : 2 by volume.
20. Ethanol.

3. Methods

3.1. Detection of Palmitoylated Proteins

3.1.1. Vaccinia Virus-Based Expression of Foreign Genes for Acylated Proteins Mammalian Cells

The Vaccinia virus/T7-RNA-Polymerase expression system requires cloning of the gene of interest in an expression plasmid, which contains the promotor for the RNA-polymerase from bacteriophage T7 (e.g., pTM1; ***6***). Mammalian cells do not express T7 RNA-polymerase and therefore they are infected with a recombinant vaccinia virus containing the gene for the polymerase (e.g., vTF7-3, ***6***). Vaccinia virus has a broad host range and many different cell types can be used for expression. The T7 promotor is very strong, causing a high rate of synthesis of the foreign protein. One disadvantage of this system is the severe and rapid cytopathic effect caused by the virus infection, which might prevent longer-term functional studies with the expressed protein. Media and material in contact with the virus should be autoclaved or disinfected (e.g., with hypochlorite, 70% ethanol, etc.). Gloves should be worn while handling virus and virus-infected cells.

The time schedule given below is for monkey kidney cells (CV-1) and it might differ for other cell types. The quantities given in **Subheadings 3.1.1.** and **3.1.2.** are for labeling of one subconfluent cell monolayer grown in a plastic-dish with a diameter of 3.5 cm (approx 1×10^5 –1×10^6 cells).

1. Wash the cells twice with medium without FCS.
2. Infect the cells at a multiplicity of infection (m.o.i.) of 10 plaque forming units (pfu)/cell with recombinant vaccinia virus vTF7-3 diluted to 200 µL with medium without FCS (*see* **Note 1**).
3. Incubate at 37°C in a CO_2 incubator for 2 h.
4. Mix 10 µL of Lipofectin (Life Technologies) with 90 µL medium without FCS (*see* **Note 2**).
5. Dilute 1–3 µg of pTM1 vector DNA with medium without FCS to a final volume of 100 µL (*see* **Note 2**).
6. Carefully mix diluted Lipofectin and vector DNA solutions and incubate at room temperature for at least 15 min to allow DNA/Lipofectin complexes to form.
7. Meanwhile, remove the vaccinia virus inoculum from the dish and carefully wash the monolayer once with medium without FCS.
8. Add 400 µL medium without FCS to the Lipofectin/DNA mixture, swirl, and carefully pipet the mixture onto the cells.
9. Place in CO_2 incubator at 37°C for 2–4 h.
10. Replace medium containing the Lipofectin/DNA mixture with labeling medium containing ^{3}H-palmitic acid (*see* **Subheading 3.1.2.**).

3.1.2. Metabolic Labeling of Cells with ^{3}H-Palmitate

1. Transfer 500 µCi ^{3}H-palmitic acid to a polystyrene tube and evaporate the solvent (ethanol or toluene) in a Speed-Vac centrifuge or with a gentle stream of nitrogen (*see* **Note 4**).
2. Redissolve ^{3}H-palmitic acid in 2.5 µL ethanol by vortexing and pipetting the droplet several times along the wall of the tube. Collect the ethanol at the bottom of the tube with a brief spin.
3. Add ^{3}H-palmitic acid to 500 µL tissue-culture medium (*see* **Note 5**), vortex and add the labeling medium to the cell monolayer.
4. Label cells for 1–16 h (*see* **Note 6**) at 37°C in an incubator. During longer labeling times, a slowly rocking platform is helpful to distribute the medium equally over the cell monolayer.
5. Place dishes on ice, remove labeling medium, wash cell-monolayer once with ice-cold PBS (1 mL) and lyse cells in 800 µL RIPA-buffer for 15 min on ice.
6. Transfer cell lysate to an Eppendorf tube and pellet insoluble material for 30 min at 14,000 rpm in an Eppendorf centrifuge (*see* **Note 7**).
7. Transfer supernatant to a fresh Eppendorf tube. Add antibody and Protein-A-Sepharose (30 µL) and rotate overnight at 4°C (*see* **Note 8**).
8. Pellet antigen-antibody-Sepharose complex (5000 rpm, 5 min), remove supernatant, add RIPA-buffer (800 µL), and vortex.
9. Repeat **step 8** at least twice.
10. Solubilize antigen-antibody-Sepharose complex in 20 µL of nonreducing 1X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (*see* **Note 9**). Heat samples 5 min at 95°C. Pellet Sepharose beads (5000 rpm, 5 min).

11. Load the supernatant on a discontinuous polyacrylamide-gel. SDS-PAGE should be stopped before the bromophenole blue has reached the bottom of the gel.
12. Agitate the gel for 30 min in fixing solution. Treat gel with scintillator as described by the manufacturer. All the commercially available scintillators are suitable for detection of palmitoylated proteins. We usually use the salicylate method as follows: Agitate the fixed gel for 30 min in distilled water and then for 30 min in 1 *M* sodium salicylate, adjusted to pH 7.4.
13. Dry the gel on Whatman 3MM filter paper and expose in a tightly fitting cassette to X-ray film at –70°C (*see* **Note 10**). Kodak X-OMAT AR film (Rochester, NY) is supposed to be most sensitive.

3.1.3. Chloroform/Methanol Extraction of ^{3}H-Palmitic Acid-Labeled Proteins

Denaturing SDS-PAGE is usually sufficient to separate proteins from lipids, which run just below the dye front and appear as a huge spot at the bottom of the fluorogram. Some proteins have a strong affinity for phospholipids or other fatty acid containing lipids. If the binding of only a small amount of lipids were to resist SDS-PAGE, this would simulate palmitoylation. To exclude possible noncovalent lipid binding, immunoprecipitated samples should be extracted with chloroform/methanol prior to SDS-PAGE and the amount of chloroform/methanol-resistant labeling should be compared with a nonextracted control.

1. Label cells with ^{3}H-palmitate and immunoprecipitate protein as described.
2. Solubilize antigen-antibody-Sepharose complex in 30 µL phosphate-buffer (10 m*M*, pH 7.4; supplemented with 0.1% SDS), pellet Sepharose beads, and dispense 2×15 µL of the supernatant into two Eppendorf tubes.
3. Add 300 µL chloroform/methanol (2:1) to one tube. Vortex vigorously and extract lipids for 30 min on ice. The unextracted sample also remains on ice.
4. Pellet precipitated proteins for 30 min at 14,000 rpm in an Eppendorf centrifuge precooled at 4°C. Carefully remove the supernatant, air-dry the (barely visible) pellet, and resuspend it in 1X nonreducing SDS-PAGE sample buffer. Add 5 µL 1X concentrated sample buffer to the unextracted sample.
5. Proceed with SDS-PAGE and fluorography as described.

If the fatty acids are noncovalently bound, the ^{3}H-palmitic acid labeling of the extracted sample should be drastically reduced compared to the control sample.

3.1.4. Hydroxylamine and Mercaptoethanol Treatment

Two types of fatty acid linkages have been described in acylated proteins, an amide bond in myristoylated proteins and an ester-type linkage in palmitoylated proteins. Whereas amide-linked fatty acids are resistant to treatment with hydroxylamine, the esters are readily cleaved. Treatment with hydroxylamine, adjusted either to neutral or basic pH, can also be used to discriminate between

thioesters to cysteine and oxygenesters to serine or threonine. Under alkaline conditions (pH 9.0–11.0) hydroxylamine cleaves both thio- and oxygenesters, whereas at neutral pH (pH 6.5–7.5) thioesters are selectively cleaved. A thioester-type linkage can be further verified by its susceptibility to reducing agents, especially at high concentrations and temperatures. However, not all thioesters are equally sensitive to reducing agents (*see* **Note 9**).

Hydroxylamine treatment is usually done on gels containing ^{3}H-palmitate labeled samples:

1. Run an SDS-PAGE with four samples of the ^{3}H-palmitate labeled and immunoprecipitated protein. Each sample should be separated by two empty slots from its neighbors.
2. Fix the gel, wash out the fixing solution with destilled water (2 × 30 min).
3. Cut gel into four parts containing one lane each.
4. Treat two parts of the gel overnight under gentle agitation with 1 *M* hydroxylamine, pH 7.0 and pH 10.0, respectively. The remaining two gel parts are treated with 1 *M* Tris, adjusted to the same pH values (*see* **Note 3**).
5. Wash out the salt solutions with dest. H_2O (2 × 30 min). Remove cleaved fatty acids by washing with dimethylsulfoxide (DMSO, 2 × 30 min) and wash out DMSO with dest. H_2O. Proceed with fluorography treatment. Reassemble the gel parts before drying and expose to X-ray film.

Mercaptoethanol treatment is done prior to SDS-PAGE:

1. Immunoprecipitate ^{3}H-palmitate labeled protein.
2. Solubilize protein in 100 µL 1X nonreducing sample buffer (2 min, 95°C).
3. Pellet Sepharose beads. Make 5 aliquots of the supernatant, add mercaptoethanol to a final concentration of 5, 10, 15, and 20% (v/v). Mercaptoethanol is omitted from one sample. Dithiothreitol (DTT) can also be used at concentrations of 50, 100, 150, and 200 m*M*.
4. Heat samples for 10 min at 95°C. Centrifuge for 15 min at 14,000 rpm. Some proteins may precipitate after treatment with reducing agents and are pelleted. Exclude by analysis (e.g., by Western blotting or ^{35}S-methionine labeling) that a decrease in the ^{3}H-palmitate labeling is not due to its aggregation. Should this be the case, treatment with reducing agents needs to be done at lower temperatures (1 h, 50°C).
5. Proceed with SDS-PAGE and fluorography as described.

3.2. Analysis of Protein-Bound Fatty Acids

During metabolic labeling ^{3}H-palmitic acid is often converted into other ^{3}H-fatty acid species and even into ^{3}H-amino acids before incorporation into proteins. Thus, labeling of a protein with ^{3}H-palmitic acid does not necessarily prove that palmitate is its only or even its major fatty acid constituent

and therefore its actual fatty acid content has to be analyzed. Furthermore, identification of the ^{3}H-palmitate derived labeling as a fatty acid is additional proof for its acylation. We describe a simple protocol feasible in laboratories without expensive equipment for lipid analysis. The method uses acid hydrolysis of ^{3}H-palmitate-labeled proteins present in gel slices, extraction of the released fatty acids with hexane and thin-layer chromatography (TLC) to separate fatty acid species.

1. Label your protein with ^{3}H-palmitate or another ^{3}H-fatty acid as long as possible to allow its metabolism. Proceed with immunoprecipitation and SDS-PAGE as described.
2. Localize the protein by fluorography and cut out the band. Remove the scintillator by washing with dest. H_2O (hydrophilic scintillators, e.g. salicylate, Enlightening) or DMSO (hydrophobic scintillators, e.g., En^{3}Hance, PPO). 2×20 min are usually sufficient. Wash out the DMSO with dest. H_2O.
3. Cut the gel into small pieces, transfer them into glass ampoules, and dry them in a desiccator.
4. Add 500 µL HCl (6 N) and let the gel swell. The gel pieces should be completely covered with HCl after swelling.
5. Tightly seal glass ampoules and incubate at 110°C for at least 16 h. Polyacrylamide and HCl form a viscous fluid at high temperatures. Add an equal amount of hexane and vortex vigorously. Separate the two phases by gentle centrifugation (5 min, 2000 rpm). Most of the polyacrylamide is sedimented to the bottom of the vessel.
7. Remove the upper organic phase containing the fatty acids with a Pasteur pipet and transfer it to conical glass vessels. Leave behind the traces of polyacrylamide that are present between the two phases. Repeat extraction of fatty acids with hexane and combine upper phases.
8. Concentrate pooled organic phases in a stream of nitrogen to a volume of 20 µL.
9. Draw a line with a soft pencil on the concentration zone of the TLC-plate (HPTLC RP 18 from Merck, Darmstadt), approx 1 cm from the bottom. Apply your sample carefully in a spot as small as possible. Apply reference ^{3}H-fatty acids (^{3}H-myristate, ^{3}H-palmitate and ^{3}H-stearate) on a parallel spot (*see* **Note 11**).
10. Put TLC-plate in an appropriate glass chamber containing the solvent system (acetonitrile/glacial acetic acid, 1 : 1). Take care that the samples do not dip into the solvent.
11. Develop chromatogram until the solvent front has reached the top of the plate (approx 50 min).
12. Air-dry plate under a hood. Measure radioactivity on the plate with a radiochromatogram-scanner. Alternatively, spray plate with En^{3}hance, air-dry completely, and expose to X-ray film. Detection of ^{3}H-fatty acids by fluorography of the TLC-plate requires long exposure times and is only feasible with a protein band easily visible in the SDS-gel after 3–5 d of film exposure.

3.3. Determination of a Possible Turnover of the Protein-Bound Fatty Acids

3.3.1. Pulse-Chase Experiments with ^{3}H-Palmitate

To show deacylation of a protein directly, pulse-chase experiments with ^{3}H-palmitic acid have to be performed. Deacylation is visible as a decrease in the ^{3}H-palmitate labeling with increasing chase time. The half-life of the fatty acid cleavage can also be determined from these experiments. However, ^{3}H-palmitate labeling of protein can not be chased completely. Vast amounts of label are present in cellular lipids, which themselves show fatty acid turn-over, and a substantial fraction also as palmitoyl-coenzyme A, the acyl donor for palmitoylation. The following protocol is designed to minimize these problems.

1. Label several cell monolayers for 1 h with ^{3}H-palmitic acid as described.
2. Remove labeling medium. Wash monolayer twice with 1 mL medium containing fatty acid free bovine serum albumin (0.1%). Albumin will extract some of the remaining unbound fatty acids.
3. Add 1 mL medium supplemented with 100 µ*M* unlabeled palmitic acid. Palmitic acid is stored as a 100 m*M* stock solution in ethanol and is diluted 1 : 1000 into the cell culture medium. Unlabeled palmitate will compete with ^{3}H-palmitate for the incorporation into protein.
4. Lyse one cell monolayer immediately and chase remaining cells for different periods of time (e.g., 20, 40, 60 min up to 4 h) at 37°C.
5. Wash and lyse cells and proceed with immunoprecipitation as described.

3.3.2. Cycloheximide Treatment

Treatment of cells with appropriate concentrations of cycloheximide prevents protein-synthesis immediately and nearly quantitatively, but has no obvious effect on palmitoylation per se. Thus, strong ^{3}H-palmitate labeling of a protein in the absence of ongoing protein synthesis is taken as an indication for reacylation of a previously deacylated protein. However, this issue is more complicated than it seems at first glance. Palmitoylation is a posttranslational modification. Therefore, freshly synthesized proteins continue to incorporate ^{3}H-palmitate until all molecules have passed their intracellular site of palmitoylation. This takes approx 10–20 min for proteins transported at a fast rate along the exocytotic pathway and their ^{3}H-palmitate incorporation decreases during this time. In contrast, ^{3}H-palmitate incorporation into a previously deacylated protein is not dependent on the labeling time after cycloheximide addition. It is therefore advisable to compare the ^{3}H-palmitate labeling of a protein at different time points after blocking protein synthesis.

1. Add 6 µL cycloheximide to 6 mL cell-culture medium from a 50 mg/mL stock in ethanol to reach a final concentration of 50 µg/mL. Add 1 mL medium to

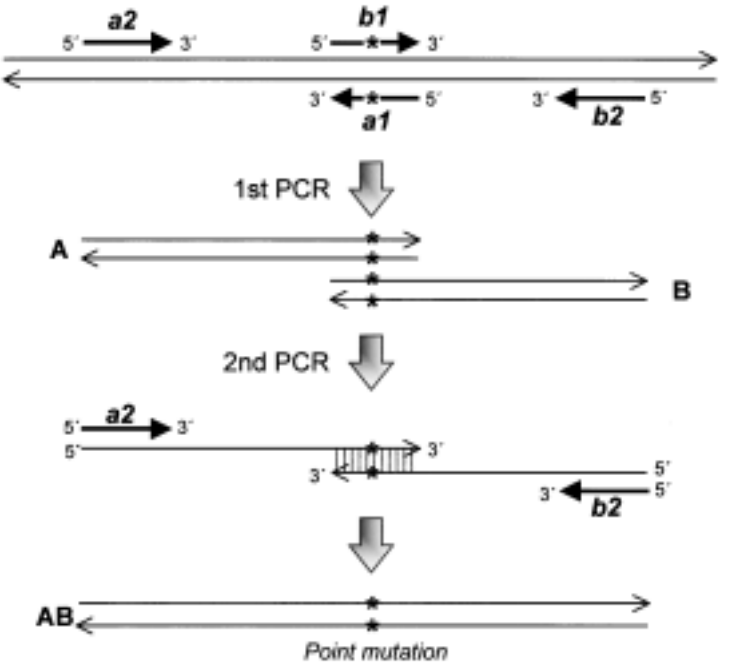

Fig. 1. Principle of introducing site-specific mutations into DNA fragment. In this case PCR is used to introduce a mutation near the overlapping ends of the targeted DNA fragments (first PCR). In the second PCR step, the mutation is placed inside the resulting DNA molecule distant from either end. The asterisk indicates the mismatch in primer b1 and a1 resulting in a mutation in the amplified DNA-products.

cell monolayers and incubate at 37°C. One monolayer should not be treated with cycloheximide.

2. Label cells with ^{3}H-palmitic acid for 1 h, either immediately or 5, 10, 20, 30, and 60 min after cycloheximide addition. The labeling medium should also contain cycloheximide (50 μg/mL).
3. Proceed with immunoprecipitation, SDS-PAGE, and fluorography as described.

3.4. PCR-Based Mutagenesis of DNA Sequences

The PCR-based mutagenesis approach is based on the fact that sequences added to the 5′-end of a PCR primer become incorporated into the end of the resulting molecule. By adding the appropriate sequences, a PCR-amplified segment can be made to overlap sequences with another segment. In the second PCR this overlap serves as a primer for extension resulting in a recombinant molecule. A schematic overview of the method is shown in **Figs. 1** and **2**.

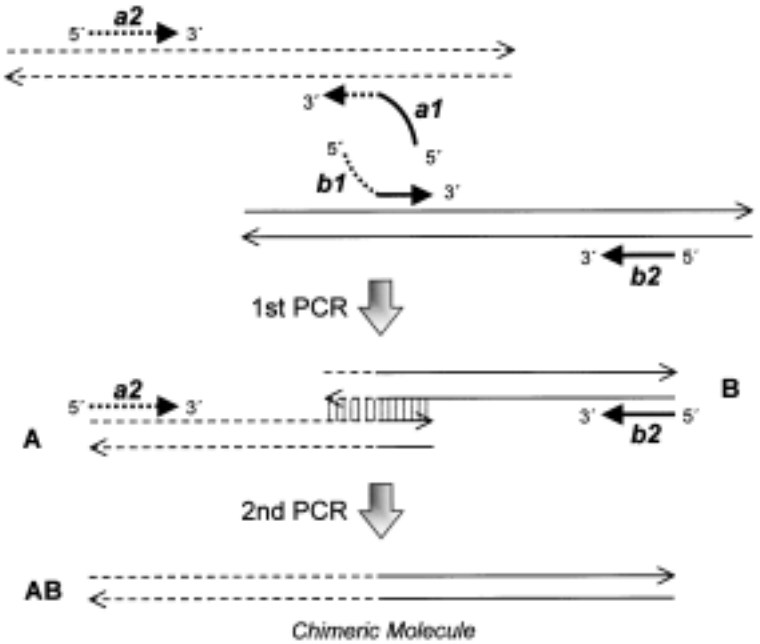

Fig. 2. Outline of overlap extension PCR application for the recombination of DNA molecules. Incorporating sequences complementary to the other gene into the 5′-end of a primer makes it possible to generate PCR products that overlap with the other gene segment. These segments can then be recombined during the second PCR step to produce the desired chimeric molecule.

1. Mix the following components for two different PCR reactions in separate autoclaved sample tubes to amplify products **A** and **B** (*see* **Table 1**).
2. Vortex the mixtures and briefly spin down in a microcentrifuge.
3. Cycle for 25–35 rounds (initial denaturation at 94°C for 2 min, denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, primer extension at 72°C for 2 min, final extension at 72°C for 10 min).
4. Run the reaction on an agarose gel (1% to 1.5%) and cut out the bands that have the appropriate size for **A** and **B**.
5. Purify the DNA fragment from the gel slice. We routinely use a JETsorb kit (Genomed, Germany) for DNA extraction from agarose gel (*see* **Note 16**).
6. Pipet the reactants for the second PCR reaction into new sample tubes and subject to thermal cycling as in **step 3** above:

Template A	50 ng to 1 µg
Template B	50 ng to 1 µg
Primer ***a2***	0.1–1 µ*M*
Primer ***b2***	0.1–1 µ*M*

Table 1
Components for the Two PCR Reactions to Amplify Products A and B

	PCR products	
Components	**A**	**B**
Template (*see* **Note 12**)	50 ng to 1 µg of appropriate gene	50 ng to 1 µg of appropriate gene
Primer ***a1*** (*see* **Note 13**)	0.1–1 µ*M*	–
Primer ***a2***	0.1–1 µ*M*	–
Primer ***b1***	–	0.1–1 µ*M*
Primer ***b2***	–	0.1–1 µ*M*
10X PCR buffer (*see* **Note 14**)	10 µL	10 µL
10X dNTPs (*see* **Note 15**)	10 µL	10 µL
Taq polymerase	0.5–2.5 U	0.5–2.5 U
H_20 bidest	To 100 µL	To 100 µL

10X PCR buffer	10 µL
10X dNTPs	10 µL
Taq polymerase	0.5–2.5 U
H_2O bidest	to 100 µL

7. Run the reaction on an agarose gel (1% to 1.5%) and cut out the bands that have the appropriate size for **AB**. Purify the DNA fragment from the gel slice.

The purified DNA can be used for subcloning into a vector suitable for further applications.

3.5. Cell-Free Palmitoylation of Proteins

Cell-free palmitoylation of a protein will give hints if acylation requires an enzyme. An effective palmitoylation assay might also be a starting point to characterize and purify the palmitoylating enzyme. The acceptor protein is incubated with the acyldonor ^{3}H-Pal-CoA in the presence or absence of an enzyme source. The samples are then subjected to SDS-PAGE and fluorography to check for incorporation of ^{3}H-palmitate. The protocols described in **Subheadings 3.1.3.** and **3.1.4.** can be used to verify covalent incorporation of the label into the protein. A mutant with the fatty acid binding site deleted or substituted is also a useful control to verify that the fatty acids are attached to the same site(s) that are palmitoylated in vivo. In the following section we describe our standard protocols to prepare ^{3}H-Pal-CoA and acceptor proteins. We also mention some considerations that have to be taken into account to set up an initial cell-free palmitoylation-experiment.

3.5.1. Preparation of ^{3}H-Pal-CoA

^{3}H-Pal-CoA is commercially available, but is expensive and has a low specific activity. We therefore prepare our own ^{3}H-Pal-CoA using acyl-CoA synthetase, Coenzyme A, ATP, and ^{3}H-palmitic acid.

1. Mix into glass vessels:
   a. Acyl-CoA synthetase (10 µL from a 10 mg/mL stock in phosphate-buffer), pH 7.0
   b. Coenzyme-A (15 µL from a 10 m*M* stock in aqua dest.)
   c. ^{3}H-palmitic acid (125 µCi, 50–60 Ci/mmol, dissolved in 25 µL ethanol)
   d. ATP (2.5 µL from a 100 m*M* stock, 1 m*M* final concentration)
   e. $MgSO_4$ (5 µL of a 100 m*M* stock, 5 m*M* final concentration)
   f. Phosphate buffer, pH 7.4, to give a final volume of 250 µL.
2. Incubate for 10 min at 28°C.
3. Stop the reaction by adding 1 mL acetonitrile/1 *M* phosphoric acid (9:1). Acyl-CoA synthetase precipitates and is pelleted with a centrifugation at 5000*g* for 10 min.
4. Extract the supernatent with 1 mL of toluene. Let the glass vessels stand until the two phases are separated.
5. Discard the upper phase containing unreacted ^{3}H-palmitic acid. The toluene-extraction is repeated twice.
6. Remove the lower phase, which contains ^{3}H-Pal-CoA but also traces of toluene. Wash the glass vessels with 200 µL TNE containing 0.1% Triton X-100. This wash contains the highest concentration of ^{3}H-Pal-CoA and is used in the palmitoylation-assay.
7. Determine the radioactivity of the ^{3}H-Pal-CoA preparation by liquid-scintilation counting. 1 µL usually contains 50,000–200,000 counts per minute.
8. Check the purity of the ^{3}H-Pal-CoA preparation using TLC with Silica 60 plates (Merck, Darmstadt) and butanol/acetic acid/aqua dest. (8:3:3) as solvent system. Run commercial PalCoA as a standard in parallel lane.

3.5.2. Preparation of Acceptor-Proteins

Proteins purified from several sources have been used successfully in a cell-free palmitoylation assay ***(1,12–17,19,20)***.

3.5.2.1. Purification of Proteins Using Overexpression of Fusion Proteins in *E. coli*

This is the method of choice to purify proteins that are not very abundant in their natural environment. Several expression and purification systems are commerially available (Qiagen, Pharmacia). The gene is expressed as a fusion protein equipped with a tag (e.g., 6 × Histidine, Glutathion Transferase), which can be used in a rapid and simple one-step affinity-chromatography puri-

fication. *Escherichia coli* cells lack the machinery for protein-palmitoylation and therefore the proteins are expressed in a nonpalmitoylated version, which can be used directly in the palmitoylation assay. One disadvantage is the inability of *E. coli* cells to express transmembrane proteins in a soluble form. Therefore hydrophobic domains, which often contain putative fatty acid linkage sites or modulate protein-acylation, have to be deleted prior to expression. Also expression products from *E. coli* are never glycosylated, which could be a critical modification in acylated glycoproteins for stabilizing conformation.

3.5.2.2. Purifiction of Proteins from Enveloped Viruses

We routinely purify palmitoylated glycoproteins from the membrane of enveloped viruses. Semliki forest virus (SFV), which contains two palmitoylated glycoproteins (E1 and E2) is grown overnight in baby hamster kidney (BHK) cells until a severe cytopathic effect becomes apparent. Cell debris is then removed by centrifugation of the culture medium at 3000*g* for 30 min. Virus particles are pelleted from the supernatant at 30,000*g* for 3 h. The viral glycoprotein (E1 and E2) is then solubilized from the viral membrane and depalmitoylated with hydroxylamine.

1. Resuspend the virus pellet in 250 µL TNE-buffer.
2. Add an equal amount of TNE with 2% Triton X-100 and incubate for 60 min with agitation at room temperture.
3. Pellet the viral core proteins at 100,000*g* for 60 min.
3. Add hydroxylamine to the glycoprotein containing supernatant to give a final concentration of 1 *M* and incubate for 60 min with agitation.
4. Equilibrate a PD-10 column with TNE containing 0.1% Triton X-100. Apply the sample to the column and collect 500 µL fractions.
5. Place 10 µL of the fraction in a microtiter plate well and add 50 µL of a hemoglobin solution. A color changes from red to brown indicates that the fractions contain hydroxylamine. These fractions are discarded because hydroxylamine interferes with the assay.
6. Check 10 µL of each fraction by SDS-PAGE and Coomassie-staining.
7. Pool the protein containing, hydroxylamine-free fractions determine protein content with an aliquot in a protein assay (Bio-Rad assay) and store aliquots at –80°C.

3.5.3. Cell-Free Palmitoylation Assay

Obviously the conditions vary for each acceptor-protein and enzyme-source under investigation. Therefore only general considerations are mentioned that have to be taken into account for the set up of an initial palmitoylation experiment.

Acceptor-protein: 1–10 µg of purified and deacylated protein (*see* above).

Lipid-Donor: 1×10^5–1×10^6 counts/min of ^{3}H-Palmitoyl-Coenzyme A prepared as described in **Subheading 3.5.1.**

Enzyme source: approx 10 µg of protein, either microsomes, detergent extracts of microsomes, or fractions derived from ion-exchange chromatography.

Detergent: Diverse nondenaturing detergents, for example Triton X-100 and Cholate are compatible with in vitro palmitoylation. Octylglucosid is often inhibitory, probably because its carbon chain competes with fatty acid transfer. The concentration of the detergent varies with the experiment. Nonenzymatic palmitoylation is extremly sensitive to detergent and therefore its concentration should be as low as possible (e.g., 0.001%). Our partially purified enzyme preparation works optimally at a Triton X-100 concentration of 0.025%, probably because it is an integral membrane protein requiring detergent to keep it soluble.

Buffer: We use TNE-buffer, but other buffers also work. No specific requirements for calcium or other ions have been reported. Low concentrations of reducing agents (e.g., 1 m*M* DTT) often enhance palmitate incorporation, probably because they reduce disulfide-bonds, thereby making additional cysteine residues available for fatty acid transfer. High concentrations of reducing agents are inhibitory because they cleave thioester-linked fatty acids.

1. Incubate enzyme source, acceptor protein, and ^{3}H-Palmitoyl-Coenzyme A with buffer in a final volume of 50–250 µL for 30–60 min at 28°C or 37°C.
2. Precipitate samples with chloroform/methanol (1 : 2, 250 µL for a 50 µL reaction) to remove noncovalently bound fatty acids. Pellet precipitated proteins for 20 min at 15,000*g*. Remove the supernatant, wash the pellet once with ice-cold ethanol and centrifuge again. Air-dry the pellet before adding nonreducing SDS-PAGE sample buffer.
3. Subject samples to SDS-PAGE under nonreducing conditions and fluorography. Film-exposure times vary from overnight to several weeks.
4. Use appropriate control reactions, e.g., samples without enzyme source, samples with SDS- or heat-denatured enzyme and samples incubated on ice, to proof the enzymatic character of the reaction.

4. Notes

1. To amplify vaccinia virus, seed a maxi Petri dish (15 cm diameter) with 1×10^7 CV-1 cells in medium supplemented with 5% FCS. Incubate overnight at 37°C and at 5% CO_2. Remove media, wash cells twice with medium without FCS, and then infect cells with vTF7-3 vaccinia virus at a low m.o.i., i.e., less than 1 pfu/cell for 1 h at 37°C. We routinely use 0.1 pfu/cell diluted with medium to a final volume of 1 mL. Discard inoculum into disinfectant and replace with 10 mL medium supplemented with 2% FCS. Incubate cells for 48 h at 37°C in a

CO_2 incubator. After this incubation, discard the medium and resuspend the cells in 1 mL medium without FCS. Freeze/thaw the cells in liquid nitrogen and a 37°C water bath two times and centrifuge at 14,000 rpm for 1 min in Eppendorf centrifuge to remove cell debris. Store the virus-containing supernatant in small aliquots at –80°C.

2. For diluting lipofectin and for preparing the DNA/Lipofectin mixture use a polysterene tube, not the polypropylene Eppendorf tube.
3. Hydroxylamine sometimes disintegrates if the pH is adjusted too quickly. Dissolve hydroxylamine in ice-cold dest. H_2O and put the solution in an ice-bath. Add solid NaOH lentils one by one and under permanent stirring until the desired pH is reached. Rapid pH changes and the appearance of a brown color in the normally colorless hydroxylamine solution are indications for a disintegration of hydroxylamine. In this case the solution has to be discarded.
4. 100 µCi - 1 mCi ^{3}H-palmitic acid/mL cell-culture medium are usually used for the labeling of acylated proteins. Tritiated fatty acids are supplied as solutions in ethanol or toluene at concentrations too low to add directly to the medium. Due to its cytotoxicity, the final concentration of ethanol in the labeling medium should not exceed 0.5%. Concentration of ^{3}H-palmitic acid can be done in advance and the concentrated stock should be stored at –20°C in tightly sealed polystyrene tubes. Concentration and storage in polypropylene (e.g., Eppendorf tubes) should be avoided because this can result in irreversible loss of much of the label on the tube.
5. Use standard tissue-culture medium for the cell line to be labeled. Possible addition of serum to the medium requires some consideration. Serum contains albumin, a fatty acid binding protein, which may delay ^{3}H-palmitate uptake by the cells. Thus, for short labeling periods (up to 4 h) we usually use medium without serum. However, serum does not prevent ^{3}H-palmitate labeling of proteins and can be added, if required. For long labeling periods, the presence of serum may even be beneficial, because reversible binding of fatty acids to albumin may help to distribute the ^{3}H-palmitate uptake of the cells more evenly. Serum contains several poorly characterized factors with biological activities and deacylation of particular proteins upon serum treatment of cells has been reported ***(3)***. Obviously, in these cases serum addition is detrimental.
6. The necessary time for optimal labeling of palmitoylated proteins is variable and has to be determined empirically for each protein. Proteins with a low but steady rate of synthesis and no turnover of their fatty acids should be labeled as long as possible, e.g., at least 4 h up to 24 h. The amount of ^{3}H-palmitate incorporation into these proteins increases with time until it reaches saturation. Reversibly palmitoylated proteins show an increase in their labeling intensity in the beginning until a peak is reached. Due to deacylation their ^{3}H-palmitic acid labeling then decreases with time. Proteins expressed from a viral expression vector should be labeled as long as the peak period of their synthesis prevails. This is 1–2 h for the described Vaccinia virus/T7 polymerase system.
7. Sedimentation of insoluble material at higher g-values (e.g., 30 min, 100,000*g*) sometimes causes a cleaner immunoprecipitation. Make sure that your protein

does not precipitate under these conditions. Another possibility to improve the specificity of the immunoprecipitation would be to preincubate the cell lysate with sepharose-beads, but without antibodies (1 h, 4°C). Pellet the beads and transfer the supernatant to a fresh Eppendorf tube and proceed with immunoprecipitation as described.

8. The amount of a monoclonal antibody (MAb) or antiserum necessary to precipitate a protein quantitatively from a cell lysate depends on its affinity for the antigen and must be determined empirically, but 1–5 µL of an high-affinity antiserum is usually sufficient for the conditions described here. To analyze a complete precipitation of a protein, transfer the first supernatant from **step 8** (**Subheading 3.1.1.**) to a fresh Eppendorf tube. Add antibody and protein-A-sepharose and proceed with immunoprecipitation. Antibodies of particulate subtypes do not bind to Protein-A. This has to be considered when MAbs are used. However, most of these antibodies bind to Protein-G sepharose ***(25)***.
9. The thioester-type linkage of fatty acids to cysteine residues is labile upon treatment with reducing agents. Cleavage of the fatty acids by these compounds is concentration-, time-, and temperature-dependent. Therefore, mercaptoethanol and DTT should be omitted from the sample buffer. If the protein requires reducing agents for solubilization, heating should be done as short as possible (95°C, 2 min) or the temperature should be decreased (e.g., 15 min at 50°C). Ester-linked fatty acids are highly susceptible to basic pH values above 12.0. Under these conditions the fatty acids are cleaved quantitatively and rapidly (<1 min) even at low temperatures. Therefore, basic pH values should be avoided under all circumstances. In contrast, treatment with acid pH (e.g., pH 1.0) for time periods up to 1 h is tolerated by the fatty acid bond. Acetic acid containing gel-fixing solutions do not lead to an obvious loss of ^{3}H-palmitate labeling.
10. Tritium-derived radioactivity has a very short range. Therefore, dry the gel as thin as possible and make sure that the X-ray film is in close contact with the gel. The times to detect a signal on the film are highly variable. Endogenous cellular proteins with low rates of synthesis require exposure times from several days up to 3 mo. For highly overexpressed proteins with multiple palmitoylation sites, they can be as short as several hours.
11. This TLC-solvent system separates fatty acids according to their hydrophobicity, i.e., the number of carbon atoms and double bonds. Myristic acid (C 14) runs faster than palmitic acid (C 16), which runs faster than stearic acid (C 18). Unsaturated fatty acids are not separated from saturated ones. A fatty acid with one double bond runs to the same position as a saturated fatty acid with two methylene groups less, i.e., oleic acid (18:1) co-migrates with palmitic acid (C 16:0). ^{3}H-Arachidonic acid (20:4) is not to be expected as protein-bound fatty acid after labeling with ^{3}H-palmitate, because cells cannot metabolize palmitate into arachidonate.
12. The primer/template ration strongly influences the specificity of the PCR and should be optimized empirically. Usually, a very wide range of template

concentrations up to about microgram works. However, too much template may lead to an increase in mispriming events.

13. It is the sequence and the concentration of primers that determines the success of the assay. Primers should be between 25 and 45 bases in length. For site-directed mutagenesis, the mismatched portions should be in the middle of the primer. For creation of chimeric molecules, the priming region (3′-end of primer, which act as primer on its template) and the overlap region (5′-end of primer, which overlap with the sequence to be joined) of the oligonucleotides should have similar length. The primers should have a G/C content between 40% and 60% but should not have any complementarity between 3′-ends.
14. 10X PCR buffer: 15 m*M* $MgCl_2$, 500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.3, and 0.01% (w/v) gelatin. This buffer is available from Perkin Elmer, which is one component of GeneAmp PCR Reagents Kit.
15. 10X dNTPs = 2.5 m*M* of each dNTP. Unbalanced dNTP mixture will reduce Taq fidelity. DNTPs reduce free Mg^{2+}, thus interfering with polymerase activity and decrease primer annealing.
16. PCR fragments purified from gel by different DNA extraction kits are often contaminated with silica beads, which strongly inhibit the subsequent PCR reaction. Therefore, it is advisable to purify DNA fragments again by phenol/chloroform extraction and ethanol precipitation.

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12

Immunoblotting Methods for the Study of Protein Ubiquitination

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1. Introduction

Ubiquitin is a highly phylogenetically conserved, 76 amino acid, 8.6 kDa, compact, globular polypeptide that appears to be ubiquitously expressed in every living cell, including plants as well as animals, as predicted by Goldstein almost 25 years ago ***(1)***. The ATP-dependent, enzyme-catalyzed, reversible attachment of one or more ubiquitin molecules to eukaryotic proteins is known as ubiquitination ***(2–6)***. Monoubiquitinated histone H2A was the first ubiquitinated protein to be discovered ***(7)***, although the function of H2A ubiquitination remains enigmatic to this day. Ubiquitin is linked to proteins by the formation of an isopeptide bond between the ε-amino group of select lysine residues in targeted substrate proteins and the C-terminal glycine carboxyl group of the ubiquitin molecule ***(7)***. The attachment of a single ubiquitin to several different lysine residues in a targeted protein is known as multiubiquitination, while polyubiquitination refers to the successive addition of many self-linked ubiquitin molecules to form linear or branched ubiquitin chains at one or more lysine residues in the target protein. For a thorough discussion of ubiquitin terminology and nomenclature, *see* **ref. *3***.

Protein ubiquitination is catalyzed by a complex, hierarchical enzymatic cascade consisting of an E1 ubiquitin-activating enzyme coupled to three classes of E2 ubiquitin-conjugating and several divergent families of E3 ubiquitin-ligating enzymes ***(3,5,6,8–10)***. This enzymatic machinery activates ubiquitin (E1) and determines substrate specificity (E2 and E3) for protein ubiquitination through recognition of several different ubiquitination-targeting signals ***(11–13)***. Conversely, there are dozens of enzymes in two

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distinct families that cleave ubiquitin from mono-, multi-, and polyubiquitinated proteins ***(14,15)***.

Within the past five years, it has become increasingly evident that protein ubiquitination plays a key role in both normal physiological and pathological conditions by modulating diverse processes such as cell growth, development, reproduction, gene expression, antigen presentation, apoptosis, and possibly even neoplastic transformation ***(5,6)***. Perhaps most importantly, ubiquitination participates in the irreversible degradation of many short-lived regulatory proteins, as well as mutated, damaged, or otherwise improperly folded proteins by the multicatalytic proteasome ***(4,5,16–18)***. Consequently, an explosive interest in the posttranslational modification of proteins by ubiquitination has occurred within many diverse scientific disciplines.

Some regulatory proteins whose functions are controlled in large part by ubiquitination and proteasomal degradation include: the tumor suppressor p53 ***(19,20)***, several mitotic cyclins ***(21)***, β-catenin ***(22)***, c-Fos ***(23)***, c-Jun ***(24)***, N-Myc ***(25,26)***, NFκβ ***(27)***, and Hif-1α ***(28)*** transcription factors, as well as the Iκβ family that negatively regulates NFκβ ***(29,30)***. Some examples of mutated or misfolded proteins that are ubiquitinated and degraded by the proteasome include: the mutated (ΔF508) cystic fibrosis transmembrane-conductance regulator ***(31)***, CD3-δ subunits of the lymphocyte T-cell receptor complex ***(32)*** and mutated, presumably misfolded, subunits of the Sec61p endoplasmic reticulum protein translocation apparatus of yeast ***(33)***. Recently, protein kinase C (PKC) activation was found to trigger its own ubiquitination and suicidal degradation by the proteasome pathway ***(34)***. Additionally, a number of plasma-membrane receptors including platelet-derived growth factor B-receptor (PDGFB-R) ***(35)***, growth hormone receptor (GHR) ***(36)*** and the epidermal growth factor receptor (EGFR) ***(37)*** are susceptible to ligand-induced ubiquitination. Interestingly, as a consequence of ubiquitination, these particular GHRs are internalized and degraded by lysosomal proteases, instead of being degraded by the proteasome (for a review, *see* **ref. *38)***.

In some cases, reversible ubiquitination acts as a switch to regulate the functions of proteins without promoting their degradation. Some examples are the Iκβ kinase complex ***(39)***, the nucleosomal histones H2A and H2B ***(40–43)***, and Ste2 protein, which is required for endocytosis in yeast ***(44)***.

Ubiquitin exists in many chemical forms within cells. It is synthesized as latent head-to-tail proubiquitin polymers with various C-terminal extensions ***(45)*** and as fusion proteins with two ribosomal protein subunits ***(46–48)***. These latent ubiquitin proforms must be enzymatically cleaved into monomers before ubiquitin can be conjugated to proteins. As first described by Haas and coworkers ***(49)***, ubiquitin forms stable thiol ester intermediates with E1 ubiquitin-activating, E2 ubiquitin-conjugating, and several of the growing number of E3 ubiquitin-

ligating enzymes ***(8,50,51)***. Ubiquitin also adventitiously reacts with thiols like glutathione and amine nucleophiles, from which the ubiquitin must be salvaged before being used by ubiquitin-conjugating enzymes ***(52)***. Polyubiquitin chains conjugated to proteins destined for degradation are at least transiently bound to the S5a subunit of the proteasome ***(53)***. However, instead of being degraded, the bound ubiquitin chains are recycled through the action of a proteasome subunit with ubiquitin hydrolase activity that cleaves the chains to ubiquitin monomers ***(54)***, possibly with assistance by other ubiquitin-processing proteases. In the nucleus, ubiquitin is extensively bound to nucleosomal histones H2A and H2B. In fact, H2A is probably the most abundant constitutively ubiquitinated protein in eukaryotes ***(55)***, although monoubiquitination clearly does not target H2A for degradation ***(40)***. It is within this complex and very dynamic biochemical milieu that cellular protein ubiquitination must be studied.

The reader is directed to recent excellent reviews on the enzymology of protein ubiquitination ***(6,56)*** and deubiquitination ***(14,15)***. Other reviews stress the cellular consequences of protein ubiquitination ***(4,5,16,17,57)***, the multiple roles that protein ubiquitination plays in cell-cycle regulation ***(21)*** and the participation of protein ubiquitination in a growing list of pathological conditions. These include Alzheimer's disease (AD), several other less prevalent neurodegenerative diseases ***(58)***, and possibly even cancer ***(6,59)***.

Knowledge of protein ubiquitination has advanced almost exponentially within the past several years, however, many important questions remain to be answered. How exactly does phosphorylation of some proteins target them for ubiquitination? What role does protein ubiquitination play in the complicated biochemistry of apoptosis? Are caspase-cleaved proteins recognized as substrates for ubiquitination? What is the function of nondegradative reversible monoubiquitination of histones and other proteins? Does unregulated protein ubiquitination play a role in the etiology of neurodegenerative diseases where excessive protein ubiquitination has been observed, and if so, can therapeutic strategies be designed to reverse or prevent protein ubiquitination? Recent evidence has suggested that proteasome inhibitors have promise as a new class of anticancer drugs. Are ubiquitinated proteins reasonable candidates for molecular targeting in the treatment of human malignancies? Are proteasome inhibitors cytotoxic because they stabilize ubiquitinated key regulatory proteins like cyclins or transcription factors? Is it possible to create chaos in rapidly proliferating tumor cells by specifically inhibiting ubiquitinating or deubiquitinating enzyme activities? Advances in the measurement of protein ubiquitination by immunoblotting, including the development of antibodies that recognize specific ubiquitin-protein conjugates, like ubiquitinated p53 or ubiquitinated NFκβ, will provide researchers with the tools to answer these and other important questions.

In this chapter, we provide a detailed practical method for measuring ubiquitinated proteins by immunoblotting (Western-blotting) techniques, based on the original method described by Haas and Bright ***(60)*** and reviewed by Wilkinson ***(61)***. We offer specific, hopefully helpful, guidelines and point out several pitfalls that should be avoided. We also included brief comments on anti-ubiquitin immunohistochemical procedures. Other specialized methods, such as ubiquitin radioimmunoassay ***(62)*** or enzyme-coupled ubiquitin assay ***(63)***, are not widely used to measure protein ubiquitination, and consequently, these methods are not discussed here.

2. Materials

2.1. Sample Preparation

1. Phosphate-buffered saline (PBS): Dissolve 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in ~800 mL of purified water, adjust the pH to 7.4 with HCl, then dilute to 1.0 L.
2. TNESV lysis buffer: 50 m*M* Tris base, pH 7.5, 1% (v/v) Nonidet P-40 detergent, 2 m*M* ethylenediaminetetraacetic acid (EDTA), 100 m*M* NaCl, 10 m*M* sodium orthovanadate, supplemented with 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL leupeptin, and 10 μg/mL aprotinin just before use. 100 μ*M* ALLnL or other proteasome inhibitor can be added to prevent degradation of ubiquitinated proteins in cell lysates and 10 m*M* iodoacetamide and 10 m*M* N-ethylmaleimide can be added to inactivate ubiquitin-cleaving isopeptidases.
3. Reducing gel-loading buffer: 100 m*M* dithiothreitol (DTT), 50 m*M* Tris-HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 0.1 % (w/v) bromphenol blue tracking dye ***(64)***.

2.2. Immunoprecipitation

1. Antibody selection (*see* **Notes 1** and **2**): Fortunately a wide selection of monoclonal and polyclonal anti-ubiquitin antibodies are available from commercial sources. These include Affinity Research Prods. (Exeter, UK), Zymed Labs. (San Francisco, CA), NovoCastra (Newcastle, UK), Sigma Chem. Co. (St. Louis, MO), Santa Cruz Biotech (Santa Cruz, CA), StressGen (Victoria, BC, Canada), Research Diagnostics, Inc. (Flanders, NY) and Boehringer Mannheim (Indianapolis, IN). Many investigators have also generated excellent and unique anti-ubiquitin antibodies in their laboratories, and these may be available for use by others upon request.
2. Antibody reconstitution buffer: sterile PBS containing 0.1% (w/v) sodium azide as a preservative.
3. Protein A-coated Sepharose microbeads. Rehydrate protein A-immobilized sepharose CL-4B beads (1.5 g) (Pharmacia Biotechnology, Piscataway, NJ) overnight in 50 mL of TNESV buffer containing 1% (w/v) bovine serum albumin (BSA) at 4°C with rotation to keep the beads in suspension. The beads are centrifuged (1000$g \times 5$ min), then washed twice in the same buffer by resuspend-

ing and centrifuging. 20 µg of rabbit anti-mouse IgG (RAM) (ICN/Cappel Biomedical, Aurora, OH) is added to a 10-mL aliquot of the washed resuspended beads (equivalent to 0.3 g of dry beads), and they are rotated at 4°C for an additional 2 h. Finally the RAM-coated beads are washed three times in TNESV and stored at 4°C. Antibody-linked protein A-coated microbeads for immunoprecipitation are also available from several commercial suppliers.

4. Rotator for tubes used for immunoprecipitations.
5. Laemmli's sample loading buffer: 80 m*M* Tris base, adjusted to pH 6.8, 100 m*M* DTT, 2% (w/v) SDS, 10% (v/v) glycerol, with a small amount of bromphenol blue indicator dye (0.1–0.2% [w/v]) ***(64)***.

2.3. Gel Electrophoresis

1. Full-sized or mini-gel electrophoresis chamber and power supply.
2. 10% Resolving polyacrylamide gel: Mix 10 mL of 30% (w/v) acrylamide 0.8% (w/v) N, N′-methylene bisacrylamide mixture (ProtoGel, National Diagnostics, Atlanta, GA), 7.5 mL of 1.5 *M* Tris buffer pH 8.8, 12.5 mL of water, 0.3 mL of 10% (w/v) SDS, 0.1 mL of 10% (w/v) ammonium persulfate solution, and 20 µL of N, N, N′, N′-tetra-methyl-ethylenediamine (TEMED) (Bio-Rad, Hercules, CA), in this order.
3. Water-saturated n-butanol.
4. Whatman 3MM or similar type of paper.
5. Polyacrylamide stacking gel: Mix 1.5 mL of 30% (w/v) acrylamide 0.8% (w/v) bisacrylamide mixture, 2.5 mL of Tris buffer, pH 6.8, 6.0 mL of deionized water, 0.1 mL of 10% (w/v) SDS, 0.1 mL of 10% (w/v) ammonium persulfate, and 8 µL of TEMED.
6. Laemmli's sample loading buffer: 80 m*M* Tris base, adjusted to pH 6.8, 100 m*M* DTT, 2% (w/v) SDS, 10% (v/v) glycerol, with a small amount of bromphenol blue indicator dye (0.1–0.2% [w/v]) ***(65)***.
7. Tris-glycine-SDS gel-running buffer: 25 m*M* Tris base, 192 m*M* glycine, 0.1% (w/v) SDS, the final pH is 8.3.
8. Pre-stained molecular-weight protein standards are available from a variety of commercial suppliers. We use prestained broad range standards from BioRad that include: 250, 150, 100, 75, 50, 37, 25, 15, and 10 kDa protein standards.

2.4. Electrophoretic Transfer of Ubiquitinated Proteins to Membranes

1. Wet-type vertical gel transfer box.
2. Nitrocellulose or polyvinylidene difluoride (PVDF) membranes.
3. For nitrocellulose or PVDF membranes, use Towbin wet-transfer buffer: 25 m*M* Tris base, 0.15% (w/v) SDS, 0.2 *M* glycine, and 20% (v/v) methanol. Do not adjust the pH.
4. An alternative transfer buffer that is especially useful for transferring free ubiquitin is CAPS buffer: 25 m*M* cyclohexylaminopropanesulfonic acid, pH 10.0, and 20% (v/v) methanol.

5. Gel-soaking buffer: 63 m*M* Tris base, pH 6.8, 2.3% (w/v) SDS, and 5% (v/v) 2-mercaptoethanol.

2.5. Heat-Activation of Ubiquitinated Proteins on Immunoblots

1. Autoclave or hot plate to boil water.

2.6. Blocking and Probing the Immunoblots for Ubiquitinated Proteins

1. Blocking Solution: 5% (w/v) fat-free dry milk in 10 m*M* Tris base, pH 7.5, 50 m*M* NaCl, 2.5 m*M* disodium EDTA with 0.1% (v/v) Tween-20 detergent.
2. Antibody diluting solution: 2.5% (w/v) fat-free dry milk in TNE wash solution.
3. TNE wash solution: 10 m*M* Tris-HCl, pH 7.5, 2.5 m*M* disodium EDTA, 50 m*M* NaCl with 0.05% (v/v) Tween-20 detergent.
4. Horseradish peroxidase (HRP)-conjugated secondary antibodies are available from several commercial manufacturers. The secondary HRP-conjugated antibody must immuno-react in a species-specific fashion with the primary antibody. We use HRP anti-rabbit (from donkey) NA934, HRP anti-rat (from goat) NA935 and HRP anti-mouse (from sheep) NA931 from Amersham Pharmacia Biotech (Piscataway, NJ) in our laboratory (*see* **Note 3**).
5. Whatman 3MM paper.
6. Luminol-based chemiluminescence immunoblot development solution kit (commercially available from Pierce (Rockford, IL) or Amersham (Piscataway, NY) (*see* **Note 3**).
7. Quality film, for example, X-OMAT AR film purchased from Eastman Kodak (Rochester, NY).
8. Film cassettes.
9. Dark room with automatic film developer.

3. Methods

By far, the majority of investigators currently studying protein ubiquitination rely on anti-ubiquitin immunoblotting to detect ubiquitin-protein conjugates. The method and variations described here is adapted, with modifications, from an original seminal description for the immunochemical detection of ubiquitin-protein conjugates by Haas and Bright ***(60)***. Measuring ubiquitination of a specific protein is usually accomplished by first immunoprecipitating the targeted ubiquitinated protein from cell lysates or from a purified, reconstituted biological system using a specific sepharose-immobilized antibody directed against the individual protein under investigation. Epitope-tagged ubiquitin can also be used both in vitro and in vivo with antibodies that recognize the epitope tag of the fusion protein for immunoprecipitating ubiquitinated proteins (*see* **Note 4**). After immunoprecipitated samples are subjected to SDS-PAGE, the proteins are electrophoretically transferred to nitrocellulose or PVDF mem-

branes, decorated with an anti-ubiquitin antibody followed by an appropriate horseradish peroxidase (HRP)-coupled secondary antibody, and ubiquitinated protein bands are detected by HRP-coupled, luminol-based chemiluminescence (*see* **Note 3**). Alternatively, ^{125}Iodinated protein A can be used in place of the HRP-coupled secondary antibody and the immuno-reactive bands are visualized by autoradiography, although this method has the technical limitations associated with the use of radioactive iodine. The benzoquinone ansamycin Hsp90-targeting drug, geldanamycin, can be used as a novel manipulation to increase the ubiquitination of Hsp90-dependent proteins within cells and facilitate their immuno-detection (*see* **Note 5**).

3.1. Sample Preparation

In general, protein polyubiquitination dramatically shortens the half-lives of many proteins because they are marked for rapid proteasomal digestion, or alternatively, some are destroyed by lysosomal proteases. In addition, linear ubiquitin chains attached to proteins are susceptible to trimming by abundant cellular ubiquitin-cleaving isopeptidases ***(14)***. At least in theory, the entire process of protein ubiquitination is reversible. Consequently, several precautions must be taken to prevent adventitious cleavage of ubiquitin from ubiquitinated proteins during sample preparation. If intact cells are used, this is accomplished by supplementing the cell lysis buffer with a cocktail of several protease inhibitors in combination with a proteasome inhibitor and a thiol-reactive agent to inactivate deubiquitinating enzymes (*see* **Notes 6** and **7**).

1. Typically, cells grown in flasks or plates are washed twice with ice-cold PBS, lysed on ice in 1.0 mL of TNESV lysis buffer with added inhibitors for 20 min. Membrane-bound ubiquitinated proteins may require the use of a "stronger" lysis buffer to extract them from their membrane anchors (*see* **Note 8**).
2. Scrape the lysed cells with plastic spatula and transfer into plastic vials to be centrifuged at 14,000*g* for 20 min. Many cytosolic and membrane-bound ubiquitinated proteins are found in the resulting clarified, detergent-soluble supernatant. The remaining pellet is mostly chromatin—where the ubiquitinated histones are found exclusively—as well as remnants of cellular membranes, cytoskeletal proteins, nuclear matrix, and detergent-insoluble protein aggregates (*see* **Note 8**). Some heavily ubiquitinated proteins may coacervate to the extent they become detergent-insoluble, and these can co-sediment in the pellet fraction ***(65)***.
3. Resuspend the detergent-insoluble pellet by sonication or vigorous mechanical homogenization before assaying for protein and electrophoretic separation of ubiquitinated proteins. Aggregated ubiquitinated proteins should dissolve when the samples are heated in the reducing gel-loading buffer.
4. If free ubiquitin is to be measured, separate samples should be processed by a different technique (*see* **Note 9**).

3.2. Immunoprecipitation

Before conducting immunoprecipitations, the protein concentration of the cell lysate or other sample should be assayed.

1. Immunoprecipitation of protein is conveniently carried out using 1.5-mL disposable microcentrifuge tubes with flip-top or screw-top caps that are available from a variety of manufacturers. It is important that all steps are carried out on ice, and a refrigerated centrifuge should also be used. Alternatively, the entire procedure can be conducted in a 4°C cold room.
2. Aliquot the lysates into prenumbered tubes. Because only a small portion of a particular protein under investigation may be ubiquitinated, a significantly greater amount of sample protein should be immunoprecipitated than would normally be used if the immunoblot were to be probed for the targeted protein, rather than for ubiquitin. For example, 50 μg of cell lysate protein is adequate to immunoprecipitate the EGFR from A431 tumor cell lysate prior to EGFR immunoblotting, but one should immunoprecipitate as much as 1.0 mg of cell of lysate to visualize the ubiquitinated forms of the EGF-receptor by anti-ubiquitin immunoblotting (*see* **Note 10**).
3. Add the primary immunoprecipitating antibody to the samples, mix gently, and incubate on ice for 60–90 min (*see* **Note 1**). Although the antibody concentration will depend on the efficiency of the specific antibody that is used, typically 2–4 μg of primary antibody per mg of sample protein is recommended to maximize the pull-down of ubiquitinated forms of the protein.
4. Add the secondary immunoprecipitating antibody to the samples. Secondary antibodies are usually linked to a support such as Protein A- or Protein G-coated Sepharose microbeads. The antibody should be directed against the same species and Ig subclass isotype as the primary antibody. If the primary antibody has been purified from immunized rabbit serum, the antibody-antigen complex will bind directly to Protein A Sepharose without the need for a secondary conjugated antibody. Usually 50–100 μL of antibody-linked beads in a 1 : 1 bead suspension in PBS or lysis buffer per mL of cell lysate is sufficient to collect essentially all of most primary antibody-antigen complexes. Of course, this depends on the amount of antigen in the sample. Some primary antibodies are available pre-linked to support beads, eliminating the need for a secondary antibody for immunoprecipitations.
5. The collection of the antigen-primary antibody complex onto the immobolized secondary antibody should be done for 1–2 h, and the samples should be constantly agitated on a rotator to prevent the antibody conjugated beads from settling. This step can usually be conveniently conducted overnight.
6. Centrifuge the tubes at 10,000*g* at 4°C for 1 min to pellet the beads.
7. Aspirate the supernatant liquid above the beads carefully using a syringe and blunt needle or similar device. Leave behind a small amount of supernatant rather than disturb the bead pellet.

8. Wash the sedimented beads with 1.0 mL of lysis buffer by vortexing and recentrifuging at 10,000*g* for 1 min. The inclusion of protease inhibitors in the wash buffer is optional and is usually not necessary because soluble proteases will have been washed away by this step.
9. Repeat the wash procedure three times for a total of four washes.
10. Elute the immunoprecipitated protein-immune complex from the beads into 50–100 μL of Laemmli reducing SDS-PAGE sample buffer.
11. Vortex the samples, place them into a boiling water bath or heating block at 95°C for 5 min, then chill on ice for 5–10 min.
12. Centrifuge the samples for 1 min at 10,000*g* to sediment the beads from the eluted immunoprecipitated proteins.
13. Aspirate the supernatants above the beads for protein resolution by gel electrophoresis.

3.3. Gel Electrophoresis

Ubiquitinated proteins can usually be resolved by standard single-dimensional SDS-PAGE using 8 or 10% polyacrylamide gels and Tris-glycine-SDS gel-running buffer. In some cases, it may be necessary to use two-dimensional gels to separate ubiquitinated forms of some proteins (*see* **Note 11**).

1. Immunoprecipitated proteins must be released from the immunoprecipitated antibody complex, heat-denatured, and linearized in Laemmli's sample loading buffer prior to SDS-PAGE.
2. Commercial, precast mini-gels or conventional 14 cm × 14 cm × 1.5 mm gels cast in the laboratory may be used to resolve ubiquitinated proteins. For a 10% resolving gel, mix 10 mL of 30% (w/v) acrylamide 0.8% (w/v) N, N′-methylene bisacrylamide mixture (ProtoGel, National Diagnostics, Atlanta, GA), 7.5 mL of 1.5 *M* Tris buffer, pH 8.8, 12.5 mL of water, 0.3 mL of 10% (w/v) SDS, 0.1 mL of 10% (w/v) ammonium persulfate solution, and 20 μL of N, N, N′, N′-tetra-methyl-ethylenediamine (TEMED), in this order. Pour the gel to a height of 11.5 cm and pipet a layer of water-saturated n-butanol (~0.5 mL) onto the top of the resolving gel to break up bubbles and exclude oxygen, which will slow the polymerization of the gel. After the gel polymerizes (~1 h), decant the butanol, rinse the gel with deionized water, and adsorb any excess water using a piece of Whatman 3MM paper as a blotter. Insert a comb to form the wells and add a 2.5 cm 4.5% acrylamide stacking gel on top of the polymerized resolving gel. The stacking gel is prepared by mixing 1.5 mL of 30% (w/v) acrylamide 0.8% (w/v) bisacrylamide mixture, 2.5 mL of Tris buffer, pH 6.8, 6.0 mL of deionized water, 0.1 mL of 10% (w/v) SDS, 0.1 mL of 10% (w/v) ammonium persulfate, and 8 μL of TEMED. Allow the stacking gel to polymerize for 1 h or more before removing the comb and fill the wells completely with 1X gel-running buffer.
3. Carefully add the immunoprecipitated samples to the bottom of the wells using "gel-loading" pipet tips. Include an aliquot of predyed molecular-weight

standards in one of the lanes to monitor the progression of electrophoresis, and to provide references to estimate the molecular weight of the anti-ubiquitin signals in the sample lanes on subsequent immunoblots.
4. Fill the gel box about one-third full with gel-running buffer, insert the loaded gel, and fill the upper buffer chamber with gel-running buffer.
5. For 10% minigels, use a constant voltage of 100–150 V for approx 1–1.5 h; for larger conventional 10% gels, use 60–65 V of current overnight for approx 12–14 h. If higher voltages are used, chill the buffer in the gel box by circulating cold water through the heat-exchange device that is usually supplied with gel boxes. The ubiquitinated forms of a protein will migrate more slowly through the gel than the unmodified protein, ideally at intervals of ~8 kDa above the normal location of the protein under investigation, reflective of the successive addition of ubiquitin molecules. In many instances, however, ubiquitinated proteins will visualize as a smear extending upward to the top of the gel (*see* **Note 12**).

3.4. Electrophoretic Transfer of Ubiquitinated Proteins to Membranes

In our experience, electrophoretic transfer of ubiquitinated proteins from SDS-polyacrylamide gels to nitrocellulose membranes by the "semi-dry" method is unsatisfactory. This is probably because many polyubiquitinated proteins have molecular weights as high as 300–400 kDa, and these do not elute from the gel efficiently. We recommend transfer of ubiquitinated proteins from gels by the "wet-transfer" method using the Tris-Glycine-SDS-methanol buffer originally described by Towbin ***(66)***.

1. Nitrocellulose or PVDF membranes should be fully hydrated in 1X transfer buffer or deionized water for 1–2 h, which will help to maximize the binding of ubiquitinated proteins to the membrane.
2. Full-sized gels should be electro-transferred for 16–20 h (overnight) at 55 V and at 4°C in a refrigerator or cold room. The transfer of ubiquitinated proteins out of mini-gels onto membranes requires less time, but these can also be transferred overnight at 25 V.
3. The electro-elution of ubiquitinated proteins and unconjugated ubiquitin from SDS-polyacrylamide gels can be enhanced by soaking the gels in 2.3% SDS, 5% 2-mercaptoethanol, 63 m*M* Tris-HCl, pH 6.8, for ~30 min prior to electro-transferring ***(43)***. This step is optional, but it should be used if a transfer problem is suspected (*see* **Note 13**).

3.5. Heat-Activation of Ubiquitinated Proteins on Immunoblots

This is an important step. The detection of ubiquitinated proteins on immunoblots can be remarkably enhanced by autoclaving the membranes in deionized water for 20 min ***(67)***. The ubiquitinated proteins on membranes can

also be activated by heating the blotted membrane at 75°C in an oven ***(60)*** or by boiling in deionized water for ~30 min (for details *see* **Note 14**).

3.6. Blocking and Probing the Immunoblots for Ubiquitinated Proteins

1. After the membrane has been heat-activated by autoclaving, submerge it in room temperature deionized water for several minutes to cool.
2. Gently rock the membrane at room temperature in a tray with sufficient "blocking solution" to completely cover it for 2 h. Alternatively, blocking the membrane may be done overnight in a cold room.
3. Incubate the membrane at room temperature with the primary anti-ubiquitin antibody diluted in "antibody diluting solution" for 1–2 h (*see* **Notes 1** and **2**). Exposing the membrane to the primary antibody may also be done overnight in a cold room with constant rocking.
4. Wash the membrane immunoblot in 1X TNE washing buffer for 30 min with 5 changes of the wash solution. It is ok to wash for longer times.
5. Incubate the immunoblot with an appropriate horseradish peroxidase-conjugated secondary antibody diluted in "antibody diluting solution" for 1–2 h with constant gentle rocking (*see* **Note 3**).
6. Repeat the wash step described in **step 4**.
7. Remove the membrane from the wash buffer and gently dry it between two sheets of Whatman 3MM paper for a few seconds.
8. Immerse the immunoblotted membrane in a small volume of freshly prepared luminol-based chemiluminescence development solution with gentle rocking for 1 min. Mix equal volumes of reagent A and reagent B according to the manufacturer's instructions.
9. Remove the membrane with forceps, allow the excess solution to drain for 10–15 s and seal the membrane inside a transparent plastic bag or plastic wrap.
10. Tape the protected membrane to the inside of a cassette and expose the immunoblot to a good quality film in the dark room. The first exposure should be for 10 s, then longer or shorter exposures should be done, depending on the quality of the first developed film.

3.7. Immunocytochemical Techniques

Although step-by-step, detailed instructions for measuring protein ubiquitination in cultured cells or in tissues by immunocytochemical techniques are not included in this chapter, we would like to provide a few brief comments that highlight the utility of this important technique.

The major limitation to the immunocytochemical approach to the measurement of ubiquitinated proteins in tissue specimens or in cultured cells is that most anti-ubiquitin antibodies cannot discriminate between free and protein-

conjugated ubiquitin. To circumvent this problem, Fujimuro and co-workers ***(68)*** developed an anti-ubiquitin monoclonal antibody (MAb) (FK1) that recognizes multi-ubiquitin chains, but does not recognize free ubiquitin. Using their specialized antibody, they found an increase in ubiquitinated proteins in the cytosol and a corresponding decrease in ubiquitinated proteins in the nucleus of heat-shocked cells ***(69)***. Although cytosolic ubiquitinated proteins were not identified in this study, the major ubiquitinated protein in the nucleus was recognized as ubiquitinated histone H2A. In another study, using an antibody (E6C5) capable of specifically recognizing ubiquitinated histone H2A, Vassilev and coworkers ***(70)*** examined the ubiquitin status of this histone in a variety of cultured cells by indirect immunocytofluorescence and by electron microscopic immunocytochemistry. They observed that ubiquitinated histone H2A was highly up regulated in SV40-transformed fibroblasts and keratinocytes and that ubiquitinated H2A was provocatively localized in clusters within the nucleus. Because only about 10% of the total nucleosomal H2A is ubiquitinated ***(7)***, this observation indicates that specialized regions of chromatin may have very high percentages of nucleosomes with ubiquitinated H2A. Interestingly, the level of ubiquitinated histone H2B has been shown to be coupled to ongoing transcriptional activity ***(71)***.

In a study designed to examine the subcellular localization of unconjugated ubiquitin, Schwartz and co-investigators ***(72)*** used an antibody that exclusively recognizes free ubiquitin. They found free ubiquitin predominantly localized in the cytoplasm and nucleus, but free ubiquitin was absent from the membranous rough endoplasmic reticulum, Golgi complex, and secretory vesicles. These observations suggest that cells may regulate protein ubiquitination by excluding free ubiquitin from specialized compartments where it might interfere with vital protein functions.

Immunocytochemical identification of ubiquitinated proteins in tissues may have considerable value in evaluating pathological states where abnormal protein ubiquitination occurs. While the underlying mechanisms and the identities of potentially many target protein substrates remain to be discovered, abnormal or unregulated protein ubiquitination has been found by anti-ubiquitin immunocytofluorescence in several neurodegenerative pathologies, such as Alzheimer's, Parkinson's, Huntington's, and Creutzfeld-Jakob diseases ***(6,58)***. Thus, in some diseases, the immunohistochemical identification of ubiquitinated proteins within cells of tissue sections may have considerable diagnostic value. As with anti-ubiquitin immunoblotting, the development of antibodies that preferentially recognize specific ubiquitinated proteins will undoubtedly extend the utility of this technique.

4. Notes

1. Antibody selection. In general, most anti-ubiquitin antibodies do not discriminate between free monomeric, polymeric, or protein-conjugated ubiquitin. In our studies, we use lyophilized, rabbit polyclonal anti-ubiquitin antiserum purchased from Sigma and reconstitute the entire contents of the vial into 1.0 mL of sterile PBS containing 0.1% (w/v) sodium azide as a preservative. Small aliquots should be stored frozen at –70°C. Stock antibody solution prepared in this way should be diluted 1/1000–1/2000 for anti-ubiquitin immunoblotting. After dilution of the stock antibody, however, even when refrigerated, this antibody is not stable beyond a few days. Therefore, freshly diluted antibody solutions are recommended. We have also used anti-ubiquitin antibodies purchased from Zymed Labs and Santa Cruz Biotech for anti-ubiquitin immunoblotting in our laboratory with good results. Anti-ubiquitin antibodies have been raised with specificity for multi-ubiquitin chains ***(68)*** or polyubiquitin chains ***(69)*** and these have the desirable feature of not recognizing free monomeric ubiquitin. Conversely, at least one antibody has been generated that recognizes monomeric ubiquitin ***(72)*** to the exclusion of ubiquitinated proteins. Although an antibody that specifically recognizes mono-ubiquitinated histone H2A has been generated ***(70)***, antibodies that recognize other specific ubiquitinated proteins, like ubiquitinated p53 or ubiquitinated NFκβ, have not yet been developed. In general, when characterizing the ubiquitination of a protein by immunoblotting, it is advisable to verify the initial observations by probing a companion blot with another anti-ubiquitin antibody, preferably one from another species that recognizes a different epitope.
2. Antibody epitope masking by ubiquitin. In some cases, the addition of multiple copies of ubiquitin may interfere with the recognition of the protein epitope by the antibody. If an initial attempt to determine the ubiquitin status of a particular protein is inconclusive, epitope masking by polyubiquitin chains may have occurred. In this situation, it is worthwhile to attempt to immunoprecipitate the target protein with an alternative antibody that recognizes a different epitope or to use a polyclonal antibody (PAb) if these are available. Epitope masking by ubiquitination theoretically can also occur on immunoblots.
3. Detection reagents. The most sensitive visualization technique for anti-ubiquitin Western blotting is luminol-generated chemiluminescence. Both Amersham (Piscataway, NJ) and Pierce (Rockford, IL) chemical companies sell easy-to-use chemiluminescence detection kits. This technique requires that the primary antibody be decorated with a species-appropriate horseradish peroxidase (HRP)-conjugated secondary antibody, which catalyzes light generation via the hydrogen peroxide-dependent, HRP-catalyzed oxidation of luminol. Occasionally, the light signal may be too intense when the reagents in these kits are used full strength. In this situation, the mixed chemiluminscence detection reagents can be diluted 1:1 to 1:5 with deionized water. This will attenuate the light output from the

immunoblot. An alternative technique is to place an undeveloped film between the immunoblot and the detection film. This procedure will diminish an overly strong signal approximately fivefold.

4. Use of epitope-tagged ubiquitin. In some instances, the specificity and sensitivity of ubiquitinated protein measurement may be increased through the use of epitope-tagged ubiquitin ***(73)***. Ubiquitin that has been covalently linked to hemaglutinin, c-Myc protein, hexahistidine, biotin, or even glutathione transferase can be added to in vitro biological systems, such as reticulocyte lysate, that contains the enzymes necessary to ubiquitinate proteins. The epitope-tagged ubiquitin competes with unmodified cellular ubiquitin for enzymatic conjugation to the newly synthesized or exogeneously added purified proteins. Highly efficient antibodies that recognize epitope tags have been developed, and these can be used to immuno-select epitope-tagged ubiquitinated proteins, as well as the unconjugated tagged ubiquitin. Following separation of the immunoprecipitated, epitope-tagged ubiquitinated proteins by SDS-PAGE, immunoblotting with an antibody directed toward the particular protein in question can be used to detect the ubiquitinated forms. This technique has been used to demonstrate ubiquitination of the short-lived MATα2 yeast transcription regulator ***(74)***, c-Jun transcription factor ***(24)***, and the p27 cyclin-dependent kinase inhibitor ***(75)***.

   Epitope-tagged ubiquitin can be transiently introduced into cells by transfection with a vector containing an insert coding for a fused epitope-tagged ubiquitin such as hemaglutinin (HA)-ubiquitin. Immunoprecipitation with an anti-HA antibody will efficiently isolate HA-tagged ubiquitinated proteins from cell lysates, and these will be detected as higher molecular-weight forms by immunoblotting with an antibody against the particular protein. Alternatively, immunoprecipitation of the protein under study, followed by immunoblotting with an anti-tag antibody should reveal ubiquitinated protein as a "ladder" or a "smear." We have used this technique to detect ubiquitination of the plasma membrane tyrosine kinase, erbB2 ***(76)***.

5. Disruption of protein chaperones with geldanamycin. Many newly-synthesized proteins, as well as some long-lived, mature proteins, are stabilized by molecular chaperone complexes containing heat-shock proteins Hsp70 and Hsp90 ***(77)*** or GRP-94, an Hsp90 homolog found in the endoplasmic reticulum ***(78)***. Ubiquitination of Hsp90- and GRP94-stabilized proteins can be stimulated by disrupting the client protein-heat shock protein-chaperone complexes with the Hsp90-binding benzoquinone ansamycin, geldanamycin ***(77,79)***. Treating cells with geldanamycin has been shown to greatly increase ubiquitination of the ErbB2 membrane receptor tyrosine kinase ***(76)***, c-Raf-1 serine-threonine kinase ***(80)***, certain mutated forms of p53 ***(81)***, and the mutated cystic fibrosis transmembrane conductance regulator protein ***(82)***. Consequently, geldanamycin can be used as an experimental tool to stimulate the ubiquitination of proteins that depend on Hsp90 chaperone interactions for their stability. Treating cells with geldanamycin combined with a proteasome inhibitor should further increase

the likelihood of accumulating ubiquitinated forms of proteins that are normally stabilized by Hsp90 chaperone complexes.

6. Prevention of enzyme-catalyzed deubiquitination in cell lysates. Adding a peptide aldehyde protease inhibitor such as N-acetyl-leucyl-leucyl-norleucinal (ALLnL) or Z-leucyl-leucyl-leucyl-CHO (MG132), at 50–100 μ*M*, to the cell lysis buffer will prevent unwanted proteasomal digestion of ubiquitinated proteins during sample preparation. Although lactacystin is more potent as well as being an irreversible covalently binding proteasome inhibitor ***(83)***, it is probably far too expensive to be practical for this purpose. Cells also contain two major families of ubiquitin-specific thiol proteases, called Ubp (ubiquitin-processing proteases) and Uch (ubiquitin carboxy-terminal hydrolases) ***(10,15)***, and these enzymes can slice ubiquitin from proteins in cells and cell lysates. The inclusion of thiol-reactive N-ethylmaleimide or iodoacetate at 10 m*M* in the lysis buffer will inactivate these ubiquitin-cleaving enzymes by alkylating their active site cysteine residues ***(61,84,85)***. Additionally, this class of proteases is sensitive to manganese ion, and strong inhibition of their deubiquitinating activity by 5 m*M* manganese in vitro has been reported ***(86,87)***. Ubiquitin aldehyde, a potent inhibitor of ubiquitin chain-cleaving hydrolases, is now available from Boston Biochem, Cambridge, MA), and this agent should help to prevent inadvertent deubiquitination. Chilling all buffers and the cell lysates and working on ice also helps to inactivate ubiquitin-cleaving enzymes and prevent unintentional enzymatic cleavage of ubiquitin from proteins.
7. Use of proteasome inhibitors to promote cellular accumulation of ubiquitinated proteins. Proteasome inhibition usually stabilizes ubiquitinated proteins that normally are rapidly degraded via the ubiquitin-proteasome pathway ***(2,50,88)***. In situations where the normal half-life of the protein is very short, proteasome inhibition may be absolutely necessary in order to detect ubiquitination. ALLnL at 100 μ*M*, 50 μ*M* MG132, or 10 μ*M* lactacystin will effectively cross the plasma membrane of cells in culture and significantly diminish proteasomal degradation of ubiquitinated proteins. Because ALLnL also inhibits calpains as well as the proteasome, N-acetyl-Leu-Leu-Met-H peptide should be used as a negative control to rule-out alternative proteolytic pathways, since it has inhibitory activity toward cathepsins and calpains, but does not inhibit the proteasome. Most recently, boronic acid-containing peptides have been developed by Proscript (Cambridge, MA), and these are potent inhibitors of the proteasome ***(89)***. Affinity Research Products recently offered several new proteasome inhibitors. The first, designated MG262 is the boronic acid derivative of MG132 and purportedly has 200,000-fold greater specificity for the proteasome than for cathepsin B. Epoxomicin, is a new highly specific and irreversible inhibitor of the chymotrypsin-like activity of the proteasome, but it has essentially no inhibitory activity toward nonproteasomal proteases ***(90)***. A third inhibitor is called aclacinomycin A, an anticancer agent that also inhibits the chymotrypsin-like activity of the proteasome ***(91)***.

Although proteasome inhibitors have been very useful in the study of protein ubiquitination and degradation, proteasome inhibitors should be used with

caution because of their inherent cytotoxic properties. ALLnL and lactacystin are lethal to many cells in culture within 12–24 h at the concentrations that are required to inhibit the proteasome. Proteasome inhibitors initiate apoptosis through a yet-to-be-defined mechanism ***(92–94)***, although cell death usually does not occur before 8 h. In addition, both ALLnL and lactacystin inhibit messenger RNA transcription and DNA replication by as much as 50% within 4 h ***(43)***, raising the likely possibility that the cellular level of some proteins might even be diminished by proteasome inhibitors because of transcriptional inhibition.

On the other hand, because the signals for promoting protein ubiquitination seem to be independent of proteasomal protease activity ***(11–13)***, blocking the degradation of proteins by inhibiting the proteasome does not automatically promote their ubiquitination. The multiple proteasomal proteases are localized inside the lumen or "barrel" of the proteasome, while the S5a polyubiquitin binding subunit is part of the 19S regulatory complex that sits at the entrance to the proteasome interior ***(53)***. Therefore, completely inhibiting the multiple proteasomal protease activities does not ensure that ubiquitin chains will remain attached to proteins when they interact with the proteasome regulatory complex. They may still be susceptible to cleavage from proteins by proteasome-associated ubiquitin hydrolase activity ***(54)*** or by other deubiquitinating enzymes that are independent of the proteasome. There at least two families of deubiquitinating enzymes that cleave ubiquitin from proteins ***(14)***, and these enzymes should retain full activity in the presence of proteasome inhibitors.

8. Immunoprecipitation of membrane-bound and aggregated ubiquitinated proteins. In certain cases, including 1% (w/v) SDS in the TNESV lysis buffer will help to solubilize ubiquitinated proteins from cell membranes. Following centrifugation, the clarified lysate should be diluted 10-fold in SDS-free buffer to decrease the concentration of SDS to 0.1% prior to attempting to immunoprecipitate the protein under study. This dilution is necessary to prevent SDS denaturation of the immunoprecipitating antibody.
9. Measurement of unconjugated ubiquitin. In addition to ubiquitin-protein conjugates, it may be necessary to measure unconjugated ubiquitin in biological samples. Since it is heat-stable and denaturation-resistant, unconjugated ubiquitin can be separated from the majority of ubiquitinated proteins by scraping cells into a small volume (0.5–1.0 mL) of deionized water and boiling the sample for 10 min. Many ubiquitinated and nonubiquitinated proteins become denatured, rapidly coagulate, and can be easily separated from free ubiquitin by low-speed centrifugation. An additional benefit is that boiling rapidly denatures deubiquitinating enzymes including the proteasome and prevents any artifactual increase in free ubiquitin in the sample. Unconjugated ubiquitin remains in the supernatant fraction, and it can be separated from any remaining ubiquitinated proteins by 16% or 18% polyacrylamide SDS-PAGE. Soaking SDS-polyacrylamide gels in 2.3% SDS, 5% 2-mercaptoethanol, 63 m*M* Tris-HCl, pH 6.8, buffer for 30 min prior to electro-transferring to the nitrocellulose membrane and using 25 m*M* CAPS, pH 10.0, with 20% (v/v) methanol transfer buffer will facilitate the

elution of free ubiquitin from the gel to the membrane surface. As with ubiquitinated proteins, autoclaving membrane-bound ubiquitin before immunoblotting greatly enhances its immune detection, at least with the anti-ubiquitin PAb purchased from Sigma. It should be noted that although monomeric ubiquitin has a molecular weight of 8.6 kDa, ubiquitin usually behaves on SDS-PAGE as if it were a 5.5–6.5 kDa polypeptide.

10. Equivalent sample immunoprecipitation. Because ubiquitinated proteins are short-lived, any treatment that increases protein ubiquitination, with the exception of proteasome inhibitors, has the potential to dramatically decrease the cellular level of ubiquitin-targeted proteins. Therefore, care should be taken to immunoprecipitate equal amounts of the target protein under investigation from control and drug-treated or otherwise manipulated cells. Increasing amounts of cell lysate should be added to a fixed amount of antibody in a preliminary experiment to insure that the immunoprecipitating antibody is saturated with its antigen. Antibody saturation will permit valid comparisons of the extent of ubiquitination of the protein under investigation from sample to sample. Alternatively, normalization of the ubiquitin signal to the quantity of the target protein, both ubiquitinated and nonubiquitinated, in the same sample—as determined by separate immunoblotting—can be done, although this technique may be difficult because anti-ubiquitin immunoblots will frequently appear as smears.
11. Two-dimensional gel electrophoresis to improve the resolution of ubiquitinated proteins. Certain ubiquitinated proteins, including ubiquitinated histones, resolve poorly by conventional SDS-PAGE. For these, it may be necessary to use an alternative gel system such as an acetic acid-urea-Triton X-100-polyacrylamide (AUT) gel and electrophoresis in 0.9 *M* acetic acid, which separates proteins according to charge ***(95)***. Two-dimensional gel techniques may even be necessary to cleanly resolve some ubiquitinated proteins. In this situation, the sample should be first separated by AUT gel electrophoresis, followed by standard SDS-PAGE in the second dimension ***(41)***. For ubiquitinated histones, the aforementioned two-dimensional technique will resolve mono-ubiquitinated H2A, H2B, H2A.Z, as well as the small amounts of multi-ubiquitinated H2A and H2B ***(95,96)***.
12. Smearing on anti-ubiquitin immunoblots does not indicate a failed experiment. Ideally, an anti-ubiquitin Western blot of samples immunoprecipitated using an antibody against the protein under investigation should appear as discrete bands, at intervals of ~8 kDa, extending upward from the expected location of the unmodified protein. This "ladder" of bands with slower mobility results from the successive addition of monomeric ubiquitin molecules to targeted proteins to form chains containing as many as 20 or more ubiquitin molecules (an addition of 170 kDa mass). More often, however, anti-ubiquitin immunoblots reveal ubiquitinated proteins as an unappealing smear stretching toward the top of the membrane. This phenomenon is especially true for many membrane-anchored proteins that are heavily glycosylated. For example, anti-ubiquitin immunoblots of the glycosylated plasma membrane tyrosine kinase, ErbB2 ***(76)*** or the EGFR

(37) will invariable be smeared, no matter how thoroughly the immunoprecipitations might be washed.

N-linked oligosaccharide chains of various lengths and heterogeneous ubiquitin chains, possibly conjugated to more than one lysine residue within a protein, make it difficult to resolve ubiquitinated glycosylated proteins into sharp bands by SDS-PAGE. In some cases, incubating cell lysates with endoglycosidase-F to cleave oligosaccharides from glycosylated and ubiquitinated proteins may reduce the smearing and improve the quality of the immunoblots ***(97)***. On the other hand, cytosolic nonglycosylated ubiquitinated proteins like Iκβα ***(29)***, p53 ***(20)*** and β-catenin ***(26)*** frequently appear smear-like rather than as a "ladder" of bands in anti-ubiquitin immunoblots. Smears have also been obtained when epitope-tagged or biotin-conjugated ubiquitin have been used to probe for the ubiquitination of c-Jun ***(24,98)***, the p27 cyclin-dependent kinase inhibitor ***(75)***, Iκβ ***(30)*** and hypoxia-inducible transcription factor 1α ***(99)***.

Collectively, these observations indicate that smearing is a frequent characteristic of anti-ubiquitin immunoblots. The smearing most likely is a consequence of different-length ubiquitin chains attached to a substrate protein at a single site, several different length polyubiquitin chains conjugated to multiple lysine residues within the protein, or the combination of both types of ubiquitin modifications. Sometimes bands can be seen within the smears on anti-ubiquitin immunoblots, and these bands probably represent the more abundant forms of a particular ubiquitinated protein.

13. Reducing proteins in the gel to enhance transfer to membranes. Exposing gels to 2.3% SDS, 5% 2-mercaptoethanol, 63 m*M* Tris-HCl, pH 6.8, for 30 min facilitates the efflux of ubiquitinated proteins from gels. This is because the 2-mercaptoethanol reduces protein disulfide bonds that may have reformed in the gel during electrophoresis, the SDS makes the proteins more soluble and the soaking in this solution causes the gel to expand considerably. This step can also be used to enhance the transfer of unconjugated ubiquitin from gels.
14. Heat-activation of ubiquitin on membrane blots. SDS-denatured ubiquitin conjugated to hemocyanin or gamma globulin is frequently used as the immunogen to generate anti-ubiquitin antibodies. Consequently, antibodies generated by this method may fail to recognize epitopes in native, nondenatured ubiquitin, whether it is free or conjugated to proteins. In this case, heat-activation of ubiquitinated proteins on membranes is essential to their immuno-detection.

    More than a decade ago, Swerdlow and coworkers ***(67)*** reported the novel observation that boiling or autoclaving nitrocellulose membranes containing free ubiquitin or ubiquitinated proteins from HeLa cell lysates improved their immune-reactivity by nearly 50-fold. These investigators suggested that heat-denaturation of ubiquitin reveals latent antigenic sites that are hidden within the mostly globular intact ubiquitin molecule. Ubiquitinated proteins bound to the surface of the membrane used for immunoblotting should therefore be heat-activated by autoclaving. Importantly, heat-activation should be done prior to blocking to prevent the heat-denaturation of milk or other blocking proteins,

which will interfere with subsequent immunoblotting. Membranes should be autoclaved for 30–40 min, sandwiched between several sheets of Whatman 3MM paper, at the bottom of a glass tray containing deionized water. Autoclaving tends to wrinkle the membrane somewhat, but do not autoclave the membrane between glass plates. Unfortunately, this will severely corrugate the blot, possibly because of trapped steam or uneven heat transfer from the glass to the membrane, making subsequent immunoblotting difficult, if not impossible.

Autoclaving the membranes increases the reactivity of many anti-ubiquitin antibodies for ubiquitin and thereby greatly intensifies the resulting immunoblotting signal. However, this procedure may not be universally effective for all anti-ubiquitin antibodies. A pilot experiment should be conducted to determine whether autoclaving the membrane increases the immune reactivity of a particular antibody toward ubiquitinated proteins.

If an autoclave is not available, the ubiquitin on membrane blots can be heat-activated by boiling them in a glass tray for at least 30 min. An alternative treatment to promote ubiquitin epitope exposure and increase the sensitivity of anti-ubiquitin immunoblots is to immerse the membranes containing ubiquitinated proteins in 6 *M* guanidine-HCl, 20 m*M* Tris-HCl, pH 7.5, 1 m*M* PMSF, and 5 m*M* DTT for 30 min at room temperature, before blocking ***(21)***. Heat-activation and guanidine-activation probably denatures ubiquitin and facilitates the recognition of latent ubiquitin epitopes by the anti-ubiquitin antibody ***(67)***. Heat-activation frequently intensifies the anti-ubiquitin immunoblot signal 20-fold or greater.

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13

Analysis of Methylation and Acetylation in *E. coli* Ribosomal Proteins

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1. Introduction

A wide variety of posttranslational modifications ***(1)*** of expressed proteins are known to occur in living organisms. Although their presence in an organism cannot be predicted from the genome, these modifications can play critical roles in protein structure and function. The identification of posttranslational modifications can be critical in understanding the functions of proteins involved in important biological pathways and mass spectrometry offers a fast, accurate method for observing them. This chapter describes the procedure for analyzing ribosomal proteins of *Escherichia coli* by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Owing to their role as the center of protein synthesis in cells, ribosomes are a popular target of research. Many of the steps involved in the translation of messenger RNA into proteins have been elucidated. Nevertheless, the ribosomes of prokaryotes, which are somewhat simpler than those of eukaryotes, contain 3 ribonucleic acids and over 50 proteins intricately bound together into large and small subunits and much about their structure remains unknown. A molecular level understanding of the role that ribosomal proteins play in the translation process remains to be attained ***(2,3)***. Because they are present in all organisms, the RNA and protein components of ribosomes provide the material for fingerprinting and evolutionary studies ***(4–7)***. The alteration or absence of ribosomal proteins conveys structural, genetic, and functional information ***(7)***. Nine *E. coli* ribosomal proteins are reported to be posttranslationally modified by acetylation or methylation ***(7)***; others are believed to be carboxylated ***(8)*** and one thio-methylated ***(9)***. In general the roles of these modifications are not

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understood ***(10)***. Despite its limited speed, two-dimensional gel electrophoresis has been the preferred method for resolving ribosomal proteins derived from a single source ***(2,11,12)***. Unfortunately, the resolution of this technique is not sufficient to distinguish most posttranslational modifications.

Mass spectrometry is an optimal method for detecting mutations and modifications in biological macromolecules. Two methods developed in the 1980s, electrospray ionization ***(13)*** and MALDI ***(14)***, have made it possible to record mass spectra of large nucleic acids, proteins, and carbohydrates. Recent progress in TOF instrumentation ***(15–17)*** has improved the quality of MALDI mass spectra to the point where the masses of molecules smaller than about 30,000 Dalton can typically be measured to an accuracy on the order of 1 Da or better. Since up to 45% of the mass of rapidly growing *E. coli* cells corresponds to ribosomes, and up to 21% of the cellular protein content is ribosomal ***(7,18)***, these cells make an ideal system for studying ribosomal proteins. The method presented in this chapter describes the analysis of *E. coli* ***(19)*** ribosomes. However, it can be extended to ribosomes from other organisms ***(20)*** and to other well-characterized biological systems. Ribosomes were extracted from bacteria and their mass spectra were recorded, allowing for the detection and identification of 55 of the 56 ribosomal proteins known to be present in *E. coli*. Previously assigned posttranslational modifications were easily observed by comparing measured masses to masses calculated from the amino acid sequences of known ribosomal proteins.

2. Materials

2.1. Ribosome Extraction

1. Luria broth (LB) growth medium: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) sodium chloride in aqueous solution.
2. Refrigerated centrifuge capable of 100,000*g* centrifugal force.
3. Mortar, pestle, alumina.
4. Buffer A: 20 m*M* Tris-HCl, 10.5 m*M* magnesium acetate, 100 m*M* ammonium chloride, 0.5 m*M* EDTA, 3 m*M* β-mercaptoethanol, pH 7.5.
5. Buffer B: same as buffer A, except 0.5 *M* ammonium chloride instead of 100 m*M*.
6. Buffer E: 10 m*M* Tris-HCl, 5.25 m*M* magnesium acetate, 60 m*M* ammonium chloride, 0.25 m*M* EDTA, 3 m*M* β-mercaptoethanol.
7. DNase.
8. Sucrose.
9. 6–8 kDa MWCO dialysis tubing.

2.2. MALDI Sample Preparation

1. 0.1% Aqueous trifluoroacetic acid (TFA).
2. 10% Aqueous TFA.
3. Acetonitrile.

4. MALDI matrix: sinapinic acid (SA, 3,5-dimethoxy-4-hydroxycinnamic acid).
5. Calibration proteins: bovine ubiquitin, horse heart cytochrome c, bovine carbonic anhydrase.
6. 10- and 30-kDa MWCO centrifugal filters.
7. TOF mass spectrometer equipped with MALDI source.

3. Methods

3.1. Ribosome Isolation (see *Note 1*)

1. Grow *E. coli* cells in ~1.8 L of LB growth medium to mid-log phase (~12.5 h, monitor by OD at 600 nm).
2. Harvest cells by centrifugation at 10,000*g* and 4°C for 10 min (should yield about 5 g of wet cells).
3. Transfer cells to mortar and freeze cells at –20°C.
4. Break up cells by adding 12 g of alumina and grinding with pestle until a sticky paste is obtained.
5. Add 15 mL of buffer A and a few crystals of DNase.
6. Transfer mixture to centrifuge tube and centrifuge at 10,000*g* and 4°C for 15 min to remove alumina and membranes. Retain the supernatant.
7. Centrifuge supernatant at 30,000*g* and 4°C for 45 min to remove more cell debris and alumina. Retain the supernatant.
8. Prepare 12 mL of 1.1 *M* sucrose in buffer B. Add 3 mL to each of 4 clean centrifuge tubes. To each tube, layer ~4 mL of sample supernatant over sucrose cushion. Centrifuge at 100,000*g* and 4°C for 15 h. Remove and discard supernatant. Remove and discard the brown flocculent material (top portion of pellet), leaving clear, colorless, gel-like pellet.
9. Suspend each pellet in 1 mL of buffer A. Repeat **step 8**, this time layering resuspended pellet over sucrose cushion.
10. Suspend each pellet in 1 mL of buffer E. Dialyze (3×) entire sample (4 mL total) against 1 L vol of buffer E using 6–8 kDa MWCO membrane tubing to remove excess sucrose.
11. Store samples frozen at –80°C (*see* **Note 2**) in buffer E prior to mass spectrometric analysis.

3.2. Mass Spectrometry

1. Prepare 10 mg/mL sinapinic acid matrix solution by dissolving 3.0 mg of the solid in 200 µL of 0.1% TFA plus 100 µL of acetonitrile (*see* **Note 3**).
2. Acidify ribosomes by adding 1 µL of 10% aqueous TFA to 9 µL of ribosome solution (*see* **Note 4**).
3. Prepare calibration protein solutions by dissolving separately 1 mg of each protein in 1 mL of distilled, deionized water.
4. Mix 1 µL of TFA-treated ribosome solution with 9 µL of the matrix solution (*see* **Note 5**).
5. Prepare the calibration protein/matrix mixture by adding 1 µL of each calibration protein solution to 9 µL of matrix solution.

Table 1
Posttranslational Modifications Observed in *E. coli* Ribosomal Proteins by MALDI Mass Spectrometry

Subunit	Sequence mass (Da)	Measured mass (Da)	Difference (Da)	Modification
S5	17472.3	17514.8	+42.5	Acetylation
S11	13713.8	13727.7	+13.9	Methylation
S12	13605.9	13651.3	+45.4	β-methylthiolation
S18	8855.3	8897.0	+41.7	Acetylation
L3	22243.6	22257.2	+13.6	Methylation
L7	12164.1	12206.7	+42.6	Acetylation
L11	14744.3	14870.2	+125.9	9 methylations
L12	12164.1	12174.4	+10.3	Methylation
L31	7871.1	6971.1	–900.0	Cleavage of –RFNIPGSK from C-terminus
L33	6240.4	6254.1	+13.7	Methylation

6. Apply 1 µL of the ribosome/matrix mixture to the sample probe and allow to air-dry.
7. Apply 1 µL of the calibration protein/matrix mixture to the sample probe near the ribosome samples and allow to air-dry.
8. Acquire MALDI mass spectra, externally calibrate spectra (*see* **Note 6**), and measure masses of observed peaks.
9. (Optional) Improved mass calibration may be achieved by using several observed peaks and their known sequence masses to internally calibrate the mass spectrum.
10. Compare masses measured by mass spectrometry to the masses calculated from the amino acid sequences of known proteins. **Table 1** shows such a comparison for *E. coli* ribosomal proteins ***(19)*** (*see* **Note 7**).

4. Notes

1. This method is optimized for the extraction of intact ribosomes from *E. coli* cells. An extensive compilation of ribosome extraction procedures for other organisms can be found in **ref. *2***. Alternatively, other biological systems should also be amenable to MALDI mass spectral analysis, provided their protein amino acid sequences are well-characterized. In all cases, special care should be taken to avoid adding chemicals to biological samples that either suppress ionization or create adducts in the MALDI process ***(21)***. The authors recommend avoiding large amounts of urea (>3 *M*) and sodium dodecyl sulfate (>1%) and even small amounts of sodium, potassium, sulfate, and phosphate ions. Substituting ammonium, citrate, trifluoroacetate, and acetate ions (when appropriate) should lead to improved MALDI ion yield.

2. Samples may also be refrigerated at 4°C for short-term storage (hours to days) or frozen at –20°C for medium-term storage (days to weeks). Extended storage (months) should be done at –80°C. Best results are normally obtained by preparing MALDI samples immediately, prior to any intermediate storage.
3. For *E. coli* ribosomes, the most useful spectra have been obtained using sinapinic acid as the matrix. Nevertheless, other MALDI matrices may be used for analysis. Alternative matrices include ferulic acid (FA, 4-hydroxy-3-methoxycinnamic acid) and α-cyano-4-hydroxycinnamic acid (CHC). Varying the matrix can change the relative peak heights for a given sample. Thus the intensities of individual peaks may be enhanced (or diminished) simply by using a different matrix, without otherwise changing the sample.
4. This acidification precipitates ribosomal RNA from the intact ribosomes, improving MALDI signal for ribosomal proteins.
5. Since MALDI analysis of mixtures is commonly known to discriminate against the ionization of larger molecules in favor of smaller components, one may wish to enhance high-mass ion signal by removing low-mass components with either a 10- or 30-kDa MWCO centrifugal filter prior to mixing the TFA-treated ribosome solution with matrix solution.
6. Singly and doubly charged ions of calibration proteins such as bovine ubiquitin, horse heart cytochrome c, and bovine carbonic anhydrase are normally observed. Masses of their singly-charged ($M+H^+$) ions are 8565.88 Da, 12361.15 Da, and 29023.2 Da, respectively. Other well-characterized proteins may also be used for calibration.
7. In *E. coli*, methionine aminopeptidase ***(22)*** is known to cleave the N-terminal methionine residue when amino acids with small side chains (alanine, cysteine, glycine, proline, serine, threonine, and valine) are in position 2, next to methionine. This loss occurs in 34 of the 55 observed *E. coli* ribosomal proteins. Mass shifts due to the loss of N-terminal methionine residues are not included in **Table 1**.

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14

Identification of In Vivo Protein Phosphorylation Sites with Mass Spectrometry

Jun Qin and Xiaolong Zhang

1. Introduction

Protein phosphorylation, principally on Ser, Thr, and Tyr residues, is one of the most common post-translational cellular regulatory mechanisms ***(1–4)***. Phosphorylation can modulate enzyme activity, alter its affinity to other proteins, and transmit signals through kinase cascades that often are branched and interactive. To understand the molecular basis of these regulatory mechanisms and to dissect signal-transduction pathways, it is necessary to determine the sites that are phosphorylated in vivo. Mass spectrometry is in the process of becoming the method of choice for the identification of protein phosphorylation sites ***(5)***. This mass spectrometric approach offers at least four major advantages over the conventional biochemical and genetic approaches. First, it is accurate. There is no ambiguity once the phosphorylation site is identified through mass spectrometric sequencing of the phosphopeptide. Second, it is fast. The cycle of identifying phosphorylation sites is typically a few days. Third, it does not require prior knowledge of phosphorylation of the protein of interest. Phosphorylation sites can be identified even for proteins that the consensus phosphorylation sites of their up stream kinases are not known. Fourth, it does not require ^{32}P labeling.

The practical sensitivity of mass spectrometry-based approaches is lower than radioactivity or immuno methods. With modern mass spectrometry using both matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), the inherent sensitivity of the mass spectrometer is high, typically in the low fmol to high attomol range for the detection of peptides. This sensitivity is comparable to those immuno-methods, such as Western blotting.

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In contrast to immuno-methods, mass spectrometry has little selectivity. It is difficult to identify the phosphopeptide ions in minute amount in a background of highly abundant ions of other peptides or contaminants. How to introduce and increase selectivity for the detection of phosphopeptides is therefore one of the key elements for the success of identifying phosphorylation sites with mass spectrometry. Methods were developed and showed success to different degrees ***(5–8)***, but none of them has the exquisite sensitivity of the radioactivity or immuno-methods.

Site-specific phosphorylation stoichiometry is useful information, but this information cannot be obtained in general by mass spectrometry. Ionization efficiency and peptide-extraction efficiency can be different for phosphopeptides and their unphosphorylated counterparts, therefore biological mass spectrometry is usually not quantitative. It is perhaps possible to calibrate the response of phosphoryated and unphosphorylated peptides using synthetic peptides, but the peptide-extraction efficiency during in gel digestion for these two forms can be difficult to evaluate.

As regulatory proteins are often expressed only at low levels, any method for determining phosphorylation sites must include steps to purify phosphoproteins with small losses and yet high purification resolution (also referred to as purification fold) to yield partially purified proteins. IP followed by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) seems to be the method of choice at present. The methods for the determination of phosphorylation sites must also include steps to recover, identify, and prepare phosphopeptides in a form that is suitable for MS analysis with minimal loss. These steps for the method described in this chapter are usually composed of: (1) in gel digestion and extraction of the resulting peptides, (2) identification of the phosphopeptides using a phosphatase ***(9)***, and (3) sequencing of the phosphopeptides using on-line liquid chromatography electrospray tandem mass spectrometry (LC/ESI-MS-MS). With this approach, an in vivo phosphorylation site can be identified and more phosphorylation sites can be located to a stretch of amino acid residues using as little as 200 fmol of fully phosphorylated protein. This allows us to study in vivo phosphorylation of regulatory proteins in the signal-transduction pathways with reasonable effort. We will detail this process in the **Subheading 3.** In theory, the processes of identifing in vivo and in vitro phosphorylation sites are identical, but in practice, there exists a major difference in which the in vivo phosphorylated samples isolated from endogenous proteins are usually truly limited. There is usually only enough sample that permits one attempt for mass spectrometric measurement, therefore the procedure for mapping in vivo phosphorylation sites must be highly reliable in addition to high sensitivity and high selectivity.

2. Materials

2.1. In Gel Digestion of Phosphoproteins

1. Coomassie Blue (CB) destain solution: 25 m*M* ammonia bicarbonate in methanol : water (1 : 1, v : v), pH 8.0–8.5.
2. Gel piece cleaning solution: 10% acetic acid in methanol : water (1 : 1, v : v).
3. Digestion buffer: 50 m*M* ammonia bicarbonate in water, pH 8.0–8.5.
4. Modified sequencing grade trypsin (Boehringer Mannheim): 250 ng/μL in high-performance liquid chromatography (HPLC) water.
5. Sequencing grade Asp-N (Boehringer Mannheim): 100 ng/μL in HPLC water.
6. Acetonitrile, methanol, and water, all HPLC grade.
7. 1 mL Pipet tips.
8. 0.5 mL Eppendorf tubes.
9. A Benson burner.
10. An aspirator.
11. A SpeedVac concentrator.

2.2. Identification of Phosphopeptides

1. Calf intestine phosphatase (CIP) (New England Biolabs): diluted to 0.1 U/μL in 50 m*M* ammonia bicarbonate.
2. An 80°C heating block.
3. A MALDI linear TOF mass spectrometer with delayed extraction (Perseptive Biosystems).
4. DHB working matrix solution: one time (in winter) and two times (in summer) dilution of the saturated solution of 2,5-dihydroxybenzoic acid in acetonitrile : water (1 : 1, volume).

2.3. Sequencing of Phosphopeptides

1. Mobile phase A: water : methanol : acetic acid (94 : 5 : 1, volume).
2. Mobile phase B: water : methanol : acetic acid (15 : 84 : 1, volume).
3. An electrospray ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA) coupled on-line with a capillary HPLC (Magic 2002, Michrom BioResources, Auburn, CA). An 0.1×50 mm-MAGICMS C18 column (5-μm particle diameter, 200 Å pore size).

3. Methods

3.1. In Gel Digestion of Phosphoprotein

1. After conventional SDS-PAGE, a CB stained gel piece containing 50 ng or more of protein is cut out and destained in CB destain solution (0.4 mL) in a 0.5 mL Eppendorf tube until the gel piece turns clear. A change of destain solution after 1 h helps speed-up the destain process.
2. The destained gel piece is then soaked overnight in the gel piece cleaning solution (0.4 mL) at room temperature.

3. The gel piece is further cleaned in water (0.4 mL) for 4 h and finally cleaned in the digestion buffer (0.4 mL) for 30 min.
4. The digestion buffer is discard and the cleaned, wet gel is grounded to a fine powder in 10 μL of digestion buffer with a fire-sealed pipet tip (*see* **Note 1**). Two μL of 250 ng/μL of trypsin in water is added, and the digestion tube is centrifuged briefly on a microcentrifuge and digestion is carried out at 37°C overnight (*see* **Note 2**).
5. To extract the peptides, three volumes of acetonitrile of the digestion solution is added and the tube vortexed for 2 min. The tube is centrifuged briefly and the solution is carefully removed with a gel-loading pipet tip.
6. **Step 5** is repeated for a second extraction. Ten μL of the digestion buffer is added to the gel piece, and the tube is vortexed for 2 min. Then 30 μL of acetonitrile is added and the tube vortexed for 2 min again. The tube is centrifuged and the solution removed (*see* **Note 3**).
7. The extracted peptides are pooled and dried completely in a SpeedVac concentrator with medium heat.
8. The dried peptides are redissolved in 10 μL of 50% acetonitrile for MALDI/TOF analysis (*see* **Note 4** and **Note 5**).

3.2. Identification of Phosphopeptides

This method uses phasphatase treatment of the peptide mixture followed by MALDI/TOF mass spectrometry to identify the phosphopeptides. The protocol for the phosphatase treatment of the peptide mixture is the following.

3.2.1. Phosphatase Treatment of Peptides

1. Two μL of the dissolved peptide solution is mixed with 5 μL of 0.1 U/μL of CIP (New England Biolabs; diluted from 10U/μL in storage buffer by 50 m*M* NH_4HCO_3).
2. The resulting mixture is incubated for 30 min at 37°C (the CIP digestion buffer supplied by the manufacturer is not used), then dried on a SpeedVac concentrator.
3. The dried peptides are redissoved in 2 μL of acetonitrile:water (1:1, volume) (*see* **Note 6**).
4. If the digestion enzyme is Asp-N, the peptide mixture is heated in an 80°C heating block first for 10 min to inactivate Asp-N, which is usually still active after in gel digestion. There is no need to heat the peptide mixture if trypsin is used.

3.2.2. MALDI–TOF Mass Spectrometry

A 1.2-meter MALDI–TOF mass spectrometer with delayed extraction (Voyager-DE, Perseptive Biosystems, Framingham, MA) is usually used. Data are collected with an external 2-GHz digital oscilloscope (Tektronix, Houston, TX). Two spectra are taken for each sample, one under conditions optimized

for high mass resolution (to measure masses with high accuracy) and one under conditions optimized for sensitivity (to observe the weaker peaks and peaks with higher molecular weights). The high resolution spectrum is often acquired with another reflector instrument (the Voyager-DE-STR) to achieve a mass accuracy of better than 10 ppm. For the linear instrument, masses are calibrated internally with an added calibration standard (bradykinin fragment 1–5) and a peptide derived from trypsin autolysis. Masses of <2000 Dalton can be isotopically resolved with the external 2 GHz oscilloscope so that even weak peaks can be measured with better than 0.2 Dalton mass accuracy. Most of the peaks in the spectrum of the tryptic digest can be easily assigned to unique peptides predicted from the protein sequence and the specificity of trypsin or to peptides formed by autolysis of trypsin. Peaks that cannot be accounted for in this way are candidates for modified peptides or contaminates and those whose observed masses are 80 Dalton (or multiples of 80 Dalton) higher than calculated for a predicted tryptic peptide are tentatively assigned as phosphopeptides. This assignment is confirmed by the absence of these peaks from the MALDI–TOF spectrum of the same peptide mixture after treatment with CIP and the appearance of new peaks that are 80 Dalton (or multiples of 80 Dalton) lower in mass. In the event of that: (1) the stoichiometry of phosphorylation is low, (2) a peak that is 80 Dalton lower in mass exists prior to CIP treatment, or (3) a new peak appears without the disappearance of a peak of 80 Dalton higher in mass, the assignments of the phosphopeptides is considered only tentative, and further confirmation from tandem mass spectrometry is needed (*see* **Subheading 3.3.**). This step removes any ambiguity of peptide assignment due to mass degeneracy in which another unphosphorylated peptide happens to have the same mass as the phosphopeptide, or to other unknown modifications that might give rise to an unphosphorylated peptide with exactly the same mass as the phosphopeptide. The protocol for MALDI–TOF measurement is the following.

1. Aliquots of 0.5 μL of the peptide mixture before and after CIP treatment and 0.5 μL of the working matrix solution spiked with an added calibration standard (bradykinin fragment 1–5) of a final concentration of 10 fmol/μL are mixed, respectively, on the sample plate and dried in air prior to MS analysis.
2. When taking mass spectra on the linear instrument, first set the instrument to single-shot mode to survey the landscape of the entire sample with a higher than usual laser setting. After finding a spot that gives more peptides and stronger signal, lower the laser setting and acquire the spectrum with 64 laser shots. Make sure that at most only a few strongest peaks can saturate the digital oscilloscope setting. This spectrum will be the high resolution spectrum. Then increase the laser setting to saturate all the dominate peptide peaks and acquire another spectrum with 64 laser shots. This spectrum is the high-sensitivity spectrum.

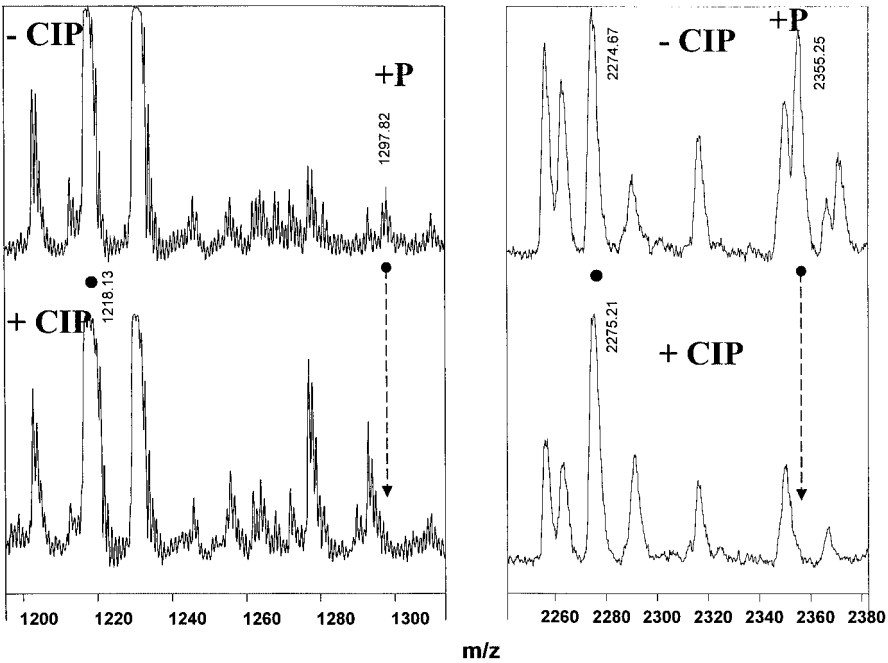

Fig. 1. Portions of MALDI–TOF spectra of an in-gel trypsin digestion of human Stat5A when activated by interleukin-2 (IL-2) that identify the phosphopeptides. Phosphopeptides are labeled with +P.

A different method file may have to be used for acquiring the high-sensitive spectrum.

3. The high-resolution spectrum is calibrated with the masses of the calibration standard (monoisotopic mass) and a trypsin autolysis peak (an average mass can be used).
4. The low-resolution spectrum is calibrated with the aid of the high-resolution spectrum by choosing two peaks that are not saturated. Either calculated masses or measured masses from the high-resolution spectrum can be used.
5. To identify the phosphopeptides, high-resolution and low-resolution spectra of before and after the CIP treatment are overlaid, respectively (*see* **Fig. 1**). The phosphopeptides are tentatively identified with the three criterions listed above (*see* **Notes 7–9**).

3.3. Sequencing of Phosphopeptides

An electrospray ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA) coupled on-line with a capillary HPLC (Magic 2002, Michrom BioResources, Auburn, CA) is used to sequence the phosphopeptide for the

identification of phosphorylation sites. An 0.1×50 mm-MAGICMS C18 column (5-μm particle diameter, 200 Å pore size) with mobile phases of A and B with a gradient of 2–98% of mobile phase B over 2.5 min followed by 98% B for 2 min at a flow rate of 50 μL/min. The flow is split with a Magic precolumn capillary splitter assembly (Michrom BioResources) and 1 μL/min directed to the 100 μm column. The steep gradient and narrow column results in narrow HPLC peaks (eluting in as short a time as 10 s) of small volume (0.2 μL), and thus high peptide concentration, which enhances the ESI/MS response.

The ion trap mass spectrometer is operated in an unusual fashion, i.e., in a constant collision-induced dissociation (CID) mode for the entire LC run with the mass spectrometer set to acquire CID spectra on a single m/z value. The charge state of the precursor ion is not measured directly, rather it is calculated by inspection of the sequence of the phosphopeptide identified in the MALDI–TOF measurements (usually it is doubly or triply charged for a tryptic peptide).

During a MS/MS measurement, a doubly or triply charged phosphopeptide is isolated in the ion trap and subjected to CID. Peptide bonds are cleaved to generate fragment ions that contain short stretches of amino acids (labeled "y" for the C-terminal fragment ion and "b" for the N-terminal fragment ion ***[10]***). In the simplest application of this method, a fragment ion with mass 80 Dalton higher than the calculated mass of the expected fragment ion contains the phosphorylation site. And if the smallest phosphorylated fragment ion contains only one phosphorylable amino acid (Ser, Thr, or Tyr), then the phosphorylation site is identified. However, fragmentation of phosphopeptides in an ion trap is more complicated than this simple picture. Phosphopeptides that contain phosphoserine or phosphothreonine predominantly lose the elements of H_3PO_4 ***(11)***, resulting in a loss of mass of 98 Dalton (49 Dalton for a doubly charged ion). Similarly, fragment ions containing the phosphate generated by CID also can lose the elements of H_3PO_4, creating ions with masses 98 Dalton less than the predicted mass. Thus, this 98-Dalton loss also serves as a signature for phosphopeptides. However, this loss of phosphate in an ion trap mass spectrometer is more complicated than stated here and is phosphoamino acid- and charge-state-dependent ***(11)***. The protocol for running LC/MS-MS experiments is the following.

1. Before running the real sample, check the LC/MS set-up. A mock run with a standard peptide solution is carried out first to ascertain that the LC/MS set-up is fully optimized. Pay attention to the noise level as well as the response of the standard peptides. We use an injection of 100 fmol bombesin as a standard test. A signal-to-noise ratio of 200–500 is considered acceptable for the optimization of the LC/MS instrument (the peak response for the +2 charged peak at m/z

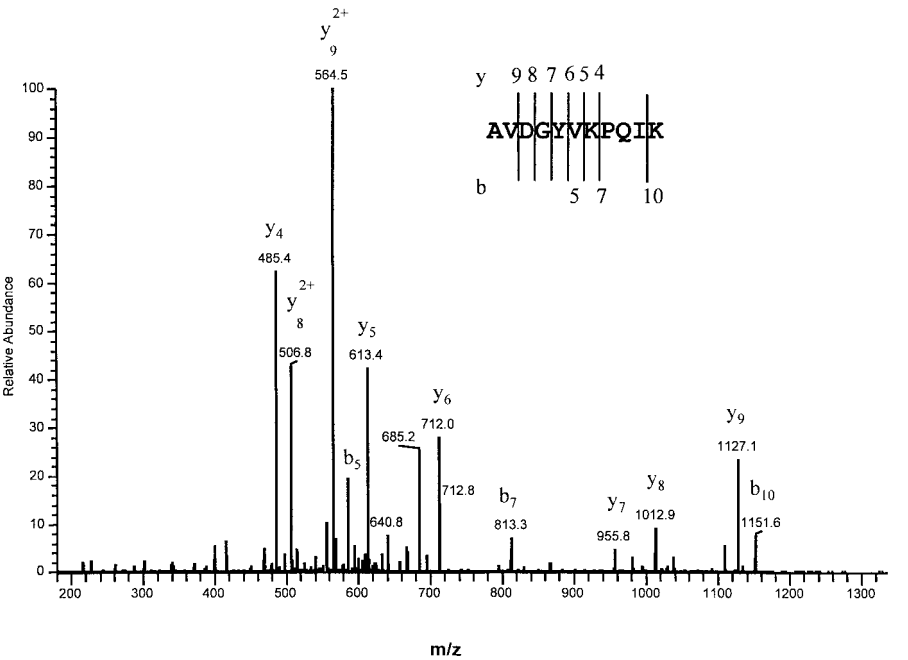

Fig. 2. The LC/MS-MS spectrum of the doubly charged small phosphopeptide as identified in **Fig. 1**. Y is the residue that is identified as phosphorylated.

810 is 5–10 × 10^7 and the overall noise level from m/z 400–1000 before the peptide eluting from the column is ~2 × 10^5).

2. Analyze the sequence of the putative phosphopeptides to determine the most probable charge states. Usually the most probable charge state is the number of R, K, and H plus one (*see* **Note 10**).
3. Calculate m/z value for the most probable chare state and set the mass spectrometer to acquire a CID spectrum on this one m/z value during the entire LC run (*see* **Note 11**).
4. Dry the peptide mixture that is dissolved in 50% acetonitrile in a SpeedVac. Dissolve the sample in 7 µL of 10% mobile phase B. Inject 2 µL sample for acquiring a CID spectrum. If the final concentration is estimated to be >20 fmol/µL (this information can also be derived from the MALDI–TOF measurement), the peptides can be further diluted. This way, more phosphopeptides (if there are any) can be sequenced.
5. For data analysis, examine all peaks in the chromatograph. CID spectra derived from phosphoserine and threonine containing peptides can usually be easily identified by looking for dominant loss of the moiety of H_3PO_4. But for phosphotyrosine containing peptides (*see* **Fig. 2**), this dominant loss can vary depending on the charge state (*see* **Note 12**).

4. Notes

1. A Benson burner is used to soften the tip of a 1 mL-pipet tip. The softened tip is then pushed against a piece of clean microscope glass slip to seal the tip.
2. More trypsin is needed if modified sequencing grade trypsin from Boehringer is used. We observed that the Boehringer bovine trypsin yields fewer autolysis products and is less active than the Promega porsin trypsin. A recent batch (after October 1998) of trypsin from Boehringer showed significantly low activity but less autolysis, therefore the digestion time is prolonged to overnight accordingly.
3. No acid is used. We did not see any improvement in peptide extraction yields upon addition of TFA, acetic acid, or formic acid at a concentration up to 10%. The addition of acids tends to prolong drying time for the extracted peptide. However, using 100% formic acid can sometimes extract more peptides from gel pieces that have been extracted twice using this protocol. As a last resort to increase sequence coverage (to recover more peptides), the gel pieces after two extraction can be further extracted with 20 μL formic acid for 10 min followed by addition of 60 μL of acetonitrile for 10 min.
4. For copper-stained gels, the same protocol is followed except that the destained step is carried out using copper destain solution as supplied by the manufacture following its suggested protocol. We found that proteins (<10 ng) that can only been seen with silver stain but not copper or CB stain are not enough for the determination of phosphorylation sites. Many seemingly weak silver stained bands can actually be observed with copper and CB staining.
5. The step of reduction/alkylation is omitted in this protocol. We observed inconsistent results of recovering Cys-containing peptides by reduction/alkylation after the protein has been separated in SDS-PAGE. It seems that Cys-containing peptides are best recovered when in-gel digestion is carried out right after SDS-PAGE. We often found that reduction/alkylation after SDS-PAGE separation did not help recovering Cys-containing peptides. However, alkylation before running SDS-PAGE can sometimes help recovering Cys-containing peptides. (Consult **ref. *12***.)
6. For higher reproducibility, a control sample of the extracted peptide can be mock-treated with heat inactivated CIP (the CIP solution is heated at 80°C for 10 min) so that final compositions of the CIP treated sample (dephosphorylated) and mock treated sample (still phosphorylated) are the same. Reproducible MALDI/TOF spectra can be better obtained this way.
7. Care must be taken for data analysis. Weak peaks cannot be overlooked. The reproducibility of ions in MALDI–TOF at m/z <1000 is poorer than that of ions of higher m/z. Special considerations have to be given in this mass range. The three criterions are followed and phosphopeptide assignments are tentative until proven by the subsequent LC–MS–MS measurements.
8. A linear MALDI–TOF mass spectrometer is often preferred for the detection of phosphopeptides because phosphopetides tend to decompose in the reflector mode. The linear mass spectrometer also offers higher sensitivity than the reflector instrument, which is important for the identification of weaker peaks

corresponding to phosphopeptides. Weaker peaks are best identified when mass spectra are taken under the high-sensitivity condition.

9. A very weak peak at m/z 1297.82 disappears after CIP treatment and there is a peak at m/z 1218.13, which is saturated in the spectrum. As they differ by 80 Dalton, this weak peak is tentatively identified as a phosphopeptide.
10. The measured mass corresponds to a tryptic phosphopeptide with the sequence of AVDGYVKPQIK, derived from human Stat5A. The number of R, K, and H are 2, and normally a +3 charge will be assumed. But since this peptide is small and the two Lys residues are too close to each other, the +2 charge state is chosen. For smaller tryptic peptides (m <2000 Dalton), it is always safe to pick the +2 charge state, as their CID spectra are easier to interpret than those of the +3 charge state.
11. For the +2 charge state, m/z was calculated as 649.5. The mass spectrum was set to acquire CID spectra on this m/z value with an mass isolation window of 4 m/z units and relative collision energy 28%. (for m/z >750, 33% relative collision energy is better and for m/z >1200, 45% relative collision energy yields better results).
12. For a more comprehensive account of fragmentation patterns of phosphopeptides in an ion trap mass spectrometer, consult **ref. *11***.

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Analysis of Tyrosine-O-Sulfation

Jens R. Bundgaard, Anders H. Johnsen, and Jens F. Rehfeld

1. Introduction

Tyrosine O-sulfation was first described almost fifty years ago as a post-translational modification of fibrinogen ***(1)***. In the following thirty years it was considered to be a rare modification affecting only a few proteins and peptides. However, in the beginning of the 1980s tyrosine (Tyr) sulfation was shown to be a common modification and since then, an increasing number of proteins have been shown to be sulfated. The target proteins belong to the classes of secretory, plasma membrane, and lysosomal proteins, which reflects the intracellular localization of the enzyme catalyzing Tyr sulfation, the tyrosylprotein sulfotransferase (TPST). TPST is a type II integral membrane glycoprotein residing in the *trans* Golgi network, which utilizes adenosine 3′-phosphate 5′-phosphosulfate (PAPS) as a co-substrate and sulfate donor. Recently, two isoforms of this enzyme have been identified and cloned and their cDNA appear to be ubiquitously present in tissues ***(2–4)***. Moreover, homologous genes are present in lower eukaryotes such as a the nematode *Caenorhabditis elegans*, fruitfly, and zebrafish.

The biological function of Tyr sulfation is modulation of protein-protein interactions. For instance, Tyr sulfation is necessary for binding of the peptide hormone cholecystokinin (CCK) to the CCK_A receptor ***(5)***; it is critical for interactions between p-selectin and PSGL-1 (P-Selectin Glycoprotein Ligand-1) ***(6)***; between hirudin and thrombin ***(7)***; and it modulates HIV-1 the ability of HIV-1 virus to enter cells via the chemokin receptor CCR5 and CD4 ***(8)***. In addition, sulfation has been reported to modulate proteolytic processing ***(9)*** and secretion rates ***(10)*** of secretory proteins. From these functions, it follows that it is not only important to detect Tyr sulfation, but also to identify

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the sulfation site in order to establish effects on the activity of the protein. Prediction of sulfation sites using a consensus sequence based on structures around known sulfation sites can be suggestive ***(11)***, but needs experimental confirmation. Furthermore, it is possible that nontypical structures may be at least partially sulfated ***(12)***.

Traditionally, Tyr sulfation has been analyzed by incorporation of radiolabeled sulfate into target cells followed by purification of the target protein. Subsequently, the protein is degraded enzymatically or by alkaline hydrolysis followed by thin-layer electrophoresis to demonstrate the presence of radioactively labeled tyrosine. These techniques have been described in detail previously ***(13)***. The aim of this chapter is to present alternative analytical methods of Tyr sulfation that are independent of radioisotope incorporation prior to analysis. Thus, sulfation of protein or peptide fragments can be demonstrated by a combination of chromatography and specific assays. This has several advantages. First, depending on the assay used, the analysis can be quantitative. Second, problems with incorporation of isotope and the high degree of incorporation of radiolabeled sulfate into carbohydrate moieties that may interfere with detection, can be avoided. In addition, it allows two ways of identification of sulfated tyrosines depending on purity of the protein.

One strategy demands purified protein using mass spectrometry or chromatography, whereas another uses Tyr sulfate sensitive antisera to distinguish between sulfated and nonsulfated forms in a crude extract. We provide protocols for establishment of sulfation specific antisera and give examples of chromatographic systems that are useful for analysis of Tyr sulfation.

Establishment of a specific immunoassay is costly and time-consuming and initiation of such a process demands an interest exceeding the mere confirmation of the modification. If such an assay is desirable, a number of considerations have to be taken into account. The size of an antibody binding site is complementary to that of a peptide ligand (or epitope) of 4–6 amino acid residues ***(14)***. Within such an epitope the exact structure of each residue profoundly influences the binding to the antibody ***(15)***. Moreover, the protein or peptide structures surrounding the particular epitope also modulate binding affinity considerably ***(16)***. Accordingly, it is not surprising that attempts to raise antibodies that specifically bind a single O-sulfated Tyr residue as such—irrespective of neighboring structures—have failed (Håkanson, R. and Huttner, W. B. personal communications, and our own results). The discrepancy with the success to raise antibodies against the structurally similar phosphorylated Tyr residues ***(17)*** is at present unexplained. In accordance with the aforementioned consideration and with prevailing immunochemical theories, antibodies that recognize sulfation of a given Tyr residue consequently have to be raised against the entire epitope containing the sulfated tyrosine, i.e.,

a peptide sequence of approx five or six amino acid residues. It has, however, proven difficult to raise high-titer and high-affinity antibodies or antisera against peptides of less than 10 residues ***(18)***. Therefore, a peptide of approx 10 residues containing the desired epitope in an otherwise expedient sequence should be designed, synthesized, and used for immunization. Hence, measurement of Tyr sulfation by immunochemical methods requires precise knowledge of the sequence and structure surrounding the O-sulfated residue in a peptide or protein. It is necessary to consider different strategies in the design of appropriate radioimmunoassays (RIA) (*see* **Note 1**).

Sulfation of a protein or peptide introduces a highly acidic group that alters its local physical and chemical properties such as conformation, hydrophilicity, and pK-value. This can be utilized to separate sulfated and nonsulfated forms by various chromatographic techniques, e.g., ion-exchange chromatography and reverse-phase high-performance liquid chromatography (RP-HPLC) at near neutral pH under which the conditions for ionization of the sulfate group induces the maximum difference between the two forms (*see* **Note 2**). Also, removal of the sulfate group by digestion with arylsulfatase combined with chromatographic analysis of the peptide before and after the digestion can reveal the presence of Tyr sulfate. Often, identification of sulfated Tyr within a protein requires digestion of the protein into smaller peptides followed by separation of the fragments and isolation of the relevant peptide. A number of proteinases may be used, for example trypsin or the endoproteinases Lys-C, Glu-C, and Asp-N, the choice being partly dependent on the individual protein. The details for these techniques are beyond the scope of the present chapter, but may be found in other volumes of this series. These analytical methods can profit from the availability of specific antisera or be carried out using pure proteins.

Having a purified protein offers alternative ways of analysis of Tyr sulfation. The sulfate ester bond is labile and especially susceptible to acidic hydrolysis. Thus, sulfated Tyr can not be identified by protein sequence analysis due to the acidic environment during the analysis. Instead, nonderivatized Tyr will result. Likewise, the standard acidic hydrolysis used for amino acid analysis is not feasible; instead, demonstration of the presence of intact Tyr sulfate in a peptide was originally obtained by basic hydrolysis prior to amino acid analysis ***(19)***. This technique has a limited sensitivity due to an inevitable background. Alternatively, nondestructive digestion can be obtained by aminopeptidase M or a combination of aminopeptidase M and prolidase followed by amino acid analysis ***(20)***.

However, these techniques are not used much anymore. In the recent years mass spectrometry (MS) of peptides and proteins has undergone a dramatic development, resulting in increased sensitivity and precision. MS in combina-

tion with protein-sequence analysis can disclose the presence of a sulfated Tyr. Thus, if the identified sequence contains Tyr and the measured molecular mass is 80 Dalton higher than the calculated value, it is a good indication that the Tyr is sulfated. However, phosphorylation also results in an 80 Dalton increase of the molecular mass (*see* **Note 3**). It should also be noted that the sulfate group might be lost during ionization in the mass spectrometer, resulting in a false-negative result. Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), the loss of sulfate can be turned into a diagnostic advantage since the loss is much more pronounced in the positive mode (and dependent on the irradiation energy) ***(24)***. Hence, comparison of spectra obtained in positive and negative linear mode of the same sample will show a vast difference in the yield of the sulfated and nonsulfated species (*see* **Note 4**), while the picture will be more complex in the reflected mode (*see* **Note 5**).

2. Materials

2.1. Generation of Tyr Sulfation-Sensitive Radioimmunoassays

2.1.1. Preparation of Immunogen (Common for All Three Coupling Methods) (see ***Note 6***)

1. 5 mg Sulfated peptide.
2. N, N-dimethylformamide.
3. 25 mg Bovine serum albumin (BSA).
4. 0.05 *M* sodium phosphate buffer, pH 7.5.
   For carbodiimide-coupling:
5. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl.
   For glutaraldehyde-coupling:
6. 500 g/L Glutaraldehyde.
7. One Sephadex G-10 column with a fraction collector.
   For maleimidobenzoyl-succinimede ester-coupling:
8. 25 mg m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS).
9. One Sephadex G-25 column with a fraction collector.

2.1.2. Immunization

1. 6–8 Rabbits.
2. 8.5 g/L Saline buffer.
3. Freund's complete adjuvant.
4. Freund's incomplete adjuvant.

2.1.3. Preparation of Tracer (see ***Note 7***)

1. Synthetic peptide.
2. HPLC apparatus with reverse-phase column (Aquapore C-8 column, RP-300, 220×4.6 mm, 7 μm bead size) or similar and fraction collector.

3. Ethanol or acetonitrile (HPLC grade).
4. 1.0 mL/L Trifluoroacetic acid (TFA; HPLC grade in water).
5. 0.02 *M* Barbital buffer, pH 8.4.

2.1.3.1. Iodination Using Chloramin T

1. I^{125} (Amersham IMS 30, specific activity 16.85 mCi/μg of iodine).
2. 0.05 *M* phosphate buffer, pH 7.5.
3. Disodium sulfite (Na_2SO_3).
4. Chloramine T.

2.1.3.2. Iodination Using the Bolton Hunter Reagent

1. Bolton and Hunter reagent for protein iodination (Amersham IM 5861).
2. 0.2 *M* Sodium borate buffer, pH 8.5 (mixture of 0.2 *M* $Na_2B_4O_7$ adjusted with 0.2 *M* H_3BO_3).
3. 0.1 *M* Sodium borate buffer with 0.2 *M* glycine, pH 8.5 (prepared from the buffer above).

2.1.4. Radioimmunoassay Procedure

1. Synthetic peptide for standard (*see* **Note 8**).
2. Disposable plastic tubes.
3. 0.02 *M* Barbital buffer, pH 8.4, containing 1 g/L BSA.
4. Activated charcoal.
5. Blood plasma. (This is a mixture of buffer and outdated human plasma from Blood Banks in 0.02 *M* sodium phosphate, pH 7.4. The optimal concentration normally varies between 10–50% plasma.)
6. γ-Scintillation counter.

2.2. Analysis of Tyr Sulfation Using FPLC-Based Ion-Exchange Chromatography (see *Note 9*)

1. An FPLC system equipped with an ion-exchange column, e.g., an 5/5HR MonoQ anion-exchange column (Pharmacia) and a fraction collector.
2. Buffer A: 50 m*M* Tris-HCl, pH 8.2, added 10% (v/v) acetonitrile.
3. Buffer B: 50 m*M* Tris-HCl, pH 8.2, added 10% (v/v) acetonitrile and 1 *M* NaCl.
4. Both buffers are filtered through a 0.45-μm filter, degassed, and stored at 4°C where they are stable for months.

2.3. Analysis of Tyr Sulfation Using Reverse Phase HPLC

1. An HPLC instrument with gradient formation capability and equipped with a suitable RP column (C8 or C18).
2. Solvents: Stock solution of 0.5 *M* ammonium acetate in water, filtered through 0.45-μm filter (can be kept for several months at 4°C). Solvents A and B are prepared by addition of 20 mL/L of H_2O and acetonitrile, respectively (both HPLC grade), to form 10 *M* ammonium acetate.

2.4. Analysis of Tyr Sulfation Using Mass Spectrometry

1. Instrument: A mass spectrometer for MALDI-TOF MS.
2. Matrix solution: Prepare a 20 mg/mL solution of α-cyano-4-hydroxycinnamic acid in 30% (v/v) acetonitrile/0.1% (v/v) TFA in an Eppendorf tube and centrifuge; use the supernatant as matrix solution. This matrix is the first choice for peptides below 5 kDa but others may be useful such as 2,5-dihydroxybenzoic acid (DHB) and for large peptides 3,5-dimethoxy-4-hydroxy-*trans*-cinnamic acid (sinapinic acid). Premade matrix solutions ("MALDI-grade") are also commercially available (Hewlett-Packard).

2.5. Arylsulfatase Treatment

1. Arylsulfatase type VIII (Sigma S 9754, 20–40 U/mg).
2. Acetate buffer: 0.2 *M* sodium acetate, pH 5.0.
3. 0.2% Sodium chloride.

3. Methods

3.1. Generation of Tyr Sulfation-Sensitive Radioimmunoassays

3.1.1. Preparation of the Immunogen

3.1.1.1. Carbodiimide Coupling

1. Dissolve 5 mg peptide hapten in 1 mL of N, N-dimethylformamide.
2. Dissolve 25 mg BSA in 2.5 mL of 0.05 *M* sodium phosphate, pH 7.5.
3. Conjugate by the addition of 125 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl to give molar ratios between the peptide, albumin, and ethylcarbodiimide of 1 : 0.1 : 40. Mix the reagents and incubate for 20 h at 20°C.
4. Divide the mixture into six portions and store at –20°C until immunization (stable up to 1 yr).

3.1.1.2. Glutaraldehyde Coupling

1. Dissolve 2.5 mg of sulfated peptide hapten together with 7.5 mg BSA in 5 mL of 0.05 *M* sodium phosphate, pH 7.5.
2. Conjugate by dropwise addition of 100 µL of 500 g/L glutaraldehyde.
3. Mix the solution and incubate for 4 h at 20°C.
4. Apply the mixture to a calibrated Sephadex G-10 column and elute at 20°C with 0.05 *M* sodium phosphate, pH 7.5, in fractions of 1 mL.
5. The void volume fractions containing the conjugate are then pooled, divided into six portions, and stored at –20°C until immunization (stable for months).

3.1.1.3. Maleimidobenzoyl-Succinimide Ester Coupling

1. Dissolve 5 mg sulfated peptide hapten together with 25 mg of BSA in 10 mL 0.05 *M* sodium phosphate buffer, pH 7.5.
2. Dissolve 25 mg of m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) in 1 mL N, N-dimethylformamide.

3. Conjugate by dropwise addition of the MBS-solution to the sulfated peptide/BSA solution.
4. Stir at room temperature for 30 min.
5. Free MBS is separated from the activated albumin by Sephadex G-25 gel filtration (eluted at 20°C with 0.05 *M* sodium phosphate, pH 7.5, in fractions of 1 mL).
6. Add the sulfated and cysteinylated peptide to the MBS-activated BSA and stir the mixture stirred for 3 h at room temperature and a pH of 7.5.

3.1.2. Immunizations

1. The first portion of the antigen is suspended in 8.5 g/L saline to a volume of 5 mL and carefully emulsified with an equal volume of Freund's complete adjuvant. We recommend emulsification by pushing the saline-adjuvant forwards and backwards between two connected 10-mL syringes until a drop of the emulsion remains coherent and condensed on a larger water surface. Randomly bred rabbits are used for immunizations in series of 6–8 rabbits each.
2. Two subcutaneous injections of the mixture are given over the hips in amounts corresponding to 40 μg hapten per animal.
3. Five or more booster injections using Freund's incomplete adjuvant (prepared as described in **Step 1**) are then administered simultaneously in all rabbits at 8-wk intervals, using one-half of the initial dose of antigen per immunization.
4. The rabbits are bled from an ear vein 10 d after immunization. Sera from the bleedings are separated and stored at –20°C.
5. The antiserum obtained is evaluated (*see* **Note 10**).

3.1.3. Preparation of Tracer for Radioimmunoassay (see ***Note 7***)

3.1.3.1. Iodination Using Chloramine T

1. Dissolve 500 μg peptide in 1 mL 0.05 *M* phosphate buffer, pH 7.5. Aliquot in 10 μL portions and store at –20°C.
2. Dilute 2 mCi I^{125} (~20 μL) with 85 μL 0.05 *M* phosphate buffer, pH 7.5 (final activity about 200 μCi/10 μL).
3. Dissolve 5 mg chloramine T in 10 mL 0.05 *M* phosphate buffer, pH 7.5.
4. Dissolve 2 mg disodium sulfite in 10 mL 0.05 *M* phosphate buffer, pH 7.5.
5. Mix 10 μL peptide, 10 μL I^{125}, and 10 μL chloramine T gently. After 30 s the reaction is arrested with 25 μL disodium sulfite. Shake gently. If more tracer is prepared, do iodination in batches and pool the preparations.
6. Purify the tracer by HPLC. Fractions with maximal counts of reactivity is diluted with 0.02 *M* barbital buffer, pH 8.4, to a final activity of 10^6 cpm/mL. Store the tracer in 500 μL aliquots at –20°C.

3.1.3.2. Iodination Using the Bolton Hunter Reagent

1. Evaporate the water from the I^{125} Bolton Hunter reagent under a slow flow of nitrogen.

2. Dissolve 5 μg of peptide in 10 μL water and add 10 μL 0.2 *M* sodium borate buffer.
3. Add the peptide solution to the Bolton Hunter reagent and incubate the mixture at 4°C for about 20 h.
4. Add 500 μL 0.1 borate buffer with 0.2 *M* glycine. Continue directly with HPLC purification of the tracer as described above or store at 4°C.

3.1.4. Radioimmunoassay Procedure

1. At room temperature, prepare the following mixtures of total volumes of 2.4 mL (RIA is carried out as an equilibrium system at pH 8.4, using 0.02 *M* barbital buffer, pH 8.4, containing 1 g/L BSA) in disposable plastic tubes: 2.0 mL of antiserum dilution, 250 μL of tracer solution (giving 1000 cpm, corresponding to 0.5 fmol of freshly prepared ^{125}I-labeled tracer-peptide), and 150 μL of standard solution or sample. All samples should be assayed in duplicate.
2. Incubate at 4°C for 2–5 d to reach equilibrium.
3. Antibody-bound (B) and free (F) tracers are separated by the addition of 0.5 mL of a suspension of 20 mg of activated charcoal and diluted blood plasma to each tube. The tubes are centrifuged for 10 min at 2000*g*.
4. Count the activities of the supernatant (B) and sedimented charcoal (F) in automatic γ-scintillation counters for 5 min.
5. Calculate the binding percentage as $B - [(B + F) \times D] / (B + F) - [(B + F) \times D] \times 100$, where the "damage" (D) is defined as $B \times 100/(B + F)$ in the absence of antiserum. The damage is usually 2–3%. The peptide concentration is determined from a standard curve based on known standards.

3.2. Analysis of Tyr Sulfation Using FPLC Based Ion-Exchange Chromatography

1. Equilibrate the column to start conditions and inject sample.
2. Elute with an appropriate gradient at the flow of 1 mL/min and collect fractions if necessary.
3. The eluting peptide is monitored by the UV signal recording or any appropriate specific assay. An example of an elution profile examined by radioimmunoassay is given in **Fig. 1**.

3.3. Analysis of Tyr Sulfation Using RP-HPLC

1. Equilibrate column to start conditions and inject sample.
2. Elute with an appropriate gradient.
3. Record UV signal at 214 nm and, if further analyses are wanted, collect fractions automatically at regular intervals or collect peak manually. An example of an elution profile is given in **Fig. 2**.

3.4. Analysis of Tyr Sulfation Using Mass Spectrometry

1. Samples should be in a final concentration of 0.05–5 pmol/μL, sensitivity depends highly on the nature of the peptide (*see* **Note 11**).

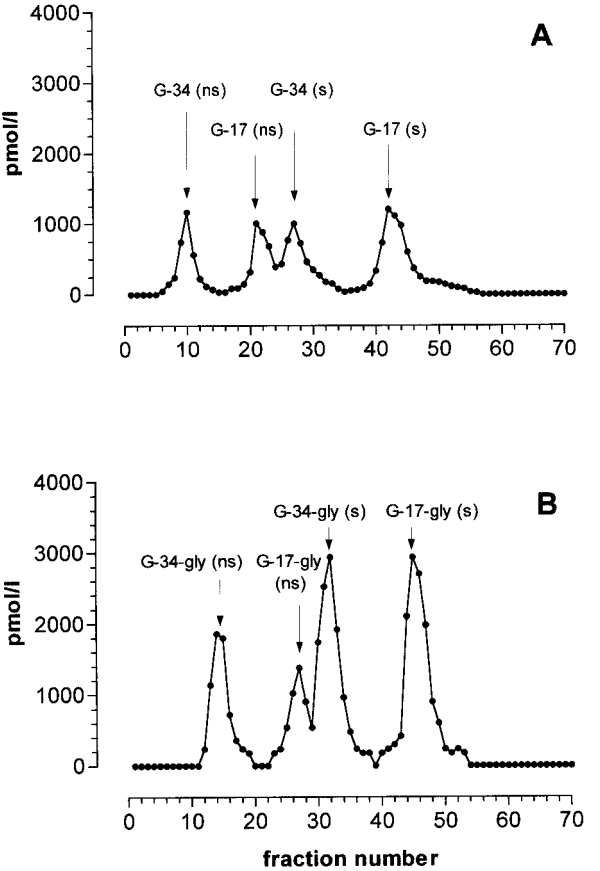

Fig. 1. Anion-exchange chromatography using a FPLC system of the main processing forms of human gastrin heterologously expressed in a hamster β-cell line, HIT. **(A)** shows carboxyamidated gastrin forms of 17 and 34 residues and demonstrates the separation of sulfated and nonsulfated forms. **(B)** shows the immediate precursor of carboxyamidated gastrin, with the free C-terminus extended with a Gly. Fractions were eluted using a linear gradient of 20–55% buffer B over 60 min at a flow of 1 mL/min. Fractions were analyzed using specific radioimmunoassays. (Elution positions can be verified using synthetic peptides or by analysis of elution positions before and after arylsulfatase treatment.)

2. Mix 0.5 µL of sample with 0.5 µL MALDI matrix solution. This can be done in a microcentrifuge tube, or many samples can be prepared consecutively at different positions in a trough made from a cut-through polypropylene tube.
3. Apply immediately 0.5 µL of the mixture to the sample target (volume may depend on the target size) and let sample dry. Do not touch the target surface with the pipet tip!

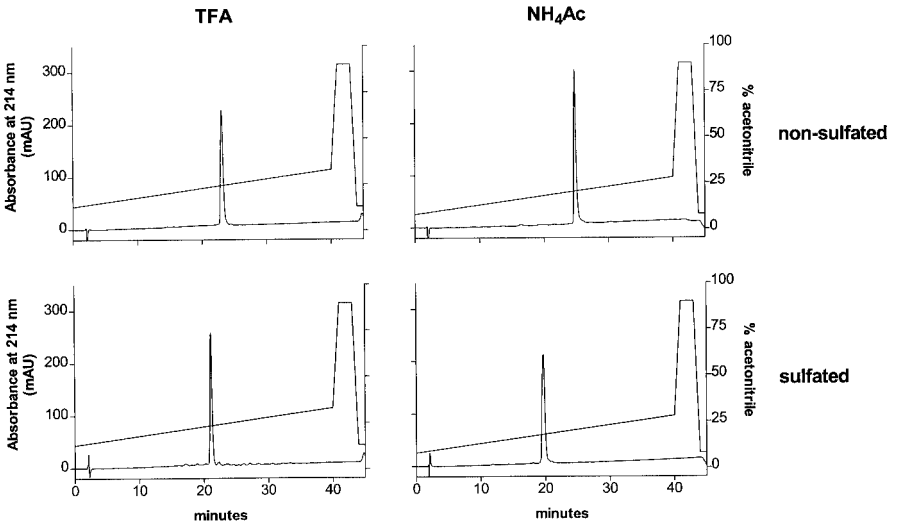

Fig. 2. HPLC separation of the model peptides, nonsulfated and sulfated shark gastrin-8 (*see* **Fig. 3**). The use of 0.1% TFA and 10 m*M* ammonium acetate (NH_4Ac) as buffering component are compared. In both cases 0.5%/min gradients from H_2O to acetonitrile were employed, starting from 13% acetonitrile in the TFA system and from 8% in the NH_4Ac system (indicated by the straight lines). While the two forms are indeed separated in the TFA system (by 1% acetonitrile) the separation is much more pronounced in the NH_4Ac system (by 2.5% acetonitrile). For bigger peptides, where sulfation influences the chromatographic behavior relatively less, use of the NH_4Ac system may be crucial for the separation of the nonsulfated and sulfated form.

4. Insert sample(s) into the mass spectrometer and record the mass spectrum according to the instrument manual.

3.5. Arylsulfatase Treatment (see *Note 12*)

1. Prepare an enzyme solution of arylsulfatase VIII of 2 mg/mL in 0.2% sodium chloride.
2. Prepare the lyophilized sample by resuspension in 400 µL acetate buffer.
3. Add 100 µL enzyme solution to the sample preparation. The final concentration of arylsulfatase is 0.4 mg/mL (equivalent to 0.55 U/mL).
4. Incubate for 3 h at 37°C.
5. Terminate the reaction by boiling for 10 min.

4. Notes

1. The strategy depends on several factors:
   a. The size of the protein or peptide;
   b. The position of the sulfated Tyr within the protein;

c. The biology of the sulfated protein or peptide (a large membrane protein, a circulating hormone, or a small neurotransmitter peptide, etc.); and
d. The concentration range in which to measure.

If the sulfated Tyr is positioned at the N- or the C-terminus, production of antibodies against a synthetic analog of the terminally sulfated decapeptide is straightforward. If, however, the sulfated Tyr is located in the middle of a protein or a long polypeptide chain, antibodies should be raised against a fragment containing the sulfated tyrosine, preferably a fragment that can be released from the protein by appropriate proteolytic cleavage. Finally, if the sulfated protein is available in substantial quantities for large immunization series, "shotgun" immunization with the entire protein and subsequent identification of epitope specificity can be attempted.

2. The presence of Met in the peptide presents a possible pitfall, since oxidation of Met to the sulfoxide (which may occur spontaneously in the presence of atmospheric oxygen) increases the hydrophilicity considerably, thus causing the peptide to elute significantly earlier than the reduced form in both a RP-HPLC and in an anion exchange chromatography system.
3. Both sulfation and phosphorylation add 80 Dalton to the molecular mass of the peptide (the exact monoisotopic values being 79.957 and 79.966, respectively). However, it is possible to distinguish the two by several criteria. Thus, under the standard conditions used for Edman sequencing peptides phosphorylated at Ser, Thr, or Tyr give no signal (although for different reasons ***[21–22]***) resulting in a blank cycle, while sulfated Tyr is hydrolyzed and seen as Tyr in almost normal yield. Furthermore, in contrast to Tyr-sulfated peptides (**Fig. 3**), phosphorylated peptides are recorded as the intact species in MALDI-TOF linear mode ***(23)***. In the reflectron mode, a typical triplet is observed from phosphorylated peptides consisting of the intact molecular ion accompanied by a major and a much less abundant fragment due to the loss of H_3PO_4 (98 Dalton) and HPO_3 (80 Dalton), respectively ***(23)***. This is in contrast to the singular loss of 80 Dalton from a Tyr-sulfated peptide (*see* **Note 5**).
4. The relative yields of the sulfated and nonsulfated form depend on the composition of the peptide. Small acidic peptides like the model peptide give almost clean peaks representing the sulfated and nonsulfated form in the negative and positive (linear) mode, respectively (**Fig. 3**), while larger and more neutral peptides give a more mixed picture.
5. A MALDI-TOF mass spectrum obtained in the negative mode using reflector will show the correct molecular ion and an additional peak at a distance less than 80 Dalton below the value for the molecular ion (**Fig. 4**). This is due to rupture of the sulfate ester bond in the linear flight tube after the peptide was accelerated but before the peptide reaches the reflector, and the calibration for the reflector requires ions with full accelerating energy. The decrease in molecular mass due to sulfate loss observed in the reflected mode is instrument dependent, but once established it can also be used as an indicator of a sulfated peptide.
6. The tyrosine 0-sulfated peptide, synthetic or purified, has to be available in mg amounts. After dissolution in an appropriate buffer, the peptide is coupled to a

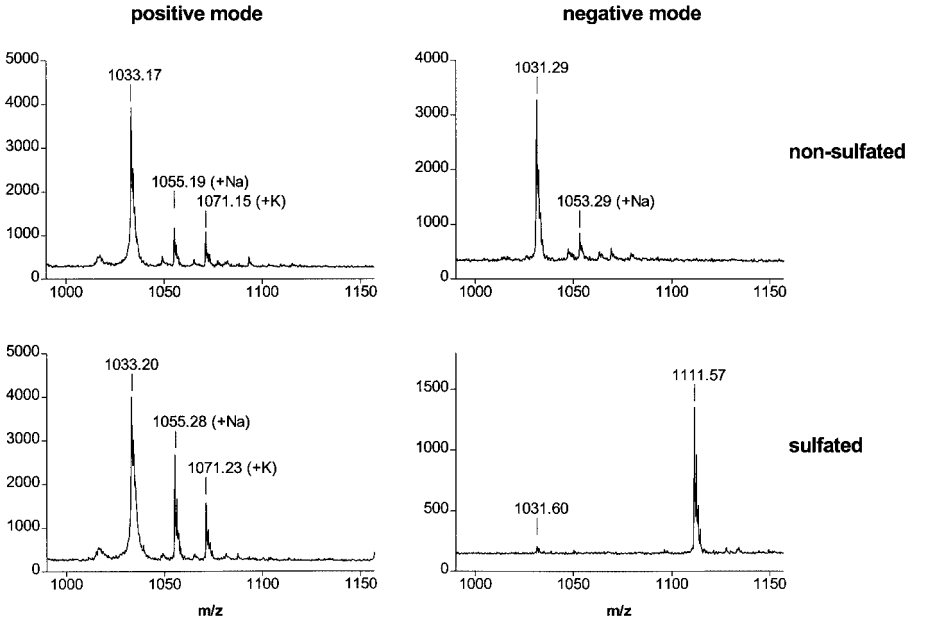

Fig. 3. Mass spectra obtained in the positive and negative mode in a mass spectrometer equipped with delayed extraction (Bruker Daltonik Biflex). As model peptides were used nonsulfated and sulfated shark gastrin-8 (Asp-Tyr(SO3)-Thr-Gly-Trp-Met-Asp-Phe-NH2) with the theoretical monoisotopic molecular masses 1032.40 and 1112.36 Dalton, respectively. In the positive mode the measured m/z values represent the protonated molecular ion (+1.01) and to a certain degree the Na^+ and K^+ adducts, while the deprotonated form (–1.01) is recorded in the negative mode. In this example the intact sulfated peptide is only observed in the negative mode together with a small fraction of the desulfated form. However, *see also* **Note 3**. *y*-axis, number of collected ions.

protein carrier in amounts that ensure a coupling ratio of approx 5–10 molecule haptens per carrier molecule. There are several useful carrier candidates. We recommend BSA (approx 5 mg/mg hapten decapeptide). BSA is easily available, inexpensive, and in our experience as effective as any other protein carrier. When peptide hapten has to be synthesized, we recommend synthesis of an analog of the genuine hapten equipped with a N- or C-terminal cysteinyl residue. Which terminus or end depends on the position of the sulfated tyrosyl residue (**Fig. 5**). The peptide hapten is then coupled to a free amino group in the carrier protein through the terminal cysteine using maleimidobenzoic acid N-hydroxysuccinimide ester as coupling reagent ***(26)***. Again, depending on the position of the sulfated tyrosyl residue, conventional coupling using either ethylcarbodiimide or glutaraldehyde may also be used ***(26)***.

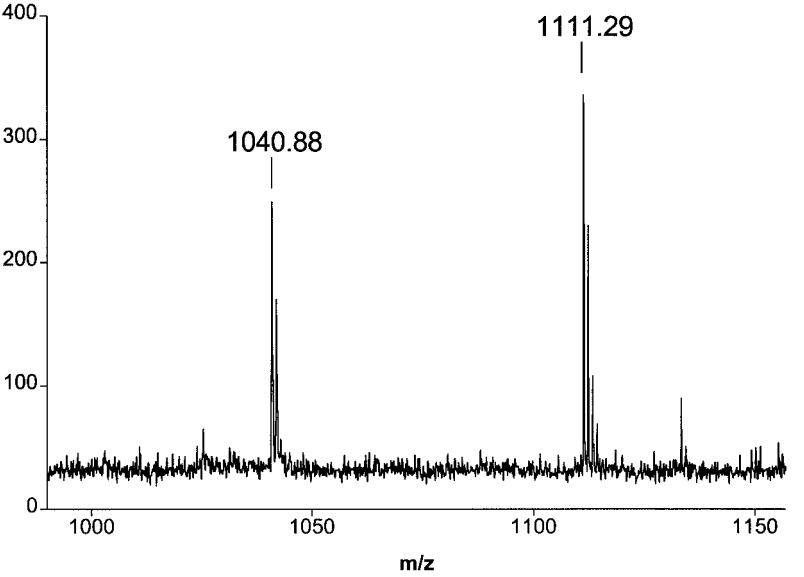

Fig. 4. Mass spectrum of sulfated shark gastrin-8 (*see* **Fig. 3**) obtained in the negative reflected mode. The peak at m/z 1111.29 represents the intact peptide (theoretical value 1112.36–1.01) while the peak at 1040.88 represents the in-flight desulfated peptide. The difference between the two is considerably less than the expected 80 (*see* **Note 2**). Note that the resolution is higher in the reflected mode than in the linear mode (**Fig. 3**).

7. With directional N- or C-terminal carrier-coupling (**Fig. 5**), it is important to design a tracer that corroborates and further advances the antibody specificity achieved. Since ^{125}I is the preferred RIA-isotope, and since iodine easiest is coupled to tyrosyl-residues, we recommend synthesis of a tyrosylated hapten decapeptide in which the additional tyrosyl-residue for iodination is placed in the same position as cysteine in the hapten analog, i.e., at the terminus opposite to the epitopic part of the hapten containing the O-sulfated tyrosine (**Fig. 5**). In this way, iodinated tyrosine will not interfere with the antibody binding. There is no risk for iodination of the O-sulfated tyrosine-residue. The sulfonate group blocks incorporation of iodine. Natural occurrence of an additional unsulfated tyrosyl in the short hapten sequence has not been experienced so far; but in such situation a new labeling strategy has to be delineated, based on the exact positions of the additional and the sulfated tyrosyl residue.

   The tyrosine-extended peptide analog (**Fig. 5**) (4 nmol) can be monoiodinated using the mild chloramine-T method, previously described ***(26)***. If mild oxidation damages the peptide (containing for instance methionyl residues), it is possible to use the nonoxidative Bolton-Hunter iodination ***(27)***. Subsequent purification

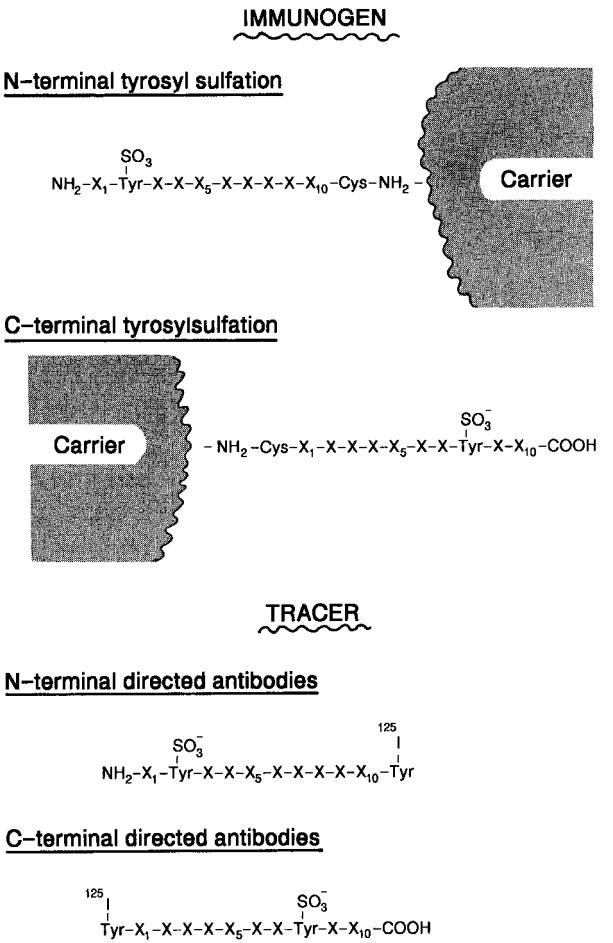

Fig. 5. The structure of immunogens used for production of antibodies directed against tyrosyl O-sulfated peptides (upper part of the figure), and the structure of corresponding radioactive tracers (lower part of the figure).

on RP-HPLC ensures a high specific radioactivity of the tracer. To evaluate the chromatographic separation of labeled and nonlabeled peptides, 1 mL of the monoiodinated peak fraction is mixed with 10 pmol of the relevant tyrosine-extended peptide and reapplied to the HPLC column as described. Both the radioactivity of the labeled peptides and the immunoreactivity are measured. Using the assumption that iodinated and unlabeled peptides are measured with identical affinity, the specific radioactivity of the tracers can be determined by self-displacement ***(28)***. The essence of the described procedure is to ensure

monoiodination without oxidative damages to the peptide. High specific radioactivity is then achieved by efficient chromatographic purification.

8. As standard substance for RIA-measurements using antibodies specific for sequence containing a sulfated tyrosyl-residue, it is straightforward to use a peptide corresponding to the hapten decapeptide. If, however, this decapeptide in some way is partly or completely hidden within the structure or conformation of the genuine tyrosyl sulfated protein, or if the antibody requires that epitope has a free N- or C-terminus (**Fig. 5**), it may be necessary to release the hidden epitope by cleavage with proteases that expose the epitope for antibody binding ***(29)***. If the naturally occurring tyrosyl-sulfated protein or polypeptide is recognized without cleavage, the protein may as well be used as standard, if available in sufficiently pure form.
9. In this example the peptide analyzed are acidic (the sequence of gastrin-17 is: pQGPWLEEEEEAYGWMDF-NH_2) for which reason an anion-exchange column is used. Tyr sulfation predominantly occurs in acidic regions of proteins and peptides, so often in similar conditions this will be appropriate. However, it is also possible to use cation-exchange chromatography to separate peptide forms, or alternatively, if the net charge of the fragments are neutral, the pH of the buffers may be adjusted.
10. Four characteristics of sera from the immunized animals have to be examined:
    a. The titer is defined as the antiserum dilution that binds 33% of the 0.5-fmol tracer at equilibrium.
    b. Affinity is expressed by the "effective" equilibrium constant ($K°_{eff}$), determined as the slope of the curve at zero peptide concentration in a Scatchard plot ***(30)***.
    c. Specificity is determined in percentage as the molar ratio of the concentrations of the sulfated standard peptide, the unsulfated peptide and other related peptides that produce a 50% inhibition of the binding of the tracer.
    d. Homogeneity of the antibodies with respect to binding kinetics is expressed by the Sips index ***(31)***. An index of 1 indicates homogeneity of both the tracer and the antiserum in the binding, otherwise seen only for monoclonal antibodies (MAbs) ***(16)***. The ability of the peptides to displace tracers from the antisera may be tested in peptide concentrations of 0, 3, 10, 30, 100, 1000, 10,000, and 100,000 pmol/L.
11. Samples in HPLC buffers are preferable (0.1% TFA in 30% acetonitrile is ideal for MALDI-TOF). Salt and buffer concentrations should be kept as low as possible. Volatile buffers may be removed in a vacuum centrifuge and the dried sample redissolved in 0.1% TFA/30% acetonitrile. Most detergents, especially SDS, will completely abolish the signal.
12. It is our experience, that the arylsulfatase treatment is tricky and sometimes is very inefficient. We therefore advice repetition of experiments with negative outcome and, if possible, the inclusion of a positive control peptide with the experiments.

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16

α-Amidated Peptides

Approaches for Analysis

Gregory P. Mueller and William J. Driscoll

1. Introduction

1.1. Biologic Importance of α-Amidation

α-Amidation is a terminal modification in peptide biosynthesis that can itself be rate-limiting in the overall production of bioactive α-amidated peptides. More than half of the known neural and endocrine peptides are α-amidated and in most cases, this structural feature is essential for receptor recognition, signal transduction, and thus, biologic function. All 20 naturally occurring amino acids serve as terminal amides in the spectrum of α-amidated peptide messengers ***(1)***. A list of selected α-amidated peptides is presented in **Table 1**.

1.2. Mechanism of Peptide α-Amidation

Peptide α-amidation is catalyzed by peptidylglycine α-amidating monooxygenase (PAM), a bifunctional enzyme localized to the *trans*-Golgi network and secretory granules of neural and endocrine tissues (**Fig. 1**). In a two-step process, PAM generates α-amidated peptides from their inactive glycine-extended precursors. Peptidylglycine α-hydroxylating monooxygenase (PHM; EC 1.14.17.3) catalyzes the formation of a peptidyl-α-hydroxyglycine intermediate, which is rapidly converted to α-amidated product and glyoxylate by peptidyl-α-hydroxyglycine α-amidating lyase (PAL; EC 4.3.2.5) (**Fig. 2**). In this sequence, PHM is rate determining and requires ascorbate, molecular oxygen, and copper for activity ***(1–5)***. α-Amidation results in a dramatic change in the physicochemical properties of the peptide by removing the

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Table 1
Representative α-Amidated Peptides of Diverse Biologic Systems

System/peptide	α-Amidated residue	Peptide length	Functions/properties
Nervous system			
Gonadotropin Releasing Hormone (GnRH)	Gly	10	Hypothalamic horome/ neurotransmitter
Corticotropin Releasing Hormone (CRH)	Ile	42	Hypothalamic hormone/ neurotransmitter
Growth Hormone Releasing Hormone (GH-RH)	Leu	44	Hypothalamic hormone/ neurotransmitter
Thyrotropin releasing hormone (TRH)	Pro	3	Hypothalamic hormone/ neurotransmitter
Cholecystokinin Octapeptide (CCK-8)	Phe	8	Neurotransmitter
Neuropeptide Y (NPY)	Tyr	36	Neurotransmitter
Calcitonin Gene Related Peptide (CGRP)	Phe	37	Neurotransmitter
Vasoactive Intestinal polypeptide	Asn	28	Neurotransmitter
Substance P	Met	11	Neurotransmitter, representative tachykinin
Pituitary			
Oxytocin	Gly	9	Parturition and nursing
Vasopressin	Gly	9	Renal water reabsorption and vasoconstriction
α-Melanotropin (α-MSH)	Val	13	Melanocyte dispersion/skin coloration
Gastrointestinal tract			
Secretin	Val	27	Stimulates pancreatic exocrine secretion
Cholecystokinin (CCK-33)	Phe	33	Stimulates pancreatic exocrine secretion
Multiple proposed functions at many sites			
Adrenomedullin	Tyr	52	Neurotransmitter/hormone/ autocrine
Amphibia peptides			
Bombesin	Met	14	Promotes proliferation of cells and secretion
Dermenkephalin	Asn	7	Isolate from frog skin, opioid peptide
Sauvagine	Ile	40	Frog corticotropin releasing hormone (CRH)
Insect peptides			
Adipokinetic hormones (several forms)	Thr, Gly, Trp	6–10	Regulate lipid utilization/ neuropeptides
Pheromone Biosynthesis Activating Neuropeptide 1	Leu	33	Reproduction pheromone
Melittin	Gln	26	Venom toxin
Chlorotoxin	Arg	36	Venom toxin
Schyllatoxin	His	31	Venom toxin
Others			
Calcitonin	Pro	32	Calcium conservation in mammals
Egg laying hormone	Lys	36	Aplysia, reproduction
Conotoxins (several forms)	Cys, Ala, Tyr	13–26	Mulluscan toxins
Extendin-3	Ser	39	Amoeba, locomotion

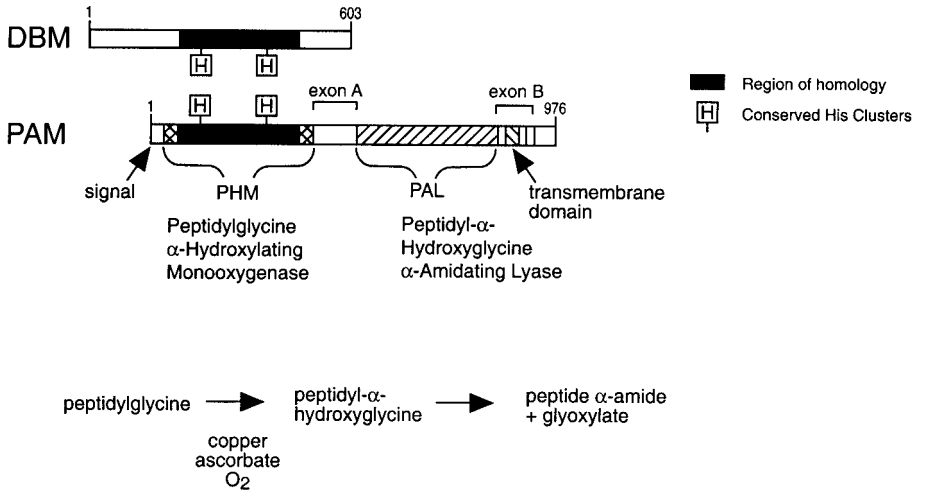

Fig. 1. Structural and functional organization of the rat PAM precursor protein. DBM, dopamine beta monooxygenase. PAM and DBM are type II, copper, ascorbate, and molecular oxygen-dependent monooxygenases. Conserved, histidine rich, consensus metal binding sites [H] convey copper dependence to both enzymes ***(1,5,6)***.

ionizable, free carboxyl group and leaving a nonionizable α-amide. These changes provide the basis for specific analytical procedures.

1.3. Overview of the Procedures Used to Investigate α-Amidated Peptides

A flow diagram for the procedures used in studying α-amidated peptides is presented in **Fig. 3**. The most powerful method for measuring α-amidated peptides is radioimmunoassay (RIA) (*see* **Note 1**). The distinctive structure of the carboxy terminal amide enables the development of remarkably specific and sensitive immunoassays. α-Amide-specific RIAs can generally detect 10–50 fmol of peptide per reaction with absolute specificity for the C-terminal α-amide. Because the antibody recognition epitope generally constitutes only 4–6 amino acids at the carboxy terminal, variations in the length and sequence of the N-terminal cannot be distinguished by the assay. Accordingly, structurally related peptides, which normally arise from the differential processing of a common precursor, can crossreact in the RIA. The peptide of interest can be separated from these crossreacting species by high-pressure liquid chromatography (HPLC) before RIA analysis. Mass spectrometry (MS) provides the most effective means for confirming the identity of an isolated peptide.

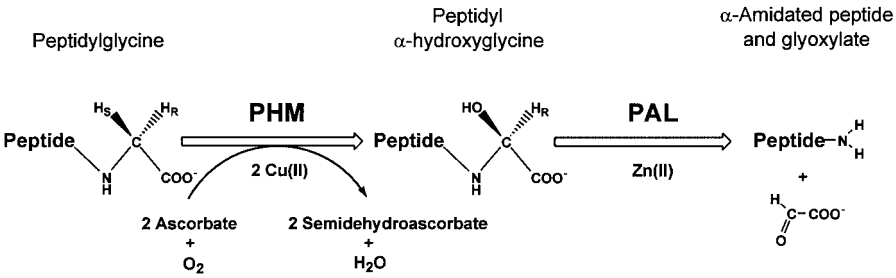

Fig. 2. Chemistry of α-amidation.

2. Materials

2.1. Radioimmunoassay (RIA) for an α-Amidated Peptide

2.1.1. Generation of a Primary Antibody

1. Synthetic α-amidated peptides for use as haptens, standards, and radiolabeling.
2. Carrier protein: keyhole limpet hemocyanin or bovine thyroglobulin (Sigma, St. Louis, MO).
3. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Pierce Chemical Co., Rockford, IL).

2.1.2. Immunization

1. Freund's complete and incomplete adjuvants.

2.1.3. Preparation of Radiolabeled Peptide

1. ^{125}Iodine, sodium salt.
2. 50 m*M* Phosphate buffer, pH 7.0.
3. Reaction tubes, borosilicate glass, 12 × 75 mm.
4. Iodo-Beads (Pierce Chemical Co.).
5. Sep-Pak C_{18} cartridges (Waters Chromatography, Division of Millipore, Marlboro, MA).
6. Sep-Pak mobile phase reagents, 0.1% trifluoroacetic acid (TFA) in water and acetonitrile.

2.1.4. Sample Preparation

1. 0.1 N Acetic acid (5.75 mL glacial acetic acid in 1 L).
2. Homogenization system: sonicator, Polytron or Potter-Elvehjem.
3. Refrigerated centrifuge capable of generating 10,000*g* at 4°C.

2.1.5. The Working RIA

1. 50 m*M* Phosphate buffer, pH 7.0.
2. Test tubes (disposable 12 × 75 mm borosilicate glass).

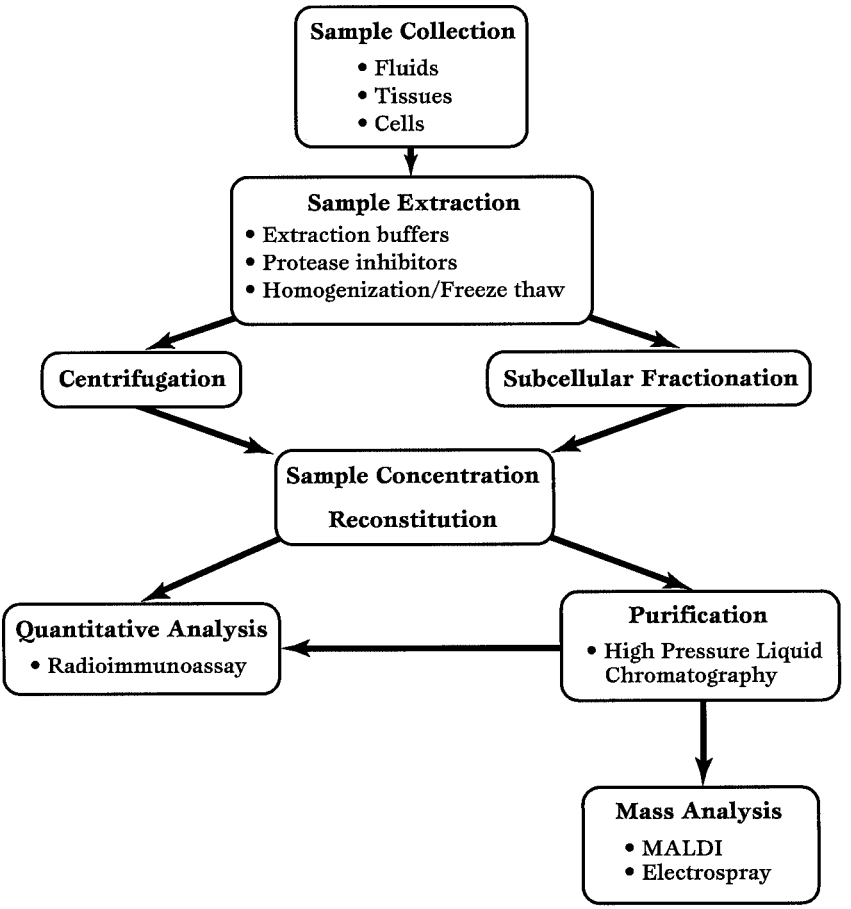

Fig. 3. Flow diagram showing procedures for studying an α-amidated peptide.

3. Bovine serum albumin (BSA) solution, 0.05–0.1% in 50 m*M* phosphate buffer, pH 7.0.
4. Heat-inactivated horse serum (GIBCO-BRL, Grand Island, NY).
5. Polyethylene glycol solution: 18% PEG (av. mol. wt 8000) dissolved in 0.05 *M* phosphate buffer, pH 7.0.
6. Refrigerated centrifuge capable of spinning assay tubes at 2500*g* at 4°C.
7. Gamma counter.

2.2. Procedures for the Identification/Verification of α-Amidated Peptides

2.2.1. High-Pressure Liquid Chromatography (HPLC)

1. HPLC system equipped with a C_{18} column and UV absorbance detector (*see* **Note 2**).

2. HPLC mobile phase reagents (water containing 0.1% TFA and acetonitrile containing 0.1% TFA).

2.2.2. Dot-Blot Western Analysis

1. Nitrocellulose (Schleicher and Schuell, Keene, NH).
2. 50 m*M* Tris-HCl, pH 7.6, 0.8% NaCl, 0.1% Tween-20 (TBST).
3. Sodium azide.
4. Blocking solution: 1% nonfat dried milk + 1% heat-inactivated horse serum in TBST.
5. Anti-rabbit/horse radish peroxidase-linked antibody (e.g., cat. no. NA9340, Amersham Pharmacia Biotech, Piscataway, NJ).
6. Enhanced chemiluminescent reagent system (ECL™, Amersham Pharmacia Biotech).
7. Radiographic film.
8. Dark room and film developing capabilities.

2.2.3. Mass Spectral Analysis

1. Mass spectrometer: Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer and/or a HPLC-electrospray mass spectrometer (*see* **Note 3**).
2. Vacuum centrifuge.
3. 70% Isopropanol/5%TFA for MALDI-TOF.
4. Matrix solution: α-cyano-4-hydroxy-trans-cinnamic acid (Aldrich, Milwaukee, WI), 10 mg/mL in 50% acetonitrile.

3. Methods

3.1. Radioimmunoassay (RIA) for an α-Amidated Peptide

A highly specific and sensitive RIA for an α-amidated peptide can generally be developed without significant problem. Ideally, three RIAs should be developed simultaneously: one for the α-amidated peptide, a second for its C-terminal glycine-extended precursor, and a third for its free acid. Together, these tools enable one to study precursor-product relationships, the peptide's metabolism and closely related crossreacting peptide species. The critical component of all immunoassays is the primary antibody. This reagent is usually developed in rabbits against the peptide conjugated to either keyhole limpet hemocyanin or bovine thyroglobulin. Polyclonal antisera have proven so effective in peptide RIAs that little attention has been given to exploring the utility of monoclonal antibodies (MAbs).

3.1.1. Preparation of a Conjugate: Coupling with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)

While a variety of crosslinking agents are commercially available for specialized uses in preparing conjugates, coupling with EDC has proven highly successful for α-amidated peptides. EDC mediates the linking of amino groups to carboxyl groups in a one-step procedure. The C-terminal amide ensures that this end of the peptide does not participate in the coupling reaction and is therefore available for immunologic recognition.

1. For a standard conjugation reaction, dissolve 5 mg of synthetic peptide (*see* **Note 4**) and 5 mg of carrier protein in 1 mL of water. It is convenient to use a flat bottom vial that will accommodate a small stirring bar.
2. Prepare a separate stock solution of EDC at 25 mg/mL in water and adjust pH to 4.0–5.0 with 0.1 N HCl.
3. Working at room temperature with constant mixing, add 1 mL of the EDC stock solution slowly dropwise to the peptide/protein solution and allow to react for 60 min or more. Some cloudiness due to aggregation is commonly observed.
4. Dialyze the mixture overnight against water (4°C, 2 L, 2 changes) to remove unreacted EDC (*see* **Note 5**).

The usefulness of an antiserum is dependent on the quality of the immunogen. Accordingly, it is important to assess the effectiveness of the conjugation reaction prior to embarking upon the intensive and time-consuming regimen of immunization, booster injections, sampling, titer checking, and antiserum characterization. Successful conjugation can be determined by: (1) increased molecular weight of the carrier protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or mass spectrometry, (2) altered retention time of the carrier protein on HPLC, or (3) uptake of trace radioactive peptide into the conjugate product. The HPLC approach is the most convenient although aggregated material must be remove by either centrifugation or filtration (0.22 micron spin filter) prior to analysis. It can be assumed that the material in solution is representative of that in the aggregate. For highly insoluble conjugates, the product must be analyzed by SDS-PAGE.

3.1.2. Immunization

1. Dilute the dialyzed conjugate to 2 mg/mL based on initial starting weight and mix with an equal volume of Freund's complete adjuvant (*see* **Note 6**).
2. Prepare an emulsion by either sonication or repeated passages through a 20-gauge hypodermic needle using a glass syringe.

3. Administer the immunogen (1 mL/rabbit) in 10–20 subcutaneous injections. The innoculation of several animals (3–6) is recommended because individual immunoresponsiveness can vary greatly.
4. Perform subsequent booster injections with immunogen emulsified in Freund's *incomplete* adjuvant (1 mg/mL) similarly at 4-wk intervals.
5. Take blood samples by ear-vein cannulation for regular titer checks (1–2 mL; 10 d after the second and each subsequent boost) and periodic bulk antiserum harvesting (15–25 mL from a 2 kg rabbit).
6. Titer serum samples by serial dilution as described in **Subheading 3.1.9.** Maximal titers can be expected by 12–15 wk after the initial immunization. Antisera collected prior to this time may not be suitable for use in RIA because of high IgM content.

3.1.3. Preparation of Radiolabeled Peptide

Radioiodination of peptides occurs on tyrosine and histidine residues. In cases where these amino acids are not present in the peptide of interest, an analog must be synthesized with a tyrosine in the 0 position. This addition should not interfere with immunologic recognition of the α-amide, which occurs at the opposite end of the molecule.

Standard iodination procedure:

1. Working at room temperature, combine 1 μg of peptide with 1 mCi of $Na^{125}I$ in 100 μL of 50 m*M* phosphate buffer, pH 7.0.
2. Add one Iodo-Bead (a nonporous polystyrene bead containing immobilized N-chloro-benzenesulfonamide) to mediate the iodination.
3. Allow the reaction to proceed for 10 min with periodic mixing (*see* **Note 7**).
4. Separate iodinated peptide from free iodine by chromatography on a SepPak C_{18} cartridge. Precondition the cartridge with 2 mL 100% acetonitrile/0.1% TFA followed by 2 mL of 0.1% TFA in water.
5. Dilute the iodination reaction mixture to 1–2 mL with 0.1% TFA in water and pass through the preconditioned SepPak.
6. Wash the cartridge with 5 mL of water containing 0.1% TFA to remove unincorporated iodine.
7. Elute the labeled peptide with stepwise, increasing concentrations of acetonitrile in water/0.1% TFA (1.5 mL each; 10% increments). α-Amidated peptides generally elute in the range of 20% to 40% CH_3CN/0.1% TFA. Iodinated peptides are stable for 4 or more wk when stored at 4°C in SepPak mobile phase.

3.1.4. Conditions for Equilibrium Binding Reactions

The basis for an RIA is competitive binding between the sample peptide and radiolabeled peptide for the antibody-recognition site. While conditions for equilibrium binding assays vary greatly, a good starting point for establishing optimal conditions include the following:

1. Buffer: 0.05 *M* phosphate, pH 7.0.
2. Reaction tubes: borosilicate glass, 12 × 75 mm.
3. 0.5 mL Reaction volume.
4. Sample volume: variable (e.g., 10–100 µL; depending on peptide concentration).
5. Range of standard curve: 1 fmol to 10 pmol.
6. Radioactivity/reaction: 20,000 cpm ("total counts").
7. Antibody dilution: primary antiserum diluted in buffer so that 20–35% of total counts are bound specifically; normally accomplished at a final dilution of 1:10,000 to 1:50,000.
8. Incubation conditions: 24 h, 4°C.
9. Inclusion of protein: BSA (0.05–0.1%) is often included in the reaction to decrease nonspecific binding (*see* **Subheading 3.1.6.**).
10. Separation of bound from free: PEG precipitation is recommended for establishing working conditions.

3.1.5. Separation of Bound from Free by PEG Precipitation

Separation of antibody-bound from free radioactive peptide is accomplished by either precipitation with PEG, charcoal absorption, or second antibody precipitation. All three approaches should be tested although the PEG precipitation method usually works well for peptide RIAs (*see* **Note 8**). PEG precipitation works best when both the PEG reagent and assay tubes are precooled (4°C).

1. Precipitate antibody-bound counts by adding of 1 mL of 18% PEG (average mol wt 8000 dissolved in 0.05 *M* phosphate buffer, pH 7.0), vortexing, and incubating at 4°C for 30 min.
2. Centrifuge assay tubes for 45 min at 2500*g*, 4°C; higher force and longer run times should be tested for improved pellet formation.
3. Remove supernatant by vacuum aspiration using a Pasteur pipet connected by flexible tubing to a low-pressure collection vessel.
4. Count the tubes containing pellets.

3.1.6. Optimization of Conditions for Antibody Binding

The functional potential of a RIA is defined by the primary antibody's titer, affinity, and specificity. Optimal conditions for antibody binding must be determined empirically. The overall objective is to maximize specific binding and minimize nonspecific binding (less than 5% of total counts). As a basis for comparison it is recommended that initial conditions be established using the parameters defined above (**Subheading 3.1.4.**). First, the antiserum is titered by serial dilution to achieve specific binding equaling 20–30% of the total counts available. This level of binding insures that the antibody is limiting in the reaction so that both increases and decreases in specific binding can be

readily detected. Second, room temperature incubation should be tested for changes in specific binding. An increase in specific binding (>5%) indicates that the antibody performs better under this condition. Third, a time course for binding is performed to define the minimum period required to reach equilibrium. Fourth, the source and amount of protein used in the assay is optimized. Although BSA is widely used in peptide RIAs, we have often found that its replacement with heat-inactivated horse serum (HIHS) (final dilution of 10%) is more effective than BSA in reducing nonspecific binding and actually promotes antigen-antibody binding as well. In addition, the presence of whole serum enhances the effectiveness of PEG precipitation. It should be recognized that the beneficial effects of HIHS are not observed in all assays and therefore must be determine for each case. Finally, the alternative methods for separating bound from free (charcoal absorption and second antibody precipitation) should be evaluated for improvement in assay performance. It should be noted that the primary antibody may need to be periodically retitered as conditions are optimized.

3.1.7. Determination of Antibody Specificity

Specificity of the primary antibody is defined by evaluating crossreactivity of closely related peptides. Necessary determinations include those with the peptide free acid and its glycine extended precursor. Neither of these species should be detected by the antibody. By contrast, variations in the amino terminal that occur beyond the antigenic epitope generally do not alter antibody recognition. Accordingly, these peptides are equivalent to the authentic α-amidated peptide in their ability to interact with the antibody and thereby, reduce the binding of radiolabeled ligand. The extent to which N-terminally extended peptides contribute to the immunoreactivity measured in experimental samples must be determined by HPLC fractionation and subsequent RIA and mass analysis.

3.1.8. Sample Preparation

Samples to be analyzed for α-amidated peptides are usually prepared from whole tissues or cell cultures. Subcellular fractionation by differential centrifugation may be carried out prior to extraction to isolate a specific cellular organelle.

1. Extract tissue samples or cells in 10 vol of 0.1 *N* acetic acid using either sonication, Polytron, or Potter-Elvehjem homogenization. Media from cell cultures may be acidified or assayed directly. A striking exception is CCK-8-NH_2, which does not solubilize well by this procedure and is effectively extracted into 90% methanol.

2. Freeze and thaw the homogenates three times to insure complete disruption and solubilization of all intracellular compartments.
3. Centrifuge the homogenates at 10,000*g*, 4°C for 15 min to generate clarified supernatant.
4. Small volumes of the acid extracts can be added directly into RIA reactions where the buffering capacity of the assay is sufficient to neutralize the sample. Alternatively, the samples may be concentrated by lyophilization under vacuum and reconstituted in assay buffer containing complete protease inhibitor mix. Yields through an extraction protocol must be determined and shown to be reproducible. This may be performed by either RIA using known amounts of added peptide standard or by radioactive tracer.

3.1.9. The Working RIA

Each RIA consists of the following five sets of tubes performed in duplicate.

1. Total count tubes (determine the total radioactivity in each reaction).
2. Nonspecific binding or background tubes (determine the amount of total radioactivity that is measured in the absence of primary antibody).
3. Total binding tubes (determine the amount of radioactivity bound by the primary antibody in the absence of nonradioactive competing peptide).
4. Standard curve tubes (determine the effect of increasing concentrations of nonradioactive competing peptide on antibody-bound counts).
5. Unknown tubes (determine the effect of experimental samples on antibody binding).

Specific binding is calculated by subtracting the nonspecific binding from the total binding. As noted earlier, nonspecific binding should be less than 5% of total counts and specific binding between 20% and 35% of total counts. Nonspecific binding is also subtracted from all standard and unknown values as a step in data analysis. Usually a standard curve entails six or more concentrations of competing peptide. The concentrations used are selected to span the range required to reduce specifically bound counts to a few percent to total specific binding. There is an inverse relationship between the number of counts bound and the concentration of standard peptide. Plotting the data obtained on semilog paper yields a sigmoid curve having a linear segment between approx 80% and 20% of total specific binding. This portion of the curve defines the working range of the assay. Serial dilutions of experimental samples (unknowns) must parallel the standard curve for the assay to be valid. Concentrations of peptide in the unknowns are determined from the standard curve either by hand plotting or computer-based analysis. Software programs for analyzing RIA data are standard features on gamma counters. In some cases, a significant increase in assay sensitivity can be achieved by allowing

the standards and unknowns preequilibrate with the antibody before the radiolabeled peptide is introduced. The effectiveness of this approach varies among assays.

3.2. Identification/Verification of α-Amidated Peptides

3.2.1. High-Pressure Liquid Chromatography (HPLC)

HPLC is indispensable in the purification and identification of peptide messengers. As a rule, peptide α-amides and their precursors and metabolites exhibit distinctive retention/elution characteristics on C_{18} columns. Identification of a sample peptide can be tentatively assigned on the basis of its having a retention time identical to that of a known standard peptide.

1. Extract samples (tissue, cultured cells, or incubation medium) into 1% HCl; tissue and cell pellets are prepared in 10X volume, whereas media is acidified by the direct addition of concentrated acid.
2. Prepared clarified supernatants by high speed centrifugation (e.g., 10,000*g*, 15 min).
3. Inject an aliquot of the clarified supernatant onto a C_{18} column equilibrated with 2.5% acetonitrile/0.1% TFA in water containing 0.1% TFA pumped at a flow rate of 1 mL/min (*see* **Note 9**).
4. Continue washing the column under these conditions for five or more min to elute material not retained on the C_{18} column. Monitor optical absorbance at 214 nm (*see* **Note 2**).
5. Once a stable UV baseline is obtained, begin developing an increasing gradient of acetonitrile/0.1% TFA in water containing 0.1% TFA. A standard screening gradient involves a 1% per min change in acetonitrile concentration at a flow rate of 1 mL/min. Target peptides typically elute between 15% and 40% acetonitrile with recoveries that exceed 75%.

3.2.2. Detection of Peptides by Dot-Blot Western Analysis

HPLC profiles may be screened immunologically to identify peptides present in concentrations too low to detected by UV absorbance. This method provides a convenient means for quickly analyzing an entire HPLC elution profile in a semiquantitative fashion. The effectiveness of this approach, of course, depends on the ability of the peptide to adhere to the membrane and the ability of the primary antibody to interact with the bound peptide. Again, these factors must be determined empirically.

1. Collect 1 mL fractions of the entire HPLC elution profile including the initial flow-through period, during which a stable baseline UV absorbance is established.

2. Apply a 10 uL aliquot of each fraction to nitrocellulose (Nytran, Schleicher, and Schuell) that has been lined off in pencil to form a numbered grid. It may be necessary to concentrate the samples by lyophilization prior to spotting onto nitrocellulose. Allow the samples to dry completely and process the membrane following the procedures for standard Western-blot analysis ***(7)*** as follows.
3. Block the membrane at room temperature for 60 min by incubation with mixing in a solution of 50 m*M* Tris, pH 7.6, 0.8% NaCl, 0.1% Tween 20 (TBST) containing 0.1% sodium azide, 1% (wt/vol) nonfat dried milk and 1% heat-inactivated horse serum (GIBCO-BRL) (blocking buffer).
4. Add anti-peptide antiserum directly to the blocking solution to achieve a final concentration of 1 : 1000. Optimal working concentrations must be determined empirically for each antiserum.
5. Incubate the blot with mixing at either 4°C or room temperature for 4–18 h.
6. Wash the membrane four times, 15 min each, with TBST.
7. Incubate the membrane in blocking buffer containing anti-rabbit/horse radish peroxidase-linked antibody (1 : 10,000 dilution) and incubate with mixing at room temperature for 2–4 h. (Optimal antibody concentrations must determined empirically.)
8. Wash the membrane four times, 15 min each, with TBST.
9. Immunoreactive peptides are visualized with an enhanced chemiluminescent reagent system according to the manufacturers instruction.

3.2.3. Mass Spectral Analysis

Peptides purified by HPLC may be quantitated by RIA or analyzed by mass spectrometry (MS). The primary purpose for MS is to verify the identity of an isolated peptide. Two commonly used methods for the mass analysis of peptides and proteins employ different mechanisms of ionization: matrix assisted laser desorption ionization (MALDI)-MS and electrospray (ES)-MS. The reader is directed to several recent reviews for theoretical details on the procedures ***(8–10)***. Additionally, tandem HPLC-ES-MS systems allow for direct analysis of the HPLC effluent without intervening sample manipulation. For both ES-MS and MALDI-MS, the limit of detection is defined, in large part, by the ability of the peptide to ionize. Unfortunately, MS is not quantitative due to differing ionization efficiencies, which occur even under seemingly identical conditions. Nevertheless, the technique is highly sensitive, and the accuracy of mass assignment is routinely 0.01%. Accordingly, an α-amidated peptide can be readily differentiated from its glycine-extended precursor, free acid, or related analog.

3.2.4. Sample Preparation

Because very limited amounts of α-amidated peptide are normally present in biologic samples, extracts routinely require concentration by drying (Speed-

Vac). Maximum concentration and recovery are achieved when samples are dried in conical vials that allow for reconstitution in 10 uL or less. Solubilization with 70% isopropanol/5%TFA (*see* **Note 10**) is appropriate for MALDI-MS analysis. Alternatively, 1% acetic acid is preferred for electrospray MS (*see* **Note 11**).

3.2.5. Slide Preparation for MALDI-Time-of-Flight (TOF)

Peptide sample is crystallized with matrix on a stainless steel sample slide. Matrix is a small, UV-absorbing organic acid that transmits the energy from a laser pulse to the peptide and in the process, ionizes the sample by providing protons. The major ion product of a peptide is the +1 species. In the ionized state the peptide is able to "fly" down the instrument's sample path to the detector (electron multiplier). The TOF is directly proportional to mass and inversely proportional to charge. Optimal conditions must be determined empirically for each specific peptide. Important variables include: chemical matrix, ratio of sample to matrix, and the energy of the laser pulse applied.

1. Resuspend HPLC fraction in 70% isopropanol/5% TFA.
2. Spot 0.5 uL of peptide sample on the sample slide followed by an equal volume of matrix. A generally effective matrix for most peptides is α-cyano-4-hydroxy-*trans*-cinnamic acid (10 mg/mL, in 50% acetonitrile) (*see* **Note 12**).
3. After all samples have been applied with matrix, dry slides thoroughly under vacuum.
4. Analyze samples by MALDI-TOF (*see* **Note 13**).

3.3. In Vivo Models for Investigating the Biology of α-Amidated Peptides

In vivo strategies for investigating the physiology of α-amidated peptides involve three approaches: nutritional, pharmacologic, and genetic. All three approaches are designed to alter the function of PAM, either directly or via its essential cofactors, copper and ascorbate. Effective use of these experimental paradigms, however, requires the application of the methods described in this chapter. Copper- and ascorbate-deficient diets predictably decrease α-amidation in laboratory rodents and thus lower the concentrations and diminish the functions of α-amidated peptide messengers (*see* **ref. *1***). Several mechanism-based inhibitors have been developed for selectively inactivating PHM. Of these 4-phenyl-3-butenoic acid is the most effective at inhibiting α-amidation in vivo ***(11)***. In addition, chelation of copper by treatment with disulfiram has been used to pharmacologically inhibit α-amidation ***(12)***. Genetic models for investigating peptide α-amidation are currently under development. While a PAM knockout mouse has yet to be created, efforts in *Drosophila* have been successful ***(13)***.

Finally, several mouse strains bearing mutations in genes that encode critical copper transport proteins are being evaluated for defects in α-amidation ***(14,15)***. It will be interesting to see how these genetic models compare to those resulting from disulfiram treatment or nutritional copper deficiency.

4. Notes

1. Solid-phase nonradioactive immunoassays offer a convenient alternative to solution based RIAs, however, at reduced sensitivity. Additionally, purified IgG is often required for optimal performance. In general, the requirement for detecting very low levels of α-amidated peptide in biologic samples has precluded the use of these nonisotopic assays.
2. While not always available, a diode array detector provides the capability of continuously monitoring the spectral characteristics of the HPLC effluent. This information is useful for determining peak purity and can confirm the identity of peaks of interest by spectral characterization.
3. Because of the expense and complexity of mass spectrometry, most investigators carry out MS analyses in collaboration with a dedicated MS lab or core facility. MS labs commonly operate both MALDI-MS and HPLC-ES-MS instruments.
4. In the case of larger peptides, using the full length sequence for conjugation may be counter-productive. This is because the resulting antiserum could recognize epitopes toward the N-terminus and not be α-amide specific. Accordingly, it is recommended that conjugations be carried out using haptens that are 8–12 residues in length.
5. The volume of the dialysate may increase by 50–100%. Loss of conjugate by binding to the dialysis tubing is not recognized as a problem in this procedure. The formation of a light-colored precipitate of peptide/protein conjugate in the dialysate is not uncommon.
6. Considerable effort has been directed toward enhancing immunization procedures with the combined goal of improving antisera quality and limiting discomfort to the animals being inoculated. One development that has proven remarkably effective in this regard is the inclusion of colloidal gold with the immunogen. Colloidal gold alters antigen uptake and presentation in a manner that promotes the development of specific, high-titer antisera against molecules with no intrinsic antigenicity (e.g., glutamate; ***16***). Importantly, these effects are achieved with smaller amounts of immunogen. We have been successful with colloidal gold antigen using 100 μg as an immunization dose (in Freund's complete adjuvant) and 50 μg (in Freund's incomplete adjuvant) for subsequent boosts. Colloidal gold is commercially available from E-Y Laboratories (San Mateo, CA) or is easily prepared in house ***(16)***. While the specific application of colloidal gold with α-amidated peptides has not been reported, its proven effectiveness with weak antigens warrants its mention here.
7. Times and concentrations may be varied to increase or decrease labeling. In addition, the $Na^{125}I$ can be reacted with the Iodo-Bead (5 min) prior to the

addition of peptide. This enhances peptide labeling by increasing the conversion of iodide (I^-) to iodous ion (I^+), the species that actually mediates the iodination of tyrosine.

8. Perform charcoal absorption at room temperature by adding 1 mL of charcoal suspension (0.1% fine powder of activated charcoal/0.01% BSA in 0.05 *M* phosphate buffer, pH 7.0) to each reaction tube, vortexing, and centrifuging (2,500*g*, 45 min). Decant the supernatant containing antibody-bound counts into new tubes and count. It should be noted that charcoal suspensions must be stirred constantly during use and that activated charcoal can strip bound counts from antibodies of low avidity. Nonspecific binding can be reduced in some cases by increasing the concentration of activated charcoal/BSA. The second antibody precipitation method employs anti-rabbit IgG antiserum to form large immune complexes. In this case, normal rabbit serum is added to the reaction (1 : 100–1 : 300 final dilution) to generate complexes of sufficient mass for pelleting by centrifugation. Anti-rabbit IgG antisera prepared in goat or sheep are widely available from commercial suppliers. The second antibody must be appropriately titered under assay conditions to insure that it is not limiting in complex formation and that maximal precipitation of antibody-bound counts is achieved.
9. Ideally, the volume injected is kept as small as reasonably possible (e.g., 50 μL) but may exceed 1 mL under conditions where the concentration of the peptide is low. Very dilute samples may be concentrated by lyophilization and then reconstituted in a small volume (e.g., 50 μL) of 2.5% acetonitrile containing 0.1% TFA. Samples are clarified by centrifugation (10,000*g*, 15 min) and injected onto the C_{18} HPLC column.
10. An alternative strong solvent worth testing for MALDI-MS is 50% formic acid. It should be noted that formylation of amino groups (+28 mass units) can occur spontaneously under neutral or basic conditions. However, under the acidic conditions of MALDI, this modification does not occur.
11. For HPLC-ES-MS acetic acid (1%) is substituted for TFA in the HPLC mobile phase because the strong ion pairing characteristics of TFA prevent efficient ionization.
12. Alternatives include 2,5-dihydroxybenzoic acid (10 mg/mL in 50% ethanol) and 3,5-dimethoxy-4-hydroxy-*trans*-cinnamic acid (10 mg/mL in 50% acetonitrile).
13. The inclusion of ammonium sulfate can significantly enhance ionization efficiency. This effect is assessed by adding 0.5 μL of saturated ammonium sulfate (in water) to the sample and matrix mixture. The order of addition to the slide is: sample, ammonium sulfate, matrix. Volumes can be reduced correspondingly (e.g., 0.3 μL each) to limit sample spreading.

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17

γ-Glutamate and β-Hydroxyaspartate in Proteins

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1. Introduction

Vitamin K-dependent coagulation plasma proteins possess from 9–12 residues of γ-carboxyglutamic acid (Gla) distributed over a ca. 45 amino acid peptide sequence, i.e., the Gla domain, which encompasses the NH_2-terminal region. In addition, epidermal growth factor (EGF) homology units present in many of these same proteins contain β-hydroxyaspartate (Hya) residues, which is a modification decoupled from γ-carboxylation ***(1)***. The function of Gla residues in these proteins, *viz*., prothrombin, coagulation factors VII, IX, and X, along with anticoagulant protein C and protein S, is to coordinate Ca^{2+}. This results in a large conformational alteration in the proteins or peptides, which allows adsorption to membrane phospholipids (PL), an event that is critical is to their proper functions in the blood coagulation system ***(2,3)***. Less certain is the role of Hya in EGF domains, but it has been proposed that modification at this residue may negatively regulate fucosylation of these regions ***(4)***. In several proteins, these modules also interact with Ca^{2+}, but it has been shown that while the particular aspartate containing the β-OH group is critical to that interaction, β-hydroxylation of that Asp residue is not.

A particularly novel class of naturally occurring peptides, the conantokins, also contain a wide variety of posttranslational modifications, γ-carboxylation being one example. These small peptides, of up to 27 amino acids, are independent gene products found in predator snails of the species *Conus*, and function as noncompetitive inhibitors of Ca^{2+} flow through the ion channel of the N-methyl-D-aspartate (NMDA) receptor ***(5–7)***. Because of this property, drugs based on these peptides have potential application in neuropathologies associated with NMDA receptor malfunction. The amino acid sequences of three of these peptides *viz*., conantokin-G (con-G) ***(8)***, conantokin-T (con-T)

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(5), and conantokin-R (con-R) ***(9)***, have been identified to date. These peptides contain 17, 21, and 27 amino acids, in which are present 5, 4, and 4 residues of Gla, respectively.

Other proteins and peptides contain Gla, such as matrix Gla protein and osteocalcin ***(10–13)***, a protein from spermatozoa ***(14)***, proline-rich transmembrane proteins ***(15)***, and dentin ***(16)***. Hya is not only found in EGF modules of proteins that are involved in coagulation ***(17)***, but is also present in these same regions of thrombomodulin ***(18)***, complement proteins ***(19)***, and the LDL-receptor ***(18)***.

Because of their widespread distribution, quantitative detection protocols for both Gla and Hya are of importance. It is the purpose of this communication to detail a reliable method for these analyses that is employed in our laboratories.

2. Materials

2.1. Hydrolysis of Samples

1. 2.5 *M* KOH.
2. Saturated $KHCO_3$.
3. $HClO_4$ (60%; v/v).
4. 6 *N* HCl.

2.2. High-Performance Liquid Chromatography (HPLC) Analysis of Hydrolysates by Ion-Exchange HPLC

1. Preparation of OPA/ET reagent. Dissolve a total of 100 mg of o-phthalaldehyde (OPA, Sigma, St. Louis, MO) in 5 mL of methanol. Add a solution of 50 µL of ethanethiol (ET) in 10 mL of 0.15 *M* sodium borate, pH 10.5, containing 0.2% Brij-35 (Pierce, Rockford, IL), and mix the solution thoroughly. Flush the mixture with N_2 and allow to stand for at least 16 h prior to analysis (*see* **Note 1**).
2. 0.1 *M* KH_2PO_4 in 50% CH_3CN.
3. HPLC column (Nucleosil 5SB, 4 × 200 mm, 5 µ, Macherey-Nagel, Easton, PA) with Brownlee silica guard column (3.2 × 15 mm, 7 µm).
4. 20 m*M* Sodium citrate, pH 4.2, 50% acetonitrile.
5. ANSFL(Gla)(Gla)RHSS (synthesized by standard Fmoc chemistry (*see* **Note 2**; **refs. *20*,*21***).
6. *erythro*- and *threo*-β-hydroxyaspartic acids (a gift from Dr. Marvin Miller, Dept. of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN).

2.3. Gla Analysis by Reverse Phase (RP)-HPLC

1. C8 column (2.0 mm × 250 mm, 5 µ).
2. Solvent A: 110 mL of 1 *M* sodium acetate, pH 7.2, 95 mL methanol, 5 mL tetrahydrofuran, 790 mL H_2O.
3. Solvent B: 100% methanol.

2.4. Proteins and Peptides

1. Human plasma protein C (PC) (Enzyme Research Laboratories, South Bend, IN).
2. Recombinant human PC and mutants [Gla^{14}D]-PC, [Gla^{20}D]-PC and [D^{71}A]-PC expressed in human kidney 293 cells ***(22–29)***.
3. Bovine factors IX and X purified from bovine plasma ***(30)***.
4. PC-related peptide (residues 1–12 of human PC).
5. Conantokin-G and T ***(20)***.

3. Methods

3.1. Hydrolysis of Samples

3.1.1. Gla Determination

1. Place lyophilized protein (20 μg-100 μg) in a Pyrex glass tube (13 mm × 100 mm).
2. Add a solution of 220 μL of 2.5 *M* KOH to the polypropylene tube, and then flush the entire system with N_2.
3. Seal the glass tube under vacuum (*see* **Note 3**) and insert in a deep-well heating block (Pierce, Rockford, IL) at 110°C. Allow the hydrolysis to proceed for 20 h.
4. After this time, place the glass tube on ice for 10 min, break the tube, and transfer the sample to an Eppendorf tube.
5. Combine the hydrolysate with a solution of 50 μL of saturated $KHCO_3$, and subject to centrifugation (3 min) with a table-top centrifuge to remove any precipitate.
6. Treat the solution with cold $HClO_4$ (60%; v/v) with frequent mixing until a pH of 7.0 (determined by spotting on pH paper), is attained.
7. After 30 min on ice, the $KClO_4$ precipitates. Remove the supernatant by aspiration, after centrifugation (800*g*) for 10 min, and store at –20°C until needed for amino acid analysis.

3.1.2. Determination of the Hya Content

1. Place the sample (100 μg) in a Pyrex tube (13 mm × 100 mm).
2. Add 300 μL of 6 *N* HCl.
3. After flushing several times with N_2, seal the tube under vacuum and place the sample in the heating block at 110°C for 20 h for hydrolysis.
4. After this time, the HCl is removed by evaporation under N_2.
5. The hydrolysate is reconstituted in 50 μL of H_2O, and then employed for amino acid analysis.

3.2. HPLC Analysis of Hydrolysates by Ion-Exchange HPLC

These methods represent operational modifications of previously described methodology ***(20–24,31)***. The derivation of amino acids in the hydrolysate was conducted with OPA/ET reagent due to the higher stability of the fluorescent derivative.

1. Add 10 μL of OPA/ET reagent to 10 μL of protein or peptide hydrolysate and mix the solution thoroughly by vortexing.
2. After 2 min, add 20 μL of 0.1 *M* KH_2PO_4 in 50% CH_3CN.
3. A 20 μL aliquot of the sample is then injected into the HPLC column.

The separation of the OPA-conjugated amino acids is accomplished by isocratic elution on an ion-exchange HPLC column (4 mm × 200 mm, Nucleosil 5SB) at 47°C, with detection at 340 nm. The elution buffer is 20 m*M* sodium citrate, pH 4.2, 50% acetonitrile. The elution positions of Glu, Asp, Hya, and Gla are shown in **Fig. 1**. Since Glu and Asp are stable to both acid and alkaline hydrolysis, the ratios of Gla/Glu and Gla/Asp, combined with a standard curve, are used to obtain the number of Gla residues per mole of protein.

Two reference standards, a commercial amino acid standard mixture, as well as a peptide, ANSFL(Gla)(Gla)RHSS, are employed. Use of this peptide for this purpose allows for recovery values after alkaline hydrolysis to be obtained for Gla and accurate conversion factors of peak area to concentrations of this amino acid. The Gla/Asp ratio of this peptide, of 2.0 (Asn is converted to Asp during hydrolysis), and the concentration response factor of Asp from the commercial standard mixture, are employed to obtain the concentration response for Gla.

For Hya determination, standard preparations of *erythro*- and *threo*-Hya, and the commercial amino acid standard mixture are used to obtain the concentration response factors of Hya and Asp. A ratio of Hya/Asp is used to calculate the amount of Hya per mole of protein in the hydrolysate.

3.3. Gla Analysis by Reverse Phase HPLC

Depending on the equipment available to an individual laboratory an alternate method for quantitative Gla determinations is suggested using RP-HPLC. After hydrolysis in alkali and precolumn derivatization with OPA/ET, as above, Gla analyses are carried out employing a C8 column (2.0 mm × 250 mm, 5 μ) column at 46°C with detection at 340 nm. The gradient used for elution consists of various combinations of two solvent systems. Solvent A containing 110 mL of 1 *M* sodium acetate, pH 7.2, 95 mL methanol, 5 mL tetrahydrofuran, and 790 mL H_2O and Solvent B consisting of 100% methanol. The column is equilibrated against 95% A:5% B. After application of the hydrolysate, the first gradient that is used immediately steps the solvent to 85% A:15% B (time = 0). From 0–10 min a linear gradient to a limit of 80% A : 20% B is applied, followed by continued elution with 80% A : 20% B for another 5 min. Under these conditions Gla is eluted at 3.53 min with baseline separation from neighboring peaks, Asp at 4.98 min, and Glu at 5.95 min. Remaining materials are then batch eluted from the column with 2% A : 98% B for 5 min,

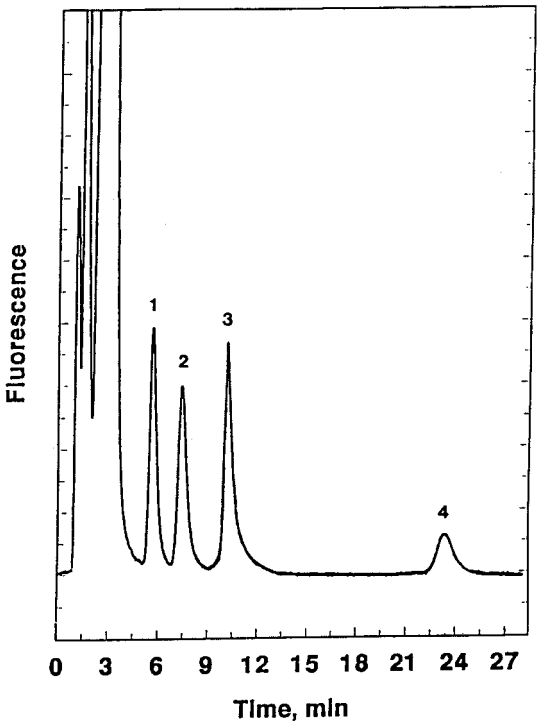

Fig. 1. The separation of acidic OPA-derivatized amino acids by anion-exchange HPLC. The separation was accomplished by isocratic elution on a Nucleosil 5SB column (4 mm × 100 mm. 5 μ) with a Brownlee silica guard column (3.2 mm × 15 mm, 7 μm). The elution buffer was 20 m*M* sodium citrate, pH 4.2, in 50% acetonitrile, at 47°C. Peak 1-Glu, peak 2-Asp, peak 3-*erythro*-β-hydroxyaspartic acid (Hya), peak 4-Gla.

after which column start buffer (95% A:5% B) is applied for 5 min. The Gla content is determined using a standard synthetic Gla-containing peptide as described previously.

3.4. Results and Conclusions

A representative example of the separation of standard OPA-derivatized acidic amino acids on a Nucleosil 5 SB column is shown in **Fig. 1**. It is seen that baseline separation of Glu, Asp, Hya, and Gla is achieved within a 26-min run time.

We have employed these analytical methods to determine Gla and Hya contents in recombinant and plasma proteins. Example chromatograms of an alkaline hydrolysate of recombinant protein C and protein C mutants, for Gla determinations, and an acid hydrolysate, for Hya determinations, are provided in

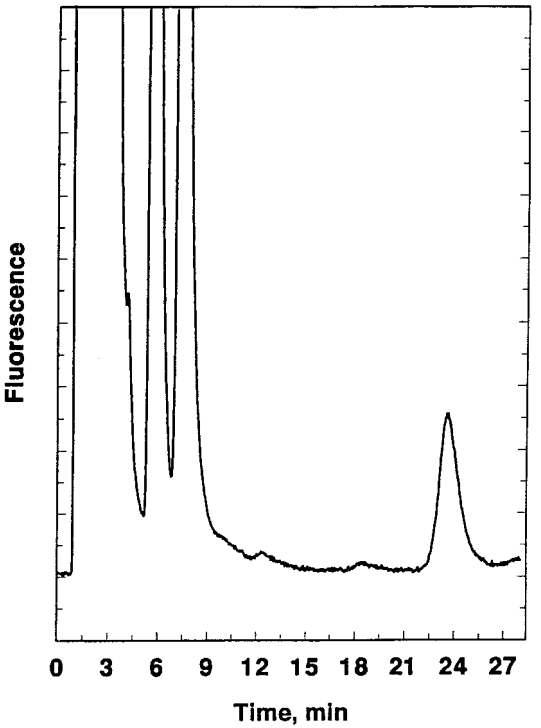

Fig. 2. HPLC chromatogram of an alkaline hydrolysate of wild-type recombinant protein C after OPA-derivatization. A quantity of 30 μg of protein was hydrolyzed with 2.5 *M* KOH at 110°C for 20 h. After neutralization with 60% $HClO_4$, 5 μL of sample, corresponding to 0.5 μg of protein were injected. The chromatography conditions are described in **Fig. 1**. The peak at approx 23 min represents Gla.

Figs. 2 and **3**. Using the approaches described earlier, quantitation of the Hya and Gla contents were determined for a variety of proteins and peptides, and a listing of the results of some of these analyses are provided in **Table 1**. The results point to the reliability of these analyses for all proteins and peptides studied.

In conclusion, reliable methodology is described for determination of Gla and Hya contents of a variety of materials, which can be performed on μg quantities of protein. Under the hydrolysis conditions described, the modified amino acids remain intact, but the exact protein hydrolysis conditions for any particular protein should probably be determined for each case.

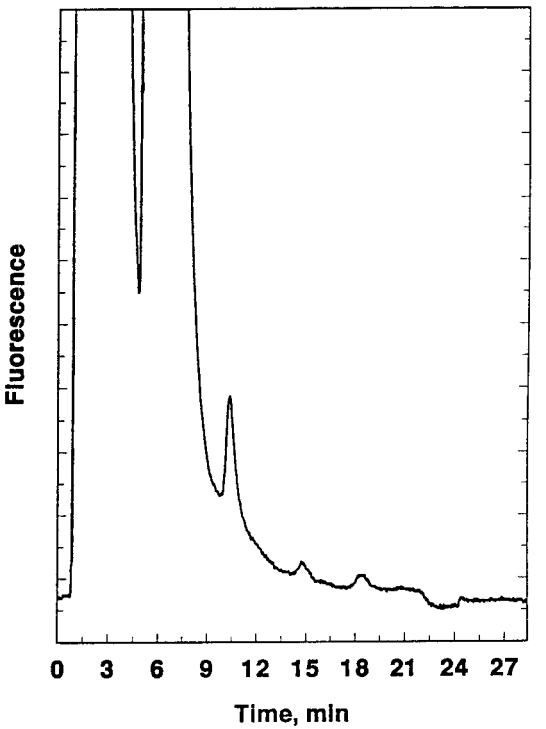

Fig. 3. HPLC chromatogram of an acid hydrolysate of wild-type recombinant protein C after OPA-derivatization. A quantity of 50 μg of protein was hydrolyzed with 6 N HCl at 110°C for 20 h, and 5 μL, corresponding to 5 μg of protein, was applied to the column under the conditions of **Fig 1**. The peak at approx 11 min represents Hya.

4. Notes

1. The OPA/ET reagent can be stored in the dark at –20°C and is useful for several months under these conditions.
2. The Nα-Fmoc-γ,γ-ditBu-L-Gla-OH was used in the peptide synthesis for placement of γ in peptide ***(20,21)***.
3. Using a Hamilton syringe, the protein solution is placed in a polypropylene tube fitted into the bottom of a pyrex 13 × 100 mm tube, which is previously stretched into an hour-glass shape using an oxygen flame. The sample is then frozen in liquid nitrogen and evacuated four times and then sealed with a flame while under vacuum.

Table 1
Gla and Hya Contents of Various Proteins

	Gla (mol/mol)		Hya (mol/mol)	
Protein/peptide	Expected	Obtained	Expected	Obtained
PC-related peptide[a]	2.0	2.0	0	0
Bovine plasma FIX	12.0	12.0 ± 0.3	1.0	1.06 ± 0.05
Bovine plasma FX	12.0	12.5 ± 0.3	1.0	1.03 ± 0.04
Human plasma PC	9.0	8.7 ± 0.2	1.0	0.92 ± 0.04
Recombinant PC[b]	9.0	8.9 ± 0.3	1.0	1.04 ± 0.04
[Gla^{14}D]-PC[c]	8.0	7.8 ± 0.3	1.0	1.20 ± 0.13
[Gla^{20}D]-PC[c]	8.0	8.2 ± 0.2	1.0	0.78 ± 0.11
[D^{71}A]-PC[d]	9.0	8.7 ± 0.2	0	0
Conantokin-G[d]	5.0	4.7 ± 0.1	0	0
Conantokin-T[d]	4.0	4.1 ± 0.1	0	0

[a]Residues 1–12 of human protein C (PC).

[b]Wild-type recombinant protein C, expressed in human kidney 293 cells.

[c]Human recombinant PC mutants. The mutation convention used is the (normal amino acid/sequence position in PC/single letter code for the new amino acid placed in that sequence position by mutagenesis), followed by PC. Adapted with permission in part from **ref. *24***.

[d]Adapted with permission from **ref. *29***.

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18

Detection of isoAspartate Residues as a Posttranslational Modification of Proteins and Peptides

Verne Schirch, Sonia Delle Fratte, and Martino di Salvo

1. Introduction

During the past two decades, deamidation of Asn residues has been frequently observed as a posttranslational modification in proteins and peptides. The determination of the mechanism of deamidation has been solved by studies on small peptides. **Figure 1** shows the proposed nonenzymatic mechanism where the peptide amide nitrogen, donated by the residue following asparagine, makes a nucleophilic attack on the side-chain amide of Asn to form a 5-membered succinimide ring. The ring can be hydrolyzed at either the β-carboximide group to form a peptide, with an Asp residue replacing the original Asn, or the succinimide can hydrolyze at the α-carboximide group to form an isoAsp residue where the peptide chain is connected to the β-carboxyl group of Asp. Several reviews on the mechanism and extent of deamidation have been published as late as 1995 ***(1–3)***. In accord with these earlier reviews, we will focus mostly on the information that has been observed since 1995.

The mechanism of deamidation appears to be nonenzymatic but dependent on the amino acid residue that follows Asn ***(3,4)***. In peptides, the sequence Asn-Gly deamidates the most rapidly but other sequences like Asn-Lys, Asn-Ala, and Asn-Ser have also been observed to deamidate ***(1,5,6)***. The rate of opening of the succinimide ring (**Fig. 1**) in peptides generates an isoAsp/Asp ratio of 3:1 ***(3)***. The rate of deamidation at pH 7.3 and 37°C is first order with a $t_{1/2}$ in the 40–70 h range for Asn-Gly sequences in small peptides. The rate of deamidation increases with pH and the presence of small bases, such as ammonia ***(4)***. There have been several reports that Asp-Gly sequences can

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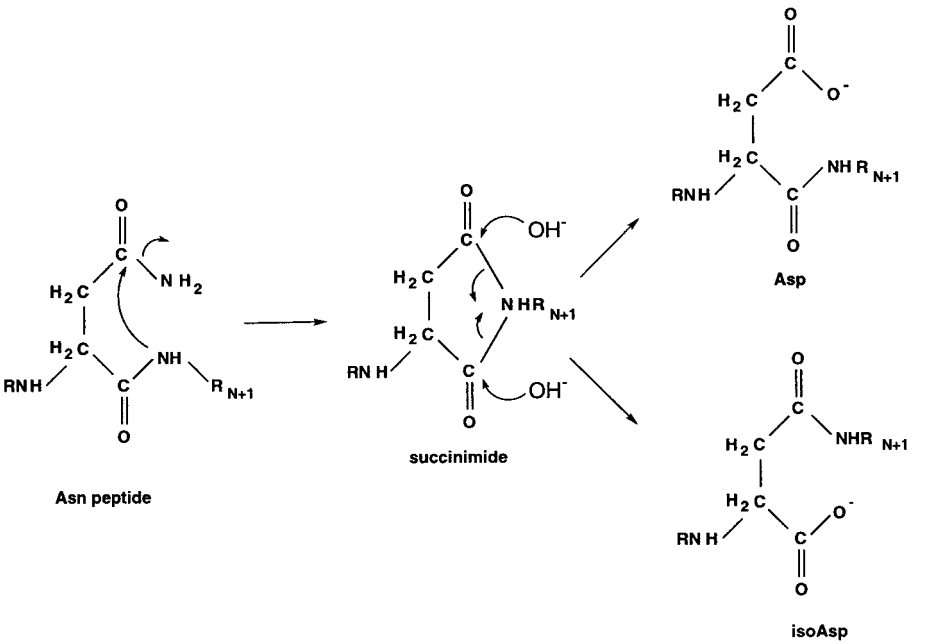

Fig. 1. Proposed mechanism for the deamidation of Asn residues in peptides and proteins. In peptides the ratio of isoAsp : Asp as products is 3 : 1.

also isomerize to isoAsp residues in peptides and proteins ***(7–10)***. Few studies have been performed on the rate of deamidation of proteins in vivo and it is possible that in some proteins the rate of deamidation is much faster than observed in small peptides.

The function of deamidation as a posttranslational modification was first considered to be a signal for aging of the protein. This was deduced from the similarity of the slow rate of deamidation and the rate of protein turnover. There is now much evidence that for many proteins deamidation does serve as a signal for protein degradation ***(8–13)***. More recent studies, however, suggest that for some proteins deamidation may have a function other than as a signal for protein turnover ***(5–7,14–16)***.

A variety of methods have been used to detect deamidation of Asn residues in proteins and peptides. Mass spectrometry is sensitive and rapid and takes advantage of the increase in one unit of mass when Asn is converted to Asp ***(5–7)***. Using peptide mass finger-printing from two-dimensional gels offers a rapid new method for using mass spectrometry to detect deamidation in proteins ***(18)***. Another advantage of mass spectrometry is that it can easily

detect the presence of the succinimide intermediate ***(7)***. Another innovation for detection of deamidation in proteins of known sequence is to make a monoclonal antibody (MAb) to a synthetic peptide of the site believed to be affected in the protein but containing an isoAsp residue in place of the Asn residue. The antibody is used to detect the presence of isoAsp in the isolated protein ***(7,8,12,13)***.

The most commonly used method to detect deamidation of Asn residues is to determine the presence of isoAsp using the enzyme L-isoaspartate O-methyltransferase (PIMT) (E.C. 2.1.1.77).

The isoAsp residue formed during the deamidation of Asn has a free α-carboxylate group. This unnatural carboxylate is a substrate for PIMT resulting in an α-carboxylate methyl ester (**Fig. 2**). This ester can nonenzymatically reform the succinimide intermediate and undergo a second round of hydrolysis to a mixture of Asp and isoAsp residues. This results in a cycle where isoAsp residues are converted to Asp residues. There is considerable evidence that in vivo this cycle operates to repair isoAsp to Asp residues ***(10,18–20)***. In some cases this repair results in restoration of catalytic activity (***5,10*** and refs. therein). There is also evidence that the methylation reaction is involved in protein degradation ***(21)***. If the isoAsp residue was generated from Asn than it is not truly a repair since the product is Asp, but if the isoAsp was formed from an Asp residue then it is a true repair mechanism.

Figure 3 illustrates the method of using PIMT activity to assay for isoAsp residues. The reaction is initiated with [C^3H_3]AdoMet to form the labeled methyl ester of the isoAsp residue. The reaction is performed in a 1.5-mL Eppendorf tube. This ester is hydrolyzed in pH 10.0 borate-SDS buffer to release tritium-labeled methanol. Separating the released methanol from the starting [C^3H_3]AdoMet is performed by vapor diffusion in a sealed counting vial containing about 5 mL of scintillation fluid. The methanol diffuses from the Eppendorf vial and dissolves in the scintillation fluid. After removing the Eppendorf tube the scintillation vial is counted.

2. Materials

2.1. Labeled AdoMet Preparation

1. S-[C^3H_3]-Adenosyl-L-methionine (14.7 Ci/mmol).
2. 0.1 *M* H_2SO_4.
3. S-Adenosyl-L-methionine chloride (*see* **Note 1**).

2.2. Isoaspartate Determination

1. Buffers: 75 m*M* NaCitrate, pH 6.2.
2. 0.5 *M* Na Borate-1% sodium dodecyl sulfate (SDS), pH 10.0.
3. PIMT, 10 mg/mL (*see* **Note 2**).

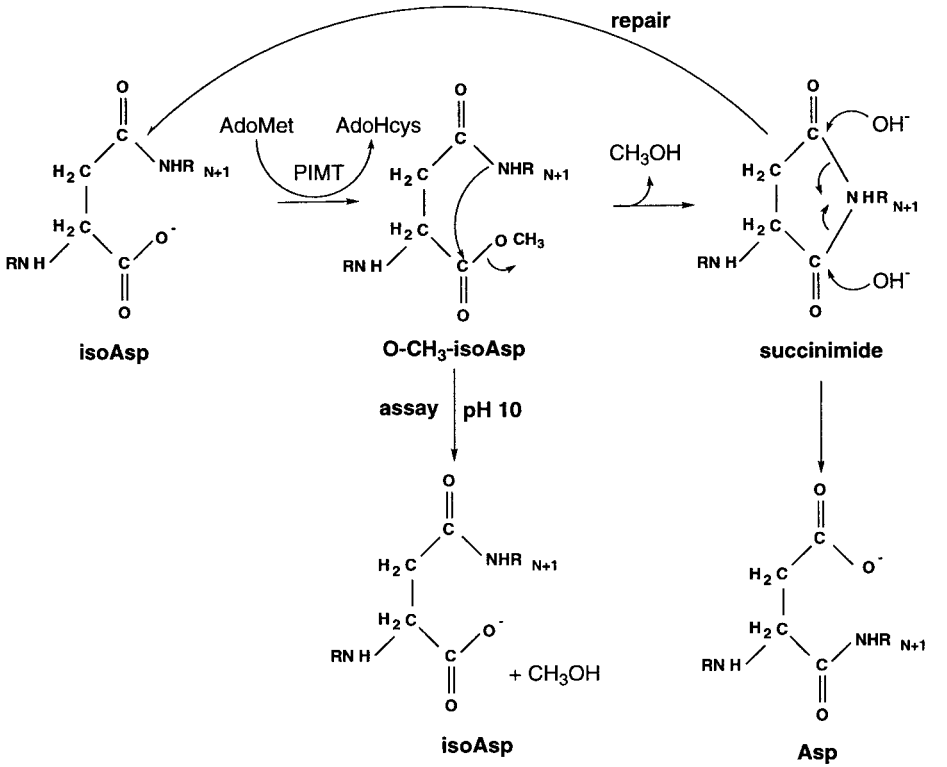

Fig. 2. Methylation of isoAsp residues by protein L-isoaspartate O-methyltransferase (PIMT). The methylated isoAsp residue can slowly reform the succinimide intermediate (*see* **Fig. 1**). This hyrolyzes to either an Asp or isoAsp residue. The isoAsp residue can be methylated again resulting in a cycle that converts isoAsp to Asp. The assay described in this report takes advantage of the alkaline liability of the isoAsp methyl ester to generate CH_3OH.

4. ScintiVerse BD cocktail (Fisher).
5. Liquid-scintillation analyzer.

2.3. Determination of Specific Activity of Labeled AdoMet Solution

1. Ac-Val-isoAsp-Gly-Ala (4 mg) in 1 mL of 10 m*M* potassium phosphate, pH 7.0, gives a 10 m*M* stock solution.
2. The stock solution is diluted 100-fold to 0.01 m*M* for the working solution (*see* **Note 3**).

2.4. Preparation of Protein Substrate

1. Protein or peptide to be analyzed in dilute buffer at approx 1 m*M* concentration (*see* **Note 4**).

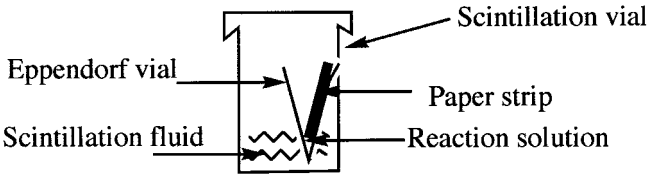

Fig. 3. Diagram of the vapor diffusion method for measuring $C[^3H]_3OH$ formed in the alkaline hydrolysis of $C[^3H]_3$-O-isoAsp.

2. Trypsin, sequencing grade.
3. Trypsin buffer: 100 m*M* ammonium acetate, pH 7.8.
4. Denaturation buffer: Trypsin buffer containing 6 *M* urea.
5. Glacial acetic acid.

3. Methods

3.1. Labeled AdoMet Preparation

1. Dissolve 2 mg of unlabeled AdoMet in 250 µL of 0.1 *M* H_2SO_4, giving a concentration of about 13 m*M*.
2. Add 5.5 µL of the unlabeled AdoMet stock solution to 100 µL of tritium-labeled AdoMet. The specific activity of this solution should be about 0.8 mCi/µmol and give 300,000–350,000 cpm/µL in a liquid-scintillation counter (*see* **Notes 5** and **6**).

3.2. IsoAsp Determination

1. In a series of 1.5-mL Eppendorf tubes add 0.2–5 nmol of isoAsp containing substrate, 5 µL of labeled AdoMet solution, 5 µL of PIMT, and add NaCitrate buffer to a final volume of 100 µL.
2. Incubate for 2 h at 30°C (*see* **Note 7**).
3. Place the reaction tubes on ice and cut off their caps. Add 100 µL of Borate-SDS buffer to stop the reaction and to denature the PIMT.
4. Using a forceps, introduce a tapered piece of filter paper so that the point is at the bottom and the top does not extend beyond the lip of the Eppendorf tube (*see* **Fig. 3**). Place the tube in a 20-mL scintillation vial containing 4 mL of scintillant.
5. Seal tightly with a cap and incubate at 30°C for at least 8 h (*see* **Note 8**).
6. Use forceps to remove the Eppendorf tubes and count the vials in a scintillation counter.

3.3. Determination of Specific Activity of Labeled AdoMet Solution

1. Perform the assay as described in **Subheading 3.2.** using as substrate increasing amounts of the standard peptide (Ac-VisoDGA) from 30 pmoles to 5 nmoles (*see* **Note 9**).

2. Construct a standard curve by plotting cpm vs pmoles of peptide. Use this graph to convert cpm of an unknown protein into nmoles of isoAsp.

3.4. Preparation of Protein Substrate

1. Dissolve 1–10 nmol of protein substrate in 1 mL of trypsin buffer containing 6 *M* urea. Incubate at room temperature for 30 min.
2. Dialyze against two changes of 500 mL of the trypsin buffer without urea.
3. Gently transfer the cloudy dialyzed solution to a clear tube and add trypsin (1:50 w:w).
4. Incubate at 37°C for 1–2 h. The cloudy solution should become clear.
5. Stop the reaction by adding 10 µL of glacial acetic acid.
6. Freeze-dry the sample. The lyophilized sample is ready to be used for isoAsp determination. It can be dissolved in assay buffer to the appropriate concentration (*see* **Note 10**).

4. Notes

1. The purity of AdoMet is usually in the 70–80% range. It should be stored at –70°C as a dry powder. It can also be stored for several months at –70°C as a solution in 0.1 *M* H_2SO_4.
2. Several expressing clones of PIMT in *Escherichia coli* are available ***(18,22)***. Purification from calf brain is another source of PIMT ***(21)***. A kit (ISOQUANT Protein Deamidation Detection Kit) containing PIMT is available from Promega (2800 Woods Hollow Road, Madison, WI 53711). PIMT can also be purchased from BioCache LLC (Biotech Center, 800 East Leigh Street, Richmond, VA 23219).
3. Most any short peptide containing isoAsp-Gly can be used as long as the Gly is not followed by an Asp or Glu. The concentration is determined from amino acid analysis. If quantitation of isoAsp in the unknown peptide or protein is not required than this peptide can be omitted. To have a positive control in the assay a 10 mg/mL solution of one of several commercially available proteins that contain isoAsp residues, such as ovalbumin, can be used ***(2)***.
4. The assay can detect as little as 0.5 nmol of isoAsp. Since many proteins have isoAsp that is 1% of the protein concentration, at least 50 nmol of protein or peptide substrate should be used in the initial experiments.
5. Because the AdoMet solution is not pure and contains some of the wrong isomer the exact specific activity of the final solution is not known. For quantitative determinations, an accurate specific activity must be determined by using a good substrate of known concentration as described in **Subheading 3.3.** If quantitation is not required this step can be omitted.
6. You can also use [$^{14}CH_3$]AdoMet in place of the tritiated compound. The advantages are both lower cost and background counts. The disadvantage is a loss in sensitivity.
7. PIMT is a sluggish enzyme whose k_{cat} value varies with the amino acid residues that flank the isoAsp. The time of incubation should be determined for each

substrate by stopping the reaction at 30-min intervals and checking that the assay is complete in the 2-h period.

8. During the 8-h incubation the methyl ester of isoAsp hydrolyzes to methanol and diffuses into the scintillation fluid. The rate of this process can be increased by incubating at a higher temperature. At 60°C an incubation of 2 h is sufficient. However, this increases the background counts. Others have increased the pH of the stopping solution to shorten the ester hydrolysis time. This also increases the background counts.
9. To obtain the specific activity of the tritiated AdoMet it is required that the isoAsp in the peptide be completely methylated. Under the conditions described this occurs at all concentrations of the peptide used in the assay.
10. This procedure will denature most proteins and trypsin digestion will convert the protein to soluble peptides. For unusual proteins other denaturants and proteases may be required to obtain a complete set of soluble peptides.

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19

Lysine Hydroxylation and Crosslinking of Collagen

Mitsuo Yamauchi and Masashi Shiiba

1. Introduction

Collagen is a large family of structurally related proteins ***(1)*** and is the most abundant molecule in vertebrates. Type I collagen, the predominant genetic type in the collagen family, is a heterotrimeric molecule composed of two α1 chains and one α2 chain and is the major fibrillar component in most connective tissues. This molecule consists of three domains: amino-terminal nontriple helical (N-telopeptide), triple helical (helical), and carboxy-terminal nontriple helical (C-telopeptide) domains (**Fig. 1**). The central uninterrupted helical domain of each chain contains more than 300 repeats of (Gly-X-Y) sequence and represents more than 95% of the polypeptide. One of the characteristic features of collagen is its extensive posttranslational modifications, most of which are unique to collagen protein. Such modifications include hydroxylation of proline (Pro) and lysine (Lys) residues, glycosylation of specific hydroxylysine (Hyl) residues, oxidative deamination of the ε-amino groups of Lys/Hyl in the telopeptide domains of the molecule, and subsequent intra/intermolecular crosslinking ***(2)*** (**Fig. 1**). The hydroxylation of Pro is catalyzed by prolyl 4-hydroxylase and prolyl 3-hydroxylase. The former reacts on Pro with the minimum sequence X-Pro-Gly and the latter appears to require a Pro-4-Hyp-Gly sequence ***(3)***. The presence of 4-Hyp (predominant form) is critical in stabilizing the triple helical conformation of collagen providing hydrogen bonds and water bridges ***(4)***. The content of Pro/Hyp in type I collagen is relatively high, representing 22–23 of the total amino acids, and 40–45% of Pro is hydroxylated (mostly 4-Hyp).

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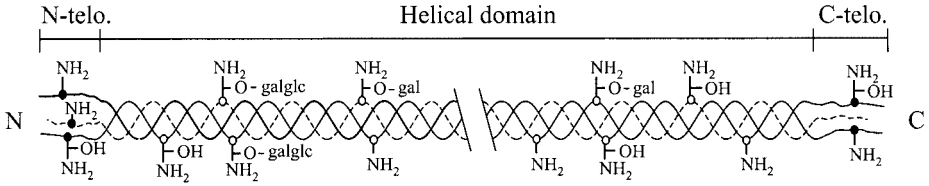

Fig. 1. Structure of type I collagen molecule. Type I collagen molecule is composed of two α1 chains (solid line) and one α2 chain (dotted line). The molecule consists of three domains: amino-terminal nonhelical (N-telo.), central triplehelical (helical), and carboxy-terminal nonhelical (C-telo.) domains. Some of the lysine residues in these domains are hydroxylated. Specific hydroxylysine residues in the helical domain can be O-glycosylated to form galactosylhydroxylysine or glucosylgalactosylhydroxylysine residues. In general, the dissaccharide form is more prevalent ***(20)*** although the monosaccharide derivative is relatively abundant in certain tissues (e.g., bone). NH_2: Lysine, NH_2 (with OH): Hydroxylysine, -gal: galactose, -glc: glucose, ●: Lysine/Hydroxylysine residues potentially converted to aldehyde.

The hydroxylation of Lys is important for glycosylation and crosslinking of collagen. In human type I collagen, there are 38 residues of Lys in an α1 chain (36 in the helical, 1 in the C- and 1 in the N-telopeptide domains) and 31 in an α2 chain (30 in the helical, 1 in the N-telopeptide and none in the C-telopeptide domains) (Sequence accession #P02452, P02464, and P08123) (**Fig. 2A**). The extent of Lys hydroxylation of collagen is much more variable than that of Pro. It varies from one genetic type to another ***(5)*** and, even within the same genetic type I collagen, it significantly varies depending on the tissue and the tissue's physiological conditions. Furthermore, a difference in the extent of Lys hydroxylation exists between the helical and telopeptide domains of a type I collagen molecule. The latter observation, together with the fact that a purified lysyl hydroxylase fails to hydroxylate the Lys residues in the telopeptide domains, led investigators to speculate that there is more than one mechanism for Lys hydroxylation of collagen ***(6,7)***. Recently, three genes encoding for isoforms of lysyl hydroxylase (Procollagen-lysine, 2-oxyglutarate, 5-dioxygenase, *PLOD 1-3*) have been cloned and characterized ***(8,9)***. Our recent study on the expression pattern of these genes during osteoblastic cell differentiation suggested that *PLOD 2* expression was associated with Lys hydroxylation of the telopeptide domains of the type I collagen molecule ***(10)***. The tissue specific pattern of Lys hydroxylation/crosslinking (*see* below) of type I collagen is likely due, in part, to differential expression of these genes by the cells in the respective tissues.

The formation of covalent intra/intermolecular crosslinks is the final step of collagen biosynthesis and is critical in providing the fibril with stability. This

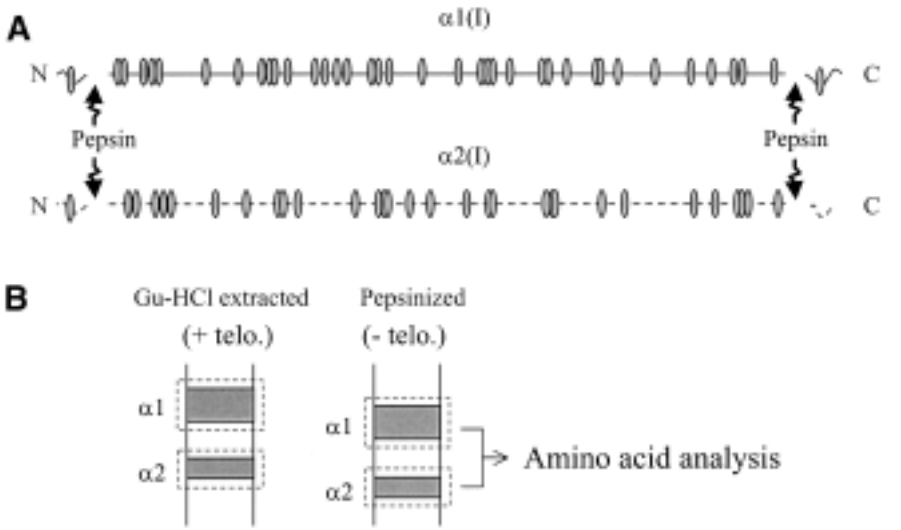

Fig. 2. **(A)** Relative distribution of lysine residues on α1 (solid line) and α2 (dotted line) chain of human type I collagen. The central straight line represents the helical domain and the curved lines at both ends the telopeptide domains. The lysine residues are indicated by ovals (⬮). The helical domains of α1 and α2 chains contain 36 and 30 residues of lysine, respectively. Note that each telopeptide contains one lysine residue except for the C-telo peptide domain of an α2 chain. The pepsin treatment of type I collagen removes the telopeptide domains. **(B)** Typical SDS-PAGE pattern of type I collagen with (left panel) and without (right panel) telopeptides. Note that α1 and α2 chains of pepsinized collagen migrate slightly faster than those of guanidine-HCl extracted collagen due to the lack of the telopeptides. Each band is excised and subjected to amino acid analysis.

process is initiated with conversion of the specific Lys or Hyl residues located in the telopeptide domains of the molecule to aldehyde (Lys^{ald} and Hyl^{ald}) by the action of lysyl oxidase ***(11)***. The aldehydes produced then undergo a series of condensation reactions involving the juxtaposed Lys/Hyl or histidine (His) residues on the neighboring molecules (**Fig. 3**). Obviously, the Lys hydroxylations in the telopeptide domains and in a certain portion of the helical domain are a key determinant for the subsequent condensation reactions. Lys^{ald} in the N-telopeptide domain, for instance, can react with another Lys^{ald} within the same molecule to form an intramolecular aldol, then matures into a tetravalent intermolecular crosslink, dehydrohistidinohydroxymerodesmosine (deH-HHMD), by involving the juxtaposed His and Hyl residues on a neighboring molecule. In skin and cornea, Lys^{ald} in the C-telopeptide domain reacts with the specific Hyl of an α1 chain on a neighboring molecule and the

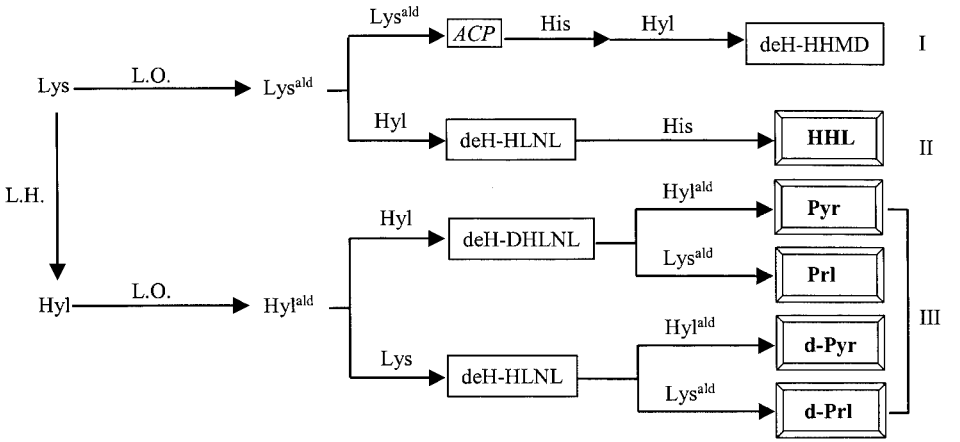

Fig. 3. Major crosslinking pathways of type I collagen. I. Predominant in non-mineralized tissue collagens in general. II. Predominant in skin/cornea collagens. III. Predominant in skeletal tissue collagens. L.H., hydroxylase; L.O., lysyl oxidase; *ACP*, aldol condensation product (Intramolecular crosslink); deH, dehydro; HLNL, hydroxylysinonorleucine; DHLNL, dihydroxylysinonorleucine; HHMD, histidinohydroxymerodesmosine; HHL, histidinohydroxylysinonorleucine; d-, deoxy-; Pyr, pyridinoline; Prl, pyrrole.

[□, Reducible crosslinks; ▣, Nonreducible crosslinks]

resulting iminium bifunctional crosslink, dehydro-hydroxylysinonorleucine (deH-HLNL), matures into a trifunctional crosslink, histidinohydroxylysinonorleucine (HHL) by involving His of an α2 chain on another molecule ***(12,13)***. The major Hyl^{ald}-derived pathway leads to the trifunctional crosslinks, pyridinoline (Pyr) and/or its lysyl analog deoxypyridinoline (d-Pyr) via the formation of the bifunctional crosslink, dehydrodihydroxylysinonorleucine (deH-DHLNL)/its ketoamine ***(14)***. A pyrrole compound is also a mature trifunctional crosslink of collagen ***(15,16)***. Although the mechanism of its formation is less clear, it likely involves both Hyl^{ald} and Lys^{ald}. Detailed chemistries of these lysyl oxidase mediated crosslinking pathways are given in several review articles ***(14,17–19)***. The stereospecificity and stoichiometry of these crosslinks at the specific molecular loci (e.g. the frequency and the relative involvement of Hyl of α1 and α2 chains) provides valuable information concerning the molecular packing structure of a fibril that seems to be tissue-specific ***(20,21)***.

Thus, the analysis for the extent of Lys hydroxylation in different domains of each α chain provides invaluable information about the cell phenotypes ***(22)***, functions of various *PLOD* genes and the tissue specific crosslinking chemistries. In the past, due to the difficulties in solubilizing and purifying

the α chains of tissue collagen, Lys hydroxylation was analyzed, in most cases, on a selective pool of tissue collagen or selective domains of α chains. In this chapter, methods we have developed for characterization of the Lys hydroxylation of the individual α chains of type I collagen with and without telopeptides and for collagen crosslinks using a cell-culture system are described. By using a cell-culture system, the matrix type I collagen deposited by the specific cells can be readily solubilized/extracted by pepsin digestion (without telopeptides) or by guanidine-HCl (with telopeptides). Based on the amino acid analysis of the individual α chains obtained from these two methods, the extent of Lys hydroxylation in helical and telopeptide domains can be calculated. Furthermore, the crosslinking profile (mostly reducible crosslinks) can be also analyzed for the same collagen matrix.

2. Materials

2.1. Cell Culture/Extraction of Type I Collagen

1. Cells obtained from human tissues.
2. Lysis buffer: 0.1 *M* Tris-HCl, 0.125 *M* NaCl buffer containing 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 1% deoxycholate.
3. Protease inhibitors: 0.5 m*M* phenylmethanesulfonylfluoride (PMSF), 5 m*M* benzamidine, 2 m*M* pepstatin A, and 1 m*M* leupeptin in dimethylsulfoxide (DMSO).
4. Phosphate-buffered saline (PBS): 6.7 m*M* potassium phosphate, pH 7.4, containing 150 m*M* NaCl.

2.2. Isolation of Type I Collagen without Telopeptide Domains

1. Pepsin (Worthington).
2. Buffer for pepsin digestion: cold 0.5 N acetic acid.
3. Buffer for collagen precipitation: cold 0.5 N acetic acid containing 0.7 *M* NaCl.
4. Instrument: Ultracentrifuge.

2.3. Isolation of Type I Collagen with Telopeptide Domains

1. Extraction buffer: 0.05 *M* Tris-HCl, pH 7.4, containing 6 *M* guanidine-HCl.
2. Instrument: Ultracentrifuge.

2.4. Separation and Blotting of α1 and α2 Chains of Type I Collagen

1. SDS gel-loading (sample) buffer: 63 m*M* Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.0025% bromophenol blue.
2. Acrylamide gel: 5% acrylamide gel (acrylamide:N, N′-methylenebisacrylamide; 29:1).
3. Transfer buffer: 10 m*M* 3-1-propanesulfonic acid, pH 11.0, and 10% methanol.
4. Polyvinylidene fluoride (PVDF) membrane.

5. Gel staining solution: 0.25% Coomassie blue and 10% glacial acetic acid.
6. Gel destaining solution: 30% methanol and 10% acetic acid.
7. Instrument: Transfer cell (Trans-Blot SD, Bio-Rad).

2.5. Acid Hydrolysis and Amino Acid Analysis

1. 6 N HCl (sequencing grade).
2. N_2 gas.
3. Centrifuge tube filter: 0.22 μm; cellulose acetate membrane.
4. Instruments:
   a. Oven (115°C) for acid hydrolysis.
   b. Speed vacuum centrifuge system to evaporate acid.
   c. Desktop microfuge for filtration of the hydrolysates.
   d. HPLC system equipped with a strong cation exchange column (AMINOSep #AA-911, Transgenomic) for amino acid analysis.
5. HPLC buffers:
   a. Buffer A: 12.5 m*M* tartaric acid disodium, 17.2 m*M* maleic acid, 2.5% isopropyl alchohol, pH 2.78.
   b. Buffer B: 0.3 *M* NaOH, 107.7 m*M* maleic acid, 48.5 m*M* boric acid, pH 9.91.
6. Ninhydrin, ninhydrin reactor (135°C).

2.6. Collagen Crosslink Analysis

1. Reducing buffer: 0.15 *M* N-trismethyl-2-aminoethanesulfonic acid (TES), 0.05 *M* Tris-HCl, pH7.4.
2. Antifoam solution.
3. NaB^3H_4.
4. Acid hydrolysis: same as **Subheading 2.5.**
5. Instruments: HPLC system linked to an on-line fluorescence flow monitor and a liquid-scintillation monitor.
6. Column: same as **Subheading 2.5., item 4d**.
7. HPLC buffer: same as **Subheading 2.5., item 5**.

3. Methods

3.1. Cell Culture/Extraction of Type I Collagen (see also *Note 1*)

1. Culture the cells obtained from human tissues with appropriate medium.
2. Maintain the cells for 4 wk with replacement of medium twice weekly.
3. Collect the cells and insoluble matrices by scraping the culture dishes.
4. Centrifuge at 2000*g* for 30 min at 4°C.
5. Remove the supernatant and add 15 mL of lysis buffer including a cocktail of protease inhibitors.
6. Incubate for 30 min at room temperature and then incubate overnight at 4°C on a rotating platform.
7. Centrifuge at 2000*g* for 30 min at 4°C.
8. Wash the residue with PBS (X3) and with cold distilled water (X1).
9. Lyophilize and store at –80°C.

3.2. Isolation of Type I Collagen α Chains Without Telopeptide Domains (see also Note 2) (Fig. 4)

1. Dissolve 2 mg of lyophilized samples (*see* **Subheading 3.1.**) in 2 mL of cold 0.5 N acetic acid.
2. Add pepsin (20% w/w) and incubate for 24 h at 4°C with constant stirring.
3. Centrifuge at 40,000*g* for an hour at 4°C.
4. Remove the residue and add NaCl gradually to the supernatants to a final concentration of 0.7 *M* while stirring.
5. Stir the solution gently for 24 h at 4°C.
6. Centrifuge at 40,000*g* for an hour at 4°C.
7. Discard the supernatant and dissolve the precipitate in 2–3 mL of 0.5 N acetic acid.
8. Dialyze against cold distilled water overnight at 4°C, lyophilize, and store at –80°C.

3.3. Isolation of Type I Collagen α Chains with Telopeptide Domains (see also Note 2) (Fig. 4)

1. Dissolve 2 mg of lyophilized samples (*see* **Subheading 3.1.**) in extraction buffer.
2. Stir for 3–4 d at 4°C.
3. Centrifuge at 40,000*g* for an hour at 4°C.
4. Dialyze the supernatant against cold distilled water for 2 d at 4°C.
5. Lyophilize and store at –80°C

3.4. Separation and Blotting of α1 and α2 Chains of Type I Collagen (see Note 3) (Fig. 2B)

1. Dissolve 100 μg of the dried samples (*see* **Subheadings 3.2.** and **3.3.**) in SDS gel-loading buffer.
2. Boil for 5 min.
3. Apply the samples to SDS-PAGE using a 5% acrylamide gel under nonreducing conditions.
4. Transfer onto a PVDF membrane with transfer buffer.
5. Stain the membrane lightly with the gel staining solution and destain with the destaining solution.
6. Excise the bands corresponding to α1 and α2 chains from the PVDF membrane.

3.5. Acid Hydrolysis and Amino Acid Analysis (see Note 4)

1. Hydrolyze the excised membrane (*see* **Subheading 3.4., step 6**) with 6 *N* HCl *in vacuo*, after flushing with N_2, for 22 h at 115°C.
2. Dry the hydrolysate under reduced pressure by a speed vacuum centrifuge.
3. Dissolve the hydrolysate in distilled water or high-performance liquid chromatography (HPLC) buffer A and filter with a 0.22-μm centrifuge tube filter.

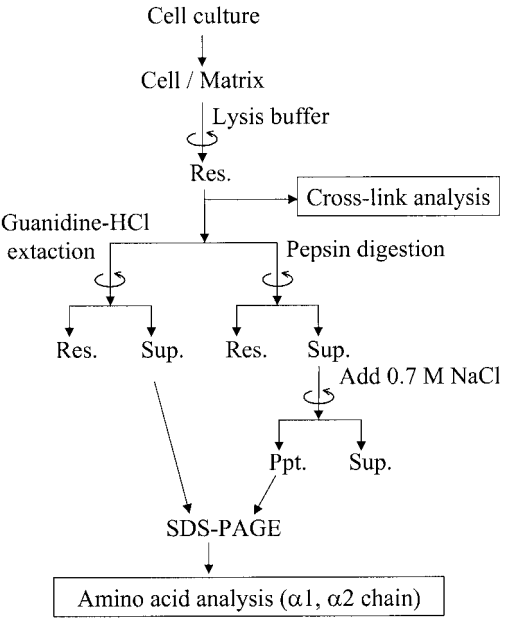

Fig. 4. Flow diagram of sample preparation for lysine hydroxylation and crosslink analysis of collagen.

4. Apply the hydrolysates to the HPLC system configured as an amino acid analyzer.
5. Elute amino acids with a combination of isocratic and linear gradients at a flow rate of 0.5 mL/min and detect the amino acids using ninhydrin for color development.
6. Analyze the Lys hydroxylation of collagen α chains based on the values of Hyl and Lys (*see* **Note 5**) (**Fig. 5**).

3.6. Collagen Crosslink Analysis (see *Note 6*)

1. Dissolve 1–2 mg of dried samples (*see* **Subheading 3.1.**) in 3 mL of reduction buffer. Add a few drops of antifoam solution. Ensure that pH of the solution is close to 7.5.
2. Add a total of 50–100 fold molar excess of NaB^3H_4 with three 5-min intervals with constant stirring and, after the third addition, continue to stir for 10 min (*see* **Note 6**).
3. Add a few drops of 50% acetic acid to drop the pH to 3.0–4.0.
4. Wash extensively with distilled water by centrifugation or dialysis to remove free NaB^3H_4 (*see* **Note 7**).
5. Lyophilize the sample and store at –80°C.
6. Hydrolyze with 6 N HCl as in **Subheading 3.5.**, **steps 1–3**.

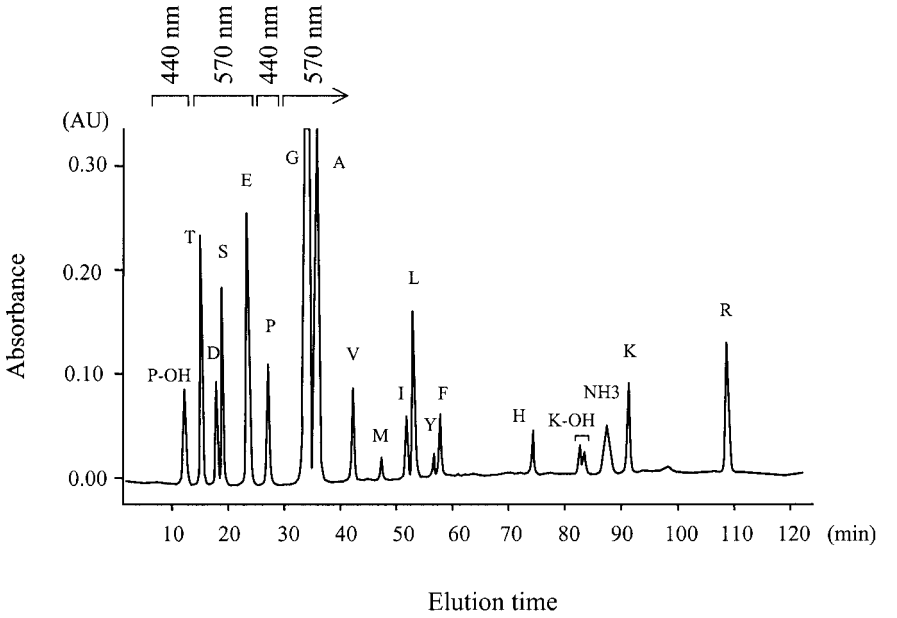

Fig. 5. An example of amino acid analysis of an α chain of type I collagen. One-letter code is used to designate amino acids. P-OH and P are measured by absorbance at 440 nm and all others at 570 nm. The extent of Lys hydroxylation is calculated by Hyl/(Lys+Hyl) and that of Pro by Hyp/(Pro+Hyp). P-OH, hydroxyproline; K-OH, hyroxylysine.

7. Determine the amount of collagen in the hydrolysate by Hyp content measured by amino acid analysis as in **Subheading 3.5., steps 4–5**.
8. Apply the hydrolysates with known amount of Hyp to the HPLC system fitted with an ion-exchange column linked to a fluorescence flow monitor and a liquid-scintillation monitor (*see* **Note 8**) (**Fig. 6**).
9. Measure the contents of reducible crosslinks and aldehydes as their reduced forms based on the specific activity of NaB^3H_4 (*see* **Note 6**). The fluorescent crosslinks (pyridinoline and deoxypyridinoline) are quantified by integrating the areas of the respective fluorescent peaks standardized by the hydrolysate of an apparently pure pyridinoline containing peptides ***(21)***.
10. The crosslinks and crosslink precursor aldehydes are calculated as a mole/mole of collagen basis based on the value of 300 residues of Hyp per collagen molecule.

4. Notes

1. Collagen samples can be stored at –80°C. Try to avoid an exposure to UV light (e.g., store the samples in an amber bottle) because of the potential photolysis of pyridinium crosslinks, though they are relatively stable in acid and when

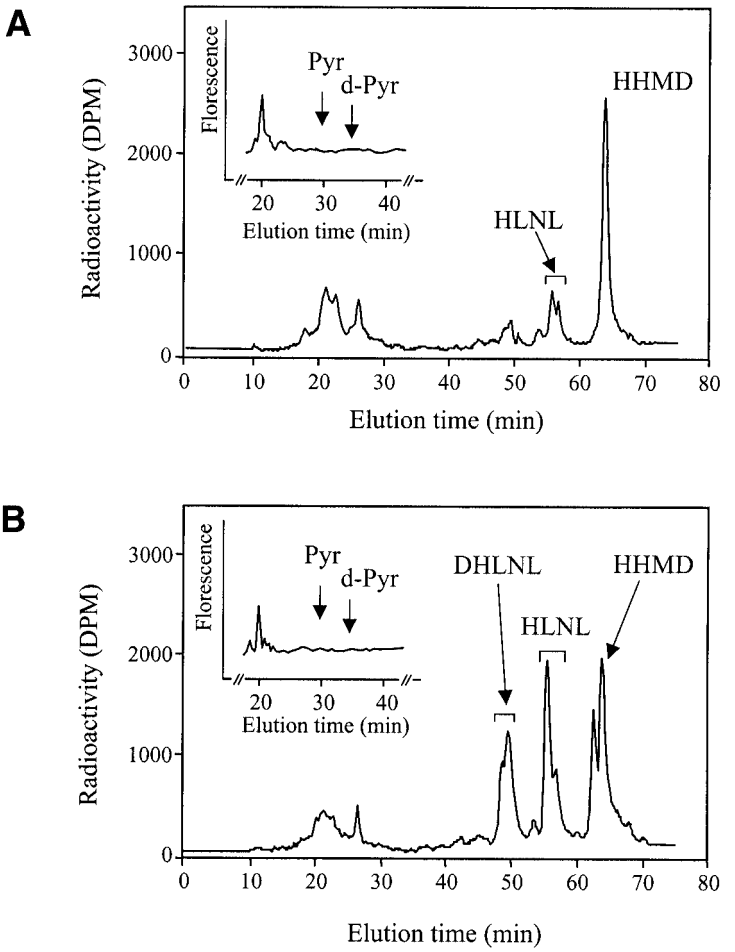

Fig. 6. Examples of crosslink analysis of collagen obtained from a cell-culture system. **(A)** Adult normal skin fibroblast. **(B)** Fetal skin fibroblasts. DHNL, HLNL, and HHMD: reduced forms of deH-DHNL, deH-HLNL, and deH-HHMD (for description of these abbreviations, *see* **Fig. 3**). The pyridinium crosslinks (Pyr, d-Pyr) were not detected in these samples (*see* inserts).

they are bound to peptide chains. Most intra- and extracellular noncollagenous soluble proteins and procollagen molecules are removed by these extraction and washing procedures.

2. Under the conditions employed, pepsin cleaves only telopeptide domains but not the helical domain. Therefore, α chains purified from a pepsin digest lack the telopeptides, while those purified from the guanidine-HCl extract do retain

them. Thus, the extent of Lys hydroxylation in each domain of each α chain can be calculated based on the ratio of Hyl to Lys of these α chains since the number of Lys residues in these domains are known (Sequence accession #P02452, P02464, and P08123) (**Fig. 2A**). This information is useful to correlate the Lys hydroxylation in these domains to the expression of various *PLOD* genes ***(10)***. By this method, however, the Lys hydroxylations between the C- and N-telopeptides of an α1 chain are indistinguishable.

3. For this procedure, α1 and α2 chains need to be well separated from each other on the gel (**Fig. 2B**). Five percent acrylamide gel is an excellent concentration for this purpose ***(10)***. Due to the loss of the samples that occurs during blotting, a relatively large quantity of the sample (about 100 μg) should be loaded on a large gel (18 × 16cm). Since type III collagen that contains inter-chain disulfide bonds can be also present in the precipitate/extract, it is important to dissolve the sample with the nonreducing sample buffer for SDS-PAGE. Otherwise the type III α chain co-migrates with an α1 chain of type I collagen.
4. The pH of the HPLC buffer should be adjusted by adding maleic acid to the solution. All collagen associated amino acids can be separated by a combination of isocratic and linear gradients from 0–98% of buffer B over a period of 2 h. The column temperature is set at 60°C (0–60 min) and 90°C (60–120 min). The color development with ninhydrin is performed at 135°C in a stainless-steel reaction coil. The temperature is maintained to ± 0.1°C with a thermostated silicone oil bath. The eluent is constantly monitored at 570 nm except for Hyp and Pro that are monitored at 440 nm. The wavelength change between 570 and 440 nm is precisely set before and after these amino acids. By this system, Hyl and Lys elute before and after NH_3, respectively (**Fig. 5**). The quantity of each amino acid is calculated based on the respective amino acid standards. Using this system, many crosslinking amino acids including Pyr, d-Pyr, DHLNL, HLNL, HHL, and HHMD can be separated from other amino acids and from each other. The extent of Pro hydroxylation in each α chain can also be calculated by Hyp/(Pro+Hyp) ***(23)***.
5. The number of Hyl residues in an α chain with or without telopeptides can be calculated based on the ratio of Hyl to Lys and the number of Lys residues in each domain (**Fig. 2A**). The number of Hyl residues in the telopeptide domains then can be calculated by subtracting the number of Hyl residues in an α chain with telopeptides from that in the α chain without.
6. Reducing procedure must be carried out in a hood. NaB^3H_4 is standardized as follows: demineralized dentin collagen obtained from unerupted bovine teeth, which has a very simple cross-link pattern (mostly DHLNL with a small amount of HLNL), is reduced with the NaB^3H_4 and hydrolyzed with constant boiling 6 N HCl for 22 h at 115°C. The hydrolysate will be dried, dissolved in water, and subjected to standardized P-2 column chromatography ***(12)***. Radioactive fractions that encompass these crosslinks (DHLNL and HLNL) are pooled and lyophilized. After dissolving in water or HPLC buffer A, an equal amount of

the sample solution is subjected to amino acid analysis (for their quantities) and crosslink analysis (for their radioactivity counts), respectively. Elution positions of these crosslinks are identified by crosslink standards ***(12)***. The ninhydrin color factor for crosslinks are obtained from amino acid analysis of apparently pure crosslinked peptides isolated from various connective tissues ***(20)***. In this manner, the specific activity of the NaB^3H_4 can be calculated and expressed in DPM/μmol.

7. This wash process can be done by repeated centrifugation or exhaustive dialysis. Either way, it is important to remove most of the free NaB^3H_4 in order to obtain a clean analysis pattern.
8. For crosslink analysis, a linear gradient of buffer B from 0–93% in 57 min (0–55% in 15 min, 55–60% in 25 min, and 60–93% in 17 min) and an isocratic gradient at 93% for the next 40 min (including wash process) at a flow rate of 0.5 mL/min are used. The column temperature is set at 58°C for the crosslink analysis and then 88°C for a column wash. The pyridinium crosslinks (Pyr and d-Pyr) are identified and quantified by a fluorescence flow monitor at excitation 330 nm and emission 390 nm. Then the eluate is mixed with scintillation fluid and the reducible compounds (i.e., reduced aldehydes, DHLNL, HLNL, and HHMD) are measured by a calibrated radioactivity flow monitor. Thus fluorescence and radioactivity are continuously monitored, recorded, and the areas of the respective peaks are integrated and calculated.

Acknowledgment

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Heterologous Expression in Endocrine Cells for Analysis of Posttranslational Modifications

Jens R. Bundgaard

1. Introduction

An attractive way of analyzing posttranslational modifications and the biological significance of these during biosynthesis, is by heterologous expression of a gene product in model cell systems. A large number of expression systems have been developed to facilitate gene transfer into cells from a wide range of species, ranging from bacteria, fungi, and insect cells to mammalian cells. Such systems have been described in details elsewhere, including in this series ***(1)***, and in many cases, developed expression systems can be purchased commercially. Each have their advantages and drawbacks, and it follows that the first step in obtaining heterologous expression is to have a clear strategy and object with the experiment to ensure that the expression system is useful to address the particular question. Hence, the cell types of choice in expression experiments depend on the type of posttranslational modification in question.

Expression in microorganisms is relatively easy to obtain, but unfortunately these are very inefficient in posttranslational processing. Therefore, insect or mammalian cells with fibroblast-like characteristics are often preferred. However, even these are not always capable of correct post-translational processing. One particularly problematic type of expression is that of secretory proteins such as peptide hormones and neuropeptides, which are extensively posttranslationally modified. Besides specific posttranslational modifications (e.g., glycosylation and tyrosine O-sulfation) of the Golgi/*trans* Golgi apparatus, these proteins require specific sorting into specialized granules in order to be correctly processed and later secreted upon specific stimulation. In these granules, bioactivation is completed by proteolytic processing and

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Table 1
Examples of Posttranslational Modifications of Proteins in Endocrine Cells Expressed Through the Regulated Secretory Pathway[a]

Posttranslational modification	Enzyme(s) involved	Intracellular localization
Endoproteolysis	Prohormone convertases (PC1and PC2)	Secretory granules
Endoproteolysis	Prohormone convertases (furin and others)	TGN, Golgi?
Carboxyamidation	Peptidylglycine α-amidating enzyme (PAM)	Secretory granules
Acetylation	N-α-acetyltransferase	Golgi, secretory granules
Exoproteolysis	Carboxypeptidases D and E Aminopeptidases	Secretory granules
Glycosylation	Several carbohydrate transferases	Golgi
Phosphorylation	Casein kinase	Golgi
Tyrosine sulfation	Tyrosylprotein sulfotransferases	*trans* Golgi network

[a]Note that some of the modifications are specific for endocrine cells, others occur in fibroblasts as well.

further modifications, such as carboxyamidation (**Table 1**). Expression of such proteins in fibroblast cell lines will result in poor posttranslational processing. Consequently, endocrine cell lines are necessary for expression studies of bioactivation of propeptides. However, most endocrine cell lines are notoriously difficult to transfect, and often it is necessary to use viral infections and/or selection of stable clones with the gene of interest inserted on the chromosome to obtain sufficient levels of expression. Obviously, this is laborious, particularly if the study includes analysis of numerous mutants affecting the post-translational processing.

The aim of this chapter is to introduce an expression system of secretory proteins via the regulated secretory pathway that is fast, simple and inexpensive. The system is based on a hamster endocrine β-cell line, HIT ***(2)***, which is efficiently transfected by simple methods and is capable of expressing high levels of products. High yields can be obtained even using transient transfection, a fact that makes the system ideal for handling of a large number of gene constructs during, e.g., mutational studies of prohormone expression. Even if stable expression is desirable, the high-transfection efficiency of the cells facilitates generation of clones. Utilization of HIT cells in analysis of peptide processing of the gut peptide hormone gastrin, has resulted in expression levels

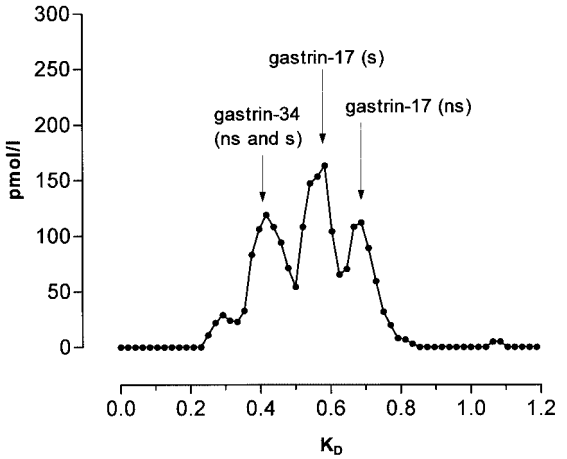

Fig. 1. Gel-filtration profile of human progastrin products after transfection of HIT cells. Boiled water extracts of 0.5 mL corresponding to approx 2×10^6 cells was applied on a Sephadex G50 (Pharmacia) column of 1×1000 mm. Peptides were eluted at a flow of 1 mL/min of VBA (0.02 *M* barbital buffer, pH 8.4 and 1 g/L BSA). Fractions were analyzed using an antisera specific of biologically active carboxyamidated gastrins. The processing pattern that contains a mixture of tyrosine sulfated and nonsulfated gastrin-17 and gastrin-34 resembles that of expression from antral G-cells.

approaching that of the antral G-cells, the natural site of gastrin expression ***(3)***. Moreover, HIT cells tend to mimic the posttranslational processing of progastrin observed in G-cells and thus reflects the natural processing pattern (**Fig. 1**). Similarly, HIT cells produce and posttranslationally process endogenous insulin ***(2)*** and cholecystokinin (**Fig. 2**) ***(3)*** correctly. Furthermore, secretion from the regulated secretory pathway in HIT cells can be assayed by the addition of secretagogues affecting the intracellular calcium (*see* **Fig. 3**). To further increase the versatility of the HIT cell-expression system, we have recently developed stably transfected HIT cells expressing the tetracycline repressor, facilitating inducible expression of up to 30-fold (a "tet-on" system ***[4]***, and unpublished results).

2. Materials

2.1. Basal Culture Techniques of HIT Cells

1. HIT-T15 cells (ATCC CRL-1777).
2. Dulbecco's Modified Eagle's Medium (DMEM) with 1 g/L glucose. Culture media is added glutamine if purchased without glutamine or glutamax.
3. Fetal bovine serum (FBS).
4. Penicillin/streptomycin solution (10,000 U penicillin/10 µg/mL streptomycin).

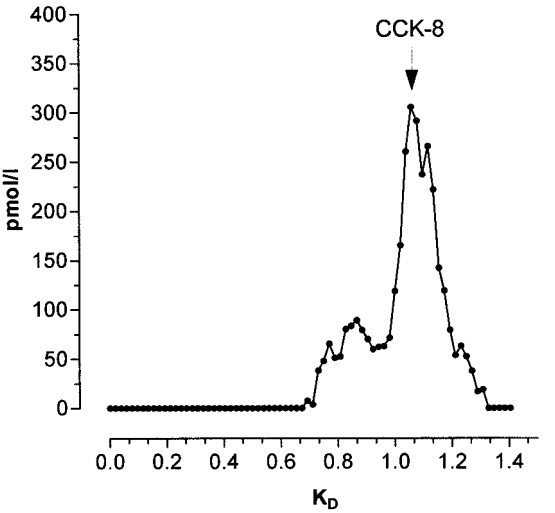

Fig. 2. Gel-filtration profile of porcine procholecystokinin (proCCK) products after transfection of HIT cells. Conditions were as described in the legend to **Fig. 1**. Fractions were analyzed using an antisera specific of biologically active carboxyamidated cholecystokinins. The cell extract contains mainly CCK-8, which is the completely processed form and the predominant form found in brain extracts in man and pig. Smaller peaks eluting at the positions of CCK-22 and possibly CCK-33 are also observed.

5. Phosphate-buffered saline (PBS): 10X stock solution: Final concentration/L in 1X solution: 137 m*M* 80 g NaCl, 2.7 m*M* 2 g KCl, 4.3 m*M* 11.5 g $Na_2HPO_4 \cdot 7H_2O$, 1.4 m*M* 2 g KH_2PO_4, pH ~7.3.
6. PBS with 2 g/L EDTA.
7. 10% CO_2 incubator.
8. Petri dishes/culture flasks for cell culture.

2.2 Freeze Cultures

1. Freeze culture vials.
2. Freeze culture buffer consisting of culture medium added 10% dimethylsulfoxide (DMSO).

2.3. Transient Transfection of HIT Cells

1. Highly purified DNA at 1 µg/µL (*see* **Note 1**). Optional: control DNA expressing a reporter protein, e.g. Green Fluorescent Protein, Luciferase, or Chloramphenicol acetyltransferase.
2. 0.25 *M* $CaCl_2$. Store as 2.5 *M* $CaCl_2$ sterile filtered through a 0.45 µm nitrocellulose filter at –20°C.

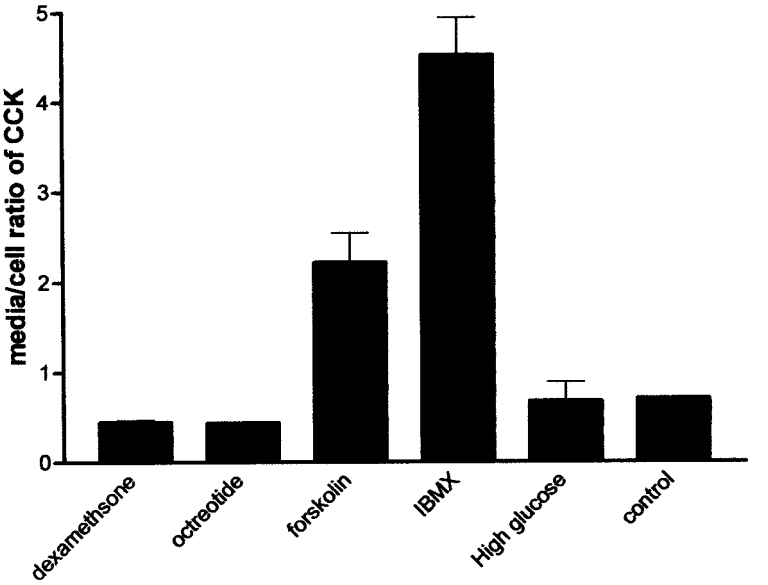

Fig. 3. Effects of secretagous on endogenous CCK secretion in HIT cells. The relative abundance of carboxyamidated CCK in culture media and cell extracts was measured using specific antisera. Cells were drug treated for 24 h except for dexamethasone treatment, which had duration of 48 h. The secretagoues increasing intracellular calcium all stimulates CCK release, IBMX being most potent. Cell cultures were treated with the following concentrations: 15 m*M* Glucose; 1 μ*M* Octreotide; 1 μ*M* dexamethasone; 10 μ*M* forskolin; 0.5 μ*M* IBMX.

3. 2X BES buffer: Prepare 800 mL solution of 50 m*M* BES (N,N-*bis*(2-hydroxyethyl)-2-aminoethane sulfonic acid); 280 m*M* NaCl and 1.5 m*M* Na_2HPO_4, pH 6.95. Adjust pH to 6.95 precisely with NaOH and add water to 1 L (*see* **Note 2**). Sterile-filter through a 0.45 μm nitrocellulose filter and store aliquots at –20°C.
4. CO_2 incubator at 35°C and 3% CO_2.
5. 2X HEPES buffer: Prepare a 800 mL solution of 11.9 g HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid); 16.4 g NaCl and 0.21 g Na_2HPO_4. Adjust pH to 7.05 precisely with NaOH and add water to 1 L. Sterile-filter through a 0.45 μm nitrocellulose filter and store aliquots at –20°C.
6. Glycerol shock solution: 10% (v/v) of glycerol in normal, complete culture medium.

2.4. Generation of Stably Transfected HIT Cells (see *Note 3*)

1. Hygromycin, Zeocin, Blasticidine, or other selectable drug.
2. Conditioned media: culture media from nontransfected HIT cells is collected and sterile filtered through a 0.45 μm nitrocellulose filter. Conditioned media

contains growth factors secreted by HIT cells that stimulate growth of colonies at low cell concentrations.
3. Multiwell culture plates.

3. Methods

3.1. Basal Culture Techniques of HIT Cell Cultures

1. HIT cells are split every 3–4 d using the following protocol. The culture media is removed by aspiration and PBS with EDTA is added (2–3 mL per 100 mm Petri dish or 5 mL per large flask). Incubate in flow bench for 1–2 min. Gently tab on the side of the flask or dish to loosen cells from the surface.
2. Collect the cells in a sterile tube and centrifuge the cells at 1500 rpm for 2 min. Remove the supernatant by aspiration.
3. Resuspend the cells in culture media and add to new flasks. Cells are split 1:3 at each passage.
4. Incubate at 37°C and 10% CO_2.

3.2. Preparation and Thawing of Freeze Stocks

1. Cells are harvested as described in **Subheading 3.1., steps 1–2**.
2. Cell pellets are resuspended in freeze culture buffer and transferred to freeze tubes.
3. Freeze the cells slowly. Preferably, the time course of freezing is about 1°C/min. In practice, we incubate at –20°C in a polystyrene foam box until the cultures are frozen. After this, cells are incubated in liquid nitrogen or in a –150°C freezer.
4. Cells stocks are thawn as quickly as possible. Take vial directly to a 37°C water bath and incubate until liquid thaws. Transfer cells to a fresh centrifuge glass and add media drop wise to avoid to abrupt osmotic changes. Centrifuge and remove the supernatant. Resuspend the cells in fresh media and plate in a culture flask or dish. After a few hours cells will attach and if many cells are dead, the media can be changed and the culture grown as usual.

3.3. Transient Transfection of HIT Cells (see *Note 4*)

Common procedure for transient transfection of HIT cells:

1. The day prior to transfection, split HIT cells into the required number of dishes using the standard protocol. For 100 mm Petri dishes, add about 2×10^6 cells per dish. It is important that cells are in exponential growth during the procedure.
2. On the day of transfection, change culture media.

Hereafter, follow either one of the specific transfection protocols:
Transfection using BES buffer *(5)*:

3. Mix 20 µg of DNA with 0.5 mL of 0.25 *M* $CaCl_2$. Add 0.5 mL of 2X BES buffer, vortex immediately to mix and incubate the solution at room temperature for 20 min.

4. Add the solution dropwise to the media of the cell culture. Swirl the plate to mix thoroughly. Incubate the plate at 35°C and 3% CO_2 overnight to allow a slow formation of precipitate.
5. The following morning, change culture media, wash the cells with PBS, and add complete culture medium. Incubate at 37°C and 10% CO_2.
6. Allow 2–4 d for maximal expression.

Transfection using HEPES buffer ***(6)***:

7. Put 0.5 mL of 2X HEPES buffer in a 10- or 15-mL conical tube. In another tube, mix 20 µg of DNA with 0.5 mL of 0.25 *M* $CaCl_2$. Bubble the HEPES buffer solution with a 1-mL pipet using a mechanical pipet while adding the DNA solution drop-wise. Vortex the sample immediately. Incubate at room temperature for 20 min.
8. Add the solution drop-wise to the media of the cell culture. Swirl the plate to mix thoroughly. Incubate the plate at 37°C and 10% CO_2 overnight.
9. The following morning, change culture media, wash the cells with PBS, and add complete culture medium. Incubate at 37°C and 10% CO_2.
10. Allow 2–4 d for maximal expression.
11. Transfection efficiency sometimes improves by glycerol shock of the cells (*see* **Note 4**). If this is desirable, **steps 4–6** are modified as follows. Four h after addition of the DNA solution to the culture, the media is aspirated and 2 mL of sterile glycerol shock solution is added. Incubate for 3 min, and then remove the glycerol solution. Wash thoroughly with PBS to remove all glycerol (2–3 times with 5 mL PBS). Add complete culture medium and incubate at 37°C and 10% CO_2 for 2–4 d to allow maximal expression.

3.4. Generation of Stably Transfected HIT Cells (see *Note 3*)

1. Follow **steps 1–5** in **Subheading 3.3.** Include nontransfected cells as a control of cell death.
2. Allow the cells to grow with complete medium for some hours at 37°C and 10% CO_2.
3. Split the cells into 5–10 Petri dishes and add selective media.
4. Incubate the cells until colonies are formed. This will take several weeks. Change media frequently in the beginning to remove dead cells. Later, as few colonies remain, add conditioned media (1 : 1 mixture with complete fresh media).
5. When colonies are clearly visible on the Petri dish, clones can be picked if desired. Take one clonal cylinder or cut off the thick end of a disposable pipet tip. Add a thin layer of sterile vacuum grease at the uncut end. Aspirate the media from the plate and put the clonal cylinders over the clones that are to be picked. Add PBS with EDTA and using a pipet, gently release the colony by flushing. Transfer the cells to a multiwell plate and grow the clones in conditioned media (*see* **Note 3**).

4. Notes

1. Both the nature of the DNA construct and the quality of the DNA used for transfection are important factors in obtaining high expression levels. Often the gene product of interest is expressed using a strong promoter like the cytomegalovirus (CMV) promoter, which is the basis of most commercially available expression vectors. These vectors also include a poly-A signal. It should be noted, that presence of an excisable intron might also improve expression levels as will the presence of a "Kozak-element" at the translational initiation site. The consensus of this element is (G/A)NN**ATG**G, where the bases in bold constitute the start codon ***(7–8)***. The purity of the transfection DNA is decisive for the outcome expression levels. DNA of sufficient quality can be prepared using DNA purification columns from vendors such as Qiagen. Protocols for preparation of high-purity DNA are described in most laboratory manuals or by the manufacturers of columns.
2. The most important factor in BES-buffered transfection is the pH conditions. Therefore, the pH of the buffer should be carefully controlled and at occasionally, we have observed variations in transfection efficiencies between batches. After preparation of new batches of buffer, therefore, we always include a transfection efficiency control in the first transfection series.
3. Several selectable drugs can be used for selection of stable clones In HIT cells. To obtain maximal efficiency, cells should be tested for survival at different concentrations of drug. The drugs may also display batch variability in efficiency. For effective selection, nontransfected cells should be killed within the first week. transiently transfected cells might survive for longer. In our hands, concentrations of 400 μg/mL of Geneticin (G418-sulfate), 6–10 μg/mL of Blasticidin S, 600–800 μg/mL of Zeocin, and 50 μg/mL Hygromycin B have proven efficient. Establishing clones is a lengthy procedure and it is crucial to work as sterile as possible throughout the procedure. Growth of clones is improved by addition of conditioned media. Conditioned media is simply media retrieved from growing HIT cells, which is sterile-filtered before addition to the clones. This media contains factors secreted by growing HIT cells and simulates the environment of more confluent cultures than the scattered clones.
4. It is vital for transfection that cells are split prior to transfection. The methods of transfection described here both use calcium phosphate precipitates to introduces the DNA into the cells. These methods tend to transfect a relatively low percentage of the cells (BES-buffered transfection typically transfects about 30% of the HIT cells). However, a relatively large amount of DNA is introduced, compared to other methods, which might be helpful for production of stable clones. Other methods using liposomes or amines, which generally transfected more cells are commercially available, but these are expensive and not necessarily better-suited for the transfection studies like those described here.

   BES-buffered calcium phosphate-mediated transfection is generally more efficient and convenient than HEPES buffer transfection. To obtain efficient transfection using HEPES buffer, it is recommended to add a glycerol chock step.

The disadvantage of BES-buffered transfection is that it is necessary to incubate the cells at a special CO_2 and temperature for maximal efficiency.

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2D-Electrophoresis

Detection of Glycosylation and Influence on Spot Pattern

Klemens Löster and Christoph Kannicht

1. Introduction

The detailed characterization of complex protein mixtures like samples from biological sources cannot be sufficiently performed by separation of polypeptides accordingly to their molecular weight as is done by conventional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1DE). For analysis of such samples, two-dimensional gel electrophoresis (2DE) is the preferable methodological approach, since it combines separation of polypeptides according to isoelectric properties and molecular weight as well. The resulting pattern of protein spots does not only provide information on composition of samples due to the complexity of a mixture of polypeptides. It delivers also a picture on the microheterogenity of polypeptides caused by posttranslational modifications. These might be of natural or artificial type and occur during biosynthetic processing of a polypeptide or within industrial scale production.

The presented method describes an experimental approach to investigate the influence of glycosylation on spot pattern of proteins: (1) A standard mixture of human plasma proteins is subjected to desialylation and deglycosylation followed by 1DE and 2DE analysis. (2) Glycosylated polypeptides are detected by lectin binding on blotted proteins using *Sambucus nigra* (SNA) and *Datura stramonium* lectin (DSA) and assigned to spots obtained by protein staining. (3) Spot patterns obtained by protein staining of an untreated, desialylated, and deglycosylated protein mixture are compared by matching gel images.

Evaluation of electrophoretic separations shows that desialylation of glycoproteins results in shifting of spot patterns for about 0.4 pH units and a

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weak decline of the relative molecular weight Mr of individiual polypeptides. Deglycosylation similarly impairs the isoelectric properties of polypeptides as desialylation does. Additionally, deglycosylated proteins are characterized by Mr reduced by 5–8 kDa. Most notably, typical trains of spots observed in 2DE are not affected neither by desialylation nor by deglycosylation, which indicates the noncarbohydrate-related character of these polypeptide isoforms.

2. Materials

2.1. Sample Preparation

2.1.1. Protein Standard Mixture

1. Fibronectin, human (F-2006, Sigma, St. Louis, MO).
2. Ceruloplasmin, human (C-4519, Sigma).
3. Plasminogen, human (P-7397, Sigma).
4. Transferrin (Siderophilin), human (T-6549, Sigma).
5. Alpha1-Antitrypsin, human (A-9024, Sigma).
6. Alpha2-HS-Glycoprotein (Fetuin), human (G-0516, Sigma).
7. Alpha1-acid-Glycoprotein (Orosomucoid), human (G-9885, Sigma).
8. Fibrinogen, human (F-4883, Sigma).

2.1.2. Desialylation

1. Thermomixer (Eppendorf, Hamburg, Germany).
2. Sialidase from *Arthrobacter ureafaciens* (Glyco, Inc., Novato, CA).
3. Sialidase incubation buffer: 500 m*M* sodium acetate, pH 5.5.
4. Centrifugal filter devices, Ultrafree 0.5, Biomax-5 (Millipore, Bedford, MA).

2.1.3. Deglycosylation

1. GlycoFree™ Deglycosylation kit (Glyco, Inc., Novato, CA).
2. Centrifugal filter devices, Ultrafree 0.5, Biomax-5 (Millipore, Bedford, MA).
3. Dialysis tube.
4. Glass syringe, 50–200 µL.
5. Freeze dryer.
6. 0.1% (v/v) Trifluoroacetic acid (TFA).
7. 0.5% (w/v) Ammonium bicarbonate.
8. Ethanol.
9. Acetone.
10. Dry ice.

2.2. Electrophoretic Separations

2.2.1. SDS-PAGE

1. 1DE sample buffer (fivefold): 300 m*M* Tris-HCl, pH 6.8, 50 m*M* DTT, 0.015% (w/v) Bromophenol blue, 50% (v/v) glycerol, 12.5% (w/v) SDS, dissolved in Aq. bidest. Store aliquots of sample buffer at –20°C.

2. Running buffer (10-fold): 250 m*M* Tris, 1.92 *M* glycine, 1% (w/v) SDS dissolved in Aq. bidest. The pH has to be adjusted to pH 8.8, before addition of SDS. Dilute 10-fold running buffer with Aq. bidest to yield onefold running buffer.
3. Gels: Precast 8–16% Tris-glycine gels 1.0 mm × 2D well or 1.0 mm × 10 wells (Novex, San Diego, CA) (*see* **Note 1**).
4. Protein marker: ProSieve Protein Markers (FMC, Rockland, ME) consisting of a set of marker proteins of 5, 10, 15, 25, 35, 50, 75, 100, 150, and 225 kDa.
5. Electrophoresis system: XCELL II Mini Cell (Novex, San Diego, CA), Power supply PowerPac 200 (Bio-Rad, Hercules, CA).

2.2.2. 2D-PAGE

2.2.2.1. First Dimension Isoelectric Focusing

1. Rehydration buffer (1.2-fold): 2.4 *M* thiourea, 8.4 *M* urea, 4.8% (w/v) Chaps, 2.4% (v/v) Immobiline solution (Amersham Pharmacia, Uppsala, Sweden) pH 4.0–7.0 or pH 3.0–10.0 (depending on the desired pH-range of focusing), 0.45% (w/v) DTT, 0.015% (w/v) Bromophenol blue, dissolved in Aq. bidest ***(1,2)***. Store aliquots of rehydration buffer at –20°C (*see* **Note 2**).
2. Paraffin oil: Dry Strip Cover Fluid (Amersham Pharmacia, Uppsala, Sweden) (*see* **Note 3**).
3. IPG-strips: Immobiline Dry Strip, pH 4.0–7.0 or pH 3.0–10.0, 70 mm, 3 mm long, 0.5 mm thickness (Amersham Pharmacia, Uppsala, Sweden).
4. Electrophoresis system: Multiphor II, supplied with Dry Strip kit, electrode wicks and Reswelling tray, Power supply EPS 3500XL (Amersham Pharmacia, Uppsala, Sweden).

2.2.2.2. Second Dimension SDS-PAGE

1. Basis solution for equilibration buffer: 6 *M* urea, 30% (w/v) glycerol, 2% (w/v) SDS, 50 m*M* Tris-HCl, pH 8.8. Store aliquots of equilibration buffer at –20°C.
2. Equilibration buffer I: Basis solution for equilibration buffer supplemented with 0.15% (w/v) DTT. This solution has to be fresh prepared.
3. Equilibration buffer II: Basis solution for equilibration buffer supplemented with 0.24% (w/v) Iodoacetamide. This solution has to be fresh prepared.
4. Equilibration tubes: Immuno Tubes 10 mL (Nunc, Wiesbaden, Germany).
5. Agarose sealing solution: 0.5% (w/v) Agarose IEF (Amersham Pharmacia, Uppsala, Sweden) in onefold Running buffer supplemented with a few grains of Bromophenol blue (*see* **Note 4**).
6. Running buffer (10-fold): 250 m*M* Tris, 1.92 *M* glycine, 1% (w/v) SDS dissolved in Aq. bidest. The pH has to be adjusted to pH 8.8 before addition of SDS.
7. Precast gels: 8–16% Tris-glycine gel 1.0 mm × 2D well (EC6045, Novex, San Diego, CA).
8. Electrophoresis system: XCELL II Mini Cell (Novex, San Diego, CA), Power supply PowerPac 200 (BioRad, Hercules, CA).

2.3. Blot Transfer

1. a. Transfer buffer for 2DE gels: 20 m*M* Tris, 150 m*M* glycine, 0.01% (w/v) SDS, dissolved in Aq. bidest.
   b. Transfer buffer for 1DE gels: 20 m*M* Tris, 150 m*M* glycine, 10% (v/v) ethanol in Aq. bidest (*see* **Note 5**).
2. Transfer membrane: Protran Nitrocellulose 0.45 µm (Schleicher und Schuell, Dassel, Germany).
3. Blotting paper: Gel blotting paper 0.37 mm, 190 g/m^2 (Roth, Karlsruhe, Germany).
4. Ponceau-staining solution (1X): Made from 10-fold Ponceau S concentrate (Sigma) by dilution with Aq. bidest.
5. Ponceau-destaining solution: 1% (v/v) Acetic acid in Aq. bidest.
6. Blotting chamber: Hoefer TE series Transphor electrophoresis unit (Amersham Pharmacia, Uppsala, Sweden).

2.4. Lectin Staining

1. Dishes for incubation of blotting membranes: Petri dish square 120 × 120 mm (Greiner, Frickenhausen, Germany).
2. Blocking solution: SuperBlock reagent (Pierce, Rockford, IL).
3. Reagents for glycan detection: SNA (*Sambucus nigra* agglutinine, specificity towards α(2-6) sialic acid bound to galactose), or DSA (*Datura stramonium* agglutinine, specificity for desialylated glycans), Digoxigenin-labeled (Roche Diagnostics, Mannheim, Germany); Sheep anti Digoxigenin antibodies, Fab fragments, Peroxidase (Horseradish)-labeled (Roche Diagnostics, Mannheim, Germany).
4. Lectin-dilution buffer: 50 m*M* Tris-HCl, 150 m*M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$ pH 7.5.
5. Washing-buffer: 10 m*M* Tris-HCl, 150 m*M* NaCl, pH 7.4.
6. Detection system: SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).
7. Autoradiographic film: BiomaxMR-1 or X-Omat AR (Kodak, Rochester, NY).
8. Processing chemicals for manual film development: GBX developer and fixer (Kodak, Rochester, NY).

2.5. Gel Staining

1. Gel staining box (Nalgene).
2. Fixing solution: 40% (v/v) Ethanol, 10% (v/v) Acetic acid, 0.018% Formaldehyde in Aq. bidest.
3. Ethanol solution: 50% (v/v) Ethanol in Aq. bidest.
4. Reducing reagent: 0.02% (w/v) Sodium thiosulfate-penta-hydrate dissolved in Aq. bidest.
5. Silver nitrate reagent: 0.016% (w/v) Silver nitrate, 0.027% (w/v) Formaldehyde dissolved in Aq. bidest. **Danger:** Formaldehyde may cause cancer and causes irritation to skin, eyes, and respiratory tract. Please refer to the safety data sheet of the manufacturer before use.

6. Developer: 5% (w/v) Sodium carbonate, 0.025% (w/v) Sodium thiosulfate-penta-hydrate, 0.015% (w/v) Formaldehyde dissolved in Aq. bidest.
7. Stopping reagent: 1% (w/v) glycine dissolved in Aq. bidest.
8. Gel-preserving solution: 10% (v/v) Ethanol, 5% (v/v) glycerol in Aq. bidest.
9. Gel-drying solution: GelDry Drying Solution (Novex, San Diego, CA).
10. Cellophane sheets (*see* **Note 6**).
11. Gel drying frames: GelAir Drying Frames (BioRad, Hercules, CA).

2.6. Gel Evaluation

1. Scanning system with transillumination unit (*see* **Note 7**).
2. Software for image processing (*see* **Note 8**).

3. Methods

3.1. Sample Preparation

3.1.1. Preparation of Standard Protein Mixture

1. Prepare protein standard mixture by dissolving proteins (*see* **Subheading 2.1.1.**) in Aq. bidest as follows: 100 µg/mL fibronectin; 100 µg/mL ceruloplasmin; 30 µg/mL plasminogen; 100 µg/mL transferrin; 50 µg/mL α1-Antitrypsin; 50 µg/mL α2-HS-Glycoprotein; 33 µg/mL α1-acid-Glycoprotein; 400 µg/mL fibrinogen.
2. Store aliquots of protein standard mixture at –20°C. Avoid repeated freezing/thawing.

3.1.2. Desialylation

This method uses enzymatic release of sialic acids in order to obtain intact desialylated proteins for further analysis. We use sialidase from *Arthrobacter ureafaciens* for its capability to split off α2-3, 6- and 8-linked sialic acids ***(3)***. The cleavage protocol consists of three steps: transfer of sample in cleavage buffer, sialidase treatment, and buffer exchange. The last step is required before submission of proteins for biochemical analysis like 2DE. Success of desialylation should be checked by 1DE/protein staining and 1DE/lectin blotting before performing a 2DE experiment (*see* **Fig. 1**).

Terminal sialylation of glycoproteins detected by SNA-binding reveals almost equal staining of the glycoprotein standard mixture (*see* **Fig. 1**, lane 4) ***(4)***. This is reasonable since plasma glycoproteins are typically sialylated.

Probing with DSA is used to detect terminal N-acetyl-galactosamine residues ***(5)***. Fully sialylated glycoproteins are not detected by DSA. DSA-reactive bands in the lectin blot of plasma proteins show incomplete sialylation. In particular, this shows the DSA reactive band at about 50 kDa (*see* **Fig. 1**, lane 6). Following removal of sialic acid residues by sialidase treatment, glycoproteins have lost SNA binding property (*see* **Fig. 1**, lane 5 vs lane 4) but strongly enhanced DSA reactivity (*see* **Fig. 1**, lane 7 vs lane 5).

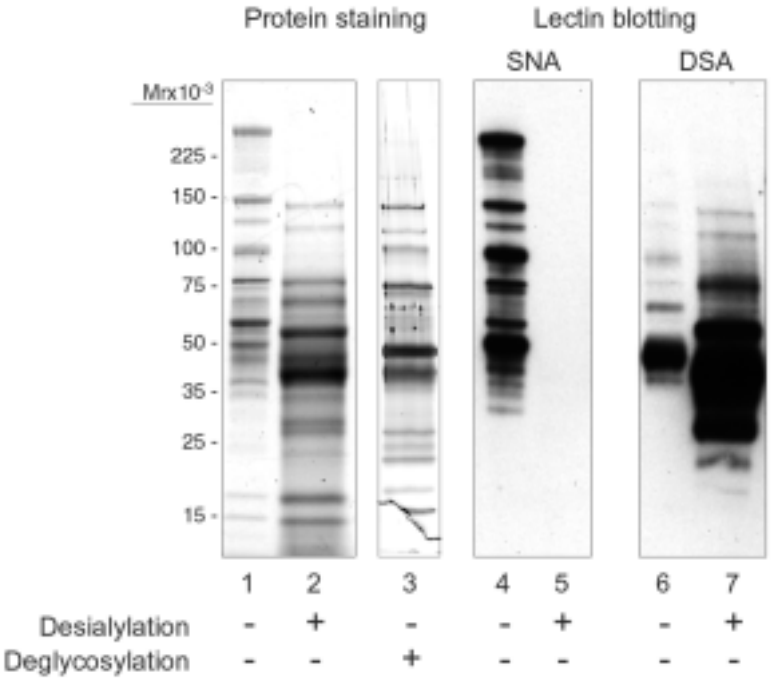

Fig. 1. 1DE analysis of the protein standard mixture following desialylation or deglycosylation monitored by protein staining (silver staining) or blotting with SNA and DSA. Lectin blotting of deglycosylated proteins is not shown since these do not contain lectin binding sites. Proteins, rel Mr: fibronectin, 280 kDa; ceruloplasmin, 150 kDa/126 kDa; plasminogen, 98 kDa; transferrin, 79 kDa; fibrinogen α chain, 75 kDa; fibrinogen β chain, 60 kDa; α_1 antitrypsin, 58 kDa; fibrinogen γ chain, 55 kDa; plasminogen heavy chain (HCh), 55 kDa; α_2-HS-glycoprotein, 49 kDa; α_1-acid-glycoprotein, 44 kDa; plasminogen HCh fragment, 39 kDa.

Besides change in lectin binding, desialylation reduces the apparent Mr of proteins as can be seen in 1DE. For example, the Mr of ceruloplasmin (150/126 kDa) and the Mr of α_1 antitrypsin (58 kDa) shift by approx 4–5 kDa to 145/121 kDa and 54 kDa, respectively (*see* **Fig. 1**, lane 2 vs lane 1). Most notably, the strong signal of DSA-reactive bands ranging between 25 and 75 kDa indicates successful removal of sialic acids, uncovering subterminal N-acetyl-glucosamine.

1. Dissolve salt-free samples in sialidase incubation buffer to a final concentration of 10–20 mg/mL.
2. If samples are not salt free, exchange buffer of protein solutions to sialidase incubation buffer by performing 3 wash cycles with centrifugal filter devices (5 kD, nominal molecular weight limit, 12,000*g*).

3. Concentrate glycoproteins by centrifugal filtration to approx 10–20 mg/mL in incubation buffer.
4. Resolve 0.2 U sialidase in 100 µL incubation buffer (*see* **Note 9**).
5. Add 50 µL sialidase solution to 50 µL protein solution.
6. Incubate for 18 h at 37°C under gentle shaking.
7. Exchange buffer to 1 m*M* Tris-HCl, 15 m*M* NaCl, pH 7.4 by performing 3 wash cycles with centrifugal filter devices before applying samples to 2DE (5 kD nominal molecular weight limit).

3.1.3. Deglycosylation

Carbohydrates may be removed from proteins by enzymatic or chemical deglycosylation. Since enzymes available for deglycosylation exhibit substrate specifity, it is difficult to achieve complete removal of carbohydrate moiety by enzymatical cleavage ***(6–8)***. Therefore we describe chemical deglycosylation of proteins by anhydrous trifluoro-methansulfonic acid using a commercially available kit ***(9)***. This method does not need special laboratory equipment.

Following a final buffer-exchange step, success of deglycosylation can be simply monitored by 1DE/protein staining and 1DE/lectin blotting, as described for the desialylation procedure (*see* **Fig. 1**). Completely deglycosylated proteins have lost capacity for lectin binding. In addition, the decline in Mr indicates to which extend the glycan moiety contributes to the Mr of a given protein (*see* **Fig. 1**, lane 3 vs lane 1).

1. Dialyze samples thoroughly against water or 0.1% TFA (*see* **Note 10**).
2. Transfer up to 1 mg protein to the reaction vial and dry sample by lyophilization (*see* **Note 11**).
3. Prepare a dry ice/ethanol cold bath.
4. Perform the deglycosylation process as described by the manufacturer (*see* **Note 12**). During the last step, the addition of ammonium bicarbonate, proteins may precipitate.
5. If proteins do not precipitate, remove the reaction mixture from protein by standard methods like dialysis or gel filtration.
6. In case of precipitation transfer reaction mixture to 2 mL conical reaction vials.
7. Add 1 mL cold acetone and chill samples to –20°C.
8. Centrifuge samples in a cooled centrifuge at 2000*g*.
9. Carefully remove supernatant and dry sample using vacuum centrifugation or lyophilization.

3.2. Electrophoretic Separations

3.2.1. SDS-PAGE

1. Mix 4 vol of sample with 1 vol of 1DE sample buffer (fivefold) and heat the mixture at 95–100°C for 5 min. If necessary, dilute the sample to appropriate

protein concentration with a diluted Tris-buffer, e.g., with 1 m*M* Tris-HCl, 15 m*M* NaCl, pH 7.4. Approximately 1 µg or 2–5 µg of total protein mixture per lane are appropriate if silver staining or lectin blotting has to be performed, respectively.
2. Prepare the electrophoresis chamber by assembling Precast gels into the XCELL II Mini Cell system and place it on a magnetic stirrer.
3. Transfer a small magnetic stir bar into the chamber and fill the chamber with onefold running buffer prepared from 10-fold stock solution by dilution with Aq. bidest.
4. Load the samples (15–20 µL of sample/well) and the molecular-weight marker (diluted 10-fold with onefold 1DE sample buffer) onto the gel. Be careful to avoid swirling the running buffer.
5. Connect the Mini Cell system with the power supply. Switch the magnetic stirrer on. Start running the gels for 30 min at a constant voltage of 120 V, and continue running at 200 V until the dye front starts moving out of the gel.
6. Disconnect the Mini Cell system from the power supply.
7. Remove the gel unit out of the Mini Cell system, open the gel unit, and remove the gel. Cut off the remaining parts of the stacking gel.
8. Transfer (*see* **Subheading 3.3.**) or stain (*see* **Subheading 3.5.**) the gels.

3.2.2. 2D-PAGE

Influence of glycosylation and sialylation on 2DE spot pattern of our standard protein mixture is shown in **Fig. 2**. Minigel 2DE analysis following desialylation and deglycosylation is performed applying protein staining and lectin staining. Individual gel imaging shows that desialylation and deglycosylation is accompanied by changes in the Mr and isoelectric point (pI) as well. Individual gel imaging clearly demonstrates contribution of the carbohydrate moiety to the physicochemical properties of polypeptides. In case of ceruloplasmin (150 kDa/126 kDa, *see* boxes within **Fig. 2**) desialylation shifts the pI by approx 0.4–0.5 pH units towards basic pH and declines the Mr by approx 1–2 kDa. Deglycosylation itself does not result in further increase of the pI but impairs the Mr of both polypeptide chains by approx 4–5 kDa. Most notably, the heterogeneity of the polypeptides as displayed by trains of protein spots is not affected indicating that the carbohydrate residues do not cause these.

We describe a 2DE-method using small, ready-made mini-gels. Since the introduction of IPG strips with short length (7 cm), ready-made mini-1DE gels can be used for the second dimension. This format has several practical advantages in comparison to large format gels. It is easy to handle, it requires only 1 d for doing a complete 2D analysis (first dimension, second dimension, blot transfer), and it safes material and working time. Furthermore, the mini-gel system shows comparable separation efficiency of polypeptides compared

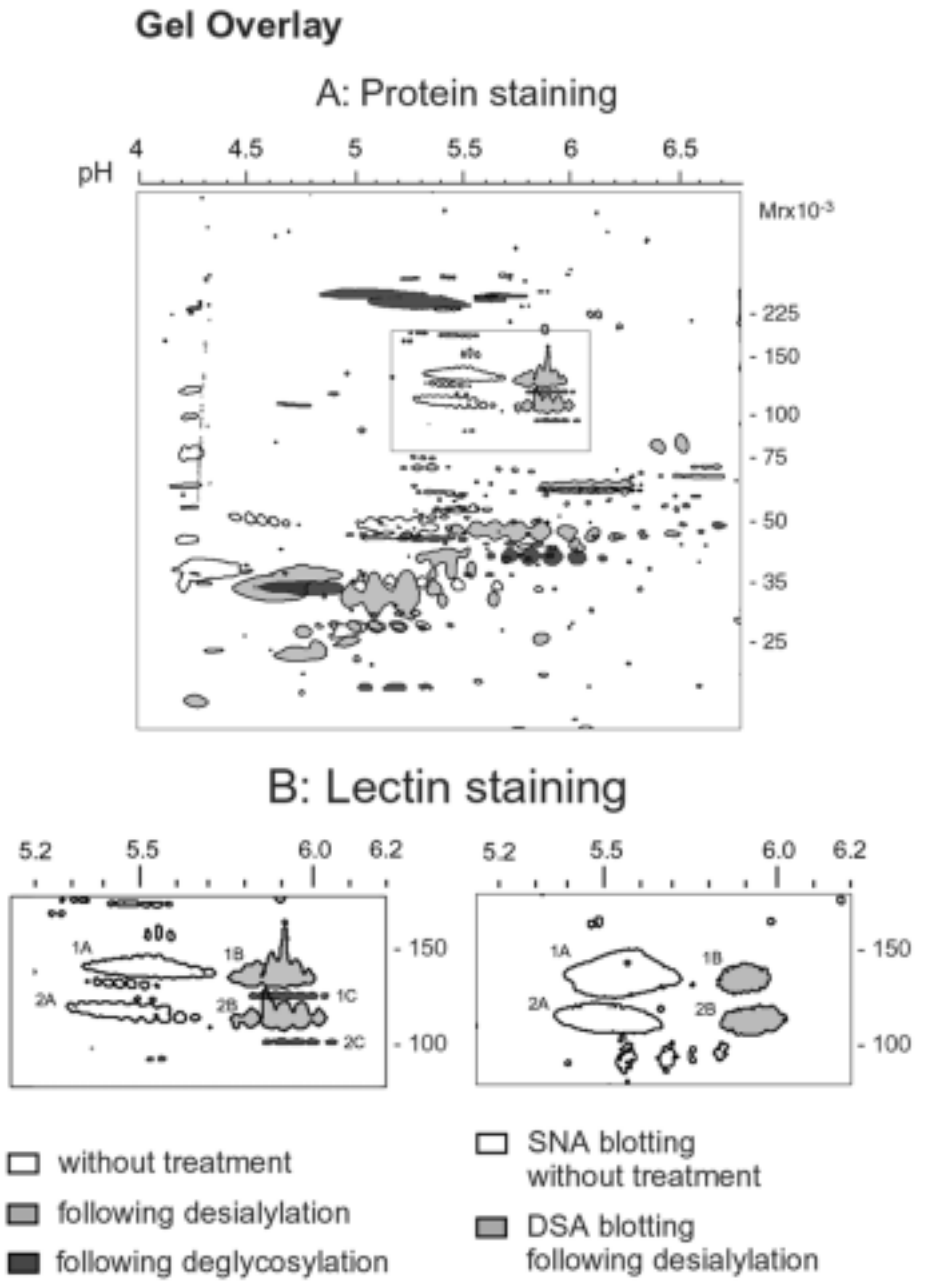

Fig. 2. 2DE analysis of the protein standard mixture following sialidase treatment or deglycosylation monitored by protein staining (silver staining) or lectin blotting. Lectin blotting of deglycosylated proteins is not shown since these do not contain lectin binding sites. The boxes within the figure encircle ceruloplasmin (1A/2A) following desialylation (1B/2B) and deglycosylation (1C/2C).

Table 1
Voltage Program for Isoelectric Focusing

Phase	[V]	[mA]	[W]	[h]	Vh
1	200	2	5	0:01	
2	3500	2	5	1:30	
3a (for IPG-strips pH 4-7L)	3500	2	5	1:30	5250
3b (for IPG-strips pH 3-10L)	3500	2	5	1:00	3500

to that of large format gels. We use this system for examination of protein mixtures of defined composition, for first determinations of pI/Mr of proteins after chemical/enzymatic modifications or for screening the reactivities of antibodies of unknown specificity. The minigel 2DE is therefore the method of choice before starting to work with a gel system of large format (i.e., $\geq 18 \times 20$ cm or even larger). Finally, application of gel imaging followed by comparison of individual images may be utilized for evaluation of changes in spot pattern between gels related to carbohydrate specific modifications.

3.2.2.1. First Dimension, Isoelectric Focusing

1. Change the buffer of samples to 1 m*M* Tris-HCl, 15 m*M* NaCl, pH 7.4. (*see* **Note 13**).
2. Mix sample (1 vol) and 1.2-fold rehydration buffer (5 vol), and incubate the mixture at 25°C for 1 h by gentle shaking. Approximately 5 µg or 5–10 µg of total protein mixture per lane are recommended if silver staining or lectin blotting has to be performed, respectively.
3. Add 165 µL of sample/rehydration buffer mixture (for 70 mm long IPG-strips) into each channel of the rehydration tray. Place IPG strips with the gel site towards the sample/rehydration buffer mixture (*see* **Notes 14** and **15**). Overlay the channels with paraffin oil until strips are completely covered. Proceed with IPG strip rehydration overnight at room temperature.
4. Drain off the paraffin oil. Transfer strips onto a sheet of thick filter paper (0.5–1 mm) and allow the oil to drain off.
5. Place and align rehydrated IPG strips into the focusing tray with the acidic and the basic site towards the anode and cathode, respectively (*see* **Note 16**).
6. Cut two electrode wicks into appropriate length depending on the number of IPG strips. Wet the wicks with Aq. bidest (60 µL Aq. bidest per cm). Carefully place moistened electrode wicks on top of the strips at each end (*see* **Note 17**).
7. Place the electrodes on top of the electrode wicks by gently pressing.
8. Pour approx 80–100 mL Paraffin Oil into the tray until all strips have been equally covered.
9. Set the temperature of the cooling bath to 20°C.
10. Start isoelectric focusing by applying the voltage program summarized in **Table 1** (for 70 mm IPG strips) (*see* **Note 18**).

11. Remove IPG strips from focusing tray. Place the strips on a sheet of 1 mm thick filter paper and allow the paraffin oil to drain off. Subsequently, transfer strips into equilibration tubes (one tube per strip). Close tubes with stoppers. At this step, strips can be stored at –20°C until performing the second dimension (1DE).

3.2.2.2. Second-Dimension SDS-PAGE

1. Prepare equilibration buffer I and II.
2. Add 5 mL of equilibration buffer I into each tube, and gently rotate the tubes for 15 min.
3. Displace equilibration buffer I by 5 mL equilibration buffer II, and continue gently rotation of tubes for 15 min. Meanwhile, heat the Agarose sealing solution (water bath or microwave oven).
4. Transfer a small magnetic stir bar into the chamber and fill the chamber with onefold running buffer prepared from 10-fold stock solution by dilution with Aq. bidest.
5. Remove equilibration buffer II, and wash the strips twice with 10 mL Running buffer.
6. Cut the IPG strip by 5 mm at the cathodic and anodic site since the sample cup of the Precast Mini 2D gel only fits to a 60 mm long strip. Carefully place the strips within the sample cup of the Precast Mini 2D gel. Avoid contact of the gel layer with the minigel plate and formation of any bubbles between the strip and the gel. Gently press the strip on top of the gel.
7. Fill the sample cup with the heated Agarose sealing solution. Be careful that the Agarose sealing solution does not enter the reference well.
8. Prepare the electrophoresis chamber by assembling Pre-Cast gels completed with IPG strips into the XCELL II Mini Cell system. Transfer a small magnetic stir bar into the chamber and place the chamber on a magnetic stirrer.
9. Fill the electrophoresis chamber with onefold running buffer.
10. Load the molecular weight marker into the reference well of the gel (*see* **Note 19**).
11. Connect the Mini Cell system with the power supply. Switch the magnetic stirrer on. Start running the gels for 30 min at a constant voltage of 120 V, and continue running at 200 V until the dye front starts moving out of the gel.
12. Disconnect the Mini Cell system from the power supply.
13. Remove the gel unit out of the Mini Cell system and open the gel unit. Cut off the remaining parts of the stacking gel and remove IPG strips and agarose.
14. Transfer (*see* **Subheading 3.3.**) or stain (*see* **Subheading 3.5.**) the gels.

3.3. Blot Transfer

1. Cut the nitrocellulose membrane and the gel blotting paper to gel size.
2. Fill the blotting chamber with cooled transfer buffer. Transfer a small magnetic stir bar into the blotting chamber and place it on a magnetic stirrer. Connect the blotting chamber to an external cooling device (*see* **Note 20**).

3. Soak the blotting paper and the blotting membrane in Transfer buffer for 2DE gels or Transfer buffer for 1DE gels (*see* **Subheading 2.3.**).
4. Prepare the blotting unit as follows: blot sponge, two sheets of blotting paper, nitrocellulose membrane, gel, two sheets of blotting paper, blot sponge. Avoid formation of air bubbles between the gel and the blotting membrane and between the stacks of blotting paper.
5. Transfer the blotting unit into the blotting chamber. The gel site has to be oriented towards the cathode.
6. Run the blot transfer at a constant voltage of 100 V for 1 h at 10°C.
7. Disassemble the transfer unit. Discard gel and blotting paper.
8. Drain the blotting membrane with Aq. bidest.
9. Control quality of blot transfer by staining the membrane with Ponceau Red: Place the nitrocellulose membrane into a dish with Ponceau-staining solution. Incubate the membrane for approx 5–10 min by gently shaking, and remove non-specific background staining by several washes of the membrane in 1% (v/v) acetic acid in Aq. bidest until the membrane turns to white. At this time, the molecular weight marker can be labeled with a pencil.
10. Destain the membrane by several incubations in washing buffer.

3.4. Lectin Staining

Gently shaking of the blot membranes is recommended. All incubations are performed at room temperature.

1. Incubate the blotting membrane for 10 min in lectin-dilution buffer followed by a 30-min incubation in Blocking solution.
2. Prepare working dilutions of the lectins: Dilute digoxigenin-labeled SNA and DSA 1:2500 in lectin-dilution buffer (*see* **Note 21**).
3. Incubate the blotting membrane in the lectin solution for 2 h at room temperature.
4. Wash membrane three times with lectin-dilution buffer for 10 min.
5. Prepare working dilutions of anti-Digoxigenin-antibody: dilute antibody 1:5000 in lectin-dilution buffer.
6. Incubate the blotting membrane in the anti-Digoxigenin-antibody solution for at least 1 h at room temperature.
7. Wash membrane five times with washing-buffer for 10 min (*see* **Note 22**).
   The procedures of **steps 8–12** have to be continued in a dark room.
8. Prepare the film processing solutions (developer, fixer). Poor the solutions into trays of appropriate size. Prepare a third tray with Aq. bidest (*see* **Note 23**).
9. Transfer the membrane into a separate dish, and add 4 mL of chemiluminescent substrate onto the membrane. Incubate the membrane for 5 min.
10. Drain off the chemiluminescent substrate. Immediately place the membrane between two sheets of plastic wrap (*see* **Note 24**).
11. Expose autoradiographic film on the blot surface for several periods of time ranging between 20 s and 5 min.

12. Develop the film by incubation in developer (approx 3 min) followed by a short wash in Aq. bidest and subsequent incubation in fixer (approx 10 min). All incubations should be performed by gently shaking.
13. Wash the film with deionized water and dry it on air.

3.5. Gel Staining

This recipe follows the protocol originally described by Blum et al. ***(10)***. Gently shaking of gels is recommended.

1. Incubate gels for 1 h 30 min in fixation reagent.
2. Wash gels three times with ethanol solution for 30 min.
3. Incubate gels for 1 min in reducing reagent.
4. Wash gels three times with Aq. bidest for 1 min.
5. Incubate gels for 1 h 30 min in silver nitrate reagent.
6. Wash gels three times with Aq. bidest for 1 min.
7. Place gels in developer and incubate for several minutes depending on progress of staining.
8. Transfer gels immediately into stopping reagent and incubate for 30 min.
9. Wash gels twice with Aq. bidest for 30 min to remove the stopping reagent.
   a. If gels shall be stored for some days in wetted form, transfer gels into gel preserving solution.
   b. If gels are to be dried, incubate gels for 30 min in gel-drying solution before placing between two sheets of cellophane sheets that have been soaked for 1 min in gel-drying solution. The cellophane/gel sandwich is placed within a gel drying frame and dried at room temperature for approx 1–2 d.

3.6. Gel Evaluation

A range of diverse image-processing software may be used for comparison of gel and immunoblot images. Among them are professional gel-matching programs that allow automatic processing, statistical evaluation, and graphic visualization of gels and matching results. However, we believe that the acquisition of this rather expensive software makes only sense if multiple numbers of gels have to be evaluated routinely. Alternatively, we recommend application of common office software for desktop publishing (e.g., AdobePhotoshop™), which allow comfortable processing of several pictures at once. In the following, a short description is given on how we evaluate gel images by use of AdobePhotoshop™ 5.5.

3.6.1. Scanning Procedure

1. Transfer the wet gel or film onto the surface of a desktop scanner.
2. Scan gels/films in transmission mode: Select gray scale (8 bit/256 gray levels), and set input to 300 ppi, scale to 100%, descreen to 250 lpi, at high sharpness.

3. Normalize the image by auto color correction.
4. Save image as 8 bit gray level image.

*3.6.2. Image Overlay (Described for Overlay of Two Different-Colored Images) (*see ***Note 25****)*

1. Open images (I1and I2) in AdobePhotoshop™ 5.5.
2. Make a copy of image I1.
3. Create a new document (D1). This document has the size of image I1.
4. Paste image I1 into document D1.
5. Make a copy of image 3.
6. Paste image I2 into document D1. This results in a picture consisting of two layers. Only one layer can be seen now.
7. Switch to RGB mode (image>mode>RGB color). Do not reduce the whole picture (layers) to background layer.
8. Color the images/layers by working with the layer tool as follows:
   a. Hide display of layer 2. Adjust the color of layer 1 (choose image>adjust>hue/saturation) by switching colorize on; hue: 0; saturation: 100; lightness: 20. Image I1 has now red coloration.
   b. Hide display of layer 1. Switch display of layer 2 on. Adjust the color of layer 2 (image>adjust>hue/saturation) by switching colorize on; hue: 120–140; saturation: 100; lightness: 20. Image I2 has now green coloration.
9. Combine both layers: Activate both layers and choose the multiplication mode (layer>layer options>multiply). Now, both images are seen at once.

   The combined images show the whole set of proteins present in both images (red stained, image I1; green stained, image I2). In case of overlapping spots/bands, color addition results in brown stained spots/bands. If necessary, the image sizes can fit each other by application of the transformation mode (edit>transform/free transformation) (*see* **Note 26**).

4. Notes

1. We generally prefer using minigels with acrylamide gradients since these give sharper bands and a better overall picture of proteins with molecular masses differing between 50–100 kDa.
2. This buffer has a rather high viscosity. Preparation of this buffer is performed at 37°C to ensure complete dissolving and mixing of all reagents. Prior use of rehydration buffer aliquots, the buffer cup is prewarmed to 25°C.
3. The paraffin oil used for covering the IPG strips during isoelectric focusing (*see* **Subheading 3.2.2.1.**) may be used for several times. We only use fresh paraffin oil when IPG strips are rehydrated.
4. We prepare 10- and 20-mL aliquots of Agarose sealing solution, which are stored at 4°C, and are used once.
5. A better cooling efficiency during blot transfer can be achieved if transfer buffers are stored at 4°C.

6. Several companies offer cellophane sheets for gel drying. We usually apply cellophane sheets used for conservation of food.
7. An office desktop scanner (300–1200 dpi; 8, 10, or 12 bit color) with transillumination unit gives sufficient optical resolution.
8. We are using AdobePhotoshop™ 5.5 for routine image processing.
9. Sialidase preparations may contain BSA as stabilizer. This may impair analysis of proteins with similar molecular weight, depending on ratio of sample and BSA concentration.
10. This step is critical for successful performance of the method. Samples have to be essentially free of salts, metal ions, and detergents. Low SDS concentrations are tolerable, if SDS concentration is kept below 2 mg/sample.
11. Samples have to be thoroughly dry for proper deglycosylation. Lyophilization should be performed <0.5 milliTorr for >24 h. Do not allow samples to be exposed to moisture after lyophilization.
12. Please pay attention to the safety data sheet given by the manufacturer. Trifluoromethanesulfonic acid causes burns on skin and eyes.
13. Since high concentrations of salts disturb separation of proteins according their isoelectric properties, it remains necessary to transfer the sample into a buffer of low ionic strength (e.g., 1 m*M* Tris-HCl, 15 m*M* NaCl, pH 7.4) or water. This should be preferably performed by gel filtration (e.g., by use of a PD10 column). In our laboratory, this method gives the best efficiency in buffer exchange. In general, avoid use of phosphate-containing solutions since these ions interfere with isoelectric focusing.
14. The cover foil of IPG strips has to be carefully removed by gently peeling up. Otherwise, parts of the acrylamid layer might be detached.
15. Prevention of air bubble formation between the IPG strip and sample/rehydration buffer mixture is absolutely necessary since the success of the isoelectric focusing strongly depends on even rehydration of the acrylamide layer.
16. It is necessary to process at least two IPG strips for isoelectric focusing. Due to the low conductivity of one IPG strip, the circuit protection of the power supply switches off the current.
17. The usage of excess of Aq. bidest impairs the resolution power of the first dimension resulting in smeared protein separation.
18. In case of samples with higher concentration of salts, we recommend application of a prefocusing step of 2 h at 200 V before starting the voltage gradient. This prefocusing step should remove salt ions and other undesired charged low molecular components of the sample.
19. For silver staining, 10 μL of 1:20 diluted (dilution made with onefold 1DE sample buffer) molecular weight marker are applied. If blot transfer is intended, 5 μL of undiluted marker are used.
20. The external cooling device may consist of an icebox filled with water/ice and a simple aquarium pump for water circulation connected to the blotting chamber by silicone tubes.

21. At least 5 mL of lectin or antibody solution are needed for soaking a membrane of 8 × 6 cm. We prefer to soak a 8 × 6 cm nitrocellulose membrane in 10 mL of solution.
22. The efficiency of washing (reduction of background staining) depends on the applied volume of washing buffer but not on the time of incubation.
23. Preparation of film processing chemicals follows the provided instructions. Storage of these reagents should be done in brown bottles in the dark at 4°C. Before starting with film processing, prewarm agents to room temperature.
24. Avoid partial drying of the membrane. This may result in signal reduction and/or elevated background staining.
25. For evaluation of silver stained gels, we recommend application of the positive mode (colored spots/bands and transparent background). This is since application of the inversion mode results in a rather high color signal of the background even in case of faint background staining of silver gels. In opposite, the homogenous background of films allows application of the inversion mode (colored spots/bands and black background).
26. Changes in gel size by image/layer transformation only results in linear changes of its dimensions.

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Tools for Functional Proteomics

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