Variation of in vitro digestibility of pea protein powder dispersions from commercially available sources

L. Jiménez-Munoz, M. Torp Nielsen, L. Roman, M. Corredig*

Aarhus University, Department of Food Science, CIFOOD Center for Innovative Foods, Agro Food Park 48, 8200 Aarhus N, Denmark

ARTICLE INFO

Keywords:
Pea protein isolate
Pea protein concentrate
In vitro digestion
Powder hydration
Free amino acid profile

ABSTRACT

With raising consumer demand for plant-derived proteins, there has been an increased interest in the utilization of pea ingredients in food formulations. It was hypothesized that differences in processing history and composition affect their colloidal properties and their breakdown during in vitro simulated gastrointestinal digestion. The gastrointestinal fate of three different commercial pea protein ingredients, two protein isolates and one less refined concentrate was compared. The concentrate dispersion showed greater solubility, different protein composition and smaller particle size than the reconstituted pea protein isolates. When heat-treated, the release of free amino groups decreased for the isolates, but increased for the concentrate dispersions. LC-TQMS of free amino acids in the intestinal digestates indicated a significantly higher release of methionine (limiting amino acid in pea protein) in the concentrates than in the isolates. This work highlights the influence of processing and composition on techno-functional and digestion properties of pea ingredients.

1. Introduction

Proteins are a key component of healthy and sustainable diets. They are not only source of dietary amino acids and bioactive peptides but play an important role in the formation of structure and texture in food. However, production of protein for food comes at an environmental cost to our planet, and therefore their origin and sourcing, processing history, and level of refinement are all important aspects to be considered to achieve the right balance between low emission diets and maximum nutritional impact for growth and healthy aging.

In the last decade there has been an increasing interest in the use of proteins sourced from legumes and pulses for food (Sozer et al., 2017), with pea protein fractions becoming increasingly popular. Several technologies can be used for their extraction and fractionation. Nonetheless, these processes alter not only the final composition of the ingredient produced (Stone et al., 2014) but also their functionality (Tanger et al., 2020).

Isolation methods are based on selective extractions and precipitations, which require changes in pH, ionic strength, washing and concentration steps, followed by drying. All these processing steps can decrease the solubility of the proteins and affect their colloidal structures (Tanger et al., 2020). The solubility is generally described as a U-shaped curve, with the highest solubility measured at low and high pH values (for example, pH 3 and pH 8, in the case of pea protein isolate). Interestingly, comparisons between laboratory extracted and commercial isolates of plant-derived proteins show a much higher level of denaturation in commercial samples, with consequences on their technological functionality, solubility and colloidal properties (Keerati-u-rai et al., 2011; Tanger et al., 2020). These same properties will also affect how the protein will destructure and form novel structures during transit in the gastrointestinal tract.

From a nutritional perspective pea proteins are rich in lysine and branched-chain amino acids (Babaoult et al., 2015), however, contain low amounts of methionine (limiting amino acid), cysteine and tryptophan (Sim et al., 2021). Pea proteins are divided into globulins and albums, with globulins being the most abundant fraction, consisting of Legumin, Vicilin and Convicilin. Legumin, approximately 360 kDa in mass, is a hexameric protein composed of varying subunits each containing an acidic and basic subunit linked by a disulfide bridge (Barac et al., 2010). Vicilin consists of three subunits interacting via non-covalent forces, of about 150 kDa. Convicilin is instead a tetramer of about 280 kDa consisting of 70 kDa subunits (Bogahawaththa et al., 2019). These proteins are extensively homologous, nevertheless, the difference lies in convicilin holding a highly charged hydrophilic sequence close to the polypeptide N-terminus, as well as containing one cysteine residue, while vicilin does not (Gonzales-Perez and Arellano, 2009). The
globulins are usually isolated by means of alkaline extraction followed by isoelectric precipitation. However, dry fractionation technologies are also available, and result in less refined ingredients, still preserving to some extent the original protein supramolecular architecture (Boukid et al., 2021). Furthermore, pea protein fractions obtained by dry fractionation technologies require less amount of energy and resources to be produced (Schuysyer et al., 2015). The fraction obtained with this technology also shows very different functional properties, such as higher water holding capacity, solubility, dispersibility, compared to spray dried protein isolates (Tanger et al., 2020; Kornet, Venenmans, Venema, va der Goot, & Meinders, 2021); while the latter tend to have lower concentrations of off-flavors and anti-nutritional components.

Protein nutritional quality is associated with the presence of essential amino acids and their digestibility. In this sense, it has been recently suggested that the digestibility of pea protein is affected by its processing history (Ma, Boye, & Ha, 2017). It is known that protein-rich foods form different structures during gastro intestinal transit, with important consequences on gastric emptying and enzymatic breakdown (Dupont et al., 2010). A recent study investigated the effect of severe processing (autoclaving, re-heating) on the extent of proteolysis of pea protein, and demonstrated an increase in digestibility caused by unfolding of pea protein subunits (Laguna et al., 2017). Furthermore, in the case of protein concentrates from quinoa (Opazo-Navarrete et al., 2019) it has been reported that the extent of aggregation may affect the accessibility of the proteolytic enzymes.

A recent study (Rivera Del Rio et al., 2020) showed slight differences in the in vitro gastric phase of pea protein isolate, expressed as extent of peptide release and degree of hydrolysis (DH). In this case, a slight reduction in digestibility was reported for samples heated to 90°C, which was then recovered after further heating (120°C). It was concluded that heating (120°C) of denatured pea protein ingredients may increase their susceptibility to hydrolysis by pepsin, improving their digestibility.

In this work, it was hypothesized that the differences in colloidal structure and solubility between different commercial fractions can affect the bioavailability and final digestibility of pea proteins. Further it was also hypothesized that heating may improve powder hydration and breakdown of the large protein aggregates, with positive consequences to the digestibility. It has been shown that a pea protein-based infant formula is highly digestible (Le Roux et al., 2020). However, the kinetics of breakdown and the structuring occurring in the gastro intestinal tract will be affected by the processing history of the protein ingredient (Jiménez-Munoz et al., 2021).

To test this hypothesis three commercial pea protein ingredients were subjected to an in vitro simulated gastrointestinal digestion. Two commercial pea protein isolates of similar composition were compared to a pea protein concentrate, obtained by dry fractionation. The protein dispersions were also subjected to an extensive heat treatment at 90°C for 15 min, and then digested using the INFOGEST static in vitro method (Brodkorb et al., 2019), as this method has been shown to be useful to address bioavailability and digestibility of proteins (Egger et al., 2017).

2. Materials and methods

2.1. Protein sources

Pea protein isolates were obtained from commercially available sources (Pisane C9, Cosucra, Warconig, Pecq, Belgium) and (Empro E86, Emsland, Emilicheim, Germany), named as PPI1 and PPI2 (un-disclosed order). PP11, consisted (‘as is’) of: 81.1 % protein, 0.94 % carbohydrates, 3.3 % ashes and 6 % moisture. PP12, consisted of (‘as is’): 80.9 % protein, 1 % carbohydrates, 3.8 % ash, 6 % moisture (according to manufacturer’s specification). A less refined concentrate, obtained by air classification (dry separation) was also used, identified as C. The concentrate was also obtained from a commercial source (Vestkorn, Holstebro, Denmark) and contained (‘as is’): 51 % protein, 15.8 % carbohydrates, 14.8 % fiber, 4.6 % fat and 5.6 % ash (per manufacturer’s specification).

2.2. Preparation of pea protein dispersions

Pea protein samples were dispersed at a final concentration of 3 % protein (w/w) in 25 mL Milli-Q water and stirred on a magnetic stirrer for one hour. Samples were then stored in the refrigerator (4°C) until use. Aliquots (25 mL) were placed in 50 mL plastic tubes and heated to 90°C in a water bath (Julabo SW22, Merck KGaA, Darmstadt, Germany), and held for 15 min at 90 °C before being immediately cooled to room temperature in ice water. Heated dispersions were named PP11-90, PPI2-90 and PPC90.

2.3. Solubility of pea protein dispersions at different pH

Protein dispersions were adjusted to various pH values, namely, pH 7.5, 6.0, 4.5, 3.0 and 2.0, using 0.5 M HCl and 0.5 M NaOH using a pH meter (PHM220, 115-230 °V, Meterlab, Radiometer Analytical SAS, Lyon, France). The suspensions were then centrifuged (Thermo Scientific, SL 40R centrifuge, Massachusetts, U.S.A.) at 3400 g and 4°C for 30 min. The supernatant was collected and protein concentration was measured using the Bicinchoninic acid (BCA) assay (Pierce BCA protein assay, Thermo Scientific, Massachusetts, USA) according to manufacturer’s instructions. Bovine serum albumin (BSA, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used as standard. Samples were measured in a microplate after incubation at 37°C for 30 min, with a Synergy 2, Biotek spectrophotometer (Thermo Scientific, Massachusetts, U.S.A.) at 562 nm. The percentage of soluble protein was expressed relative to the total protein content in the initial protein dispersion.

2.4. SDS-PAGE Electrophoresis

To estimate the distribution of the polypeptides as well as their digestion Sodium Dodecyl Sulphate - Polyaerylamide Gel Electrophoresis (SDS-PAGE) was performed. Reduced samples (both before and after in vitro digestion) were prepared by mixing 13 μL suspension with 5 μL NuPAGE LDS sample buffer (4X) (Invitrogen, Thermo Fisher Scientific, CA, U.S.A.) and 2 μL dithioerythritol (1 M DTE) as a reducing agent, making a total volume of 20 μL. Samples were incubated using a heating mixer (Thermo Fisher Scientific, CA, U.S.A.) at 95°C for 5 min and then centrifuged at 4°C and 10,000 g for 5 min (Eppendorf micro-centrifuge 5417R, Merck KGaA, Darmstadt, Germany). For analysis a 1.0 mm X 12 well 4–12 % gradient Bis-Tris gel (Invitrogen, Thermofisher Scientific, CA, U.S.A.) was used. Aliquots (10 μL) were loaded in each well and 5 μL of pre-stained protein ladder standard (PageRuler Prestained Protein Ladder, Thermo Fisher Scientific, Vilnius, Lithuania) was loaded in a separate well. The protein separation was run at 200 V for 35 min using MES-SDS running buffer as per manufacturer’s instructions (Invitrogen, Thermo Fisher Scientific, CA, U.S.A.) and images were analyzed using ChemiDoc XRS + and Image Lab software (Bio-Rad lab., CA, U.S.A.).

2.5. Microstructural analysis

Samples were stained by mixing 1.5 mL of sample with 25 μL of 1 mg/1 mL staining solution, fluorescein-5-isothiocyanate (FITC). The microstructure was then observed using a confocal laser scanning microscopy (Nikon Eclipse Ti series, Nikon Instruments Inc., Amsterdam, Netherlands). FITC absorb light ranging from 400 to 530 nm, though absorbing most efficiently at its excitation maximum at 490 nm, emitting fluorescence ranging from 475 to 650 nm, which is within the green spectrum (Bio-Rad Laboratories, 2021). The laser was therefore set at 488 nm. The samples were observed with a 20x objective (dry) and a 60x
objective (in water), with a refractive index of 1.0 and 1.33, respectively.

2.6. In vitro simulated gastrointestinal digestion

A static in vitro INFOGEST model to simulate gastrointestinal digestion was employed to test the digestibility of the different protein dispersions, before and after heating. The simulated salivary fluids (SSF), simulated gastric fluids (SGF) and simulated intestinal fluids (SIF) were prepared as previously described (Brodkorb et al., 2019). A control digestion was performed prior to the actual digestion of the samples to determine the amount of acid and base needed to adjust the gastric chyme to pH 3 and pH 7–7.5, respectively. The samples, PP11, PP12, and PPC together with the heated suspensions counterparts, PP11-90, PP12-90 and PPC90, respectively, were subjected to oral simulatated digestion by diluting 10 mL of the dispersions 1:1 (vol/vol) with SSF, 0.3 M CaCl₂(H₂O)₂, Milli-Q water, and additional Milli-Q water instead of amylase. After 2 min mixing, the oral bolus was diluted 1:1 (volume ratio) with SGF, 0.3 M CaCl₂(H₂O)₂, the gastric enzyme pepsin (from porcine gastric mucosa ≥ 250 units/mg solid, Sigma–Aldrich, Merck KGaA, Darmstadt, Germany), 1.0 M HCl to reach pH 3 and Milli-Q water to reach a final pre-calculated volume. The samples were then incubated at 37°C for 2 h and mixed with a rotator (Cole-Parmer Stuart Rotator Disk, Thermo Fisher Scientific, CA, U.S.A.) at 40 rpm.

The tubes were then placed in an ice-water bath, and NaOH added to reach pH 7–7.5, to stop pepsin activity. Samples destined to intestinal digestion were then diluted 1:1 (vol/vol) with SIG, bile salts (bile extract from porcine, Sigma–Aldrich, Merck KGaA, Darmstadt, Germany) and the pancreatic enzyme pancreatin (from porcine pancreas, Sigma–aldrich, Merck KGaA, Darmstadt, Germany), and again incubated at 37°C as described above. To examine the impact of the duration of intestinal digestion, samples were incubated either for 15 or 120 min. After the intestinal stage, the enzymatic reactions were stopped by heating at 90°C in a boiling water bath for 5 min and immediately cooled in ice water. The samples were then stored at −18°C until further analysis. All experiments were carried out in triplicate.

2.7. Size exclusion chromatography

To assess changes in size distribution of the digestates, centrifugal supernatants (see above) of undigested suspensions, gastric and intestinal in vitro stages were analyzed by size exclusion chromatography (SEC-HPLC). The chromatographic separation of the samples was performed with a HPLC system (Agilent Technologies 1100 series) equipped with a TSKgel column G2000SWXL (7.8 mm × 600 mm) (Tosoh Corp., Merck, Germany). Samples were centrifuged at 10,000 g for 30 min at room temperature, diluted 1:5 with mobile phase (see below) and filtered using a syringe filter (0.45 μm PVDF membrane, Gilson Scientific ltd). The mobile phase consisting of acetonitrile (30 %, v/v) and trifluoroacetic acid 0.1 % (v/v) was added isotratically at a flow rate of 0.5 mL min⁻¹ and the absorbance was measured at 214 nm. Integration of the chromatograms was performed using Agilent Chemstation software (Waters). The molecular weight was estimated based on the elution time of the following standards bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), aprotinin (6.5 kDa) and the amino acids histidine–leucine (0.268 kDa), phenylalanine (0.165 kDa) and glycine (0.075 kDa).

2.8. Degree of hydrolysis and free amino acid analysis

The o-phthaldialdehyde (OPA) method, which estimates the concentration of primary amines, was used to determine the amount of free amino acid groups (measured as mmol of glutamic acid equivalents) in the soluble fraction of the gastric and the intestinal digestates. The non-homogenized digestates (PP11, PP11-90, PP12, PP12-90, PPC, PPC90) were mixed with methanol (MeOH, 80 % v/v), centrifuged at 10,000 g for 30 min and then filtered using a syringe filter (0.45 μm PVDF membrane, Gilson Scientific ltd). The OPA working solution was prepared by mixing 12.5 mL of 1 M di-sodium tetraborate-decahydrate, 2.5 mL of sodium dodecyl sulfate (SDS, 10 %, w/w), 0.5 mL of OPA in ethanol (4 %, w/w), 0.5 mL of 2-mercapto-ethansulfonic acid sodium salt (Na-MES, 20 %, w/w), 1.25 mL of triton X-100 solution (10 %, w/w) and 25 mL of distilled water. The assay was carried out by mixing 232 μL of OPA working solution with 8 μL of samples, standards (L-glutamic acid, 0–8 mM) or blank (perchloric acid, 0.5 M) in a 96-well microplate and the absorbance was measured at a wavelength of 335 nm. The degree of hydrolysis was calculated with the following formula:

\[
\text{DH} \% = \frac{h - h_{\text{tot}}}{h_{\text{tot}}} \times 100
\]

where h represents the number of hydrolyzed peptide bonds and \( h_{\text{tot}} \) is the total number of peptide bonds present (Rutherford, 2010).

To estimate the residual protein concentration in the supernatant, the non-homogenized digesta were centrifuged at 5000 g for 20 min. The protein content in the supernatant of the digesta was determined by bicinechonic acid assay (BCA) (Thermo Fisher Scientific Inc., USA) using bovine serum albumin (BSA) as a standard. The absorbance was measured at 562 nm. The degree of hydrolysis was calculated as the ratio between the amount of free NH2, in glutamic acid equivalents (g/L, using 147.13 g/mol for the amino acid molecular weight) divided by the concentration of pea protein in the original suspension (g/L). This does not take into consideration the amount of endogenous protein added during the in vitro testing, nor the differences in solubility of the peptides during the different digestion stages.

In addition to the OPA assay, free amino acids were measured using a Triple Quadrupole Mass spectrometer (TQMS). The chromatographic separation of the soluble peptides was carried out on a UHPLC system (Agilent Technologies, 1290 Infinity II) and the detection and mass analysis on a Triple Quadrupole Mass spectrometer (TQMS) system (6495 Agilent Technologies) using MassHunter® (Agilent Technologies) to process the data. Samples (20 μL) were diluted with 180 μL of 0.1 M HCl, followed by the addition of 50 μL of internal standard consisting of stable isotopically labelled amino acids (“Cell Free” Amino acid mix (20 AA, U-13C, 97–99 %; U-15 N, 97–99 %) (Cambridge Isotope Laboratories, Inc)). Stock solutions of the internal standard was prepared in 0.1 M HCl and stored in vials at −20°C.

The separation was performed using an Intrada Amino Acid column, 150 × 3 mm, 3 μm (Imtakt, USA) in combination with a Van-Guard Pre-column ACQUITY UPLC BEH HILIC, 5.0 × 2.1 mm, 1.7 μm (Waters, Ireland). The analytical column was thermostatted at 35°C (±0.8°C), whilst the injection volume was 5 μL. Acetonitrile with Formic acid (0.1 %, v/v) and Ammonium Formate were used as solvents for the chromatographic separation at a flow rate of 0.6 mL/min. A gradient elution program was applied starting from 20 % (100 mM, Ammonium Formate) (t = 0 min), going to 100 % (t = 12.0 min), followed by an isotropic elution of 100 % Ammonium Formate for 2 min (t = 14 min) and by equilibration to 20 % Ammonium Formate (t = 15.0 min).

2.9. Statistical analysis

The measurements reported for protein solubility are the results of three independent measurements, while two independent measurements were used for degree of hydrolysis, HPLC-SEC and free amino acids. Statistical tests were performed using MiniTab 19, and minimum significance was set at the 5 % level (P < 0.05). A two-way analysis of variance (ANOVA) with Duncan’s multiple range test was conducted to determine the significance of the treatments. A general linear model was conducted to test significance of variables. Mean values are shown in graphs, with bars representing standard deviations. A principal component analysis (PCA) was conducted using SIMCA® 17.0.1 in order to visualize correlations and sample grouping according to their release of free amino groups as well as their degree of hydrolysis.
3. Results and discussion

3.1. Microstructure and solubility of the initial protein dispersions

The solubility of the protein suspensions as a function of pH followed the typical U-shaped behavior for pea protein reported in the literature (see Fig. S1, supplementary material). It has been previously reported that globulins, present in the highest ratio in pea, have an isoelectric point of about 4.5, hence the typical U-shaped curve of solubility (Lam et al., 2018). All treatments followed this typical behavior, regardless of the heating treatment (see Fig. S1). There were significant differences in solubility, defined as the protein concentration in the supernatant after centrifugation at 3000 g, between protein isolates (PPI1, PPI2) and concentrate samples (C), with C showing higher solubility (up to 60% solubility for pH 7.5) than PPI1 and PPI2 (Fig. S1). Although the less refined fractions may be less preferred for formulations as they may contain undesirable components such as insoluble fiber and phytates, the proteins never underwent a denaturation step, so they maintain higher solubility than the more purified isolates, as already reported (Fredrikson et al., 2001). Previous authors have also reported about 30% solubility for commercial pea protein isolates (Adebiyi and Aluko, 2011; Taherian et al., 2011), similar to the values found in the present study at pH 2 and 8, especially after heat-treatment (up to 20–30% solubility for pH 8).

Fig. 1 shows representative microstructure images of the pea protein isolates (PPI1, PPI2) and concentrates (PPC), before and after heating.
treatment at 90 °C for 15 min. There were clear differences between the colloidal structures of the two pea protein isolates (Fig. 1A-D). The dispersions showed large particle aggregates, both for the unheated (Fig. 1A,C) and heated (Fig. 1B,D), ranging from more than 100 μm to <10 μm. Some hydrated areas were noted inside the protein aggregates, represented by void spaces within the particle. Similar findings have been reported for spray dried pea protein isolates (Ben-Harb et al., 2018; Lan et al., 2019) when studying suspensions of 15 % pea protein isolate in a 1 % NaCl solution, stirred at room temperature for 1 h. These authors also reported low values of solubility for pea protein isolate dispersions, with clusters of undissolved pea protein aggregates of various sizes, with the largest up to 50 μm (Ben-Harb et al., 2018). In the present work, the two PPI, PPI1 and PPI2 showed some size differences, with PPI2 having less amount of larger particles than PPI1 in the unheated dispersions. In all cases, the protein particles were spherical in shape with a smooth surface, with some degree of concavity and some hollow areas.

By comparing the microstructure of the dispersions before and after heating, it is clear that heating improved powder hydration, and caused the formation of smaller protein clusters, with smooth surfaces and some concavities. Furthermore, the particles in the PPI heated suspensions appeared more homogeneous than those of the unheated counterparts. After heating there was a much larger population of small protein particles dispersed in the continuous phase. These findings are consistent with previous reports stating that thermal treatment results in fewer aggregates and a more homogenous protein dispersion (Ben-Harb et al., 2018). In spite of these microstructural differences there was no significant difference in the solubility behavior of the two protein isolates before heating (Fig. S1); however heated suspensions of the pea protein isolates showed higher solubility compared to unheated suspensions, confirming the role of heat in increasing the hydration and dissolution of the protein powders.

Fig. 1 (Fig. 1 E,F) also shows the CLSM images of the pea protein concentrate (PPC). The microstructure of these suspensions was clearly different from that of the spray dried isolate suspensions; these samples were more readily dispersed. The CLSM images showed no large aggregates, with most particles < 10 μm. The PPC also showed the presence of other components (darker unstained structures), that could be associated with fiber structures. PPC was indeed less refined, and contained a complex carbohydrate fraction. No differences in particle structure or distribution were perceived between Fig. 1E and 1F. Nevertheless, unlike the isolate suspensions, heating decreased the solubility of the protein concentrate (Fig. S1), presumably by exposing non-polar groups in the native structures, leading to aggregation and precipitation of newly formed aggregates.

In conclusion, the colloidal structures observed by confocal microscopy demonstrate large differences between different protein ingredients, and it is possible to hypothesize that these structural changes may impact their fate during transit through the gastrointestinal tract.

3.2. SDS-PAGE of proteins after in vitro digestion

The polypeptide composition of the protein suspensions during in vitro digestion was analyzed using SDS-PAGE as summarized in Fig. 2. The optimal pH for activity for the porcine pepsin used in the present study is ~ 2.2, which is close to the pH of gastric digestion (pH 3.0) as for Infogest protocol (Brodkorb et al., 2019). When gastric chyme reaches the small intestine pepsin is inactivated and the digestion of proteins continues due to pancreatic enzyme activity, consisting of trypsin, chymotrypsin, amylase, lipase and colipase, respectively.

All the undigested samples showed a similar electrophoretic profile, despite differences in processing conditions between the isolates and the concentrates. All samples showed distributions similar to those reported previously in the literature, from pea isolates extracted by isoelectric precipitation (Kornet et al., 2020; Tanger et al., 2020; Jiménez-Munoz et al., 2021). There were some differences between the concentrate suspensions and the isolates, with the concentrate (PPC, PPC90) showing more intense bands at approximately 110, 70, 50, 45, 40, 25 and 20 kDa and less intense bands at ~ 30 and ~ 15 kDa. On the other hand, the two isolates (PPI1, PPI1-90 and PPI2, PPI2-90) showed more intense bands at ~ 110, ~70 and ~ 50 kDa, and less intense bands at approximately 55, 40, 25, 20 and 15 kDa. The bands observed at 40 kDa (Legumin α) and 20 kDa (Legumin β) were attributed to Legumin subunits. Meanwhile, Vicilin (7S) is a 150 kDa trimer with 50 kDa subunits (Tanger et al., 2020). The bands migrating around 70 kDa are Con vicillin subunits, a tetramer of about 280 kDa (Tanger et al., 2020).

The concentrate showed less bands at Mw larger than 100 kDa, and also showed less aggregates at the top of the gel. There were some differences in the polypeptide composition of the two isolates, with PPI2 showing less legumins (bands migrating at 40 and 20 kDa) than PPI1. The most abundant globulins were present in all samples, concentrates also contained small molecular weight albumin bands (not visible for the isolates), as well as sharper bands for lipoygenase. These results show that the protein ingredient obtained through air classification (dry fractionation) is less refined and purified than the isolate obtained through isoelectric precipitation. The two isolates contained large

---

**Fig. 2.** SDS-PAGE of isolates (PPI1,PPI1-90, PPI2, PPI2-90) and concentrates (PPC and PPC90), unheated and heated at 90°C. U: Undigested; G: end of gastric phase, I15 and I120: 15 and 120 min of intestinal digestion, respectively. GE gastric extract; IE intestinal extract; Molecular weight markers in kDa are also shown. Main protein bands are identified. Equal volumes of digesta were loaded in all wells.
aggregates on the top of the gel, once again confirming the less denatured nature of the proteins in the concentrate.

All sample dispersions showed extensive protein hydrolysis after the \textit{in vitro} gastric stage. In fact, for the isolates, only a few distinct bands were present after the gastric stage. Conversely, the unheated concentrates showed the highest resistance to digestion: in the concentrate there was a noticeable difference in the residual peptides in the gastric juices before heating compared to after heating (PPC v PPC90), with heated digestates showing no intact bands of high MW. It is important to note that the denaturation temperature of pea proteins is $> 82 \, ^\circ\text{C}$ (Messien et al., 2013), temperature below the one used during the heat treatment of the protein dispersions.

Comparing the SDS-PAGE migration of the dispersions after the \textit{in vitro} gastric stage, there was no effect of heating in the protein isolates (PPI1, PPI1-90, and PPI2, PPI2-90), although there were differences in the bands between the two isolates, with PPI2 showing less migration of bands after gastric digestion compared to PPI1. The differences may result from the larger microstructure present in PPI1 compared to PPI2 (see Fig. 1) and processing history. Further, it was possible to conclude that under \textit{in vitro} conditions, pepsin was able to cleave most of the protein subunits, with the exception of convicilin, which was only partially degraded, in all isolates. Additionally, some low molecular weight bands ($<20 \, \text{kDa}$) were persistent in the gastric digestates of the pea protein isolates. The SDS-PAGE profiles were similar to those reported by Laguna et al. (2017). The authors observed bands at $\sim 40$ and $\sim 70 \, \text{kDa}$ after 120 min of \textit{in vitro} gastric digestion of a PPI, attributed to subunits of vicilin and convicilin, respectively (Laguna et al., 2017). Comparisons of the bands under gastric conditions between the protein concentrate and the two protein isolates clearly demonstrate a difference in digestibility between the different pea protein ingredients.

Fig. 2 also shows the polypeptide patterns for the suspensions after 15 and 120 min of \textit{in vitro} intestinal simulated digestion. After 120 min, all samples showed no residual intact protein. However, there were differences in the suspensions treated for 15 min under \textit{in vitro} simulated intestinal digestion. In the case of PPI1, more resistance was noted after heating. The band visible at $\sim 70 \, \text{kDa}$ after gastric digestion (G) both in unheated and heated PPI1, was no longer present after 15 min of intestinal digestion. However, in PPI1, a new band at $\sim 38 \, \text{kDa}$ was observed, also present in the pancreatic extract (see Fig. 2, G1). Further work is necessary to better identify the distribution of resistant polypeptides during the initial stages of digestion, as a function of processing history, as the behavior of PPI1 and PPI2 showed a few differences.

After prolonged intestinal digestion no bands at MW greater than 15 kDa were observed, suggesting that all large polypeptides both from pea proteins as well as endogenous from the pancreatic and gastric juices, were degraded.

Fig. 2 clearly shows that although there were some differences in the kinetics of digestion of the polypeptides, there were no residual polypeptide bands after the 120 min of \textit{in vitro} intestinal stage implying mostly the presence of small molecular weight peptides in the intestinal tract.

### 3.3. Size distribution of peptide fractions during \textit{in vitro} intestinal transit

To better understand possible differences in the peptide distributions of the various digestates, the initial suspensions, as well as the gastric and intestinal digestas of the six different pea protein suspensions were subjected to size exclusion chromatography analysis of the supernatants. Under the experimental conditions, the peptides present in the supernatants eluted in a MW range from 1 to 100 kDa. Representative elution profiles are shown in Fig. 5.2. Three regions were then quantified as percentage over total elution area, reporting an elution of peptides in three different groups: between 100 and 30 kDa, between 30 and 1 kDa, and $<1 \, \text{kDa}$. The undigested showed the largest population of soluble peptides to be larger than 30 kDa. As expected, the elution of the supernatant fraction shifted to a population of smaller molecular weight peptides during \textit{in vitro} stomach and intestinal digestion (Fig. S2). The changes in the peptide distribution (i.e. relative area distribution) over digestion time for the three MW peptide groups were quantified to better compare between the types of pea source and also to evaluate the effect of heating the suspensions, as illustrated in Fig. 3.

In all protein suspensions, as the area of the large MW peptides decreased, smaller MW peptides appeared in the chromatogram. There was no difference in the relative distribution of the peptides between pea protein isolates and concentrates in the undigested suspensions (i.e., before digestion). In all cases, the largest population was that of high MW peptides. There was a statistically significant difference between unheated and heated protein isolates in the initial, untreated suspensions (see star symbols in Fig. 3): when comparing the undigested heated and non-heated PPI1 and PPI2 suspensions, both showed a change in the MW distribution profile, as the 100–30 kDa group increased in the area % from about 30 % to 70 % after heating, in full agreement with the increase in soluble protein (Fig. S1). This indicated that the majority of the soluble material present in the unheated sample and with a colloidal size $<200 \, \text{nm}$ (as these samples were filtered) was composed of small peptides, and only after heating, the colloidal aggregates of bigger MW were disrupted.

The untreated concentrate had a statistically significant difference in the distribution profile, as the “100–30 kDa” group was already high, representing 67 % of the area ratio, and decreased when heated to 64 %, displaying the opposite behavior to isolates. The small peptide population ratio ($<1 \, \text{kDa}$) significantly increased from 8 % in PPC to 13 % in PPC90. This may suggest also that heat-induced activation of endogenous proteolytic enzymes have occurred.

During digestion, the population of small peptides, $<1 \, \text{kDa}$, (Fig. 3, triangles) grew rapidly reaching values near 100 % within the gastric step. Large MW peptides were clearly degraded by pepsin (confirming SDS-PAGE results, Fig. 2), with the appearance of an intermediate group (MW ranging from 30 to 1 kDa, Fig. 3, circles). All pea proteins showed high peptides bioavailability after the first intestinal digestion stage (115) with over 90 % of the peptides in the supernatant estimated to be below 1 kDa. These results clearly show that the totality of compounds present in the supernatant were completely hydrolyzed and consisted mostly of small peptides (e.g. tri/di-peptides) and bioavailable amino acids. Furthermore, the highest distribution of low MW peptides at 115 is proof of the rapid action of pancreatic proteases (e.g. trypsin, chymotrypsin) for cleaving peptide groups from pea proteins. There were no significant differences within treatments at 115 or 1120.

### 3.4. Degree of hydrolysis during \textit{in vitro} digestion

To understand the extent of protein hydrolysis under gastrointestinal conditions, the free amino groups were measured as mmol of glutamic acid equivalent per initial protein (Fig. S5). The degree of hydrolysis (DH) for the various treatments are summarized in Fig. 4.

All undigested pea protein dispersion showed a degree of hydrolysis between 1 % and 3 %, increasing slightly (from 2 % to 3 %) in the case of protein isolates, but in the case of the protein concentrates there were no significant changes. This results are similar to those reported in the literature for pea protein isolates (Osen, Toelstedte, Eisner & Schweiggert-Weisz, 2015).

All treatments showed a low DH after the \textit{in vitro} gastric digestion step corresponding to values between 11 % and 23 % of the protein. Increased levels were measured after the \textit{in vitro} intestinal stage, as expected because of the additional activity of trypsin and chymotrypsin. Interestingly, most of the breakdown occurred within the first 15 min of \textit{in vitro} intestinal digestion. There were some differences depending on the source of the protein, with significantly higher levels of hydrolysis observed for PPC (heated and non-heated) compared to the isolates. In a previous study, it was reported that after 120 min of intestinal digestion the DH of a commercial pea protein isolate reached 38 % (Reyraud et al., 2020). This was not the case in the present study, where more than...
60% of the protein in the isolates was already hydrolyzed after 15 min. Interestingly, non-heated PPC showed a 19% increase in DH from I15 to I120. This could indicate that the presence of fibers induced a delay in protein digestibility. Conversely, when heated, there were no substantial differences between the two intestinal timepoint (I15-I120), thus, heating not only improved protein digestibility in the concentrate but facilitated its rapid digestion. This could be due to exposure of cleavage sites in protein due to increased hydration, induced heat-denaturation of the protein and/or increased hydration of the non-digestible carbohydrates. All of the above could have facilitated the accessibility of enzymes. In addition to the fiber, concentrates also contained a lipid fraction. The effect of the lipid fraction on the digestibility was not explored in this project.

A comparison of the different digestion points of the pea protein isolates PPI1 and PPI2 showed some differences in DH for heated compared to unheated isolates, as well as different sources. For instance, at I120, both isolates showed a lower DH when heated: PPI1 decreased from 78.60% ± 0.74% to 70.33% ± 3.5% and PPI2 DH decreased from 76.8% ± 2.4% to 70.20% ± 4.2%. These differences need to be also related to the amount of soluble protein present in the samples after the gastric and intestinal stage. In the case of the pea protein isolates, the soluble protein increased from the gastric to the intestinal stage from ~12 to 14 g/L, with an increase in free amino acid equivalents. This would indicate that large peptides were still present in the isolate fractions after the intestinal stage. On the other hand, for the concentrate samples, the gastric stage contained much higher levels of soluble protein (18 and 10.5 g/L in unheated and heated dispersions, respectively) and decreasing to about 7 g/L in the intestinal stage, indicating a much more extensive hydrolysis of the concentrates. A more detailed analysis of these differences can be found in supplementary material, Fig. S4. The DH results emphasized the complexity of protein digestibility, and the necessity to analyze further any possible difference in the distribution of the digested peptides.

3.5. Free amino acids after intestinal digestion

All digested pea protein suspensions were analyzed for free amino acids per gram of protein after intestinal digestion, the data are summarized in Table 1. Regarding the free essential amino acids from pea, methionine was found to be the limiting amino acid. The concentration
of methionine in the pea protein concentrate was much greater than that found in the pea protein isolates. This significant difference in methionine content between concentrates and isolates is due to the means used for protein purification. Isoelectric precipitation separates the globulin fraction, maintaining the albumin in the soluble phase and as a result, this last fraction is poorly present in isolates. This is not the case in the dry separation process for pea protein concentrate were albumins are preserved (Schutyser et al., 2015) and hence, methionine is present in a higher amount. During digestion, pea protein concentrates released more than three times the amount of free methionine under intestinal conditions than pea protein isolates regardless of the treatment. There were no differences in the amounts of released cysteine and glycine between the isolates PPI1/I2 and PPC. The pea protein concentrate (both before and after heating) showed a higher amount of free leucine, isoleucine, phenylalanine, lysine, glutamic acid, aspartic acid and arginine per gram of protein, compared to the pea protein isolates. These findings are consistent with literature (Rubio et al., 2013).

A principal component analysis of the data including the DH and the release of free individual amino acids showed a first component explaining 75% of the data variation, and 11% in the second component (Fig. 5). The samples (heated or unheated, triangles and square symbols, respectively) after intestinal digestion (two separate replicate experiments after intestinal digestion for 120 min) are plotted in Fig. 5. While concentrates, heated and unheated are very closely clustered (right hand side) to most of the free essential amino acids, there was a significant separation of each of the two isolates, and in this case, there was a significant clustering of the effect of heating in the isolates. The application of the PCA to the free amino acids analysis allowed for a clearer picture of the differences between the samples and their treatments. It was possible to identify cluster groups based on the type of protein ingredients as well as the effect on heat treatment, in the case of the isolates.

4. Conclusions

The study of different commercial pea protein ingredients led to a better understanding not only of the colloidal differences between the dispersions, but also their behavior during gastrointestinal transit. Although the microstructure and solubility of the protein dispersion was quite different between the proteins, all of the protein dispersions were readily digested after 15 min of in vitro simulated intestinal digestion. A higher level of soluble protein was measured for the concentrates compared to the isolates, due to the difference in processing history. Heating of the dispersions caused distinct differences in the free amino acid release compared to the corresponding unheated dispersions. Although similarities were found in the degree of hydrolysis between isolates, heating decreased the amount of released free amino groups per

Table 1
Free amino acid values obtained by LC/TQ-MS measured in the digestes after in vitro intestinal digestion. PPI1 and PPI2, PPI1-90 and PPI2-90 isolates untreated or after heating treatment of the initial dispersions, PPC and PPC90 concentrates, untreated and after heating. The values are expressed as g of free amino acid per 100 g of protein in the samples. Values are reported as average of two replicates with standard deviation. TFAA: Total Free Amino acids. Different letters indicate statistically significant differences between treatments for a given amino acid.

<table>
<thead>
<tr>
<th>g of Free amino acid/100 g protein</th>
<th>PPI1</th>
<th>PPI1-90</th>
<th>PPI2</th>
<th>PPI2-90</th>
<th>PPC</th>
<th>PPC90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>–</td>
<td>3.1</td>
<td>2.0</td>
<td>± 0.3</td>
<td>0.6</td>
<td>± 0.3</td>
</tr>
<tr>
<td>Cysteine</td>
<td>± 0.4</td>
<td>0.3</td>
<td>0.2</td>
<td>± 0.3</td>
<td>0.4</td>
<td>± 0.3</td>
</tr>
<tr>
<td>Proline</td>
<td>± 0.4</td>
<td>0.05</td>
<td>0.05</td>
<td>± 0.05</td>
<td>0.1</td>
<td>± 0.4</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>± 0.4</td>
<td>0.56</td>
<td>0.6</td>
<td>± 0.6</td>
<td>0.2</td>
<td>± 0.3</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>± 0.4</td>
<td>1.2</td>
<td>0.9</td>
<td>± 0.3</td>
<td>0.9</td>
<td>± 0.3</td>
</tr>
<tr>
<td>Alanine</td>
<td>± 0.4</td>
<td>0.5</td>
<td>0.2</td>
<td>± 0.2</td>
<td>0.8</td>
<td>± 0.3</td>
</tr>
<tr>
<td>Serine</td>
<td>± 0.4</td>
<td>0.17</td>
<td>0.2</td>
<td>± 0.2</td>
<td>0.2</td>
<td>± 0.3</td>
</tr>
<tr>
<td>Arginine</td>
<td>± 0.4</td>
<td>0.6</td>
<td>0.5</td>
<td>± 0.5</td>
<td>0.4</td>
<td>± 0.3</td>
</tr>
<tr>
<td>Total Free Amino Acids</td>
<td>± 0.4</td>
<td>3.4</td>
<td>0.5</td>
<td>± 0.5</td>
<td>0.4</td>
<td>± 0.3</td>
</tr>
</tbody>
</table>

Gastrointestinal points

Fig. 4. Degree of hydrolysis, expressed as g of free glutamic acid equivalent per g of initial protein present in the dispersions. PPI1 (black), PPI2 (gray), PPC (white), solid bars, unheated, patterned bars, heated at 90°C for 15 min after in vitro gastric (G) and intestinal digestion for 15 (I15) and 120 (I120) min. Values are the average of two independent digestion runs. Bars indicate standard deviations. Different letters indicate statistical significant differences (p < 0.05).
Fig. 5. Principal component analysis carried out with all free amino acids (see Table 1). Gray circles indicate maximum free essential amino acid. Hexagonal symbols, PPI2, black symbols, PPC.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2016.12.012

References


Protein Aggregation. *Journal of Agricultural and Food Chemistry*, 61(6), 1196–1204. https://doi.org/10.1021/jf003739m


