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Protein Blends: Plant and Animal Proteins

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Abstract

Plant proteins have gained interest in food industry due to their low cost and sustainability. Subsequently, an interest on protein blends between animal and plant proteins increased. However, a partial or complete replacement of animal proteins by plant proteins leads to some undesired changes in the perception of food products, such as off-flavours. This project focuses on the molecular interactions between whey and pea proteins which determine the macroscopic functionality of protein blends. Heat stability and emulsifying and gelling capacities were the properties studied by using methods like RP-HPLC, particle size analyses (Mastersizer), SDS-PAGE and texture analysis. The mixtures of whey and pea protein showed high stability in terms of their emulsifying properties. However, the fat content had an impact on the particle size of the emulsions during storage. Heat stability studies showed that pH 6.5 (pH close to pI of some of the pea proteins) led to protein aggregation and precipitation, while increasing pH to 7.1 or 7.4 increased dispersibility of pea proteins in water. It was found that both proteins influenced each other behaviour and that the heating time increased the dispersibility of pea proteins in water. The addition of NaCl only increased protein aggregation during heating. Finally, GDL-induced cold gelation was studied as well as yoghurt formulation. A homogenization and a pre-heating step were necessary to prevent pea proteins sedimentation and obtain firmer gels. The yoghurt went from a lumpy to a creamy texture when WPI was replaced by pea proteins, but the pea off-flavours were perceived.

Keywords: Whey proteins, pea proteins, emulsification, heat stability, acid-induced cold gelation, yoghurt.

Introduction

Proteins play an important role in food processing since they are responsible for changes in structure, texture, taste and flavour, which influence the consumer perception of the products. The preference of the consumers has been changing in consequence of their awareness about the nutritional value of food and the environmental impact of food production. Therefore, the demand requires sustainable and highly nutritional products. The use of protein blends between animal and plant proteins has been rising since plant proteins are sustainable and cost effective, nevertheless less functional than animal proteins. [1] [2] [3] [4] Another problem is that a partial or complete replacement of animal proteins by plant proteins often leads to undesired changes in the texture and perception of food products. This is due to the interactions between the proteins. When two proteins are mixed together three different scenarios can take place; They can stay mixed on a molecular scale throughout the gelation process (single phase – compatibility) but the trend is usually to phase separate, segregation (bi-continuous network or spherical enclosures), or to create complexes, which eventually may separate into two phases, as well. [5] [6] [7] In the present work, the interactions between whey and pea proteins will be studied.

Milk is a main constituent on the human diet, not only consumed in its natural form, but also in the form of a wide range of dairy products. Caseins and whey proteins (also called, serum proteins) are the main groups of proteins present in milk, which represents 78.3 and 19 %(w/w), respectively, of the protein content. According to the primary structures, four different groups of caseins can be distinguished, such as αs1-casein (38%), αs2-casein (10%), β-casein (36%) and k-casein (13%). Casein is not a globular protein, opposed to whey proteins and it has an isoelectric point of 4.6. The major proteins in whey are β-lactoglobulin (β -Lg), α-lactalbumin (α-la), although bovine serum albumin (BSA) and immunoglobulin (Ig) are also part of these group of proteins. β –Lg is a globular protein and it is the major whey protein in the milk of ruminants (representing 50% of the total whey protein). It belongs to the lipocalin family, contains 162 amino acids per monomer, one free sulfhydryl group, two intramolecular disulphide bonds and it has an isoelectric point of 5.2. β-lg is a thermosensitive protein, loosing solubility and unfolding when exposed to temperatures above 72.8ºC, which is

its denaturation temperature. It molecular weight (MW) is between 18 and 20 kDa. α–la is also a globular protein and the variant present in the bovine milk (B) has a MW around 14kDa. It has eight cysteine residues in the four disulphide bonds and three aspartic acid residues which stabilize the calcium binding loop. A denaturation temperature of 65.2 ºC has been reported for this protein. [8] [9]

Pulses provide energy, dietary fiber, protein, minerals and vitamins required for human health. Recent research studies suggest that consumption of pulses may have potential health benefits, including reduced risk of cardiovascular diseases, cancer, diabetes, osteoporosis, hypertension, gastrointestinal disorders, adrenal disease and reduction of LDL cholesterol. From the nutritional perspective, pulses are of particular interest because they contain high amounts of protein (18–32%). However, the off-flavours and colour that these proteins can confer to the final products as well as the presence of anti-nutritional factors which reduce digestibility are the biggest barriers to their consumption and limit the expansion of pulse ingredients into mainstream food applications. The off-flavours in pea are mainly caused by volatile compounds, which are partly inherent to peas themselves and partly developed during harvesting, processing and storage. The most frequently flavours associated to pea are green, beany, pea, earthy, hay-like, bitter and astringent. The most abundant proteins in pea are legumin, vicilin and albumin, being the first two globular proteins. Pea legumin is a hexameric protein consisting of an acidic and basic polypeptide of ~40 and ~20 kDa, respectively, that are linked covalently through a disulphide bond. It has a molecular weight of 60 kDa and its isoelectric point is 4.8. Pea vicilin is a heterogenic trimmer protein with each subunit 50 kDa in molecular weight. Vicilin contains a high content of aspartic acid and serine. Its isoelectric point is 5.5. Two major albumin proteins have been identified in pea. The major albumin protein contains two polypeptides with molecular weight around 25 kDa, whereas the minor albumin protein contains polypeptides with 6 kDa. [10] [11] [12] [13] [14] [15] [16]

During the production of commercial food, the proteins may be exposed to a wide range of processing steps, such as thermal treatment (pasteurization or sterilization), shear (pumping, mixing homogenization), precipitation (heat, acid, salts, solvents), among others, which can modify the functional properties of the proteins, such as solubility, heat stability, water and fat holding capacity, emulsification and gelation. solubility is of primary importance due to its significant influence on the other functional properties of proteins. [17] The three main factors determining solubility of a protein are intermolecular electrostatic and hydrophobic interactions, and molar mass. [8] Thus, the pH which affects the electrostatic interactions and temperature that can lead to denaturation influences the solubility. It was found that whey proteins are much more soluble than pea proteins. [8] [17] [18] Globular proteins can be subject to denaturation, while caseins are very heat stable. Proteins are surface-active molecules and possess good emulsifying capacity and stability due to the presence of both hydrophobic and hydrophilic amino acids. The presence of protein in the oil-water interface greatly reduces the interfacial tension in emulsions and acts like an electrostatic, structural and mechanical energy barrier. In general, plant proteins form a relatively thicker interfacial layer at oil/water interfaces, compared with dairy proteins, due to their much larger molecular size and structural constraint by disulphide crosslinks. [19] [20] [21] Gelation is induced by lowering the pH of the milk. In dairy practice this is the result of the conversion of lactose into lactic acid by lactic acid bacteria. For models experiments it is much easier to mimic this process by the addition of an ester, in this case Glucono-δ-lactone (GDL), that hydrolyses to a weak acid, gluconic acid. This procedure, cold gelation, is a common practice in dairy research but also applied in certain products. In the yoghurt formulation, the common fermentation temperature used is 42 ºC. However, the use of slightly lower incubation temperatures (e.g. 40°C) leads to slightly longer gelation times and, thus, firmer and more viscous gels that are less prone to whey syneresis.

The goal of the present work is to increase the knowledge on protein blends between animal (whey proteins, milk, milk protein concentrates) and plant proteins (pea protein) by relating their molecular interaction to the macroscopic functionality (emulsification, gelation and heat stability).

Materials and Methods

Protein isolates

Whey protein isolate (WPI) with a protein content on wet basis of 94%(w/w). The pea protein isolate (PPI) used in the major experiments in the present work was produced at NIZO (PPI-A). Two other pea protein isolates were used in the last set of experiments, referred as PPI-B and PPI-C.

Milk

The milk used for the GDL experiments and for the yoghurt was pasteurized milk produced by Campina with a protein content around 3.5 %(w/w) and a fat content of 1.5 %(w/w) of which 1.1 %(w/w) is saturated.

Emulsions

Solutions with 3.5 %(w/w) of protein content and different ratios between protein and fat content were prepared. For each ratio protein:fat (1:0.5: 1:5 and 1:10) were prepared 5 different solutions that differ on the amount of WPI and PPI (WPI:PPI = 1:0; 0.75:0.25; 0.5:0.5; 0.25:0.75; 0:1). The protein powder (WPI and/or PPI) was dispersed in water during 30 minutes and the left stirring overnight in the cold room (4ºC). In the day after the sunflower oil was added in a concentration of 1.75% (w/w) followed by the pre-homogenization step – Turrax for 1 min at 10,000 rpm. The solution was then homogenized in a Homogenizer NIRO-SOAVI Panda 2K at 200/50 bar and were done 3 passages. To see which were the proteins stabilising the emulsion, 9 g were taken from each emulsion and centrifuged at 10,000 *xg* for 1 hour at room temperature to separate the fat phase from the serum phase. Then samples were taken from the fat phase, which was later analysed in RP-HPLC. An attempt to obtain a better separation between the fat and serum phase was made; The centrifugation was, sometimes, performed in the presence of 60% (w/w) sucrose solution. It was added 3g of 60% (w/w) sucrose to 6g of the emulsion to achieve a 20% (w/w) sucrose. To study the stability of the emulsions overtime, the particle size of the emulsions was analysed in day 0, 1 and 3 when possible by using the Mastersizer. 2000 from Malvern. These measurements were performed in duplicates or triplicates for each sample, depending on the reproducibility of the two first measurements.

Heat Stability

The WPI and PPI-A were dissolved in water or 50mM NaCl to obtain 3.5 %(w/w) as final protein content. Five different WPI:PPI ratios were studied – 1:0, 0.75:025, 0.5:0.5, 0.25:0.75 and 0:1. After stirring the samples for 15 minutes the pH was adjusted to 7.1 or 7.4 with NaOH (1M) or HCL (1M). Then the solutions were stirred for more 30 minutes and divided in 10 tubes with 10 mL each. This tubes were immersed in an oil bath at 120ºC and every minute during 10 minutes one of them was taken out of the bath. The samples (10 mL) were centrifuged at 4500 *xg* for 30 minutes at RT and the supernatant was removed and analyzed with a Moisture Analyzer (Sartorius YPD20-0CE) in order to calculate the dry matter present in the sample. The pellet was weighted, freeze dried and measured again when dried. The experiment was done in duplicate. Samples from the minute 1, 5 and 10 of each experiment were taken and analyzed in SDS-PAGE. The same protocol was used to the experiments with minerals, where the proteins were dispersed in 50mM NaCl instead and the minerals, calcium and magnesium, were added in the form of calcium and magnesium chloride, respectively. Two different concentrations of Ca and Mg were tested. Firstly, 0.19 and 0.02 %(w/w) and then 0.1 and 0.01 %(w/w) of calcium and magnesium, respectively in both situations.

GDL Experiments

The protocol for the experiments with GDL was improved between each trial based on the results of the previous one. The final procedure, which was concluded to be the best is the following: WPI and PPI were dispersed into the milk during 30 minutes according to the WPI:PPI ratio desired (1:0, 0.75:0.25, 0.5:0.5, 0.25:0.75 and 0:1) and to obtain 2% (w/w) of these proteins in the solution (a final protein content of 5.5 %(w/w) was reached); The pH was adjusted to 7.1 with 1M NaOH or 1M HCl; Homogenization in the Homogenizer NIRO-SOAVI Panda 2K at 400/50 bar and 1 passage; Pre-heating in a water bath at 95ºC for 20 minutes; Cooling of the samples until RT; Addition of GDL (0.5, 0.75, 1.0, 1.25 and 1.5 %w/w) and stirring for 5 minutes; Incubation in a water bath (25ºC) for 24 hours.

The homogenization and the pre-heating steps were not always performed in the order described above. Sometimes were performed the other way

around, first pre-heating followed by homogenization, and other times only one of them was performed. Different heating times (5, 10, 20 and 30 minutes) were tested until it was decided to use 20 minutes for the pre-heating step. Moreover, also the incubation temperature suffered some changes until reaching the conclusion that 25 ºC was the best; It was also tested 20 and 37 ºC. After the incubation (24h) the texture was analysed using a texture analyser (XT plus, Stable Micro Systems Ltd) and the amount of syneresis and the final pH were measured. The amount of syneresis was measured by removing the syneresis to a container and then measured on a scale.

Yoghurt

The protein powders were dispersed in pasteurized milk for 30 minutes to obtain 2 %(w/w) of total WPI and/or PPI in the solution. The amount of each protein depends on the WPI:PPI ratio that is wanted in the end – 1:0, 0.75:0.25, 0.5:0.5, 0.25:0.75 or 0:1. After that the pH was adjusted to 7.1 or 7.4 with 1M NaOH. Followed by 1 passage in the homogenizer NIRO-SOAVI Panda 2K at 400/50 bar and then the pre-heating step in a water bath at 95ºC for 20 minutes. In the next day the fermentation was performed at 42ºC. The frozen pellets of acid lactic bacteria (Thermophilic YoFlex culture) were thawed in a sterilized cup. Then the cultured was diluted 10x in 9 mL of PFZ buffer (Tritium Microbiologie BV) and vortex. The diluted culture was inoculated to 0.05% (w/w) of the undiluted culture. The pH was monitored during the fermentation and when pH 4.6 was reached the samples were taken out from the water bath, mixed and passed through a syringe to mimic the homogenization step in the stirred yoghurt. Finally, they were stored for maximum 4 days in a cold room at ~7ºC to stop the bacterial activity.

On the last set of experiments sugar was added to the solutions. Sugar was added at a 2 %(w/w) concentration and mixed together with the protein powders in the beginning of the procedure. When flavours were added, they were added after the syringe step and at 0.1% (w/w) of concentration.

In the day after the fermentation, the texture of the yoghurts was measured in the texture analyser (XT plus, Stable Micro Systems Ltd). In the most of the cases an informal tasting was performed.

The informal tastings were performed with a minimum of 3 people and maximum 7, where at least two of them were present in all the informal tastings performed during this project. The yoghurt was always tasted when it was cold, usually right after it was taken from the cold room.

SDS-PAGE

In the present work, the SDS-PAGE was performed in reducing conditions using DTT from Sigma- Aldrich. The samples were diluted with MiliQ water to 2mg/mL and then 50 µL of the diluted sample were added to 50 µL of Laemmli buffer from BIO-RAD. The buffer was previously mixed with DTT to have 15 mg DTT per mL of buffer. As it is in the presence of reducing conditions, the samples were incubated for 5 minutes at 90 ºC. Then they were centrifuged at 15,000 *xg* for 4 minutes (Eppendorf Centrifuge 5417C) and 10 or 5 µL were pipetted into each well depending if it was a 12+2 or 26 well gels, respectively. After the run is finished the gels were placed in Instant Blue (Expendeon). The gels used in the present work were 12+2 or 26 well Criterion TGX Precast Gels (BIO-RAD) with 12% gradient and the electrophoresis was performed at 200 V and sometimes on ice. The protein standard used was Precision Plus Protein Standards from BIO-RAD.

RP-HPLC

The equipment is constituted by an Ultimate 3000 pump, WPS auto sampler, column compartment and a diode array detector (ThermoFisher Scientific). The analyses were performed by inject 10 µL of the sample into a wide-pore C18 analytical column (Phenomenex) thermostated at 40 ºC. Proteins were eluted at a flow rate of 0.40 mL/min with a linear gradient of 0.10 v/v% trifluoroacetic acid (TFA) in 98 v/v% water + 2 v/v% acetonitrile to 0.10 v/v% trifluoroacetic acid in 40 v/v% water + 60 v/v% acetonitrile in 47 minutes. Data analysis was done with Chromeleon software version 7.2 (ThermoFisher Scientific). For protein solutions with 3.5 %(w/w) of protein content, 100 µL were diluted in 300 µL of Buffer E containing 20 mg/mL DTT. After 1 hour of incubation at RT, 1.5 mL of Buffer D are added and then the sample is filtered to the HPLC container over a 0.22 µm filter (Merck Millipore Ltd).

Mastersizer

To perform these measurements a few droplets of the emulsions were dropped into a compartment of the Mastersizer (Malvern Mastersizer 2000), which had water. The amount of emulsion added to the water was dependent of the laser obscuration, which should be between 9 and 10%, in order to obtain good measurements, where the particles could be described as isolate spheres. After the analysis, the program reports a graphic with the particle size distribution as a function of a volume equivalent sphere diameter.

CLSM

In the present work, the preparation of the samples consisted in adding 0.02% (w/w) of Nile Blue.

Texture Analyser

In the present work, it was used a cylindrical stainless-steel probe of 1 cm diameter. The texture analyser was calibrated with a 5 Kg weight. Compression was the test mode defined. The pre-test, test and post-test speeds used were, respectively, 1.00, 0.50 and 5.00 mm/sec. The target mode was distance defined as 20.0 mm. The trigger type it was defined as Auto (Force) with the trigger force of 0.1 g. The advanced options were off. The measurements were always done at least in duplicate and sometimes in triplicate. In the GDL experiments, the breaking point was the value used to compare the different samples, whereas in the yoghurt experiments the value used to comparison was the maximum gramme-force used.

Results and Discussion

In this section, only the results from the heat stability and stirred yoghurt experiments will be shown.

Heat Stability

All the experiments were done in duplicate so the values in the graphic are the average between the duplicates, since it was very reproducible.

pH Influence

In the first set of experiments, three different pH values were studied, 6.5, 7.1 and 7.4. However, pH 6.5 is closer to the isoelectric point of some of the pea proteins, so when pea protein is present the formation of aggregates increased and it was observed precipitation of the most pea protein (Figure 1). Thus, from now on only pH values 7.1 and 7.4 were analysed.

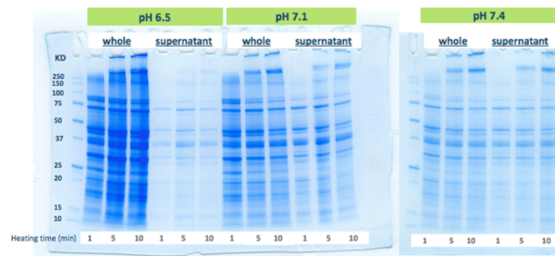

Figure 1 - SDS-PAGE from samples with ratio WPI:PPI of 0:1. The whole solution and the supernatant were analysed to minutes 1, 5 and 10 and to different pH values (6.5, 7.1 and 7.4).

Divalent Cations Effect

The heat stability of protein solutions containing 0.1 or 0.19%(w/w) and 0.01 or 0.02%(w/w), of calcium and magnesium, respectively, were studied. In both set of experiments, the system obtained after the heating step was very unstable. A gel was formed when only whey protein was in the presence of the divalent cations, even for the lower concentration of both, calcium and magnesium. As the pea protein was added to the system, the gelation doesn't occur but a lot of aggregates were formed making an unstable system as well. The gelation and such unstable system in the presence of a low protein concentration can be due to the fact of the presence of a lot of globular proteins that is not usual in this kind of products. Usually milk concentrate is used in clinical food that, besides whey proteins, contains as well caseins which, because of being heat stable, compensate the presence of globular proteins contributing to a more stable system. Furthermore, in most of the cases in this industry is used insoluble calcium and magnesium salt, which prevents aggregation of the proteins and precipitation, allowing them to stay in suspension.

Protein solutions in water and in 50mM NaCl

Visual Appearance

After the oil bath at 120ºC it was visible in all the experiments (at pH 7.1 and 7.4, in water or NaCl), that the samples with only whey protein are transparent but as pea protein was added to the system they become more turbid and yellowish (Figure 2 and Figure 3). This is explained by the presence of larger particles in pea proteins that scatter more light.

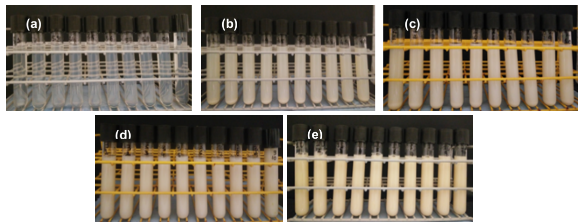

Figure 2 - Samples after the oil bath at 120ºC, from minute one (first sample on the left side of each picture) to minute ten (last sample on the right side of each sample) and five different ratios of WPI:PPI, dissolved in water and pH 7.1. From (a) to (e) is represented ratio 1:0, 0.75:0.25, 0.5:0.5, 0.25:0.75 and 0:1, respectively.

The turbidity increased as the salt was added to the system (Figure 3).

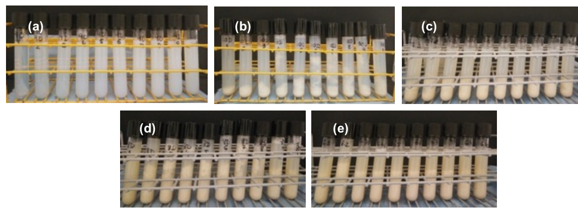

Figure 3 - Samples after the oil bath at 120 C, from minute one (first sample on the left side of each picture) to minute ten (last sample on the right side of each sample), at five different ratios of WPI:PPI, dissolved in NaCl and pH 7.1. From (a) to (e) is represented ratio 1:0, 0.75:0.25, 0.5:0.5, 0.25:0.75 and 0:1, respectively.

SDS-PAGE

With the SDS-PAGE analysis was, in general, observed an increase on aggregation with the heating time for any ratio of WPI:PPI, since the denaturation increased as the samples stayed more time in the oil bath. Even though the samples with only whey were transparent (Figure 2), they still have aggregates present, which are soluble since they are present in the whole solution and in the supernatant.

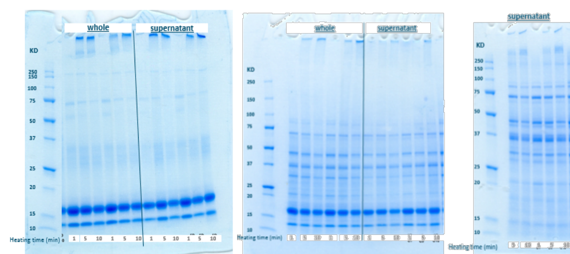

Figure 4 - SDS-PAGE from the whole solution and the supernatant at minute 1, 5 and 10 of the heating step and at pH 7.1 and protein solutions in water. From left to right, three different WPI:PPI ratios can be observed: 1:0, 0.5:0.5 and 0:1.

With the SDS-PAGE was possible to confirm the increased of aggregation and precipitation in the presence of NaCl.

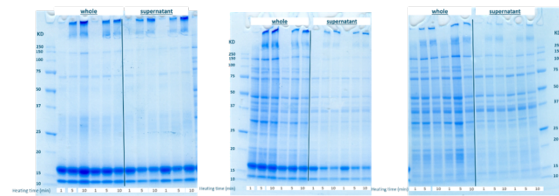

Figure 5 - SDS-PAGE from the whole solution and the supernatant at minute 1, 5 and 10 of the heating step and at pH 7.4 and proteins solutions in 50mM NaCl. From left to right, three different WPI:PPI ratios can be observed: 1:0, 0.5:0.5 and 0:1.

DM in the pellet

Regarding the dry matter present in the pellet, was observed that the heating promotes the dispersibility of the proteins in water and there is more soluble material when more whey protein is present.

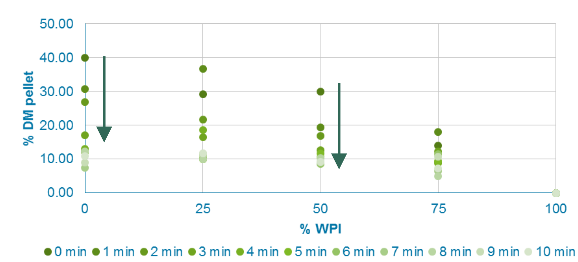

Figure 6 - Percentage of dry matter in the pellet, at pH 7.4, as a function of %WPI from protein solutions dispersed in water. Even though the measurements were done in duplicate, the standard deviation was not represented for the clarity of the results.

However, when proteins are dispersed in NaCl as more whey protein is present in the system less soluble material is present leading to an increase on the dry matter present in the pellet.

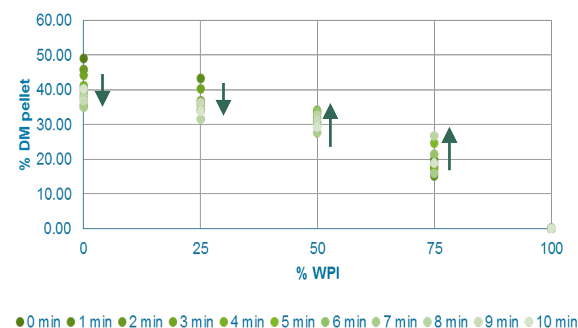

Figure 7 - Percentage of wet pellet, at pH 7.4, as a function of %WPI from proteins solutions dispersed in 50 mM NaCl. Even though the measurements were done in duplicate, the standard deviation was not represented for the clarity of the results.

Comparison

Regarding the protein solutions in water it was seen that by increasing the pH and the heating the protein dispersibility increased, being this effect

more accentuate in the present of pea proteins. Since the decrease of the percentage of dry matter in the pellet with the increase of whey protein is not linear it was also possible to conclude that both proteins, whey and pea proteins, are interfering with each other behaviour; either whey proteins are preventing pea proteins to dissociate or pea proteins are promoting the whey proteins to aggregate on its own. The last hypothesis is in accordance with what the RP-HPLC results suggested.

As the salt is added, it was observed that neither the pH or the heating time have a big impact on the wet pellet or in the dry matter in the pellet. It is also visible that the dry matter present in the pellet is higher on the presence of NaCl than in water, which explain the turbidity observed.

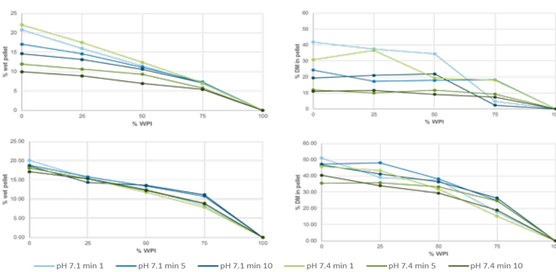

Figure 8 - Percentages of wet pellet (on the left) and dry matter in the pellet (on the right) for the proteins solutions in water (graphics above) and in 50 mM NaCl (graphics below). Even though the measurements were done in duplicate, the standard deviation was not represented for the clarity of the results.

Stirred Yoghurt

The GDL experiments allowed us to define the yoghurt protocol. The stirred yoghurt was produced by running a fermentation with acid lactic bacteria.

Effect of the Starting pH

Visual Appearance

The increase in pea protein content in the samples leads to a decrease in particle size that is possible to see in Figure 9**Erro! A origem da referência não foi encontrada.**. Even though the increase in yellow colour is not that much visible in the picture, was another effect of adding pea protein to the mixture.

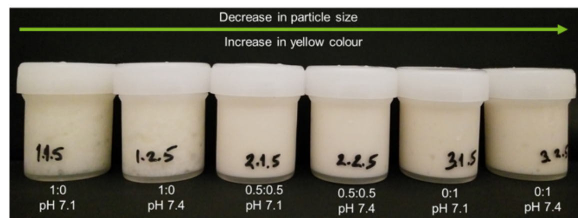

Figure 9 - Samples from the fermentation at 42ºC. Three different WPI:PPI ratios, 1:0, 0.5:0.5 and 0:1, from left to right, respectively and two different initial pH values (7.1 and 7.4) were studied.

Informal Tasting Session

Comparing the different compositions in protein, it was observed a very creamy yoghurt in the presence of pea protein whereas when only whey protein was present the structure was not homogeneous and was lumpy, similar with cottage cheese. The appearance of lumps in the presence of whey proteins suggest that the heating treatment was too harsh to these proteins, which have a lower denaturation temperature when compared to pea proteins. A hypothesis to decrease this effect is to add fat to the yoghurt, which will break the texture and maybe lead to a smoother yoghurt when whey protein is present. However, in the presence of pea protein the fat may have a negative impact since the yoghurt obtained in the presence of this protein was already very soft and, thus the presence of fat can lead to a very liquid yoghurt.

The presence of pea protein brings the downside of the cardboard taste and the astringency, which will be later try to mask with some flavours. Comparing both starting pH values, it was observed that at pH 7.4 the astringency and the pea flavour were less intense than at pH 7.1. Thus, the next experiments will be done with the starting pH of 7.4 The summary of the main characteristics is described in Figure 10.

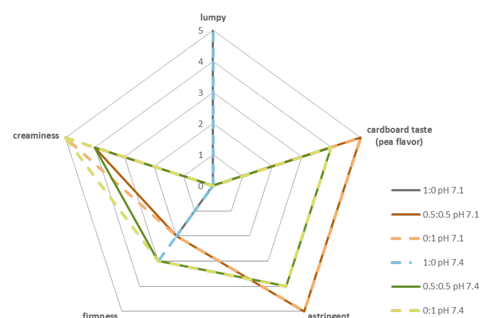

Figure 10 - Sensory profile of 6 different yoghurts. They different in WPI:PPI ratio (1:0, 0.5:0.5 and 0:1) as well as in the starting pH (7.1 or 7.4)

Overview with 5 different WPI:PPI ratios at pH 7.4

Based on the feedback of the tasting and in the gel stiffness of the previous set of experiments it was decided, from now on, to use as starting pH the pH 7.4 and to do the 5 different ratios of WPI:PPI to have an overall view of the effect of the pea protein.

Acidification rate

It is possible to see that the acidification rate is faster as more pea protein is present and dramatically changes when only 25% of the system is pea protein. It is noteworthy that when it is present more than 50% of pea protein the acidification profiles don't suffer further changes with the addition of more pea protein.

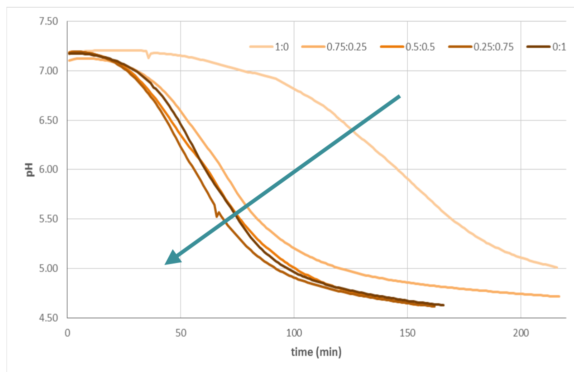

Figure 11 - Acidification rate during the fermentation. From the light to dark orange are represented the WPI:PPI ratios of 1:0, 0.75:0.25, 0.5:0.5, 0.25:0.75 and 0:1.

Texture Analysis

In Figure 12 it is possible to see that from the point that the pea protein is added to the system the texture and the firmness become completely different. It becomes much less firm and the texture becomes creamier with the pea present and less lumpy as it was in the presence of whey. It is possible to see, as it was seen in the acidification rate, that with ≥50% of pea protein the gramme-force doesn't suffer further changes.

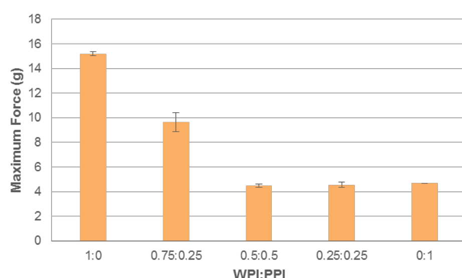

Figure 12 - Maximum force (g) achieved in the texture analyser. Five different WPI:PPI ratios (1:0, 0.75:0.25, 0.5:0.5, 0.25:0.75 and 0:1) were studied for the initial pH 7.4

CLSM

In the CLSM pictures, it is possible to confirm the texture observed in the samples before. When more whey protein is present it is possible to see the large protein aggregates (orange arrow), confirming the lumps that where visible in the yoghurt and then felt as well in the informal tasting. With the increasing of pea protein concentration (≥ 50% of the total protein content), the structure becomes more homogenous, with much smaller particles, which explains the creaminess observed in the corresponding yoghurt. In the presence of pea protein, it is still visible some insoluble particles characteristic from PPI (orange circle).

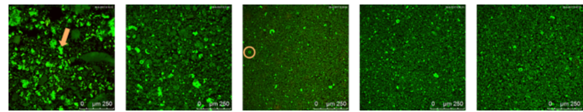

Figure 13 - CLSM pictures from the fermentation at 42ºC done to five different WPI:PPI ratios. From left to right is 1:0, 0.75:0.25, 0.5:0.5, 0.25:0.75 and 0:1 ratio of WPI:PPI, respectively.

Different Protein Sources with Flavours

The next step was study different pea protein sources and try to mask the pea off-flavours. For that three different sources of protein were used – PPI-A (the one used in the rest of the present work), PPI-B and the PPI-C. It was also added three different flavours, strawberry, lemon and raspberry, that were chosen based on an informal tasting performed with the previous samples. In that informal tasting 10 different flavours were tested with a concentration of 0.1%. From the informal tasting of the yoghurts made with the three different protein sources and the three different flavours was concluded that the lemon and raspberry mask very well the pea taste, even though the lemon peel flavour was too strong (the concentration need to be lower). In the other hand, the strawberry didn't mask that much the pea flavour.

With the PPI-C the pea flavour (however a different pea flavour compared to PPI-A) was stronger and bitter when compared with the other protein sources. The PPI-B even though was not so bitter as the PPI-C, the pea flavour was not pleasant.

Conclusions

Regarding the heat stability was concluded that pH 6.5 was too close to the isoelectric point of some of the pea proteins leading to an increase of pea protein aggregation and precipitation. Increasing heating time increased pea protein dispersibility but, at the same time, increased the formation of soluble aggregates. Whey protein and pea protein are influencing each other behavior, either whey protein is preventing the dissociation of the insoluble particles of pea protein or pea protein is promoting the aggregation of whey proteins on their own. It was observed that sodium chloride had a negative impact on the protein dispersibility.

The stirred yoghurt prepared with milk and whey proteins only showed a lumpy texture, which may be related to a too harsh heat treatment for these proteins. The texture became creamier when the protein content was ≥50% of pea protein. This creaminess was confirmed by a reduction in particle size on the CLSM, leading to a homogenous yoghurt. The initial pH was found to have a big influence in the flavour, being better at pH 7.4 when compared to pH 7.1. Besides the creaminess associated to the pea protein, astringency and cardboard taste are two characteristics that appeared when this protein is added. However, it is possible to mask the pea off-flavours with the addition of flavours e.g. raspberry and lemon.

From the emulsions and GDL experiments (not shown in this report) it was concluded that the fat content has an impact on the particle size of the emulsions; low fat content promotes the dissociation of insoluble particles of pea protein overtime, whereas by increasing the fat content the aggregation overtime is promoted. The particle size of the emulsions increased with pea protein due to the presence of fibers and insoluble materials. It was concluded that both proteins participated in the stabilization of the emulsion since they were both present on the fat phase when analyzed by RP-HPLC. In general, stable emulsions were obtained in the presence of protein blends. Focusing on the GDL experiments, it allowed to conclude that homogenization and the pre-heating step are necessary to prevent pea sedimentation and get firmer gels. However, in the presence of pea protein weak gels were still obtained.

Recommendations

In the emulsions, further work is needed to find which protein is making the primary layer around the oil droplets.

Regarding the heat stability, there is the need to find a technique to determine how pea and whey proteins are interacting with each other.

In the yoghurt formulations is recommended to repeat the last set of experiments, with the raspberry and lemon flavors and in the presence of sugar. It is also possible to try with new flavors, like blueberries which were seen to work well with soy. Then it is suggested to add cream to the yoghurt and see what is the effect on the flavor. Moreover, it is possible to try to increase the pea protein content and try with different plant proteins. On a long-term plan, stays here the suggestion to try with dairy analogues based on vegetable proteins instead of milk and try to use stevia to substitute the sugar.

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