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Effect of enzymatic hydrolysis on molecular weight distribution, techno-functional properties and sensory perception of pea protein isolates

Verónica García Arteaga[a,b], Marijose Apéstegui Guardia[b], Isabel Muranyi[b], Peter Eisner[b,c], Ute Schweiggert-Weisz[b,*]

[a] Center of Life and Food Sciences Weihenstephan, Technical University of Munich, Germany
[b] Fraunhofer Institute for Process Engineering and Packaging IVV, Germany
[c] ZIEL - Institute for Food & Health, Technical University of Munich, Germany

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ABSTRACT

Pea protein isolate (*Pisum sativum* "Navarro") was hydrolyzed with 11 proteolytic enzymes at different hydrolysis times (15, 30, 60, and 120 min) to improve techno-functional and sensory properties. The degree of hydrolysis and changes within the molecular weight distribution were used as indicators for a reduced allergenic potential. The highest degree of hydrolysis was reached by Esperase hydrolysates (9.77%) after 120 min of hydrolysis, whereas Chymotrypsin hydrolysates showed the lowest (1.81%). Hydrolysis with Papain, Trypsin, Bromelain, Esperase, Savinase, and Alcalase suggested an effective degradation of the 72 kDa-convicilin fraction. Papain and Trypsin hydrolysates showed a degradation of the 50 kDa-mature vicilin after 15 min of hydrolysis. Most hydrolysates showed a significant increase in protein solubility at pH 4.5 at all times of hydrolysis. Trypsin hydrolysates showed the highest foaming (2271%) and emulsifying (719 mL/g) capacities. The bitterness of the hydrolysates was strongly correlated ($P < 0.05$) with the degree of hydrolysis. In general, enzymatic hydrolysis improved techno-functional properties indicating their potential usage as food ingredients.

Industrial relevance: Due to their high protein content, peas are becoming an attractive ingredient for the food industry. However, pea protein isolates are often characterized by poor techno-functional and sensory properties. Enzymatic hydrolysis is known to change the molecular weight distribution of proteins. Consequently, the techno-functional and immunogenic properties might be altered selectively. In this study, enzymatic hydrolysis was applied, resulting in highly functional pea protein hydrolysates with a hypothesized reduction of main allergens. The lower bitter perception highlights their high potential as valuable functional food ingredients.

1. Introduction

The use of protein-rich raw materials for food applications has become increasingly important in recent years. Within the legume family, peas (*Pisum sativum* L.) are an auspicious raw material due to the high amounts of proteins as well as to their absence in the allergen list of *Official Journal of the European Union* (O.J.E.U., 2011). The pea protein content ranges between 20 and 30% (Koyoro and Powers, 1987), and the proteins are mainly composed of salt-soluble globulins (55–80% of the total protein) and water-soluble albumins (18–25% of the total protein). The ratio of these storage proteins depends on genetic and environmental characteristics such as maturation, fertilizers, soil nutrients and cultivation temperature (Barac et al., 2015.; Gueguen and Barbot, 1988; Nikolopoulou et al., 2007).

Depending on the production conditions, pea protein isolates (PPI) are characterized by deficient techno-functional properties, in particular, their low foaming and emulsifying capacities, and by unpleasant sensory properties. Several approaches are described in the literature for the alteration of protein structures in order to improve the techno-functional as well as the sensory properties (Adler-Nissen and Olsen, 1979; Angioloni and Collar, 2013; Buchert et al., 2010; Raksakulthai and Haard, 2003). Among them, enzymatic hydrolysis has shown to be one of the most promising methods for the modification of tailor-made protein preparations (Lqari et al., 2005; Meinlschmidt et al., 2016; Polanco-Lugo et al., 2014; Schlegel et al., 2019). Proteolytic active enzymes cleave peptide bonds, resulting in a mixture of peptides of different sizes and free amino acids (Wouters et al., 2016). Proteases are classified as endopeptidases or exopeptidases depending on their mechanism of action and catalytic site. The efficiency of the enzymatic hydrolysis mainly depends on the enzymes applied and hydrolysis

* Corresponding author.
E-mail address: ute.weisz@ivv.fraunhofer.de (U. Schweiggert-Weisz).

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conditions (time, temperature, pH) used, where the resulting cleavage products have a decisive influence on the hydrophobicity of the peptides and thereby the techno-functional properties (Singhal, Karaca, Tyler, & Nickerson, 2016). Barac et al. (2011, 2012) studied the influence of enzymatic hydrolysis (chymosin, Papain, and *Streptomyces griseus protease*) on pea protein isolates. All hydrolysates showed an improvement in protein solubility (pH 5) and better emulsifying and foaming capacities. However, they focused only on the functional properties for food application, and no attention was given regarding the sensory perception of the hydrolysates.

Enzymatic hydrolysis also affects the peculiar sensory properties of plant proteins such as the green, bitter, or astringent attributes (Adler-Nissen, 1986a; Saha and Hayashi, 2001). The extent of changes in sensory properties is attributed to the degree of hydrolysis and, in particular, to the release of low molecular weight peptides constituted of hydrophobic amino acids. This release depends on the enzyme and the substrate used (Raksakulthai and Haard, 2003; Saha and Hayashi, 2001). Humiski and Aluko (2007) demonstrated that Papain and α-Chymotrypsin hydrolysates from pea proteins were less bitter, while those hydrolyzed with Flavourzyme and Alcalase preparations resulted in an increased bitterness. On the other hand, hydrolysis of soy protein isolates with Flavourzyme showed similar bitterness to the untreated isolate (Meinlschmidt et al., 2016).

Although pea proteins are not included in the list of main allergens, there is some evidence in the literature that also pea proteins, in particular, Pis s 1 (vicilin) and Pis s 2 (convicilin), exhibit an allergenic potential (Codreanu-Morel et al., 2019; Dreyer et al., 2014; Sanchez-Monge et al., 2004). Sanchez-Monge et al. (2004) identified three major pea allergens by immunodetection, immunoblot inhibition assays and cDNAs encoding of pea vicilin. These fractions are a 63 kDa convicilin (Pis s 2), a 47 kDa mature vicilin (Pis s 1), and its 32 kDa proteolytic fragment, which are recognized by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee. The results for pea albumin (2S) potential allergens such as PA1 (6.5 kDa) and PA2 (26 kDa) are ambiguous, and the proteins are yet not recognized as allergens (Malley et al., 1976; Mierzeiewska, Mitrowska, Rudnicka, Kubicka, & Kostyra, 2008). Among the approaches to reduce this allergenic potential, enzymatic hydrolysis has been investigated in detail for different legume proteins such as peanut, soy, and lupin (Kasera et al., 2015; Meinlschmidt et al., 2016; Schlegel et al., 2019). but few data are available for pea proteins (Frączek et al., 2008; Szymkiewicz and Jędrychowski, 2008).

As mentioned above, some studies have focused on the effect of enzymatic hydrolysis on techno-functional and sensory properties of pea protein isolates and, to a lesser extent, on the mitigation of pea allergenicity. However, only the simultaneous study of all effects of proteolysis will enable the production of highly functional and appealing food ingredients where changes in the molecular weight distribution might have an effect on the reduction of allergens.

Therefore, the present study aimed to investigate the influence of enzymatic hydrolysis on the techno-functional properties such as protein solubility, emulsifying capacity, foaming capacity, and foam stability as well as the sensory profile of PPI and its hydrolysates. An indication of the degradation of the main pea allergens Pis s 1 and Pis s 2 was reached by determination of molecular weight distribution.

2. Materials and methods

2.1. Materials

Peas (*Pisum sativum* L. cultivar "Navarro") were provided by Norddeutsche Pflanzenzucht Hans-Georg-Lembke KG (Germany). Enzymes Alcalase® 2.4 L FG 1, Flavourzyme®, Neutrase®, Protamex®, and Savinase® 16 L were from Novozymes (Denmark); Trypsin, Bromelain, and Esperase® 8.0 L were obtained from Sigma-Aldrich (Germany); Chymotrypsin, Corolase® 7089 and Papain were from Merck KGaA (Germany), AB Enzymes (Germany) and Carl Roth GmbH (Germany), respectively. Broad Range™ Unstained Standard, 4–20% Criterion™ TGX Stain-Free™ Precast Gels and Coomassie Brilliant Blue R-250 were purchased from Bio-Rad Laboratories GmbH (Germany). Sodium dihydrogen phosphate, sodium dodecyl sulfate, sodium tetraborate decahydrate, o-phthaldialdehyde, and sodium monohydrogen phosphate were from Sigma-Aldrich (Germany). All chemicals used in this study were of analytical grade.

2.2. Production of pea protein isolate

Peas were dehulled and split using an underflow peeler (Streckel & Schrader KG, Germany) and separated using an airlift system (Alpine Hosakawa AG, Germany). Subsequently, the split pea seeds were milled using a pilot-plant impact mill (Alpine Hosakawa AG, Germany) with 0.5-mm-sieve insertion. The isolation of pea protein was performed according to Tian, Kyle, & Small, (1999) with few changes. An aqueous alkaline extract of the pea flour was prepared in deionized water at a ratio of 1:8 (w/v) at pH 8.0 ± 0.1 using 3.0 mol/L NaOH under constant stirring for 60 min. The protein extract was removed by means of a decanter (3,300 rpm). For isoelectric precipitation, the protein extract was adjusted to pH 4.5 using 3.0 mol/L HCl. After 60 min, the precipitated proteins were separated from the clear supernatant in an SC 20-disc separator (GEA Westfalia Separator Group GmbH, Germany) at 12,000 rpm. The isolate was neutralized with 3.0 mol/L NaOH, pasteurized (70 ± 2 °C) for 2 min and spray-dried.

2.3. Enzymatic hydrolysis of PPI

For enzymatic hydrolysis, a 9% (w/w) PPI dispersion was prepared in deionized water in a thermostatically controlled reactor with temperature and pH adjusted to the optimum conditions of each enzyme (Table 1) according to product data sheet. The enzyme to substrate ratio (E/S) was chosen according to literature. After enzyme addition, the

Table 1
Enzymes preparations used for the hydrolysis of pea protein isolate and the respective hydrolysis conditions applied.

Enzyme	E/S (%)	T (°C)	pH value (−)	Activity	Origin
Alcalase® 2.4 L FG	0.5	65	8	Serine Endoprotease	*Bacillus licheniformis*
Bromelain	0.1	50	7	Cysteine Endoprotease	Pineapple stem
Chymotrypsin	0.1	50	8	Serine Endoprotease	Bovine pancreas
Corolase® 7089	0.5	50	7	Endoprotease	*Bacillus subtilis*
Esperase® 8.0 L	0.5	65	8	Serine Endoprotease	*Bacillus sp.*
Flavourzyme®	0.5	50	7	Endo- and *exo*-protease	*Aspergillus oryzae*
Neutrase® 0.8 L	0.5	50	7	Metallo Endoprotease	*Bacillus amyloliquefaciens*
Papain	0.1	65	7	Cysteine Endoprotease	Papaya latex
Protamex®	0.5	65	7	Endoprotease	*Bacillus licheniformis* and *amyloliquefaciens*
Savinase® 16 L	0.5	50	8	Serine Endoprotease	*Bacillus*
Trypsin	0.1	50	8	Serine Endoprotease	Bovine pancreas

E/S: enzyme to substrate ratio, T: temperature.

suspension was continuously stirred and the temperature and pH were maintained constant. Aliquots of approximately 900 mL were transferred to smaller reactor vessels after 15, 30, 60 and 120 min for enzyme inactivation at 90 °C for 10 min. The hydrolysates were cooled to room temperature and neutralized to pH 7.0 ± 0.1. Aliquots of 5 mL were stored at −20 °C for a minimum of 24 h until electrophoretic analysis. The rest of the samples was lyophilized and ground for 10 s at 7,500 rpm (Grindomix GM200, Retsch GmbH, Germany). The control samples were treated with the same conditions but without the addition of the enzymes. The hydrolysis and controls were performed in duplicate.

2.4. Chemical composition

The dry matter and ash content of the samples were determined by means of a thermogravimetric method (TGA 701, Leco Instruments, Germany). The protein content was determined according to the Dumas combustion method (TruMac N, Leco Instruments, Germany) using the average nitrogen-to-protein conversion factor of N x 6.25. All analyses were performed in duplicate and according to AOAC Official Methods (AOACa, 2003; AOACb, 2003).

2.5. Determination of protein degradation

2.5.1. Molecular weight distribution

The molecular weight distribution was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions according to Laemmli (1970) with slight modifications. Briefly, depending on protein content and dry matter, aliquots between 5.8 and 7.6 µL of the liquid hydrolyzed samples were suspended in 60% (v/v) 2× Tris-HCl treatment buffer, 30% (v/v) phosphate buffer (pH 7) and 10% (v/v) HPLC water to reach a protein concentration of 5 µg/µL. The samples were heated at 95 °C for 5 min (300 rpm) prior to centrifugation at 13,400 rpm for 3 min (Mini Spin Centrifuge, Eppendorf AG, Germany). An aliquot of 3 µL was added into the gel pocket of the Bio-Rad 4–20% Criterion™ TGX Stain-Free™ Precast Gels. The Broad Range™ Unstained Standard (Bio-Rad Laboratories, Germany) was used as a standard molecular weight marker. Gels were run for 30 min at 200 V, 60 mA, and 100 W at room temperature. Staining of the gel was performed using 0.02% Coomassie Brilliant Blue R-250 solution. Finally, gel images were obtained with the Coomassie Blue Gel Doc™ EZ Imager (Bio-Rad Laboratories, Germany). The SDS-PAGE was performed in duplicate, with each sample being prepared two times independently.

2.5.2. Degree of hydrolysis

The degree of hydrolysis (DH) was analyzed according to Nielsen et al. (2001). The DH was calculated based on the total number of peptide bonds per protein equivalent (h_{tot}), and the number of hydrolyzed bonds (h) using the following equation:

$$DH = h/h_{tot} \cdot 100\%$$

The constant values used for α, β and h_{tot} factor were 1.0, 4.0, and 8.0, respectively, according to theoretical general values for unexamined raw material (Nielsen et al., 2001). The sample preparation was performed in duplicate with each preparation measured in triplicate.

2.6. Techno-functional properties

2.6.1. Protein solubility

The protein solubility was performed according to Morr et al. (1985). A 3% (w/v) sample solution was prepared in 50 mL of 0.1 mol/L NaCl solution and adjusted to pH 4.5 and 7.0 using 0.1–1 mol/L NaOH or 0.1–1 mol/L HCl. After constant stirring for 1 h at room temperature, the non-dissolved fraction was centrifuged at 13,650 rpm for 15 min at 15 °C (3 K30 Sigma Laborzentrifugen GmbH, Germany). The supernatant was then filtrated in Whatman No.1 filter paper and frozen until analysis (−20 °C). The protein content was determined using the Dumas combustion method (AOACb, 2003). The protein solubility was analyzed for all four times of hydrolysis of each sample.

2.6.2. Foaming properties

The foaming capacity and foam stability were analyzed according to Phillips et al. (1987) using a whipping machine (Hobart N50, Hobart GmbH, Germany). A 5% sample solution (w/w) was gently stirred for 15 min before whipping (580 rpm) for 8 min until the formation of stable foam. Based on the relation of the foam volume before and after whipping the foaming capacity was calculated. The foam stability was assessed as the percent loss of foam volume after 60 min. The foaming properties of each sample were analyzed after 15 min and 120 min of hydrolysis.

2.6.3. Emulsifying capacity

The emulsifying capacity was determined according to Wang and Johnson (2001). A 1% sample solution (w/w) and 125 mL of Mazola corn oil were placed in a reactor system (IKA®-Werke GmbH & Co. KG, Germany). After 1 min homogenization at 11,000 rpm using an Ultra-Turrax (IKA-Werke GmbH & Co. KG, Germany), 10 mL/min oil were added using a dispenser (IKA®- Werke GmbH & Co. KG, Germany), while measuring constantly the emulsion conductivity using a conductivity meter (LF 521 with electrode KLE 1/T, Wissenschaftliche-Technische Werkstätten GmbH, Germany). The measurement was stopped as a phase inversion was reached (< 10 µS/cm) and the volume of added oil was used to calculate the emulsifying capacity (mL oil/g sample). The emulsifying capacity was analyzed in the samples hydrolyzed for 15 min and 120 min, respectively.

2.7. Sensory analysis

2.7.1. Sample preparation

For sensory analysis, 2% solutions (w/w) of PPI and hydrolysates inactivated after 15 min and 120 min were prepared with tap water. The samples were adjusted to pH 7.0 with 1 mol/L NaOH and coded using three-digit random numbers. Water and plain crackers were provided for palate cleansing in between.

2.7.2. Sample evaluation

The sensory evaluation was conducted according to DIN 10967-1-1999. For selection of the main attributes, a ten-member trained panel evaluated attributes regarding retronasal aroma, taste and trigeminal sensation of the PPI and its hydrolysates. Attributes selected by more than five assessors were chosen for further sensory analysis such as pea-like (3-s-butyl-2-methoxypyrazine), green (hexanal), earthy (geosmin), roasted (furaneol/acetylpyridine), cooked potato (3-(methylthio-) propanal), salty, astringent, and bitter.

For sensory analysis, 20 mL of each sample were presented at room temperature, in glass cups and random order. Six samples were presented per session. The panelists assessed the sample intensities of the attributes on a 0 (not noticeable) to 10 (strongly noticeable) ranging scale. Furthermore, overall intensity (0 = not perceivable, 10 = very strong perception) and hedonic scale (0 = dislike, 5 = neutral, 10 = like) were assessed. The results are presented as the mean values among all panelists.

2.8. Statistical analysis

All results, expressed as mean values ± standard deviation of at least two measurements ($n = 2$), were analyzed by one-way analysis of variance (ANOVA). Additionally, a two-way ANOVA was used to analyze the influence of pH and time of hydrolysis on the protein solubility. The mean values were compared using Tukey's post-hoc test. The

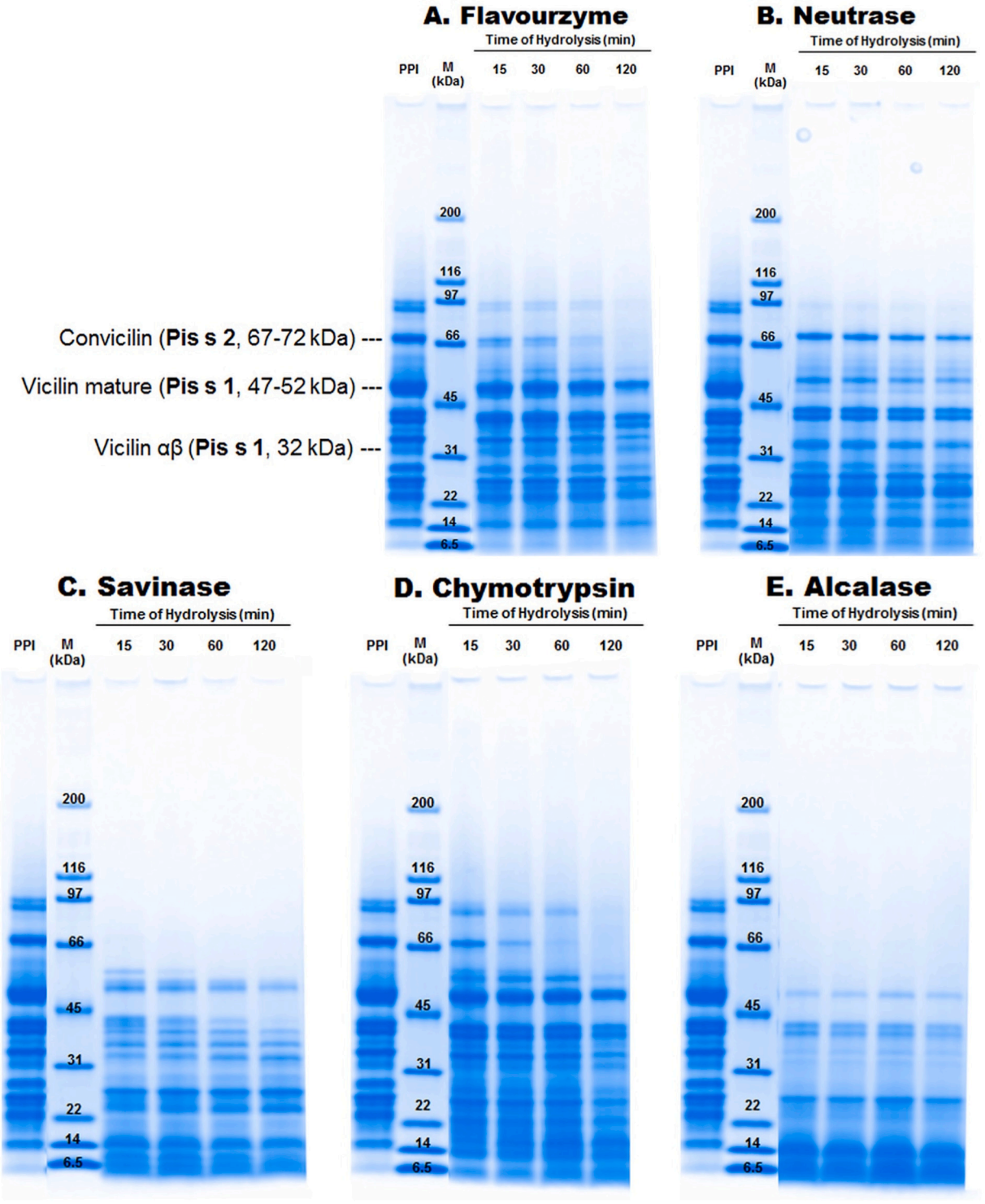

Fig. 1. Molecular weight distribution of the untreated pea protein isolate (PPI) and PPI hydrolysates (Flavourzyme, Neutrase, Savinase, Chymotrypsin, and Alcalase) obtained at different times of hydrolysis as determined by SDS-PAGE under reducing conditions. M = molecular weight standard, indicated in kilo Dalton (kDa).

relationship among DH, protein solubility, and bitterness was analyzed using the Pearson correlation coefficient. All statistical analyses were performed using OriginPro 2018b and were considered statistically significant at $P < 0.05$.

3. Results and discussion

The reference PPI showed 83%of protein, 92% of dry matter, and 5% ash content. The hydrolyzed PPI solutions showed an average protein content of 83%, dry matter of 95%, and ash content of 6%. Complete data can be found in Table A-1 in the Mendeley dataset (García Arteaga et al., 2020).

3.1. Effects of enzymatic hydrolysis on protein degradation

3.1.1. Molecular weight distribution

The molecular weight distribution was analyzed in order to investigate the effect of enzymatic hydrolysis on the pea proteins and on

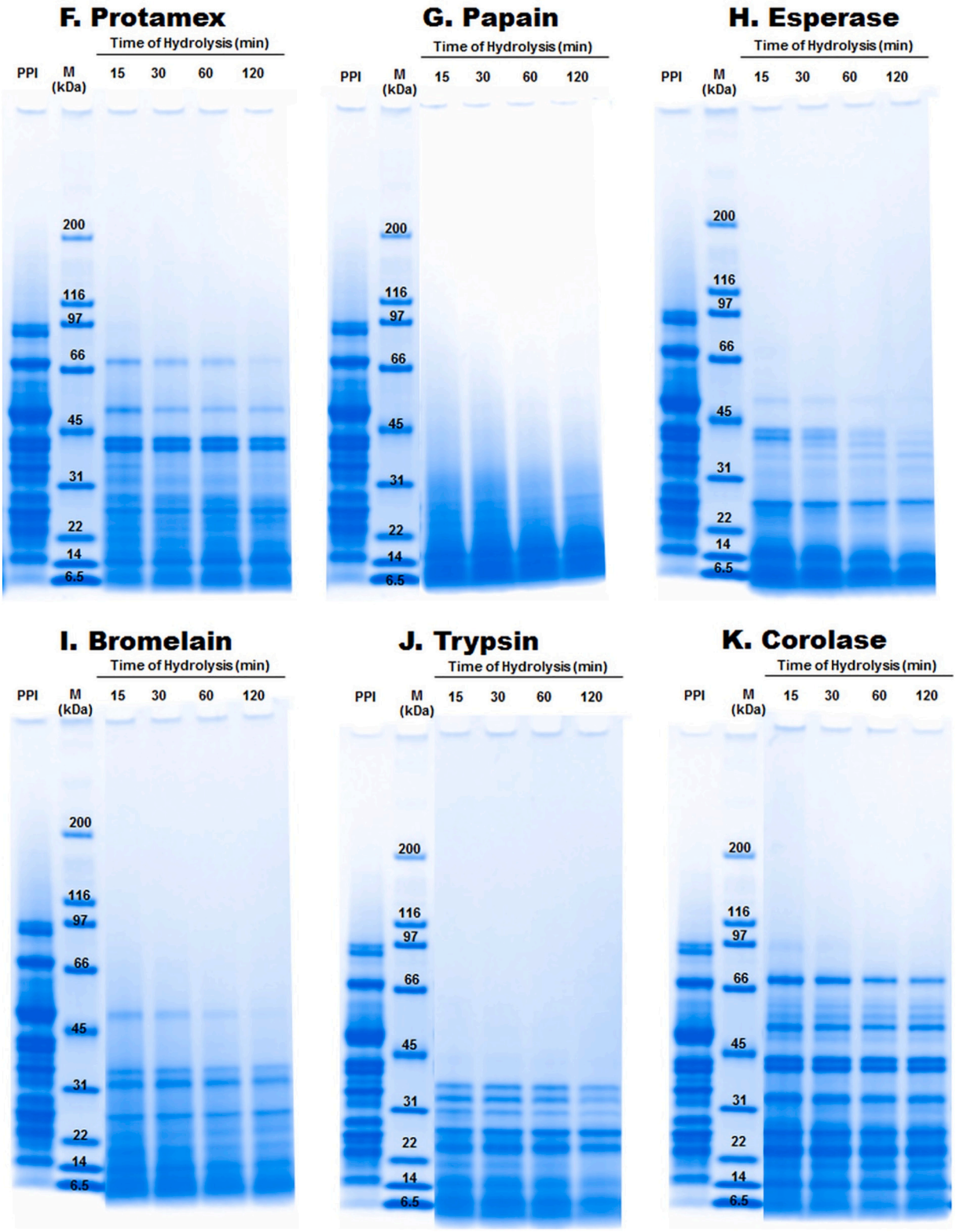

Fig. 2. Molecular weight distribution of the untreated pea protein isolate (PPI) and PPI hydrolysates (Protamex, Papain, Esperase, Bromelain, Trypsin, and Corolase) obtained at different times of hydrolysis as determined by SDS-PAGE under reducing conditions. M = molecular weight standard, indicated in kilo Dalton (kDa).

the main allergens. Protein fractions of the PPI ranged from 97 to 7 kDa (Figs. 1 and 2), which is in accordance to pea SDS-PAGE profiles available in literature (Barac et al., 2012). Enzymatic hydrolysis showed an influence on molecular weight distribution, especially regarding the high molecular weight fractions (Figs. 1 and 2).

3.1.1.1. Pis s 2 degradation. Pis s 2 (~72 kDa) was degraded almost completely after 15 min of hydrolysis with Savinase, Alcalase, Papain, Esperase, Bromelain, and Trypsin. Hydrolysis with Neutrase and Corolase showed no effect on this protein fraction; whereas hydrolysis with Flavourzyme, Chymotrypsin, and Protamex showed a

reduction of this fraction with longer times of hydrolysis. Furthermore, Flavourzyme, Neutrase, Chymotrypsin, and Corolase hydrolysates showed one neo-band at around 55 kDa, which has been previously described by Le Gall et al. (2005) as a possible cleaved-peptide of convicilin.

3.1.1.2. Pis s 1 degradation. Pis s 1 (~50 kDa) was completely hydrolyzed by Papain and Trypsin within the first 15 min of hydrolysis, whereas Esperase and Bromelain cleaved this fraction only after 60 and 120 min, respectively. Alcalase, Protamex, and Savinase reduced the Pis s 1-intensity by approximate 71%, 38%, and 20%, respectively, after 120 min of hydrolysis. The mature Pis s 1 vicilin is composed by different polypeptides such as vicilin αβ (30–36 kDa) and vicilin-γ (12–16 kDa), and the breakdown of mature Pis s 1 could result in an increase of these fractions. The vicilin αβ has been also described as one major pea allergen (Sanchez-Monge et al., 2004). Except for all Papain hydrolysates and Esperase 120-min hydrolysate, all other enzymes were unable to hydrolyze the vicilin αβ fraction, which might indicate a preservation of the allergenic potential of this protein fraction.

Different results within the same protease family might be due to substrate specificity. From the cysteine endopeptidases, Papain shows a preference for bulky hydrophobic residues, whereas Bromelain shows a preference for polar amino acids in both P1 and P1′ position (Cstorer and Ménard, 1994; Rowan, 2013). The electrophoretic results from this study suggest that the PPI probably had more of the hydrophobic residues such as leucine or glycine, which enabled Papain to cleave peptide bonds within the protein efficiently. Similarly, hydrolysates from serine proteases showed different degradation patterns suggesting different substrate specificities. Furthermore, as pea protein composition depends on the botanical variety, time of harvest, and environmental conditions, further studies of the PPI "Navarro," such as amino acid profile and protein fractioning, are necessary to understand the mechanism of action of these enzymes. A comparison of electrophoretic results in this study with those from literature is difficult as different pea varieties and enzyme conditions have been used.

Comparable to the results of Le Gall et al. (2005), the PA2 albumin fraction (26 kDa) showed resistance to proteolysis in all hydrolysates except for Papain hydrolysate. The complete and partial degradation of Pis s 2 and Pis s 1, respectively, indicates that enzymatic hydrolysis might represent an effective method to destroy the main allergens of pea proteins.

3.1.2. Degree of hydrolysis

The DH was analyzed with the OPA reagent, which forms a complex with free primary α- and ε-amino groups, which is then photometrically detected. The DH increased significantly with longer times of hydrolysis (Table 2). Among the serine proteases, Esperase hydrolysate showed the highest DH after 120 min (9.77%) followed by Alcalase (9.24%) and Savinase (8.62%) hydrolysates after 120 min of hydrolysis. Trypsin hydrolysates showed lower DH with 7.59% after 120 min of proteolysis, while Chymotrypsin hydrolysates showed the lowest DH with 1.81% after 120 min. As mentioned in the previous section, different results within the same protease family might be due to substrate specificity, however, the presence of a *Pisum sativum* Trypsin inhibitor (PSTI II) could have an influence on the hydrolysis with Trypsin and Chymotrypsin (Pouvreau et al., 1998), thus reducing their proteolytic mechanism of action. Another explanation for the low DH of Chymotrypsin hydrolysates might be the low amounts of methionine and tryptophan in pea proteins reducing the enzyme-substrate interactions (Hedstrom et al., 1992).

Although Papain and Bromelain showed noticeable changes in the molecular weight distribution, the DH values of 5.04% and 3.57% were unexpectedly low after 120 min. A reason could be an unstable and weak reaction of the OPA reagent with cysteine, as postulated by Chen et al. (1979). Hydrolysates from Protamex, Corolase, Flavourzyme, and Neutrase showed a lower increase in the DH with 4.15%, 4.65%, 4.70%, and 5.16% after 120 min of hydrolysis, respectively.

3.2. Effects on techno-functional properties

According to the molecular weight distribution and DH, the hydrolysates with the most changes in the electrophoretic profile (Papain, Trypsin, Esperase, Bromelain, and Alcalase hydrolysates) and hydrolysates with the least changes (Chymotrypsin hydrolysates) are shown in tables and figures of further sections. Complete data can be found in Tables B-1 and B-2 in the Mendeley dataset (García Arteaga et al., 2020).

3.2.1. Protein solubility

Enzymatic hydrolysis promotes the interaction of hydrophilic groups with water molecules by decreasing peptide size, hence increasing protein solubility (Wouters et al., 2016). Consequently, an increase in protein solubility could be attributed to changes in the protein structures, the release of smaller peptides and hydrophilic amino acids as well as changes in the electrostatic forces (Lam et al., 2016).

Protein solubility was analyzed at pH 4.5 (general isoelectric point of pea proteins) and pH 7.0 as well as after the four different hydrolysis times. The PPI showed a low protein solubility of 2% at pH 4.5, while the protein solubility at pH 7.0 was 51%. Fig. 3 shows the protein solubility of the different hydrolysates. Except for Chymotrypsin at 15 min and 30 min, all the hydrolysates improved protein solubility significantly at pH 4.5. Esperase hydrolysates showed the highest protein solubility at pH 4.5 and pH 7.0, with 71% and 78%, respectively, after 120 min. Trypsin, Savinase, and Alcalase hydrolysates followed

Table 2
Degree of hydrolysis of pea protein isolate (PPI, 0 min) and PPI hydrolysates after 15, 30, 60 and 120 min of hydrolysis.

	Degree of hydrolysis (%)				
	0 min	15 min	30 min	60 min	120 min
Flavourzyme	2.36 ± 0.16^{a}	2.96 ± 0.46^{a,b}	3.41 ± 0.21^{b,c}	3.88 ± 0.05^{c}	4.70 ± 0.24^{d}
Neutrase	2.36 ± 0.16^{a}	4.12 ± 0.15^{b}	4.14 ± 0.34^{b}	4.73 ± 0.15^{c,d}	5.16 ± 0.14^{d}
Savinase	2.36 ± 0.16^{a}	5.40 ± 0.57^{b}	6.45 ± 0.81^{b,c}	7.44 ± 1.04^{c,d}	8.62 ± 1.07^{d}
Chymotrypsin	2.36 ± 0.16^{a}	1.43 ± 0.70^{a}	1.50 ± 0.80^{a}	1.74 ± 0.75^{a}	1.81 ± 0.70^{a}
Alcalase	2.36 ± 0.16^{a}	7.15 ± 0.59^{b}	7.77 ± 0.69^{b}	8.40 ± 0.99^{b,c}	9.24 ± 0.28^{c}
Protamex	2.36 ± 0.16^{a}	2.79 ± 0.69^{a}	2.92 ± 0.71^{a}	3.21 ± 0.80^{a}	4.15 ± 0.67^{a}
Papain	2.36 ± 0.16^{a}	4.41 ± 0.44^{b}	4.67 ± 0.35^{b}	4.81 ± 0.33^{b}	5.04 ± 0.37^{b}
Esperase	2.36 ± 0.16^{a}	5.96 ± 0.19^{b}	7.05 ± 0.76^{b,c}	8.15 ± 0.75^{c}	9.77 ± 0.51^{d}
Bromelain	2.36 ± 0.16^{a}	2.29 ± 1.28^{a}	2.71 ± 1.05^{a}	2.48 ± 0.43^{a}	3.57 ± 0.87^{a}
Trypsin	2.36 ± 0.16^{a}	3.29 ± 0.48^{a}	4.72 ± 0.80^{a,b}	6.08 ± 0.88^{b,c}	7.59 ± 1.67^{c}
Corolase	2.36 ± 0.16^{a}	3.38 ± 0.41^{a,b}	3.94 ± 0.19^{b,c}	4.26 ± 0.67^{b,c}	4.65 ± 0.45^{c}

Results are expressed as means ± standard deviation (n = 4). Means with different letters within one row indicate significant differences (Tukey, $P < 0.05$).

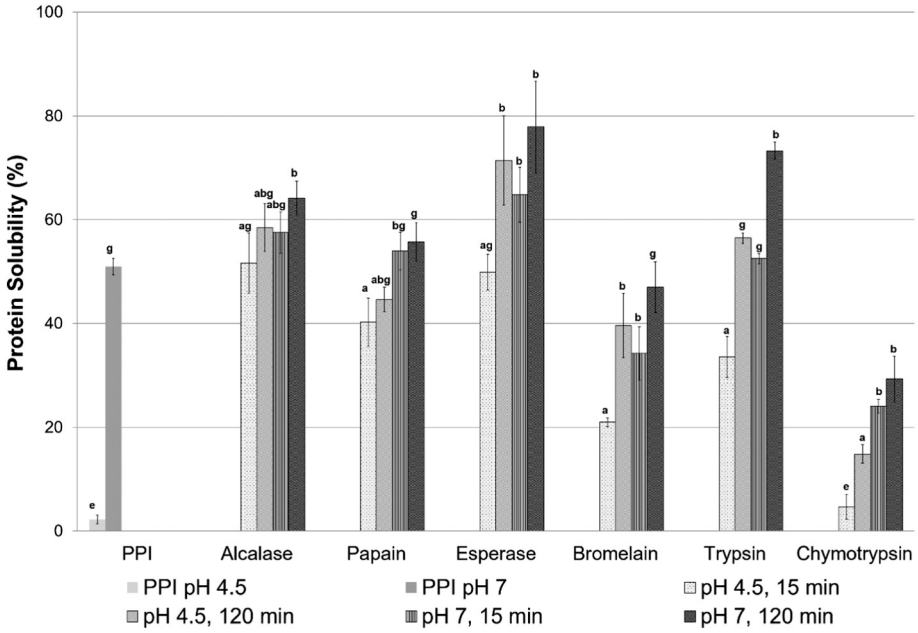

Fig. 3. Protein solubility of the untreated pea protein isolate (PPI) and PPI hydrolysates at different pH and hydrolysis times. Results are expressed as means ± standard deviation (PPI $n = 2$, Hydrolysates $n = 4$). Means with different letters within each enzyme indicate significant differences with the untreated PPI (Tukey, $P < 0.05$).

with 56%, 65%, and 59%, respectively, at pH 4.5 and 73%, 68%, and 64% at pH 7.0, respectively. Serine endopeptidases such as Alcalase, Esperase, Savinase hydrolyze peptide bonds with tyrosine, phenylalanine or leucine at the carboxyl side (Mahajan and Badgujar, 2010) which might have a positive effect on protein solubility (Adler-Nissen, 1986b; Molina Ortiz and Wagner, 2002). However, Chymotrypsin showed the lowest protein solubility at both pH 4.5 and pH 7.0, which might be attributed to previously discussed reasons in Section 3.1.2.

Moreover, Flavourzyme hydrolysates showed the second lowest protein solubility at pH 7.0, followed by Protamex, Neutrase, and Corolase. Papain (45%) and Bromelain (40%) improved significantly the solubility at pH 4.5, especially after 120 min, while at pH 7.0 their hydrolysates showed no significant difference to the PPI. Protein solubility correlated strongly with the DH after 15 min and 120 min.

3.2.2. Foaming properties

Proteins are known as good foaming agents that distribute homogeneously fine air cells, especially if they have a low molecular weight, a highly hydrophobic surface, and a low electrostatic repulsion as well as a low surface tension (Barac et al., 2015; Lam et al., 2016; Zayas, 1997).

The method used in this study considers that a sample is able to form a foam, only when no liquid remains visible in the whipping bowl directly after whipping. According to this method, the untreated PPI was unable to properly form foam, probably due to its higher molecular weight and unfolded structure. On the other hand, all hydrolysates showed a significant improvement of the foaming capacity and foam stability (Table 3). This might have been caused by changes in the molecular peptide size and surface hydrophobicity. The improvement of protein solubility is known to impact the surface hydrophobicity (Molina Ortiz and Wagner, 2002), and although the solubility was correlated with the degree of hydrolysis, there were no significant correlations between the foaming capacity and the degree of hydrolysis. Thus, to some extent, the average hydrophobicity of the released peptides might have played an essential role in foaming capacity (Lam et al., 2016). Trypsin hydrolysates showed the highest foaming capacity of 2271% after 120 min of hydrolysis, followed by the samples obtained after 15 min of Esperase hydrolysis (2237%). However, the foaming capacity decreased significantly after a 120 min treatment with Esperase (1859%). A similar tendency was observed for the foaming capacity of Savinase hydrolysates (15 min: 2013%, 120 min: 1798%),

Table 3
Foaming properties of the pea protein isolate (PPI) and PPI hydrolysates after 15 min and 120 min of hydrolysis.

	Time (min)	Foaming capacity (%)	Foam stability (%)
Alcalase	15	1940 ± 35^{a}	81 ± 6^{a}
	120	1806 ± 60^{b}	80 ± 6^{a}
Papain	15	2119 ± 72^{a}	97 ± 1^{a}
	120	2101 ± 167^{a}	97 ± 2^{a}
Esperase	15	2237 ± 124^{a}	90 ± 5^{a}
	120	1859 ± 78^{b}	74 ± 12^{b}
Bromelain	15	1710 ± 19^{a}	87 ± 5^{a}
	120	1830 ± 80^{b}	81 ± 16^{a}
Trypsin	15	2065 ± 122^{a}	93 ± 2^{a}
	120	2271 ± 19^{b}	95 ± 3^{a}
Chymotrypsin	15	1619 ± 11^{a}	88 ± 4^{a}
	120	1831 ± 18^{b}	79 ± 3^{a}

Results are expressed as means ± standard deviation (n = 4). Means with different letters within each enzyme indicate significant differences in each experiment (Tukey, $P < 0.05$).

Alcalase hydrolysates (15 min: 1939%, 120 min: 1806%), and Papain hydrolysates (15 min: 2119%, 120 min: 2101%). Flavourzyme hydrolysates showed the lowest foaming capacity of 1614% and 1611% at 15 min and 120 min of hydrolysis, respectively, followed by Corolase, Neutrase, Protamex, and Bromelain hydrolysates.

Horiuchi et al. (1978) suggested that the foam stability of enzymatic hydrolysates improves with an increase in the hydrophobic surface of the protein molecules rather than with the release of hydrophobic amino acids. In our study, Papain hydrolysates showed the highest foam stability (97%) after 15 min and 120 min of hydrolysis. On the other hand, Neutrase hydrolysates showed the lowest foam stability after 15 min (19%) and 120 min (12%) of hydrolysis, followed by Flavourzyme hydrolysate (22%) after 120 min and Protamex hydrolysate (34%) after 15 min of hydrolysis. These results suggest that the higher hydrolyzed isolates might have formed peptides with larger hydrophobic surfaces resulting in higher stabilities.

3.2.3. Emulsifying capacity

Emulsions are dispersions of two immiscible liquid phases, which are generally unstable due to high interfacial tension. Proteins have the

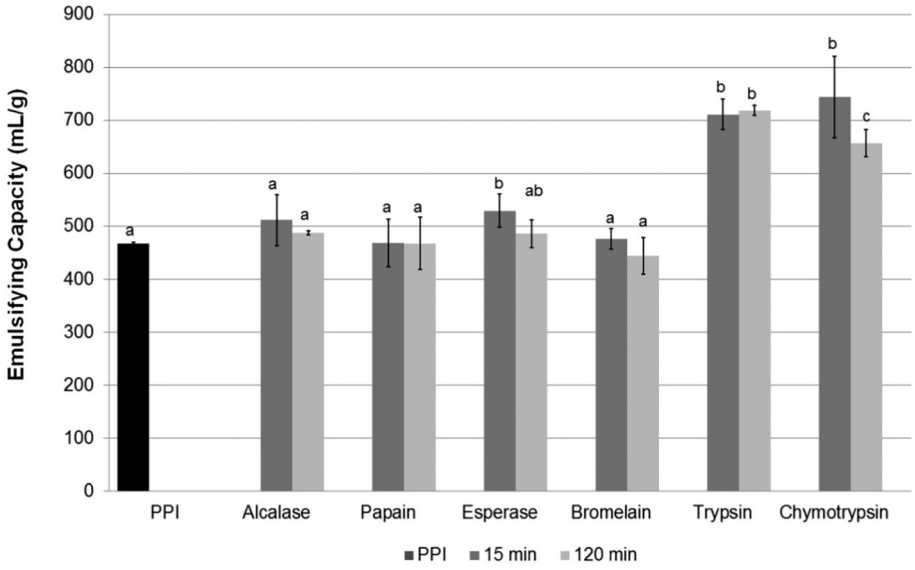

Fig. 4. Emulsifying capacity of the untreated pea protein isolate (PPI) and PPI hydrolysates after 15 min and 120 min of hydrolysis. Results are expressed as means ± standard deviation (PPI $n = 2$, Hydrolysates $n = 4$). Means with different letters within each enzyme indicate significant differences with the untreated PPI (Tukey, $P < 0.05$).

ability to reduce the tension between the two phases by redirecting their amphiphilic residues towards the water and oil phase resulting in smaller droplets.

The PPI showed an emulsifying capacity of 467 mL/g (Fig. 4). After 15 min of hydrolysis, Chymotrypsin hydrolysate showed the highest emulsifying capacity with 727 mL/g, followed by Flavourzyme (715 mL/g) and Trypsin (711 mL/g) hydrolysates. Savinase (499 mL/g) and Esperase (529 mL/g) hydrolysates showed a slight increase in the emulsifying capacity after 15 min of hydrolysis but it decreased with longer hydrolysis times (120 min). Papain, Bromelain, and Alcalase hydrolysates were not significantly different from the PPI. Hydrolysates from Neutrase and Corolase improved the emulsifying capacity significantly after both times of hydrolysis, ranging from 592 mL/g to 641 mL/g. Protamex hydrolysates showed only a significant increase in emulsifying capacity after 15 min of hydrolysis with 645 mL/g.

Negative correlations between the degree of hydrolysis and emulsifying capacity have been reported in the literature (Achouri et al., 1998; Adler-Nissen and Olsen, 1979; Klost and Drusch, 2019). Thus, the molecular protein size might influence protein-protein and protein-oil interactions. Peng et al. (2016) suggested that higher molecular weight and bigger hydrodynamic diameter of the proteins might improve emulsifying capacity. They also suggested that heat treatment and larger peptide sizes increase surface hydrophobicity, promoting hydrophobic interactions between protein-oil droplets, which result in higher emulsifying capacity. However, Barac et al. (2012) suggested that high molecular weight aggregates decreased emulsifying capacity but formed more stable emulsions.

The present study showed a weak negative correlation between the degree of hydrolysis and emulsifying capacity. The weakness of this correlation was mainly due to trypsin hydrolysates as they showed higher emulsifying capacity compared to other highly hydrolyzed samples. One explanation might be that the trypsin hydrolysates maintained the protein fractions between 35 kDa and 22 kDa. These two protein fractions might provide an amphiphilic character to the trypsin hydrolysates since the hydrolysates without these fractions presented lower emulsifying capacity. However, the emulsifying capacity of trypsin hydrolysates was also higher compared to some of the less hydrolyzed samples. Therefore, trypsin might have facilitated the unfolding of hydrophobic side chains of the pea proteins, promoting optimal interaction with the oil.

3.3. Effects on sensory properties

The retronasal aroma of the PPI resulted in attributes such as pea-like (4.2), green (2.9), earthy (1.6), roasted (2.3), and cooked potato (3.8), whereas the main taste attributes were salty (2.1), astringent (1.9), and bitter (3.0) with an overall intensity of 4.9 and a preference indication (hedonic) of 4.3. Compared to the PPI aroma profile, bitterness was the only attribute with a significant change after 15 min and 120 min (Fig. 5). Complete data can be found in Tables C-1 and C-2 in the Mendeley dataset (García Arteaga et al., 2020).

The bitter intensity of the Savinase and Alcalase hydrolysates (15 min of hydrolysis) increased significantly to 6.7 and 6.5, respectively, compared to the untreated PPI (3.0); however, with longer hydrolysis times (120 min), the bitterness of those samples was reduced to 6.5 and 5.4, respectively. On the other hand, the bitterness of Esperase hydrolysates increased significantly to a score of 6.4 only after 120 min of hydrolysis. After 15 min of hydrolysis, Bromelain (2.4), Protamex (2.5), Trypsin (2.6), and Papain (2.7) hydrolysates showed lower bitter intensities compared to the PPI followed by Chymotrypsin (3.5), Corolase (3.5) and Neutrase (3.7) hydrolysates. The lowest bitterness in the samples (2.2) was obtained by hydrolysis with Chymotrypsin and Protamex after 120 min of hydrolysis followed by Neutrase (2.4) and Corolase (2.4). These results suggested a strong correlation between the bitterness and the DH. The correlation between the DH and the formation of bitter peptides has been extensively studied (Adler-Nissen and Olsen, 1979; Meinlschmidt et al., 2016; Saha and Hayashi, 2001; Sun, 2011), where the cleavage of peptide bonds and release of small peptides with hydrophobic amino acid residues leads to an increase in bitterness.

The highest overall intensity after 15 min of hydrolysis was observed in Alcalase (5.9) and Savinase (5.7) hydrolysates; whereas Papain (4.1) and Trypsin (4.1) hydrolysates showed the lowest overall intensity. However, after 120 min of hydrolysis, Esperase hydrolysate showed the highest overall intensity of 6.9 followed by Savinase (5.9), Alcalase (5.4), and Trypsin (5.0) hydrolysates. The high overall intensity results suggest that the panelist perceived this intensity as an increase in bitterness. Accordingly, Esperase (2.9, 1.9), Savinase (1.7, 1.8), and Alcalase (1.6, 2.0) hydrolysates were the least favorite among the panelist after 15 min and 120 min of hydrolysis, respectively. After 15 min of hydrolysis, Protamex hydrolysate (5.5) was the favorite

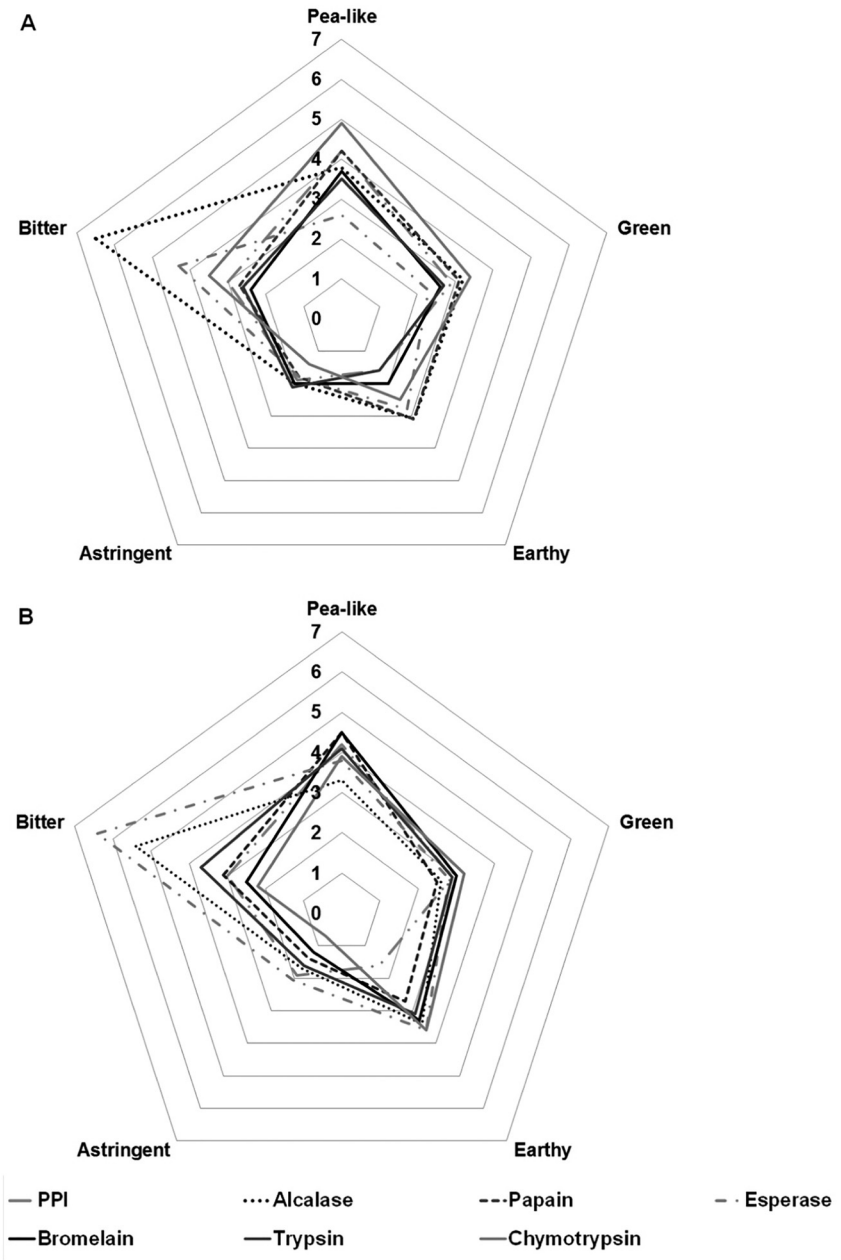

Fig. 5. Retronasal aroma and taste profile of the untreated pea protein isolate (PPI) and PPI hydrolysates after 15 min (A) and 120 min (B) of hydrolysis. Results are expressed as means (n = 10).

sample among the panelists, followed by Chymotrypsin hydrolysate (4.8), Bromelain hydrolysate (4.6), and Trypsin hydrolysate (4.2). These results suggest that bitterness is an important factor influencing the acceptance by the panelist (Fig. 6).

4. Conclusions

The present study aimed to investigate the effect of hydrolysis of PPI with different enzyme preparations on the techno-functional and sensory properties as well as on the degradation of potential allergens through changes in the molecular weight distribution. Of the 11 enzyme preparations investigated by SDS-PAGE, only Papain, Trypsin, Esperase, Bromelain, and Alcalase hydrolysates showed major changes in the molecular weight distribution with a degradation of high molecular weight peptides and an increase in low molecular weight peptides. This was particularly evident in the Papain and Trypsin hydrolysates. Although these electrophoretic results might indicate a degradation of the main pea allergens, the SDS-PAGE gives only an indication of molecular changes, and further immunological studies are necessary to evaluate a possible reduction in the allergenic potential. Most enzymes improved the techno-functional properties of the PPI, especially protein solubility at pH 4.5 and foaming capacity. Regarding sensory properties, only bitterness changed significantly after enzymatic hydrolysis. This increase in bitterness might affect their usage as a food ingredient; therefore, ongoing studies such as the combination of enzymes and fermentation of hydrolysates are being considered to reduce bitterness while maintaining improved techno-functional properties.

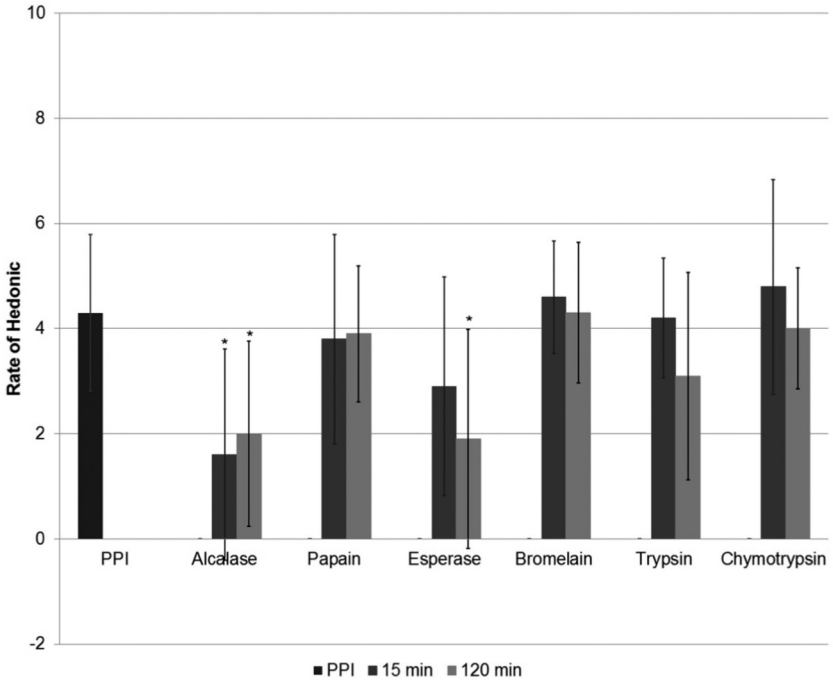

Fig. 6. Hedonic evaluation of the untreated pea protein isolate (PPI) and PPI hydrolysates after 15 min and 120 min of hydrolysis. Results are expressed as means ± standard deviation (n = 10). Means marked with an asterisk (*) indicate significant differences between the individual sample and the untreated PPI (Tukey, $P < 0.05$).

Author agreement statement

The authors hereby declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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