ENZYMATIC MODIFICATIONS OF PEA PROTEIN AND ITS APPLICATION IN PROTEIN-CASSAVA AND CORN STARCH GELS

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ABSTRACT

The interactions between starch and proteins during processing influence pasting and rheological properties of starch and produce modifications on starch gel structure. Enzymatic modifications have been proposed for overcoming the limitations of using proteins as food ingredients. This work aimed to study the impact of native and enzymatically modified pea proteins on the properties of protein–starch (from cassava or corn) gels. Pea protein isolate (PPI) was incubated with endopeptidase (AL) or microbial transglutaminase (TG). Pasting profile, rheological behaviour and water retention capacity of protein–starch gels were analyzed. Protein (native and enzymatically modified) incorporation increased the viscosity of both corn and cassava starches during gel preparation. However, the hydrolyzed protein reduced drastically the increment of viscosity of protein-starch gels. The addition of PPI led to corn starch network that shifted from an elastic-like nature to a more viscous-like, whereas the opposite effect was observed in cassava gel network. TG- and AL-treated proteins led to a decreased of both G' and G'' moduli of protein-starch gels, and AL-treated proteins showed the highest decrease on these parameters. Hydrolyzed proteins also favoured the syneresis of the protein-corn starch gel, whereas crosslinked proteins tended to reduce it. Enzymatic modifications of pea proteins affected significantly pasting and rheological properties of protein-starch gels.

1. INTRODUCTION

The development of protein-enriched products has gained considerable attention in recent years. The increase on protein content and/or improve of protein quality of food could lead to formulations with better nutritional properties. In this sense, although vegetable proteins are major components in the diet of food-producing animals, they are increasingly important in human nutrition (Colombo, Ribotta & León, 2010).

Peas (*Pisum sativum* L.) are commonly used in animal feed, being this seed most used for pig feeding in Europe. This legume is rich in protein and contains more lysine but less sulphur amino acids and tryptophan per unit of protein than soya bean meal (Gatel & Grosjean, 1990). Peas have become interesting as potential protein source in food formulation since, besides their nutritional characteristics, pea protein has good gelling properties (Nunes, Raymundo & Sousa, 2006). However, the application of pea protein in food products is limited because of its weak functionality as a food ingredient (Sun & Arntfield, 2010).

Several modifications have been proposed for overcoming the limitations of using proteins as food ingredients. Among these, protein hydrolysis can improve nutritional and texture characteristics of food proteins (MacLeod & Ames, 1988; Periago et al., 1998). Protein hydrolysis is considered a mild transformation and does not destroy amino acids; it is also specific, which allows controlled processing. Enzymatic treatment of pea flour with acid protease reduced the molecular size of the proteins exposing ionisable amino and carboxyl groups that increase the hydrophilicity of the hydrolysed proteins, which significantly improved the protein solubility at acid pH, the oil absorption capacity and the emulsification capacity of pea flours (Periago et al., 1998). Humiski and Aluko (2007) confirmed that proteolytic enzymes played a major role in determining the functional, nutritional, and bitterness properties of pea protein hydrolysates. The most desirable hydrolysates were

produced by papain and α-chymotrypsin because of reduced bitterness intensity coupled with high levels of angiotensin converting enzyme inhibition and modest free radical scavenging activities. Ribotta and Rosell (2010) showed that the soy protein hydrolysates modified the rheological and pasting parameters of different starches. Molina Ortiz and Añón (2000) reported that the solubility and ability to form and stabilize foams of soybean hydrolysates obtained from five proteases correlated well with the structural properties.

AnoOther alternative for modifying protein functionality is the crosslinking catalyzed by enzymes. Crosslinking of protein molecules can profoundly affect the textural and rheological properties of food. It has been considered as one of the most important mechanisms for engineering food structures with desirable mechanical properties (Dickinson, 1997; Gerrard & Brown, 2002). Transglutaminase (TG, proteinglutamine γ-glutamyltransferase, EC 2.3.2.13) catalyzes an acyl-transfer reaction between the γ-carboxyamide group of peptide-bound glutamine residues (acyl donors) and a variety of primary amines (acyl acceptors), including the ε-amino group of lysine residues, being the pH optimum range for activity between pH 5 and 8 (Data Sheet provided by Ajinomoto Co., Inc.Tokyo, Japan; Marco & Rosell, 2008). Crosslinking by TG was broadly studied in food protein from various sources (Han & Damodaran, 1996; Babin & Dickinson, 2001; Ramírez-Suárez & Xiong, 2003; Ribotta et al., 2010). Although pea protein isolate has limited ability to generate strong heat-induced gels (Shand, Ya, Pietrasik & Wanasundara, 2007), it was showed that TG treatment enhanced the strength and elasticity of pea protein isolated gels (Shand, Ya, Pietrasik & Wanasundara, 2008; Sun & Arntfield, 2011).

In recent years, extensive research has been carried out in order to analyze the properties of vegetable protein/starch systems (Lim and Narsimhan, 2006; Ribotta, Colombo, León & Añón, 2007; Marco et al., 2008; Ribotta et al., 2010; Colombo, León & Ribotta, 2011).

Studies involving soy protein derivatives have been far more common than those concerning other vegetable protein sources. Although utilization of pea derivatives as food ingredients is poorly applied, they could play an important role (similar to what is done with soy protein) when using them as substitutes for meat proteins or as a nutritious and functional additive (Sun et al., 2010). Extensive research exploring the functional properties of enzymatically modified food proteins has been conducted. However, the relationship between modified protein characteristics and food texture modification has not been fully elucidated. This work aimed to study the effect of pea protein enzymatic modification by protease or transglutaminase and its application on the preparation of protein-starch gels. Cassava or corn starches were utilized for determining the impact of enzymatically modified pea proteins on two different sources of starch.

2. MATERIALS AND METHODS

2.1. Materials

Native corn and cassava starches were purchased in the local market (Señor de Sipan, Argentina). Corn starch had 123 g/kg moisture, 4.1 g/kg protein, 0.2 g/kg lipid, 0.1 g/kg ash, 176 g/kg amylose and 824 g/kg amylopectin, dry basis) and cassava had 156 g/kg moisture, 4.2 g/kg protein, 0.1 g/kg lipid, 0.9 g/kg ash, 164 g/kg amylose and 836 g/kg amylopectin, dry basis). Commercial pea protein isolate (PPI) Trades SA, Barcelona, Spain) had moisture, protein, lipid, and ash contents of 67, 848, 9, and 45 g/kg (dry basis), respectively.

Food grade powder microbial TG from Streptomyces spp. from Ajinomoto Co., Inc., (100 U/g) was kindly supplied by Apliena, SA (Terrasa, Barcelona, Spain). The composition of TG was 1% enzyme and 99% maltodextrin (Safety Data Sheet). Protease from *B. licheniformis*

(AL) was kindly donated by Novozymes (Madrid, Spain). All reagents in this study were of analytical grade. The stabilizing agent for AL was glycerine and water (Safety Data Sheet).

2.2. Alcalase and transglutaminase treatments of pea protein isolates

Pea proteins (1.32 g) were dispersed into 20 mL of distilled water. The pH of the suspension was adjusted to ~ 6.5. Preliminary assays were conducted to optimize the incubation time and enzyme amount to produce extensive enzymatic reaction followed by protein solubility and electrophoresis studies. TG (0.83 TG units/gram PPI) or 30 μL of AL (49.1 mAU/gram SPI) was added to the protein suspensions. The suspensions were incubated for 5 h at 35 °C. The enzyme was inactivated by keeping the mixture in boiling water bath for 10 min and the slurry was cooled down to room temperature. Native or non-enzymatically treated PPI followed the same procedure (incubation for 5 h at 35 °C and heating for 10 min) than the enzyme treated samples except that no enzyme was added.

2.3. Protein and peptide solubility

The enzyme-treated mixtures were centrifuged (4400 x *g*, for 15 min) to precipitate insoluble protein. The supernatants were analyzed for nitrogen content (micro-Kjeldahl method AACC 46-13, AACC 2000). The reaction progress was estimated by measuring the nitrogen content of the supernatants, which was able to keep soluble in a solution of 10% trichloroacetic acid (TCA) as showed by Kong, Zhou and Qian (2007). Each determination was done in triplicate.

2.4. Electrophoresis

The enzyme-treated mixtures were centrifuged (4400 x *g*, for 15 min) to precipitate insoluble protein. The supernatants were analyzed by SDS-PAGE. It was performed using gels of T = 12% and C = 2.7%. The gels were 0.75-mm thick and consisted of a 2-cm stacking gel and an 8-cm running gel. The electrophoresis was conducted at a constant voltage of 150 V until the front reached the end of the gel (in approximately 90 min). A Mini Protean II Slab Cell

(BioRad Laboratories, Richmond, CA) was used. MW standards were obtained from BioRad (Broad range, BioRad Laboratories, Hercules, USA). Equal volumes of each extract were applied to the electrophoresis gels for quantitative comparisons. The gels were stained with 0.25% Coomassie Brilliant Blue R in methanol/water/acetic acid (4:5:1 v/v) and were distained in the same solvent.

2.5. Viscosity profile during the thermo–mechanical process

A rapid visco-analyzer (RVA) instrument (Newport Scientific, Australia) was utilized to prepare the samples and follow the apparent viscosity profile of the samples as a function of temperature and time. Corn or cassava starch (1.32 g) and the slurry from enzymatic treatment (1.32 g of PPI and 20 mL of water, pH 6.5) and 5 mL of water were placed inside the aluminium canister and the pH was again adjusted to 6.5. Mixtures of starches and protein had 4.8% w/w starch and 4.8% w/w PPI to keep a 50:50 concentration. Corn and cassava starches were also analyzed by dispersing 1.32 g of starch with 25 mL of distilled water (5.0% w/w starch). RVA corn starch Pasting Method was applied as follows: automatic stirring action was set at 960 rpm for 10 s and then slowed down to 160 rpm. The temperature of the sample was equilibrated at 50 °C, heated to 95 °C for 4 min 42 s, held at 95 °C for 3 min, cooled to 50 °C over 3 min 42 s, and then held at 50 °C for 2 min. Viscosity and temperature were recorded over time; data gathering and analysis were performed using Thermocline for Windows software, provided by the instrument manufacturer. Pasting temperature (PT), peak viscosity (PV), final viscosity (FV), breakdown (BD), and setback (SB) were obtained from the viscograms.

After the measurement of viscosity profile, the suspension was poured while hot (50 °C) into polypropylene tubes, 30 mm diameter and then cooled to room temperature (25 °C) for 24 h.

The samples were analyzed for rheological properties or stored at 4 °C for further determination of syneresis properties. Each sample was done in duplicate.

2.6. Rheological measurements

After the thermo–mechanical preparation process, the samples were kept at 25 °C for 24 h. The viscoelastic behaviour of each sample was measured in duplicate. Measurements were carried out in a controlled stress rheometer RheoStress 1 (Thermo Haake, Germany), using serrated plate–plate geometry of 60 mm diameter and 0.5 mm gap, at a temperature of 25 °C. Samples were carefully poured into the lower plate to minimize the possible breakdown of the gel network. After descending the upper plate, samples were allowed to rest for 3 min. Fresh sample was loaded for each measurement. In order to determine the linear viscoelastic region, strain sweeps (0.01–100%) were run at 1 Hz. The frequency sweeps were then performed at 0.04% over a frequency range of 0.01–10 Hz and the values of the storage modulus (G'), the loss modulus (G''), and the loss tangent (tan δ), as a function of frequency, were calculated using the Rheowin Pro Software (version 2.93, Thermo Haake). Two fresh samples of each gel lot were measured and gels were elaborated in duplicate to ensure reliable results.

2.7. Syneresis

Syneresis was measured by a centrifugation test (Ribotta et al., 2007) using a Beckman J2-MI centrifuge (Beckman Instruments, USA). Starch and starch–pea protein gels were stored seven days at 4 °C. After storage, the gels were tempered at 25 °C for 2 h and centrifuged at 1500 x g for 15 min at 25 °C. After centrifugation the free water was separated, weighed, and expressed as percentage of the total water present in the gel. Measurements were the mean of three repetitions for each duplicated gel.

2.8. Statistical analysis

The data obtained were statistically treated using analysis of variance while the means were compared by the LSD Fisher test at a significance level of 0.05 using Statgraphics Plus Software (v2.01).

3. RESULTS AND DISCUSSION

3.1. Alcalase and transglutaminase treatments

Pea protein isolates were enzymatically modified for altering the protein functionality. With that purpose pea protein were crosslinked by transglutaminase or hydrolyzed with alcalase. The enzymatic modification was followed by quantifying the nitrogen released and the electrophoretic pattern of the enzymatically modified proteins. When treated with TG, nitrogen solubility of pea protein isolates decreased by 46%, from 3.17 ± 0.33 mg/mL (native proteins) to 1.70 ± 0.13 mg/mL (TG-treated proteins), revealing the decrease of protein solubility after crosslinking. SDS-PAGE protein patterns are shown in Fig. 1. TG-treated PPI (line 2, figure 1) showed an intense band which remained at the stacking gel and an evident increase in intensity at the top of the running gel. Ya (2004) informed the formation of large molecular weight compounds when studying treatment of pea proteins with TG, and those compounds were too large to enter the on SDS-PAGE gel. In addition, TG-treated proteins showed a reduction of some bands as compared with the non-treated protein profile (lane 1, figure 1). Sun et al. (2011) showed that most of the PPI subunits cross-linked by TG are in the molecular weight range of 35-74 kDa, which corresponded to pea vicilin and legumin acidic subunit (41 kDa). Also, these authors found that low molecular weight subunits (smaller than 25 kDa) were unaffected by the enzyme. These results are in accordance with the ones obtained in the present work and confirm the formation of protein polymers of higher molecular weight with a concomitant disappearance of the lower molecular weight

polypeptides. Besides, the increase in molecular weight of PPI proteins explained the reduction of nitrogen solubility.

Regarding the treatment of PPI with alcalase, the nitrogen content on 10%-TCA supernatants increased from 0.68 ± 0.06 mg/mL (native protein) to 6.53 ± 0.28 mg/mL (AL-treated protein). Moreover, it was noted great increase of low molecular weight peptides in SDS-PAGE pattern of AL-treated PPI (line 3 figure 1), together with a disappearance of bands along the running gel (lane 3). Clearly, these results are related to the hydrolytic activity of the protease.

3.2. Pasting profile of protein-starch blends

The onset temperatures of corn and cassava starches were 64.9 ºC and 57.5 ºC (Colombo et al 2010). Heating of starch granules above the gelatinization temperature in the presence of water increases the viscosity of the system due to water absorption and swelling of starch granules. Pasting temperature (PT) obtained in the RVA can be considered the temperature at the onset of this rise in the viscosity. Viscosity increases to the point where the number of swollen-intact starch granules reaches its maximum level; this point is named peak viscosity (PV). During the holding period at 95 °C in RVA analysis the sample is subjected to mechanical shear stress, causing loss of starch granule integrity and subsequent disruption which lead to a reduction of paste viscosity, which is measured by the breakdown (BD) in RVA viscograms. As the sample is subsequently cooled down to 50 °C, reordering of amylose chains results in an increase in viscosity (which is defined as setback -SB-) until a gel is formed. Viscosity at the end of the test is called final viscosity (FV).

Cassava starch presented lower PT but higher PV and FV than corn starch (Table 1), which indicate that cassava starch has weaker granular structure and better water binding properties than corn starch. BD values of cassava starch samples were higher than those for corn

samples. Therefore, corn starch showed higher paste stability, which could be related to their low peak viscosities coupled with higher shear and temperature stability (Singh, Isono, Srichuwong, Noda & Katsuyoshi, 2008). The SB values showed higher retrogradation rate in cassava starch dispersion than in corn starch. Moreover, cassava starch showed superior thickening properties, as indicated the higher FV than corn starch.

Addition of PPI decreased pasting temperature in corn starch samples. On the other hand, PT was slightly increased by the protein isolates in cassava samples, with the exception of AL-treated samples. A similar result was recently found by Ribotta et al. (2010) when studying the addition of soy protein isolate to corn and cassava starches.

The presence of PPI increased PV, FV and SB of both starch pastes during heating-cooling process (Table 1). The effect on setback could be attributed to the reorganization of the denatured proteins from the isolates and their effect on amylose crystallization during cooling (Motoki, Nio & Takinami, 1984). A gelatinized starch suspension can be considered as a composite material comprised of a dispersed phase, swelled starch granules, in a continuous phase formed by a suspension of amylose/amylopectin (Ribotta & Rosell, 2010). The rheological properties of such system depend on the properties and the ratio of the components of the continuous phase, the interaction between them and between the dispersed phase and the matrix (Eliasson & Gudmundsson, 1996). In fact Ribotta and Rosell (2010) showed that corn gel displayed a continuous phase formed by swollen starch granules pressed against each other, while a completely disintegrated structure was identified on cassava gel. The higher paste viscosity observed in PPI-containing samples as compared to starch pastes could be due to crosslinks between hydrophilic groups of proteins and starch molecules (Goel, Singhal & Kulkarni, 1999; Ribotta et al., 2007). Although thermodynamic compatibility could also affect the pasting behaviour, viscosity results did not allow to assess that effect. In

addition, hydration and solubilisation of pea protein could affect the effective concentration of starch in the continuous phase, resulting in an increased paste viscosity (Ribotta et al., 2010). Enzyme-treated proteins produced an increase of PV and FV of the starches, but in lesser extent than non-enzyme-treated proteins. Some differences were detected between the pasting properties of starches blended with crosslinked proteins and the ones obtained with hydrolyzed proteins. AL-treated PPI led to a noticeably decrease in peak viscosity, final viscosity and setback in both starches, compared to the values obtained for the protein-starch gels. Regarding the breakdown, enzyme treated proteins reduced that parameter of protein-cassava starch gels, but no significant effect was observed in the protein-corn starch gels. The effect promoted by the crosslinked proteins was less marked than the observed with the hydrolyzed proteins. It seems that crosslinked proteins caused minor alterations on the pasting of the protein-starch gels, whereas the hydrolysis strongly modified the resulting gels. In fact, the effect on the setback was different depending on the enzymatic modification. Hydrolyzed proteins induced a dramatic decrease of the SB in both protein-starches gels, whereas the effect promoted by crosslinked proteins was barely noticeable. Therefore, hydrolyzed proteins affected in greater extent the amylose retrogradation, likely due to interactions between the low molecular weight polypeptides and the amylose chains.

From the results, it is clear that enzymatic modifications affect protein properties and therefore their interactions with starch and water. Non-treated and enzyme-treated PPI could interact with gelatinized starch components in a different way.

3.3. Rheological properties of the gels

Storage modulus (G') was higher than loss modulus (G'') throughout the whole range of frequency for both starches with and without protein isolate addition, indicating that deformations were fundamentally elastic (figure 2). G' values were almost independent of the

frequency in corn starch samples (Figure 2A), suggesting that the gel can be considered strong gel. Cassava starch gels showed a steady increase of G' with frequency (Figure 2B), behaving like weak gels (Lopes da Silva and Rao, 1999). In addition, cassava gels showed higher relative viscous component and a lower consistency when compared to corn samples, as evidenced by higher tan δ and lower G' and G'' values of the cassava gels (figure 3 and table 2). Therefore, cassava gels led to weaker structures with less gel-like character than the corn starch. Corn gel shows a continuous phase formed by swollen starch granules pressed against each other, whereas cassava gels are formed by completely disintegrated granules that yielded continuous polymer dispersion where no starch granules can be envisaged (Ribotta and Rosell 2010).

Pea protein isolate raised storage and loss moduli of both starches, affecting in greater extent the loss modulus in the case of corn starch but the storage modulus in the case of the cassava starch. The interaction between two different biopolymers can be either of segregative or associative nature, but generally in the case of proteins and polysaccharides there is a thermodynamic incompatibility (Grinberg and Tolstoguzov, 1997), thus under certain conditions, any protein-polysaccharide-water system is spontaneously demixed in two different phases. The overall effect of PPI in the starch gel would be the result of possible interactions among hydrophilic groups of proteins and starch molecules, starch and starch molecules, the self-aggregation of pea proteins, or the mutual exclusion of pea proteins and carbohydrates, which increases the effective concentration of both.

Nevertheless, results of tan δ indicated that the addition of PPI led to a corn starch network that shifted from an elastic-like nature to a more viscous-like with less gel-like character than the corn starch alone. Similar findings have been reported when rice starch gels were mixed with different hydrocolloids, indicating weaker structures where the starch network shifted

297 from an elastic-like nature to a more viscous-like (Rosell, Yokoyama & Shoemaker, 2011).
298 Conversely, in the case of cassava gel the presence of PPI resulted in more structured and
299 more solid like (lower tan δ) gel. Likely the lower pasting temperature observed for cassava
300 gel favoured the interaction of starch and proteins chains, leading to better network.
301 Therefore, the structure of the protein-starch gel must be dependent on the starch source
302 yielding more structured network of the PPI with cassava starch than with corn starch, as
303 suggests the rheological behaviour
304 The addition of hydrolyzed proteins (AL-treated PPI) on both cassava and corn gels did not
305 affect the shape of the moduli and loss tangent versus frequency curves compared to the gels
306 obtained with non-treated proteins. In opposition, the presence of PPI or TG-treated proteins
307 yielded gels that were more frequency dependent at high frequencies (figure 2 and 3). The
308 absolute values of the moduli changed significantly when PPI were enzymatically treated
309 (table 2). Both G' and G'' moduli were shifted to lower values when TG- and AL-treated
310 proteins were added to corn and cassava starch gels compared to non-enzymatically treated
311 proteins-starch gels. However, AL-treated proteins showed more pronounced decrease on
312 these parameters. The same trend was observed with the pasting properties, which agrees with
313 the positive relationship described for the viscoelastic moduli and the pasting properties,
314 namely peak viscosity, breakdown, final viscosity and also with the parameter related to
315 amylose retrogradation or setback (Rosell, Yokoyama & Shoemaker, 2011).
316 Concerning the loss tangent, the effect of enzyme-treated proteins on protein-starch gels was
317 only significant when they were prepared with cassava gels. Presumably, corn starch yields
318 stronger or more structured gels, which were less susceptible to be modified with the PPI or
319 enzyme treated PPI addition. Conversely, the enzyme treated proteins added to cassava starch
320 produced marked changes in the loss tangent. The hydrolyzed protein added to cassava starch

led to gels with higher tan δ gels. That effect could be partially related to its ability for reducing or preventing amylose retrogradation, as has been suggested for the interaction between hydrocolloids and starch (Techawipharat, Suphantharika & BeMiller, 2008). The TG-treated proteins led to protein-starch gels with similar tan δ than that of the untreated proteins-starch gel. Clearly, the effect of PPI on the viscoelastic behaviour of starch gels is completely dependent on the starch nature.

3.4. Syneresis

Water self-separation as consequence of gel network contraction is known as syneresis and is produced by the reorganization of starch molecules or retrogradation (Zheng & Sosulski, 1998). The water separated from starch gels or starch-containing products is usually viewed unfavourably since it is associated to produce product deterioration.

Cassava gels did not show water separation despite the addition of PPI during the storage period. However, syneresis was observed on corn starch gels. Only gels containing AL-treated PPI showed a significant increase in water released (Figure 4), which could be attributed to the loss of water retention capacity and the negative effect of hydrolyzed proteins on gel structure, as was previously described for pasting and rheological properties. A tendency to decrease the syneresis, although not significant, was observed in the gels containing TG treated PPI. Water released of soy protein/corn gels was decreased when soybean proteins were treated with TG (Ribotta et al., 2010) and it was related to the high water retention capacity of these proteins.

4. CONCLUSIONS

Pea proteins affected significantly the pasting behaviour of both corn and cassava starches, increasing the viscosity through the heating-cooling cycle. Enzymatically modified pea

proteins by crosslinking or hydrolysis affected the pasting behaviour of starches, having the hydrolyzed pea protein higher impact on the pasting properties than the crosslinked ones. Viscoelastic properties of protein-starch gels revealed that hydrolyzed proteins led to weaker gels. The TG-treated proteins led to protein-starch gels with similar tan δ than that of the native proteins-starch gel. Hydrolyzed proteins also favoured the syneresis of the protein-corn starch gel, whereas crosslinked proteins tended to reduce it. Clearly, the effect of PPI on starch properties was completely dependent on the starch nature and the enzymatic treatment of protein. Enzymatic changes of pea proteins could be an important tool to increase the incorporation of pea proteins in the starch-based foods.

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