HELGOLÄNDER MEERESUNTERSUCHUNGEN
Helgoländer Meeresunters. 49, 747–757 (1995)

Food control by applied biochemistry of marine organisms: comparison of proteins and metabolites from fish and invertebrate muscle

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ABSTRACT: Most fishery products consist of muscle tissue from fish and invertebrates. Differences in the molecular structure and in metabolism of muscles can be utilized to characterize and identify various seafood. Creatine and arginine were found to be useful for the differentiation between imitation crab/shrimp meat and real crustacean meat. Octopine served as an indicator for the meat of cephalopods and mussels. In order to identify the animal species of a fishery product, several electrophoretic methods were used. It depended on the type of product, whether sarcoplasmic or myofibrillar proteins were better suited. Raw products were best analysed by isoelectric focusing of sarcoplasmic proteins. Two types of sarcoplasmic calcium-binding proteins, parvalbumins of fish and soluble calcium-binding proteins of invertebrates, were especially useful for species identification. Due to their thermal stability, these proteins gave species-specific patterns for cooked products, too. Two other techniques were also investigated: urea gel isoelectric focusing and sodium dodecyl sulphate – polyacrylamide gel electrophoresis. These methods were applied in the analysis of products where the sarcoplasmic proteins had been removed by washing steps, i.e. imitation crab meat made from surimi, and of other raw and cooked products. The myosin light chains gave protein patterns that were characteristic for many species. Paramyosin, which is absent from vertebrate muscle, indicated the presence of mollusc muscle. It was shown that the determination of arginine kinase activity enabled differentiation between raw fish muscle and invertebrate muscles.

INTRODUCTION

A very large number of different marine organisms are used for human consumption. Relevant control of the nutritional and sensory quality of seafood by means of chemical analysis, and of the correct labelling of products by means of protein electrophoresis (Shaklee & Keenan, 1986) requires some knowledge of the biochemistry of marine fishes and invertebrates.

Most fishery products, fillets, squid rings or shrimp meat, consist of muscle tissue. Some types of seafood, surimi and other fish mince products (Hall & Ahmad, 1992), may contain mixtures of muscle tissue from several species. Surimi, originating from Japan, is an intermediate product prepared from minced fish muscle by washing, dehydrating and stabilizing the myofibrillar proteins (Lee, 1984). After these processing steps, surimi is stored frozen until used for the manufacture of a large number of products, like kamaboko (boiled fish paste) or imitation crab meat.

The present work gives some examples of how different metabolites and specific

proteins of fish and invertebrates can be used for the classification and species identification of products.

MATERIALS AND METHODS

Samples of fish and invertebrates

Samples of fish, Norway lobster, *Nephrops norvegicus*, and northern shrimp, *Pandalus borealis*, were collected on research cruises. Deep frozen seafood (shrimp, squid, mussel meat, imitation crab meat and other products) were obtained at the local fish market. Live green crab, *Carcinus maenas*, was given by Dr. Siebers; frozen green crab and Chinese crab, *Eriocheir sinensis*, were supplied by W. Wienbeck; live common cockle, *Cerastoderma edule*, was made available by Dr. Meixner. Other products were bought in fish shops after being judged to be of high quality.

Chemical analysis

Perchloric acid extracts

Minced tissue (20 g) was thoroughly homogenized for 2 min with 180 ml of cooled aqueous perchloric acid (0.6 M) using a commercial food blender (Krups 3 Mix 3000) or the Ultra-Turrax. The mixture was filtered through a folded filter paper.

Creatine and arginine

The perchloric acid extracts were analysed for creatine by enzymatic analysis as described previously (Oehlenschläger & Rehbein, 1990). Arginine was determined by the same procedure, replacing creatine kinase by arginine kinase (Sigma, St. Louis).

Octopine

Octopine was measured in neutralized perchloric acid according to Gäde (1985) using octopine and octopine dehydrogenase supplied by Sigma.

Arginine kinase

The activity of arginine kinase was measured using a coupled test system with pyruvate kinase and lactate dehydrogenase as auxiliary enzymes (Livera & Shimizu, 1989).

Extracts were prepared by mixing 5 g ot muscle with 45 ml of precooled 100 mM Tris-HCl/1 mM 3-mercapto-propane-1,2-diol, pH 8.0, using an Ultra-Turrax. The homogenate was centrifuged for 30 min at 5 °C at 38000 × g_{max}.

The unit of enzyme activity, U, is defined as the quantity of enzyme that converts 1 µmol of subtrate in 1 min.

Isoelectric focusing (IEF) of water soluble proteins

Servalyt Precotes (Serva, Heidelberg) were used for IEF of sarcoplasmic proteins and handled according to the instructions given by Serva. After IEF, the gels were washed with 20 % weight per volume (w/v) trichloroacetic acid for fixing of proteins and removal of ampholytes. The staining solution contained 0.1 % (w/v) of Serva Violet 49 dissolved in

a mixture of methanol/acetic acid/water, 25/10/65, v/v/v; this solution was also used for destaining.

Preparation of extracts: 5 g of muscle were cut into small pieces and homogenized with 15 ml of pre-cooled distilled water by means of an Ultra-Turrax. The total mixing time, including 2 interruptions, was 2 min; the speed of rotation increased gradually, and warming of the mixture was avoided.

The homogenate was centrifuged using the Eppendorf 5412 Table Centrifuge for 4 min at room temperature (12000 rpm = 8000 × g).

Urea gel IEF

CleanGels (Pharmacia, Uppsala) were used for urea IEF. The gels were rehydrated with a solution containing 8 M urea/0.5 % (w/v) Servalyt 3–10/2.5 % Servalyt 4–6. Fixing, staining and destaining were performed as described above; the destained gels were impregnated by shaking in a solution containing 1 % (w/v) glycerol (87 %, w/w) in distilled water.

Preparation of extracts: 2 g of muscle were cut into small pieces and homogenized with 20 ml of 8 M urea/1 % (v/v) 3-mercapto-propane-1,2-diol/20 mM Tris-HCl, pH 8.0, by means of an Ultra-Turrax. The mixture was kept overnight in a refrigerator (10 °), then homogenized again and centrifuged at 10 °C and 38000 × g_{max} for 30 min.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

CleanGels 5/10 were used for SDS-PAGE following the instructions given by Pharmacia, with the exception of the staining procedure.

Proteins were stained by the following method: (1) The gels were shaken for 20 min in a mixture of methanol/acetic acid/water, 50/10/40, v/v/v, for fixation of proteins and removal of SDS. (2) For staining, the gels were shaken for 15 min in 0.2 % (w/v) Serva Blue R dissolved in fixing solution. (3) Destaining was performed by shaking the gels in a mixture of methanol/acetic acid/water, 20/10/70, v/v/v, until the background was nearly colourless. (4) The destained gels were impregnated by shaking them for 10 min in 5 % (w/v) glycerol. For each step, a volume of 200 ml of solution was used.

The gels were dried at room temperature and then protected by covering with a polyester foil.

Preparation of extracts for SDS-PAGE: 1 g of muscle was cut into small pieces and homogenized with 20 m l of 3 % (w/v) SDS/1 % (v/v) 3-mercapto-propane-1,2-diol/ 80 mM Tris-HCl, pH 6.8, by means of an Ultra-Turrax. The mixture was shaken for 2 h in a waterbath at 60 °C. After cooling to room temperature, the mixture was centrifuged for 30 min at 20 °C at 38000 × g_{max}.

Protein determination

The protein content of extracts used for both types of IEF was measured by means of a Coomassie dye-binding assay (Bio-Rad Protein Assay, Bio-Rad, Richmond).

For protein determination in extracts made with SDS buffer, a modified biuret method, tolerant to SDS and 3-mercapto-propane-1,2-diol, was used (de Wreede & Stegemann, 1981).

Bovine serum albumin served as a protein standard in both methods.

RESULTS AND DISCUSSION

Comparison of guanidino compounds of fish and invertebrates

In recent years, surimi-based seafoods experienced good acceptance in the USA and are now entering the European market. The consumption of imitation crab meat and similar products is expected to rise to nearly 40000 tonnes in 1992 (de Franssu, 1991). Limited amounts of real crab meat are often added to the imitation products for enhancement of quality and price.

In search of a rapid and simple method for the detection and identification of invertebrate meat in imitation products and the differentiation between these products and authentic crab, shrimp, squid or mussel meat, we measured the concentration of guanidino compounds. Creatine and arginine have been found in high amounts in fish and invertebrate muscle, respectively (Konosu & Yamaguchi, 1982). The concentrations of creatine and arginine in a number of fishery products are presented in Table 1.

Raw or cooked tail muscle of shrimps and prawns, as well as the leg and claw muscle of crabs, are rich in arginine (mean value: 438 mg/100 g wet weight, range: 40–697 mg/100 g). The low value for one shrimp muscle may be due to washing procedures. On the other hand, the content of creatine was very low in all invertebrate muscles listed in Table 1.

In contrast to this distribution of guanidino compounds, creatine predominated in light muscle of fish: the mean concentration of creatine in several food fishes was 425 mg/100 g, whereas the concentration of free arginine was about one percent of this value.

The concentration of creatine and arginine in imitation crab meat is given in Table 2. Low and varying amounts of creatine indicate the pronounced washing of the fish flesh during processing. The concentration of arginine was found to be higher than expected for washed fish flesh, indicating the addition of crab or shrimp meat or extracts from such meat to surimi. It was demonstrated by SDS-PAGE and urea IEF, that at least the samples 3 and 4 contained additional proteins to the muscle proteins of Alaska pollack (Schildhauer, 1991).

Analysis of seafood cocktails gave us cause to include meat of cephalopods and mussels in our study. Besides creatine and arginine, octopine was also determined, because it might be an indicator of molluscan meat (Konosu & Yamaguchi, 1982). Octopine, N^{2-}(1-carboxyethyl)-L-arginine, is formed by reductive condensation of pyruvate and arginine in anaerobic glycolysis during short bursts of muscle activity (Gäde, 1985).

In cooked muscle of cephalopods and in raw or cooked mussel meat (being a mixture of different tissues), varying amounts of octopine were detected (Table 1). The concentration ranged from 0 to 102 mg/100 g wet weight (102 mg/100 g = 4 mmol/kg), possibly depending on the physiological stress the animals suffered during catch and storage (Gäde, 1985). Recently, another opine, tauropine, and D-lactate were found to be useful as indicators of the metabolic stress during transport and storage of live New Zealand abalone (*Haliotis iris*) (Baldwin et al., 1992). Enforced exercise and prolonged exposure to air resulted in accumulation of taurine and D-lactate in the foot and adductor muscle

The tail muscle of two shrimps also contained a low amount of octopine (Table 1). If this result can be confirmed by analysis of more samples, octopine can be used as an

Table 1. Content (mg/100 g wet weight) of arginine, octopine and creatine in edible tissues of marine invertebrates and fishes. One extract of each sample was analysed in duplicate for each of the three metabolites. R = raw, C = cooked tissue

Species or product name	Tissue	Arginine	Octopine	Creatine
Shrimps & prawns				
Shrimp[P]	Tail muscle, C	91	8	7
Giant prawn[P]	Tail muscle, R	697	4	•
Giant tiger prawn[P]	Tail muscle, R	676	•	•
Giant prawn[P]	Tail muscle, R	541	•	•
Giant prawn[P]	Tail muscle, R	535	•	•
Shrimp, from Asia[P]	Tail muscle, C	40	•	7
Shrimp, crevette[P]	Tail muscle, C	235	•	4
Shrimp, gamba[P]	Tail muscle, C	451	•	27
Giant shrimp, *Penaeus* sp.	Tail muscle, R	690	•	70
Common shrimp, *Crangon crangon*	Tail muscle, C	147	•	6
Greenland shrimp, *Pandalus borealis*	Tail muscle, C	294	•	15
Crabs				
Mitten crab, *Eriocheir sinensis*	Leg muscle, R	607	•	••
Green crab, *Carcinus maenas*	Leg muscle, R	500	•	••
Snow crab, *Chioneocetes opilio*	Claw muscle, C	625	•	5
Cephalopods				
Squid[P]	Skinned rings, C	18	33	••
Squid[P]	Skinned rings, C	••	7	••
Cuttlefish[P]	Skinned strips, C	36	102	••
Mussels				
Mussel, Ireland[P]	Mussel meat, C	156	3	••
Clam, Thailand[P]	Mussel meat, C	11	••	5
Common muscle, *Mytilus edulis*	Mussel meat, R	67	26	8
Common muscle, *Mytilus edulis*	Mussel meat, C	95	••	••
Common cockle, *Cerastoderma edule*	Mussel meat, R	31	•	5
Fishes				
North Atlantic hake, *M. merluccius*	Light muscle, R	4	•	500
Cod, *Gadus morhua*	Light muscle, R	••	•	441
Alaska pollack, *Theragra chalcogramma*	Light muscle, R	••	•	470
Blue whiting, *Micromesistius poutassou*	Light muscle, R	4	•	372
Saithe, *Pollachius virens*	Light muscle, R	••	•	343

[P]: Commercial product, species unknown
•: not determined
••: below limit of detection (3 mg/100 g wet weight)

indicator for molluscan meat, only if the concentration is above a certain level, which still has to be established.

Comparison of proteins of fish and invertebrates used for species identification in products

The determination of metabolites like arginine, creatine, octopine, trimethylamine oxide (for distinction between marine and freshwater animals) (Hebard et al., 1982) or

Table 2. Content (mg/100 g wet weight) of arginine and creatine in imitation crab meat and other surimi derived products. One extract of each sample was analysed in duplicate for arginine and creatine

No.	Product name	Arginine	Creatine
1	Krabby a la Romana	8	33
2	Surimi prawns a la Romana	11	36
3	Ocean gourmet chunks	19	5
4	Ocean gourmet chunks smooth cut	24	7
5	Surimi lobster chunks	•	32
6	Fish crab meat sticks	11	6

• below limit of detection (3 mg/100 g wet weight)

urea (for distinction between elasmobranchs and teleosts) (Konosu & Yamaguchi, 1982) cannot be applied for the differentiation of animals on the level of species. In food chemistry, electrophoresis of proteins is nowadays mostly used to identify the species which have been processed (Kaiser et al., 1981). Regarding the analysis of muscle-based

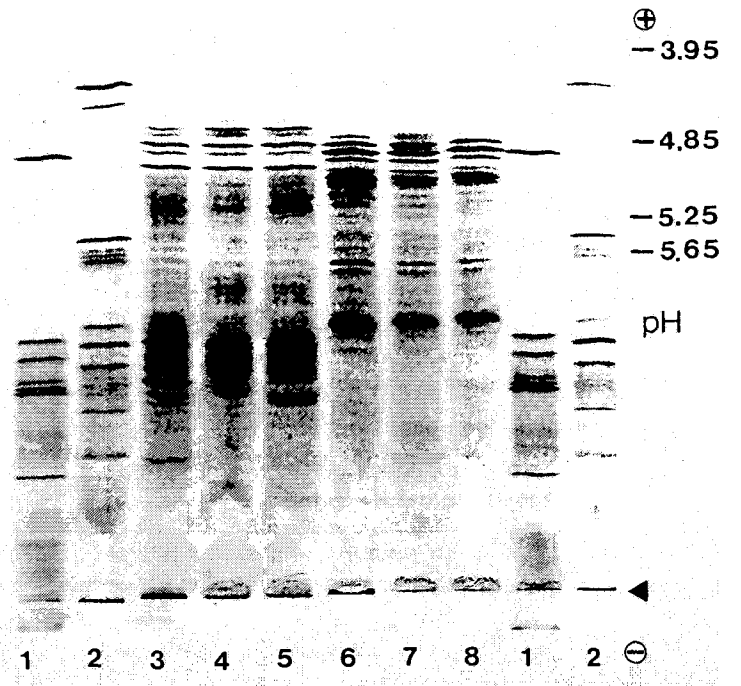

Fig. 1. Patterns of sarcoplasmic proteins of fish and shrimp. The water soluble proteins from raw muscle of (1) redfish, (2) cod, (3–5) three specimens of Norway lobster and (6–8) three specimens of northern shrimp were separated by IEF using Servalyt Precote 3–10, 150 µm. 7.5 µl of extract, containing 7.5–11 mg/ml of protein, was applied to the gel at the position marked by the arrow. The pH-gradient was determined by means of the protein standard VII (Merck, Darmstadt)

fishery products, it depends on the type of product, whether sarcoplasmic or myofibrillar proteins are better suited for this purpose.

Raw, as well as cooked, smoked, cured or canned products have been successfully analysed by isoelectric focusing (IEF) of sarcoplasmic proteins (Rehbein, 1990a). Figure 1 shows the protein patterns for raw muscle of fish, shrimp and Norway lobster; the patterns are species specific. The acidic proteins, having pIs between 4 and 5, are heat-stable (Figure 2) and can be used to identify the species in the case of cooked or canned fish products (Rehbein, 1990b). They belong to the class of calcium-binding proteins (Gerday, 1982; Rehbein, 1992).

Light muscle of fish contains high amounts of parvalbumins (up to 5 mg/g wet weight). Parvalbumins have a low molecular weight (10–12 kD) and their amino acid composition is characterized by a high content of phenylalanine and acidic amino acids. Tyrosine, tryptophan, histidine, proline, cysteine and methionine are found only in small amounts or not at all, and most parvalbumins have an unusual UV spectrum, which can be applied for their identification (Gerday, 1982).

Parvalbumins are restricted to vertebrates. In many invertebrate phyla, the function-

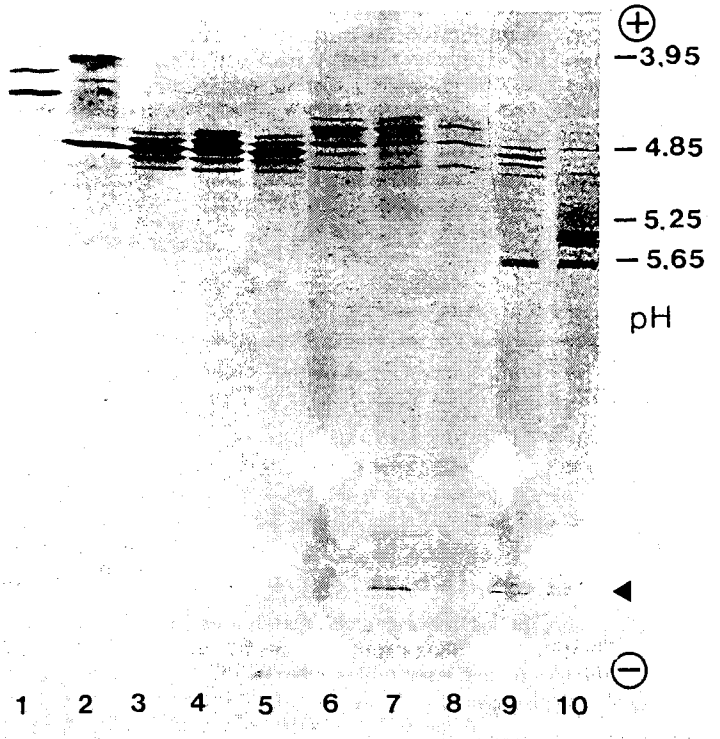

Fig. 2. Heat-stable sarcoplasmic proteins of fish and shrimp. Raw extract was heated (15 min at 95 °C), and clarified by centrifugation. 7.5 µl of supernatant, containing 2–4 mg/ml of protein, was analysed by IEF with Servalyt Precote 3–10, 150 µm. The position of the applicator strip is marked by the arrow. Samples: (1) cod, (2) redfish, (3–5) three specimens of northern shrimp and (6–8) three specimens of Norway lobster. Sample 9 and 10 are extracts prepared from cooked meat (20 min at 75 °C) of northern shrimp (9) and Norway lobster (10)

ally similar soluble "sarcoplasmic calcium-binding proteins" (SCPs) have been detected, having the monomer molecular weight of 20–22 kD (Wnuk et al., 1982). SCP have been found in the muscle of lobster, shrimp, crab, mussel *Mytilus edulis*, but not in squid, cuttlefish or octopus (Wnuk et al., 1982).

Parvalbumins and SCPs exhibit extensive polymorphism between species, a very useful property for electrophoretic species identification (Rehbein, 1992).

Raw and cooked flesh of fish, shrimp or crab, as well as fishery products, where the sarcoplasmic proteins have been removed by washing steps during processing, have been analysed by urea IEF or SDS-PAGE to identify the species (Scobbie & Mackie, 1988; Wei et al., 1990; Civera & Parisi, 1991). By use of urea or SDS as extractive, not only residual sarcoplasmic proteins, but also myofibrillar proteins are solubilized (Fig. 4). Figures 3 and 4 give examples for protein separation by urea IEF and SDS-PAGE. Specific protein patterns have been obtained with both techniques for the three fish species, with a clearer distinction between salmon and trout by urea IEF. As the origin of some of the commercial invertebrate samples is not certain at the species level, it can here only be stated that mussel meat, and flesh of cephalopods and shrimps gave different protein patterns with urea IEF. In SDS-PAGE, the differentiation between species mainly relies on the differences in molecular weight of the myosin light chains (Seki, 1976). SDS-PAGE of myofibrillar/sarcoplasmic proteins had not the same power of discrimination as

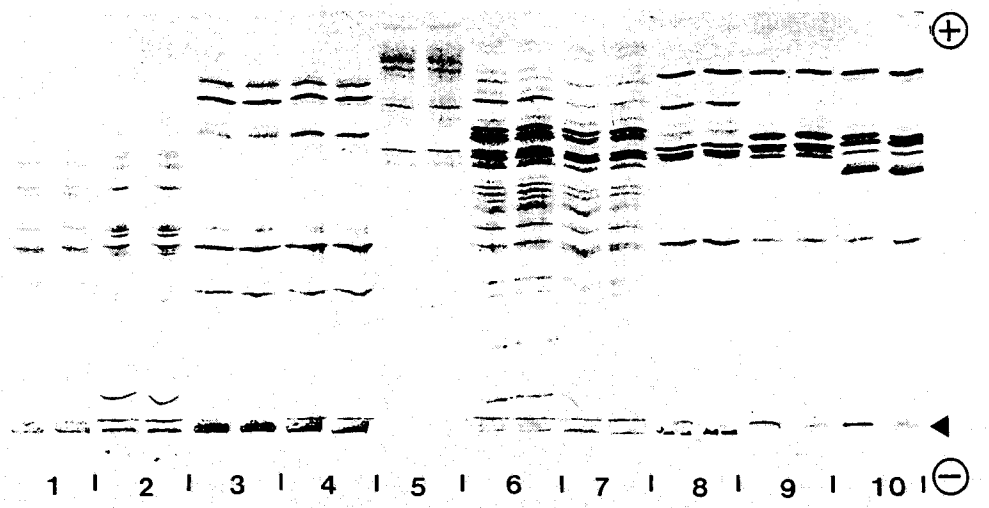

Fig. 3. Urea gel IEF of muscle proteins from fish and invertebrates. Raw (R) or cooked (C) muscle was extracted with 8 M urea/1 % (v/v) 3-mercapto-propane-1,2-diol/20 mM Tris-HCl, pH 8.0. The extracts, containing sarcoplasmic and myofibrillar proteins (in the case of mussels, proteins from other tissues, too), were analysed by IEF, using a CleanGel rehydrated with 8 M urea/0.5 % (w/v) Servalyt 3–10/2.5 % Servalyt 4–6. 7.5 µl of extract from the following samples were applied in duplicate to the gel at the position marked by the arrow: (1) *Mytilus edulis*, R, protein content: 8.2 mg/ml; (2) mussel meat from Ireland, presumably *M. edulis*, C, 14.1 mg/ml; (3) squid, skinned rings, C, 8.8 mg/ml; (4) cuttle fish, skinned strips, C, 9.7 mg/ml; (5) IEF calibration kit, range 2.5–6.5 (Pharmacia, Uppsala); (6) giant prawn, R, 14.8 mg/ml; (7) shrimp, C, 10.9 mg/ml; (8) *Pollachius virens*, saithe, R, 17.3 mg/ml; (9) *Salmo salar*, Atlantic salmon, R, 21.7 mg/ml; (10) *Oncorhynchus mykiss*, rainbow trout, R, 19.5 mg/ml. Volt hour product: 6479 Vh

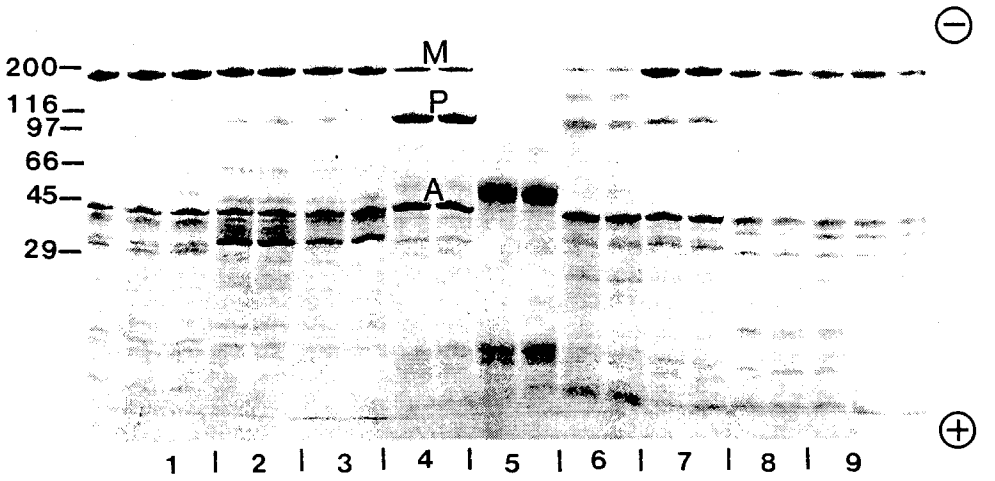

Fig. 4. SDS-PAGE of muscle proteins from fish and invertebrates. Raw (R) or cooked (C) muscle was extracted with 3 % (w/v) SDS/1 % (v/v) 3-mercapto-propane-1,2-diol/80 mM Tris-HCl, pH 6.8 and analysed by SDS-PAGE, using a CleanGel (stacking gel: T = 7.5 %; separating gel: T = 10 %). 10 µl of extract from the following samples were applied in duplicate to the gel: (1) *Pollachius virens*, saithe, R, 9.5 µg of protein; (2) *Salmo salar*, Atlantic salmon, R, 9.9 µg; (3) *Oncorhynchus mykiss*, rainbow trout, R, 8.9 µg; (4) mussel from Ireland, presumably *M. edulis*, adductor muscle, C, 9.1 µg; (5) mussel from Ireland, mantle, C, 10.8; (6) squid, skinned rings, C, 11.1 µg; (7) cuttle fish, skinned strips, C, 10.6 µg; (8) king prawn, R, 7.1 µg; giant shrimp, R, 9.2 µg. Myosin heavy chain (M), paramyosin (P) and actin (A) were identified using the SDS molecular weight markers (MW-SDS-200) from Sigma (St. Louis). The molecular weights corresponding to the positions of the marker proteins in the gel are shown on the left side of the figure.

IEF of sarcoplasmic proteins, but the method was found to be reliable for the identification of many fish species and *Nephrops norwegicus* (Scobbie & Mackie, 1988).

SDS-PAGE has the advantage that proteins can be identified easily by their molecular weight (Fig. 4). For example, the presence of muscle from molluscs can be demonstrated by paramyosin, which is absent from vertebrate muscle (Kantha et al., 1990).

The enzyme arginine kinase (EC 2.7.3.3) was used as another tool for the differentiation between muscle food from invertebrates and fishes. As is to be expected (Livera & Shimizu, 1989), high activities were measured in extracts of raw crustacean muscle (Table 3), whereas the enzyme activity was hardly detectable in fish muscle. Unfortunately, the enzyme activity was destroyed by heating the muscle of prawn to 60 °C (Table 4), preventing the application of the arginine kinase test to the analysis of imitation crustacean meat (Table 3).

However, recently it was found that thermal denaturation did not result in loss of antigenic properties of arginine kinase (Verrez et al., 1992). Polyclonal antibodies against lobster (*Homarus vulgaris*) muscle arginine kinase were used to detect crustacean muscle (snow crab) in heated mixtures of surimi from blue whiting *Micromesistius poutassou* and crab meat by the immunodot technique.

Using the same technique, but working with anti-paramyosin antibodies, it was possible to differentiate crustacean and mollusc tissues. Crustacean meat exhibited no

Table 3. Arginine kinase activity in raw (R) or cooked (C) muscle of shrimp, crab and fish, and in imitation crab meat

Fishery product: species and tissue	Arginine kinase activity (U/mg protein)
Penaeus sp., giant prawn, tail muscle, R	44.4
Carcinus maenas, green crab, leg muscle, R	11.5
E. sinensis, mitten crab, leg muscle, R	11.7
Ch. opilio, snow crab, claw muscle, C	0.04
Th. thynnus, blue fin tuna, light muscle, R	0.04 / 0.03 (–arg)•
C. harengus, herring, light muscle, R	0.05 / 0.05 (–arg)
Th. chalcogramma, Alaska pollack, light m., R	0.09 / 0.12 (–arg)
Fish crab meat, C during processing	0.07
Imitation crab meat, C during processing	0.05
Imitation crab meat, C during processing	••
Crab meat, C during processing	0.13

• Arginine was omitted from the assay, but might be present in low concentration in the extract (50–200 µl) tested
•• Below limit of detection (delta E_{340} less than 0.01)

Table 4. Thermal inactivation of arginine kinase. Tail muscle of giant prawn *Penaeus* sp. was mixed with the twofold amount of water. The homogenate was heated for 30 min at different temperatures (40–90 °C), cooled and extracted for determination of specific arginine kinase activity (U/mg) protein). The activity in the extract from the unheated sample was 27.9 U/mg protein

Heating temperature (°C)	Arginine kinase activity (% of activity in unheated sample)
40	106
50	5.4
60	0.1
70	0.2
80	0.2
90	0.4

immunological reactivity, in contrast to meat of squid *Todarodes sagittatus*, clam *M. mercenaria* and grooved carpet shell *Venerupis decussatus*.

Acknowledgements. My thanks go to G. Kress, R. Kündiger and T. Schmidt for skilful performance of the experiments. I thank Dr. Meixner, Dr. Siebers and W. Wienbeck for delivering samples of mussel and crab.

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