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Yellow pea flour and protein isolate as sources of antioxidant peptides after simulated gastrointestinal digestion

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Abstract

Although peas are widely consumed legumes throughout the world, the bioactivity of the peptides released by the gastrointestinal digestion has not been sufficiently studied so far. The objective of the present work was to evaluate the potential of flours and protein isolates obtained from two varieties of yellow peas as sources of antioxidant peptides. Flours and protein isolates were prepared and submitted to a simulated gastrointestinal digestion. Protein hydrolysis degree (TNBS method) and protein solubility (in phosphate buffer saline, pH = 7.4) values were independent on the starting material. Antioxidant activity measured by oxygen radical absorbance capacity (ORAC) and hydroxyl radical averting capacity (HORAC) showed no differences between varieties. A lower activity was registered for protein isolates with respect to flours in the case of HORAC, which could be associated with a loss of molecules with molecular masses lower than 43 kDa in the protein isolates. A significant increase in activities was evidenced by both methods after gastrointestinal digestion, except in the case of HORAC activity of flours. Digested from protein isolates presented a greater ratio of molecules smaller than 1.4 kDa and a lower ratio of those larger than 6.5 kDa with respect to digested flours, according to electrophoresis and gel filtration chromatography studies. Results suggested that the presence of other components or/and the initial state of proteins would affect proteolytic attack of digestive enzymes. Both, pea flours and protein isolates, present interesting potential as antioxidants food ingredients.

KEYWORDS
antioxidant properties, flour, gastrointestinal digestion, protein isolate, yellow peas

1 | INTRODUCTION

In recent years, the role of dietary proteins as physiologically active components has been exhaustively studied, demonstrating that they can be the source of biologically active peptides. These peptides are inactive within the sequence of parent protein and can be released during food processing or gastrointestinal digestion. Once peptides are released, they may cause different physiological actions such as antioxidant activity. Studies of structure–activity relationship have shown that physic-chemical and structural features, such as charge, amino acid sequence, molecular size, and hydrophobicity, may determine the bioactivity of peptides (Sarmadi & Ismail, 2010). Conditions in the gastrointestinal tract, such as activity of digestive enzymes and pH values might influence the structures and functions of the released peptides (Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011). The ability of peptides to resist the

enzymatic attack is related to their amino acid composition due to the specificity of digestive enzymes. Gastrointestinal digestion can be in vitro simulated by different methodologies that try to mimic physiological conditions (temperature, agitation, pH, enzyme activities, and fluids composition) and the sequence of events in the gastrointestinal tract. Static (or biochemical) methods are the simplest ones and include two or three digestion steps (oral, gastric, intestinal) which products stay in one only reactor (Minekus et al., 2014).

Pea (*Pisum sativum*) seeds are an important source of nutrients and healthy compounds (20% to 26% w/w protein, 1% to 3% w/w lipids, 46% to 50% w/w carbohydrates, and 14% to 18% w/w fiber) providing approximately 317 kcal/100 g of grain (Zulet & Martínez, 2001). Peas are a source of ingredients such as flours, protein isolates, starches, and fiber, which are of increasing importance in the design of healthy foods and foods for special diets (Agboola, Mofolasayo, Watts, & Aluko, 2010). As a negative aspect, peas contain anti-nutritional factors, including α-galactosides, trypsin inhibitors, and phytates, which concentrations differ widely between varieties, and whose elimination is essential to improve the nutritional quality. There are simple and economical processing techniques capable to effectively remove anti-nutritional factors, such as soaking, cooking, and germination (Vidal-Valverde, Frias, & Valverde, 1992).

Diets rich in dry peas have showed to be effective in decreasing the incidence of colon cancer, type-2 diabetes, LDL-cholesterol, and heart disease (Roy, Boye, & Simpson, 2010). As for other legumes, these beneficial effects have been related to micronutrients, phytochemicals, and bioactive peptides. Inhibitory activity of the angiotensin converting enzyme (ACE), antioxidant activity, antitumor activity, among others, have been described for peptides released from legume proteins by using diverse proteases and proteolysis conditions (López-Barrios, Gutiérrez-Uribe, & Serna-Saldívar, 2014). In this sense, it has been reported that the nonhydrolyzed pea protein showed no ACE inhibitory activity, but this activity was observed after in vitro gastrointestinal digestion (Barbana & Boye, 2010; Jakubczyk & Baraniak, 2014).

Both flours and pea protein isolates are ingredients used in food formulation. The presence of diverse components and the complexity of the matrix could have an effect on the gastrointestinal digestion processes. The aim of this work was to study the effect of the simulated gastrointestinal digestion on flours and protein isolates from two varieties of yellow peas, focusing on the release of peptides with antioxidant activity. In this way, the potentiality of both types of ingredients as sources of antioxidant molecules was evaluated.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2′-Azo-bis-(2-methylpropionamidine) dihydrochloride (AAPH), trypsin from porcine pancreas (91.4 U/mg), and α-amilase from *Bacillus subtilis* (57.4 U/mg) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pepsin 1:15000 5X NF standards and porcine Pancreatin 4X-100USP were from MP Biomedicals LLC (Solon, OH, USA). Bovine bile salts (B3883) and fluorescein sodium were from Fluka (Steinheim, Germany). Other reagents were of analytical grade.

2.2 | Preparation and characterization of samples

2.2.1 | Pea flours (*F*)

Two varieties of yellow peas (*Pisum sativum*) were used: variety Yams and variety Navarro. Both were cultivated in 2016 in the province of Buenos Aires (Argentina). Flour was obtained by grinding the unpeeled whole peas in an Udy mill using a 1-mm mesh.

2.2.2 | Pea protein isolate (*I*)

Protein isolates were obtained from flours of the two pea varieties by a protocol adapted from Makri, Papalamprou, and Doxastakis (2005) and Qayyum, Butt, Anjum, and Nawaz (2012). Flour was defatted by lipid extraction with hexane (overnight, room temperature). Defatted flour was dispersed (10% w/v) in MilliQ water, and pH was adjusted to 9.5, agitating for 40 min at room temperature. After centrifugation (11,000×*g*, 20 min, 4°C), soluble proteins were precipitated by adjusting the supernatant to the isoelectric pH (pH = 4.5) and centrifuging (10,000×*g*, 20 min, 4°C). The precipitated proteins were suspended in MilliQ water, neutralized (pH = 7), freeze-dried, and stored at 4°C.

2.2.3 | Antitrypsin activity evaluation

Seeds were soaked in tap water for 4 h. Soaked seeds were boiled (seed to water ratio: 1:5 w/v) for 30 min, and, finally, they were dried at 54°C overnight (Khattab & Arntfield, 2009). Flour was obtained from the thermally treated seeds (*Ft*) according to Section 2.2.1. Antitrypsin activity of samples of *F*, *Ft*, and *I* of the two varieties of peas was evaluated. Dispersions (10% w/v) of these samples in phosphate buffered saline (PBS) solution (pH = 7.4) were prepared, kept overnight at 4°C, and then centrifuged (21,380×*g*, 25 min). Antitryptic activity was determined in the supernatant according to Manassero, Vaudagna, Sancho, Añón, and Speroni (2016) by using porcine trypsin (1,350 enzymatic units per milliliter) and denatured hemoglobin as enzyme substrate.

2.2.4 | Centesimal composition

Centesimal composition of flours and protein isolates was determined using the following methods: water: drying at 105°C to constant weight; lipids: Soxhlet method; ash: 550°C (AOAC, 1990); carbohydrates: antrona method after complete acid hydrolysis; proteins:

micro-Kjeldahl method followed by a modified Berthelot colorimetric method (Tabbaco, Meiattini, Moda, & Tarli, 1979), *f* = 5.6 g protein/g N (Gueguen & Barbot, 1988).

2.2.5 | Soluble protein determination

Suspensions (10 mg·ml^{-1}) of samples in PBS (pH = 7.4) were prepared by agitation at 500 rpm (1 h, 37°C) (Thermomixer Eppendorf) and then centrifuged (10,000×*g*, 10 min, room temperature) (Hermle, Labortechnik GmbH, Germany). Soluble protein concentration in the supernatant was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.3 | Simulated gastrointestinal digestion

2.3.1 | Static gastrointestinal digestion

The protocol of Minekus et al. (2014) with some modifications was applied on flours and protein isolates to obtain the corresponding digests (*Fd*, *Id*). *Oral phase*: Samples (about 5 g *F* or 1 g *I*, in order to have comparable amounts of proteins) were homogenized with 3.5 ml of electrolite solution of the simulated salival fluid (SSF, pH = 7); 0.5 ml of α-amylase solution in SSF (26 mg·ml^{-1}), 25 μl 0.3 mol·L^{-1} $CaCl_2$, and 975 μl H_2O were added (all reactives preincubated at 37°C). The mixture was agitated and incubated 2 min at 37°C (Thermomixer Eppendorf, 350 rpm). *Gastric phase*: The oral solution was mixed with 7.5 ml of the electrolyte solution of the simulated gastric fluid (SGF, pH = 3), 1.6 ml of pepsin solution in SGF (47.8 mg·ml^{-1}), and 5 μl 0.3 mol·L^{-1} $CaCl_2$, adjusting the pH to 3 with 2 eq·L^{-1} HCl and adding water to complete 10 ml of SGF. The mixture was incubated for 2 h at 37°C with agitation (Thermomixer Eppendorf, 350 rpm). *Intestinal phase*: Gastric solution (20.0 ml) was mixed with 11.0 ml of the electrolyte solution of the simulated intestinal fluid (SIF), 5.0 ml of pancreatin solution in SIF (15.3 mg·ml^{-1}), 2.5 ml of bovine bile salts (150 mg·ml^{-1}), and 40 μl 0.3 mol·L^{-1} $CaCl_2$; the pH was adjusted to 7 with 1 mol·L^{-1} NaOH and water was added to complete 20.0 ml of SIF. The mix was incubated for 2 h at 37°C (Thermomixer Eppendorf, 350 rpm). After that, enzyme activities were inactivated by treatment at 85°C for 10 min. Electrolyte solutions for SSF, SGF, and SIF were prepared according to Minekus et al. (2014).

2.3.2 | Protein hydrolysis degree (HD)

HD was measured by the 2,4,6-trinitrobencenesulfonic acid (TNBS) method (Adler-Nissen, 1979; Orsini Delgado, Tironi, & Añón, 2011). HD was calculated as follows: HD% = 100 × ($[-NH_2]_h - [-NH_2]_0$)/($[-NH_2]_\infty - [-NH_2]_0$); where [-NH2] indicates the concentration of free amino groups in the nonhydrolyzed ($_0$) or the hydrolyzed ($_h$) samples. $[-NH_2]_\infty$ was estimated according to $[-NH_2]_\infty = 1/M_{aa} \times (1 + f_{Lys}) \times C_{prot}$, where M_{aa} is the average molecular weight of amino acids (169.42 g·mol^{-1}), f_{Lys} is the proportion of lysine (1/17.8) (values calculated from amino acid composition of peas; Boye, Zare, & Pletch, 2010), and C_{prot} is the protein concentration.

2.3.3 | Glycine-SDS-PAGE

Freeze-dried samples were dispersed in sample buffer (0.0625 mol·L^{-1} Tris, 2% SDS, 10% v/v glycerol) and centrifuged before loading in the gel (Laemmli, 1970). Runs were performed in a Mini-Protean II (BIO-RAD) equipment using 120 and 40 g·L^{-1} acrylamide for separating and stacking gels, respectively. The electrophoresis runs were carried out at constant current (30 mA per gel) and at room temperature. Gels were stained with Coomassie Brilliant Blue R-250 (1 g·L^{-1}). Silver staining was performed to increase analytical sensitivity (Blum, Beier, & Gross, 1987).

2.3.4 | Tricine-SDS-PAGE

Freeze-dried samples were dispersed in sample buffer (0.375 mol·L^{-1} Tris-HCl, pH = 7, 75 g·L^{-1} glycerol, 30 g·L^{-1} SDS, 0.125 g·L^{-1} Coomassie blue G-250) and centrifuged before loading (Schägger, 2006). Runs were performed using 160 (6 mol·L^{-1} urea), 100, and 40 g·L^{-1} acrylamide for separating, spacing, and stacking gels, respectively. The following buffer solutions were used: 1 mol·L^{-1} Tris-HCl, 1 g·L^{-1} SDS, pH = 8.45 (gel buffer); 0.1 mol·L^{-1} Tris-HCl, pH = 8.9 (anode buffer); 0.1 mol·L^{-1} Tris, 0.1 mol·L^{-1} tricine, 1 g·L^{-1} SDS, pH = 8.25 (cathode buffer). The electrophoresis runs were carried out in a Mini Protean II Dual Slab Cell (BIO-RAD) at variable current (30 to 100 mA per gel after entering the separation gel) and at room temperature. Gels were fixed and stained with Coomassie Brilliant Blue R-250 (1 g·L^{-1}) and silver.

2.3.5 | Gel filtration FPLC chromatography

Soluble fractions (see Section 2.2.5) were analyzed in an ÄKTA purifier (GE Healthcare, Uppsala Sweden) equipment using two different molecular exclusion columns. Superdex 75 10/300 GL column (exclusion limit: 10^5, separation range: 3–70 kDa) (GE Healthcare) was calibrated with blue dextran (exclusion volume, V_o = 7.3 ml), albumin (67 kDa), ovalbumin (9.2 kDa), chymotrypsin (25 kDa), ribonuclease (19 kDa), and aprotinin (6.5 kDa), obtaining the following calibration equation: log molecular weight (MW) = 1.96 − 2.30 × K_{av}, where $K_{av} = (V_e - V_o)/(V_t - V_o)$, V_e is the elution volume, V_o is the void volume, and V_t is the total volume of the column (V_t = 24 ml). Superdex Peptide 10/300 GL (GE Healthcare) (optimal separation range < 10 kDa) was calibrated with blue dextran (exclusion volume V_o = 7.60 ml), aprotinin (6.5 kDa), vitamin B12 (1.85 kDa), and hippuric acid (0.18 kDa) obtaining the following calibration curve: log MM = 4.84 − 3.30 × K_{av}, where $K_{av} = (V_e - V_o)/(V_t - V_o)$, V_e is the

elution volume, V_o is the void volume, and V_t is the total volume of the column (V_t = 24 ml). Samples were filtered by a 0.45-μm nylon filter. Samples (200 μl) were loaded and eluted with PBS buffer at 0.4 or 0.5 ml/min for Superdex 75 and Superdex 30, respectively. Detection by absorbance at 210 nm was performed.

2.4 | Antioxidant activity

Antioxidant activity of soluble fractions (see Section 2.2.5) was evaluated by the oxygen radical absorbance capacity (ORAC) and the hydroxyl radical averting capacity (HORAC) assays using previously optimized protocols in our lab (Orsini Delgado, Galleano, Añón, & Tironi, 2015). Scavenging % was plotted versus the protein concentration of the samples; each concentration point was measured by duplicate. Curves were fit by linear regression or nonlinear regression using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Nonlinear regression was performed according to the Sigmoidal dose–response (variable slope) equation: Y = bottom + (top − bottom)/(1 + 10^((LogIC$_{50}$-X) * hillslope)), where X = log (concentration) and Y = % radical inhibition. In order to normalize curves, bottom was constrained to a constant value of 0 and top was constrained to a constant value of 100. The concentration that inhibits the 50% of radicals (IC_{50}) was obtained.

2.5 | Statistical analysis

Differences between samples (antitrypsin activity, HD%, protein solubility, IC_{50} values) were analyzed by one way analysis of variance multiple comparisons. Significant differences ($p < 0.05$) among mean values were evaluated by the Tukey test (GraphPad Prism 5.0, USA).

3 | RESULTS AND DISCUSSION

3.1 | Flours (*F*) and protein isolates (*I*) characterization

Flours were prepared from raw seeds (*FY* and *FN*) as well as from boiled seeds (*FYt* and *FNt*) of the two yellow pea varieties (Yams and Navarro). Inhibition of the trypsin activity by dispersions (10 mg·ml^{-1}) of all these samples was evaluated. Dispersions obtained from untreated flours showed values between 20% and 30% of inhibition of the trypsin activity (TI %). When values were normalized by the protein content of samples, *FN* presented a significantly lower ($p < 0.05$) specific inhibition than *FY* (0.8 ± 0.3 and 1.7 ± 0.1 TI %·mg protein^{-1}, respectively). Vidal-Valverde et al. (2002) analyzed 18 varieties of Spanish peas, which presented a broad range of antitrypsin activity (measured by a different methodology than in the present work). Differences were attributed to the diversity of climatic and soil conditions during cultivation. The authors also informed that antitrypsin factor in peas was 10 times lower than in soy. Different processing treatments such as dehulling, soaking, cooking, fermentation, and germination have been used to reduce antinutritional factors in food legumes. Frequently, one method is not enough, and the combination of two or more process is required. Soaking is used to remove soluble antinutritional factors, whereas thermal treatment is useful to inactivate heat-sensible factors such as antitrypsin factors due to the thermal denaturation of these proteins. In our case, antitrypsin activity of flours was strongly reduced after soaking and boiling treatment of seeds (0.36 ± 0.08 and 0.33 ± 0.08 TI %·mg protein^{-1} for *FYt* and *FNt*, respectively). Khattab and Arntfield (2009) demonstrated a complete reduction of antitrypsin factors when applying a treatment similar to that of this work in Canadian yellow peas. In addition, protein isolate dispersions presented very low antitrypsin activity (0.278 ± 0.005 and 0.164 ± 0.004 TI %·mg protein^{-1} for *IY* and *IN*, respectively), indicating that antitrypsin factors were lost in a high proportion during the protein isolate preparation. In agreement with this, Olivera-Castillo et al. (2007) informed a reduction of several antinutritional factors (trypsin inhibitors among them) during the preparation of protein isolate of cowpea by alkaline extraction and isoelectric precipitation.

Composition of flours and protein isolates are shown in Table 1. Ash and lipid contents in flours of both varieties were comparable with values previously informed for Canadian peas (Boye et al., 2010). Total carbohydrate values were similar between both varieties and also with respect to those obtained indirectly (by difference) in Canadian peas (60.3%; Boye et al., 2010). Probably, these high values determined after extensive hydrolysis included some fiber components. Legumes are important sources of proteins. Protein content values registered were about 21% wet basis (w.b.) (23.5% dry basis [d.b.]) for *FN* and 18% w.b. (20% d.b.) for *FY*, without significant differences between them ($p > 0.05$). Boye et al. (2010) informed a comparable value of 21% w.b. (24.6% d.b.) for Canadian yellow peas. Protein isolates presented a diminution of about 85% in the content of glucides and an increment of ash with respect to flours. Protein content of pea isolates could be variable according to the processing conditions applied: 84.9% d.b. (Fernandez-Quintela, Macarulla, Del Barrio, & Martinez, 1997), 80.7% d.b. (Shand, Ya, Pietrasik, & Wanasundara, 2007), and 81.7% d.b. (Boye et al., 2010), as some examples. In our case, proteins were concentrated about four times in isolates with respect to flours, presenting values of 84.7% and 85.9% d.b. for *IY* and *IN*, respectively. Protein recovery (g protein in *I* with respect to 100 g protein in *F*) was 56% and 61% for *IY* and *IN*, respectively. These values were comparable with those (55%) previously obtained for Boye et al. (2010).

According to these results, the only slight difference registered between the two varieties of yellow peas was a higher antitrypsin factor activity both in the flour and in the protein isolate of Yams variety. However, it is important to remark that antitrypsin activity was low in both flours and even much lower in soaked and thermally treated flours and in protein isolates, suggesting that, for these peas, antitrypsin factor is not a major problem from a nutritional point of view.

TABLE 1 Centesimal composition (g × 100 g^{-1} wet basis) of yellow pea ingredients

Variety	Sample	Moisture	Ash	Proteins	Carbohydrates	Lipids
Yams	*FY*	10.4 ± 0.1^{b}	2.7 ± 0.5^{a}	18 ± 1^{a}	70 ± 3^{c}	2.2 ± 0.2^{a}
	IY	6.8 ± 0.1^{a}	4.1 ± 0.2^{b}	79 ± 3^{b}	8.4 ± 0.1^{a}	nd
Navarro	*FN*	10.5 ± 0.5^{b}	3.0 ± 0.1^{ab}	21 ± 1^{a}	69 ± 4^{c}	2.2 ± 0.2^{a}
	IN	9.2 ± 0.4^{b}	3.6 ± 0.1^{ab}	78 ± 4^{b}	10.0 ± 0.4^{b}	nd

Note: Pea flours: *FY* and *FN*. Protein isolates: *IY* and *IN*. nd: not determined. Carbohydrates are expressed as glucose. Values are expressed as the mean ± standard deviation (SD). Different letters in the same column indicates significant differences ($p < 0.05$).

TABLE 2 Protein-related parameters of yellow pea ingredients and their gastrointestinal digests

Variety	Sample	Hydrolysis degree (%)	Total protein (TP) (% w/w)	Soluble protein (SP) (mg·ml^{-1})	Solubility (g SP·100 g TP^{-1})
Yams	*FY*	-	18 ± 1^{ab}	1.3 ± 0.1^{a}	70 ± 3^{a}
	DFY	29 ± 2^{a}	16 ± 2^{a}	1.2 ± 0.1^{a}	77 ± 10^{a}
	IY	-	79 ± 3^{e}	5.3 ± 0.3^{c}	67 ± 5^{a}
	DIY	35 ± 2^{a}	46 ± 0^{d}	3.6 ± 0.3^{b}	79 ± 7^{a}
Navarro	*FN*	-	21 ± 1^{bc}	1.5 ± 0.1^{a}	69 ± 4^{a}
	DFN	32 ± 2^{a}	24 ± 1^{c}	1.5 ± 0.1^{a}	64 ± 4^{a}
	IN	-	78 ± 4^{e}	5.5 ± 0.2^{c}	70 ± 4^{a}
	DIN	33 ± 3^{a}	39 ± 3^{d}	2.9 ± 0.1^{b}	75 ± 6^{a}

Note: Pea flours: *FY* and *FN*. Digests from pea flours: *DFY* and *DFN*. Protein isolates: *IY* and *IN*. Digests from pea protein isolates: *DIY* and *DIN*. Values are expressed as the mean ± SD. Different letters in the same column indicates significant differences ($p < 0.05$).

3.2 | Simulated gastrointestinal digestion

Gastrointestinal digestion of flours and protein isolates was simulated by applying a static protocol based on the INFOGEST action (European Union) with modifications; it includes oral, gastric, and intestinal phases. Our main focus of analysis was the protein digestion. The protein hydrolysis degree (HD) is shown in Table 2. Values (about 30%) did not present significant differences ($p > 0.05$) neither between pea varieties nor between *F* and *I*, suggesting that factors such as the presence of other components, the conformational state of proteins or the presence of different levels of antitrypsin factors did not influence the overall level of proteolysis. Barbana and Boye (2011) obtained simulated gastrointestinal digestion from lentil protein concentrates; although the digestion conditions applied were different from those in the present work, the HD values (29%, TNBS method) were comparable. In other way, we have applied the present digestion protocol to amaranth flour and protein isolate, obtaining higher HD values (37% and 43%, respectively; Rodríguez, García Fillería & Tironi, unpublished), suggesting a minor in vitro digestibility of pea proteins with respect to amaranth proteins.

The protein contents of gastrointestinal digests are also shown in Table 2. Protein solubility (PBS, 10 mg sample·ml^{-1}) was analyzed before and after digestion. Proteins in flours presented a solubility of about 70% with respect to total protein, without significant differences between pea varieties ($p > 0.05$). Under the extraction conditions used, albumins and globulins should be solubilized. Protein isolates showed a protein solubility value similar ($p > 0.05$) than flours. In agreement with this, Ladjal-Ettoumi, Boudries, Chibane, and Romero (2015) informed a protein solubility of 65% at pH = 7 and 70% at pH = 8 for Algerian pea protein isolates. After simulated gastrointestinal digestion, none of the samples showed significant changes ($p > 0.05$) in protein solubility (Table 2). These results suggest that the proteins attacked by digestive enzymes were mainly those that were soluble in PBS before proteolysis. However, other variables that could affect protein solubility cannot be ruled out, such as treatment at 85°C carried out to inactivate the digestive enzymes could cause aggregation and insolubilization of some—especially hydrophobic—polypeptides or peptides, and this effect could be different between flours and isolates and their digests; Lowry's method can underestimate the presence of amino acids, dipeptides, and tripeptides, and in this way, soluble protein determined in the digests could be undervalued. Similar results have been previously obtained in amaranth flour and protein isolate and their digests (Rodríguez, García Fillería & Tironi, unpublished).

Polypeptide composition of samples was analyzed by SDS-PAGE. Flours from both varieties of peas presented similar electrophoretic profiles (Figure 1a, lane 1 and lane 2). Band 1 would correspond to legumin subunits (60 kDa), bands 2, 3, and 4 could be attributed to vicilin subunits (43–53 kDa) (Shevkani & Singh, 2015); the presence of free acid and basic legumin subunits (about 37 and 25 kDa, respectively) could not be discarded. Bands 5 to 14 would correspond mainly to albumins. According to Lu, Quillien, and Popineau (2000), pea albumin fraction contains polypeptides of 30 and 14 kDa and other smaller ones. SDS-PAGE profiles of protein isolates were comparable with those of flours (for both pea varieties), evidencing only a diminution of band 11 (Figure 1a, lane 3 and lane 4). In the case of the

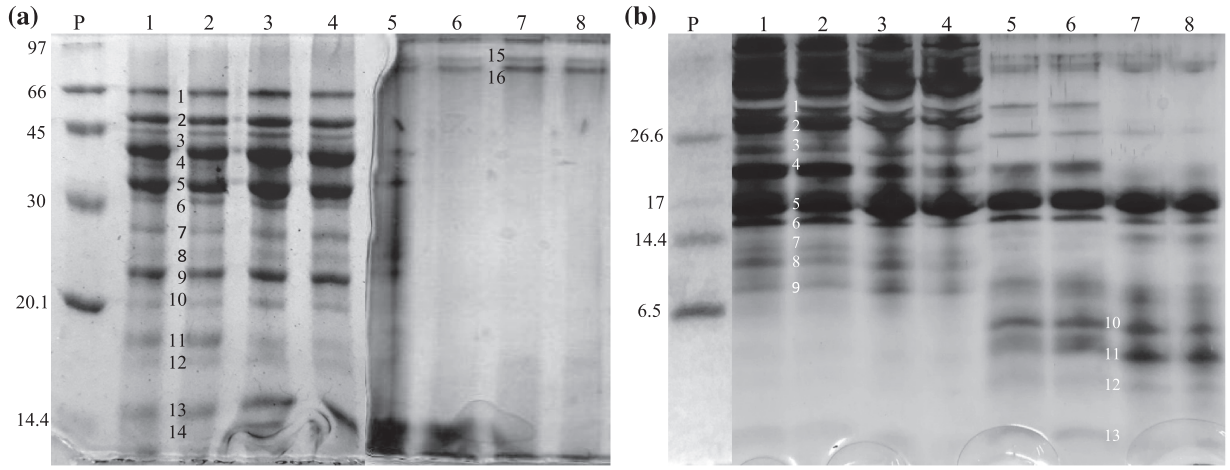

FIGURE 1 (a) Electrophoresis SDS-PAGE of samples solubilized in electrophoresis buffer: (1) *FY*; (2) *FN*; (3) *IY*; (4) *IN*; (5) *DFY*; (6) *DFN*; (7) *DIY*; (8) *DIN*; (P) low molecular weight standard. Lanes 1 to 4 and P were stained with Coomasie Brilliant Blue, lanes 5 to 8 were stained with silver. (b) Electrophoresis tricine-SDS-PAGE of samples solubilized in electrophoresis buffer: (1) *FY*; (2) *FN*; (3) *IY*; (4) *IN*; (5) *DFY*; (6) *DFN*; (7) *DIY*; (8) *DIN*; (P) very low molecular weight standard. All lanes were stained with silver

gastrointestinal digests (Figure 1a, lane 5 to lane 8), all bands corresponding to molecular weights (MW) greater than 14 kDa disappeared almost completely, and two new bands appeared (15 and 16, MW greater than 60 kDa) that could be attributed to hydrolysis products. Molecules smaller than 14 kDa also appeared especially in flour digests (Figure 1a, lane 5 and lane 6). According to the SDS-PAGE, the majority of the digestion products would correspond to low molecular weight molecules that escape from the gel. Tricine-SDS-PAGE gels were performed in order to analyze the low molecular weight peptides. Both, flour and protein isolates, presented diverse polypeptides/peptides with MW lower than 30 kDa (Figure 1b, lanes 1 to 4). Protein isolates showed a diminution in the intensity of some bands corresponding to polypeptides greater than 17 kDa in comparison with flours (Figure 1b, lanes 1 to 4). Gastrointestinal digests presented a strong decrease of all bands corresponding to MW > 17 kDa, more evident in case of protein isolates, and also of the band 8 (about 10 kDa) (Figure 3, lanes 5 to 8). Bands with MW between 14.4 and 17 kDa (5 and 6) and others with MW about 8 kDa (9) remained, suggesting that these peptides were resistant to the simulated gastrointestinal process. New bands (10 to 13) with MW lower than 6.5 appeared after digestion of *F* and *I* (Figure 1b, lanes 5 to 8).

Polypeptide composition of the soluble fractions was analyzed by gel filtration chromatography (FPLC). By using a Superdex 75 column (exclusion limit = 100 kDa), it was possible to observe similar profiles for flours from both varieties of peas; the largest proportion of the total area corresponded to peak 1 (MW > 100 kDa, globulins oligomers) and peak 2 (43–64 kDa, legumin subunits, vicilin subunits, albumins). In addition, molecules with MW between 25 and 43 kDa (peak 3), 11 and 16 kDa (peak 5), 4 and 7 kDa (peaks 6 and 7), and 2.7 to 4 kDa (peak 8), corresponding mainly to albumins, were present (Figure 2a,b). After simulated gastrointestinal digestion, peaks 1, 2, 3, 4, and 5 (MW > 11 kDa) diminished due to protease attack, and molecules with MW in the range of 2.5 and 10 kDa (peak IV) and between 1 and 2.5 kDa (peaks V and VI) increased (Figure 2a,b). Chromatograms of soluble fractions from protein isolates showed a different relative proportion of molecules with respect to flours, with a greater ratio of peaks 1 + 2 and lower proportion of the area of the other peaks. These results showed again a partial loss of low molecular weight polypeptides (<43 kDa) during the protein isolate preparation (Figure 2c,d). After gastrointestinal digestion, molecules smaller than 11 kDa decreased and new peaks appeared: peak III (3.2 to 11.5 kDa), peak V (2.5 to 3.2 kDa), peak VI (1 to 1.7 kDa), and peaks IV and VII (MW < 1.7) (Figure 2c,d). A Superdex 30 column (optimal separation in the range of MW < 10 kDa) was used in order to analyze low MW peptides. Chromatograms obtained from flour soluble fractions presented a peak corresponding to MW greater than 10 kDa, molecules greater than 6.5 kDa (peak 3), and four small peaks (MW between 2 and 0.1 kDa) (Figure 3a,b). The simulated gastrointestinal digestion produced a diminution of the peaks 1, 2, and 3 (MW > 6.5 kDa) and the appearance of molecules in the range of 0.6–4.3 kDa (peak III), and between 0.1 and 0.5 kDa (peaks IV and V) corresponding to peptides of about four amino acids to free amino acids, which could also include other kinds of molecules of low molecular weight released by gastrointestinal digestion (Figure 3a,b). In the case of protein isolates, peptides lower than 6.5 kDa were much less abundant than in flours (Figure 3c,d). After gastrointestinal digestion, peaks corresponding to MW > 6.5 kDa diminished and diverse peaks associated to molecules in the range of 0.1–7.1 kDa appeared, the more relevant between 0.6 and 4.3 kDa (peak III). Also in this case, molecules with very low MW could be evidenced (peak VIII) (Figure 3c,d).

3.3 | Antioxidant activity

The antioxidant activity of soluble fractions of flours and protein isolates before and after simulated gastrointestinal digestion was evaluated. ORAC assay measures hydrophilic chain-breaking antioxidant

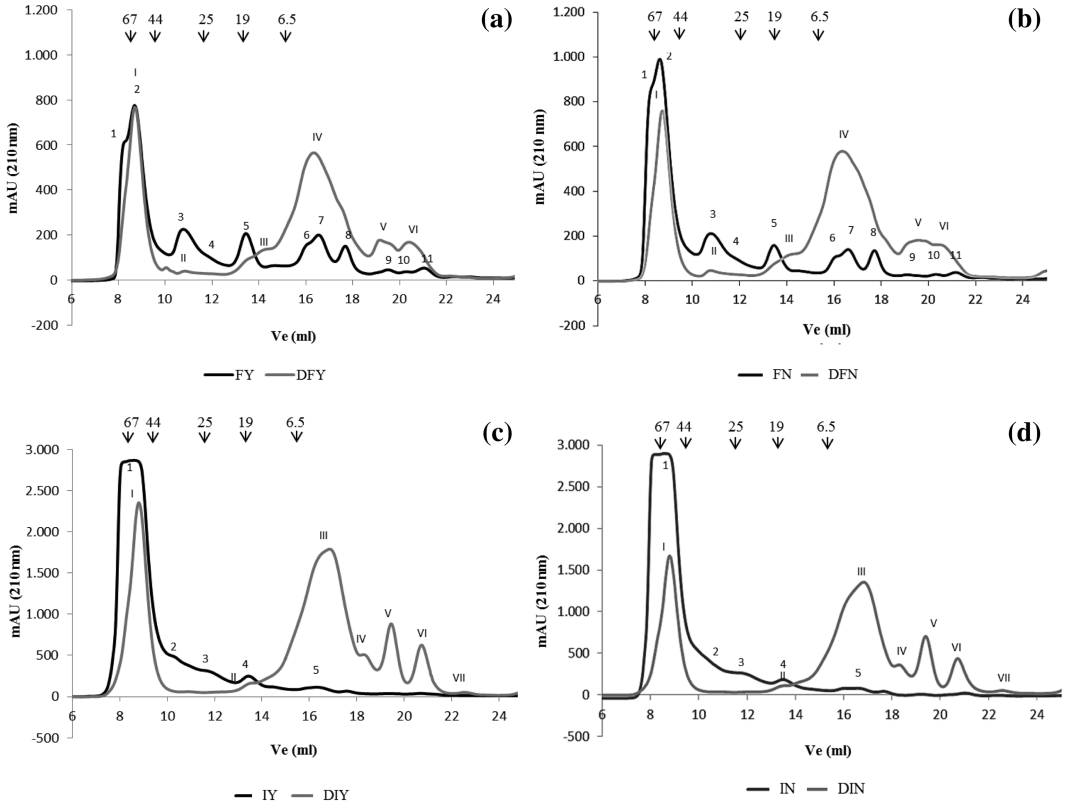

FIGURE 2 Gel filtration chromatograms using a Superdex 75 column (separation range: 1 to 70 kDa). Molecular weight markers are shown

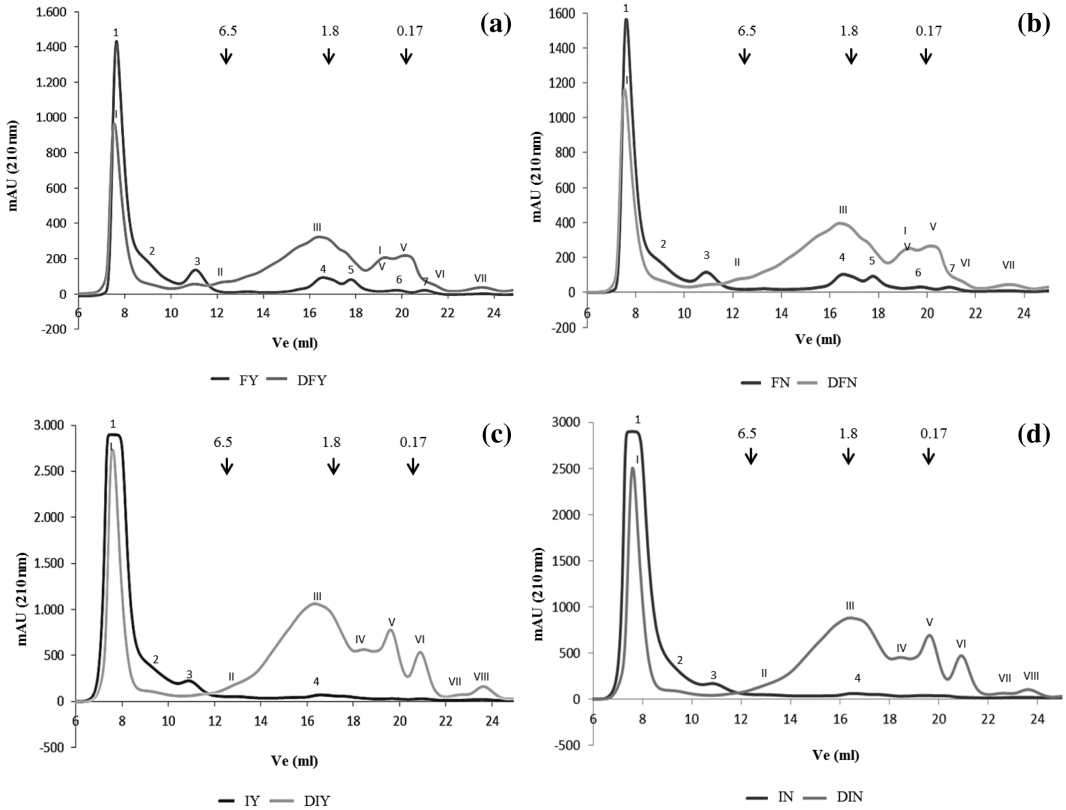

FIGURE 3 Gel filtration chromatograms using a Superdex 30 column (optimal separation range < 10 kDa). Molecular weight markers are shown

TABLE 3 Antioxidant activity (ORAC and HORAC assays) of yellow pea ingredients and their gastrointestinal digests

Variety	Sample	IC_{50} ORAC ($mg \cdot ml^{-1}$)	IC_{50} HORAC ($mg \cdot ml^{-1}$)
Yams	*FY*	0.24 ± 0.08^{b}	5 ± 1^{a}
	DFY	0.08 ± 0.01^{a}	3 ± 1^{a}
	IY	0.31 ± 0.02^{b}	9 ± 2^{b}
	DIY	0.07 ± 0.00^{a}	4 ± 1^{a}
Navarro	*FN*	0.29 ± 0.08^{b}	4 ± 1^{a}
	DFN	0.07 ± 0.00^{a}	3 ± 1^{a}
	IN	0.26 ± 0.06^{b}	10 ± 3^{b}
	DIN	0.07 ± 0.01^{a}	4 ± 1^{a}

Note: Pea flours: *FY* and *FN*. Digests from pea flours: *DFY* and *DFN*. Protein isolates: *IY* and *IN*. Digests from pea protein isolates: *DIY* and *DIN*. Values are expressed as the mean ± SD. Different letters in the same column indicates significant differences ($p < 0.05$).
Abbreviations: HORAC, hydroxyl radical averting capacity; ORAC, oxygen radical absorbance capacity.

capacity against ROO• radicals induced by AAPH, which proceed as a classic hydrogen atom transfer mechanism (Ou, Hampsch-Woodil, & Prior, 2001). Dose–response curves (ROO• scavenging % versus peptide concentration) were obtained by adjusting the data according to Sigmoidal dose–response (variable slope) equation, and IC_{50} values were calculated (Table 3). This parameter did not present significant differences ($p > 0.05$) between flours and protein isolates. This fact indicated that flours did not contribute additional soluble components capable to modify the ROO• scavenging activity with respect to the protein isolates, suggesting that this activity is due mainly by polypeptides or other components associated with the protein fraction. HORAC assay, in which the oxidative degradation of fluorescein occurs by hydroxyl radicals generated by the Fenton reaction (Ou et al., 2002), was also performed. Dose–response curves presented a linear fitting in this case. The yellow pea variety had no influence ($p > 0.05$) on the IC_{50} values of flours and neither on those of protein isolates (Table 3). In contrast to what was observed in the ORAC assay, HORAC activity showed differences between flours and protein isolates. Thus, protein isolates presented greater ($p < 0.05$) IC_{50} values (lower HORAC potencies) than flours. ORAC and HORAC assays reflect different action mechanisms: ORAC evaluates the capacity to scavenge peroxyl radicals (hydrogen atom transference), whereas HORAC evaluates mainly the capacity to chelate metals inhibiting the formation of hydroxyl radicals (Ou et al., 2002). A possible explanation to the observed behavior may be given by certain differences in the composition of both ingredients. As was previously described, they presented some differences in the molecular composition with a diminution of molecules smaller than 43 kDa in the case of the protein isolates. The present results suggested that these lost components would have relevant activity as metal chelator but not as radical scavengers.

Gastrointestinal digests exhibited significantly lower IC_{50} values in ORAC assay ($p < 0.05$) than the starting materials in all cases, without significant differences among them. These results indicated than the digestion process produced an increment of about four times in the ORAC activity of flours and protein isolates by releasing antioxidant compounds, presumably peptides, although the presence of other antioxidant components could not be discarded. IC_{50} values for HORAC assay did not present significant differences ($p > 0.05$) for flours before and after simulated gastrointestinal digestion (Table 3). However, digestion from protein isolates showed a significant decrease ($p < 0.05$) in the IC_{50} value, indicating an increment of HORAC activity by release of active molecules (Table 3). The rate of increment (between two and three times) was lower than in the case of the ORAC assay (between four and five times). A similar behavior had been observed in amaranth protein isolate and its gastrointestinal digestion (Orsini Delgado et al., 2015). So, active molecules could be different for each assay.

According to the present results, pea flour contained HORAC (metal-chelating) active molecules and the gastrointestinal digestion process was not able to increase this activity. The loss of low molecular weight molecules during the preparation of the protein isolates would be related to a diminution of HORAC activity but not of ORAC activity. Gastrointestinal digestion of protein isolate released molecules presenting both activities. The flour digests showed some differences in the molecular composition with respect to the protein isolate digests: a greater proportion of molecules with MW > 6.5 kDa and lower proportion of molecules smaller than 1.4 kDa in the former ones (*DFY* and *DFN*). These facts indicated the presence of different peptides in the different types of digests, which exhibit different sequences, structures, physicochemical properties, and variable amino acids in key positions for the antioxidant activity, and consequently can exert different action mechanisms with variable efficiency. Pownall, Udenigwe, and Aluko (2010) studied the antioxidant activities of peptide fractions obtained from high-performance liquid chromatography separation of a hydrolysate of pea protein isolate prepared by thermolysin action. They demonstrated that the more hydrophobic fractions exhibited the strongest radical scavenging and metal chelating activities; however, hydrophobic character did not seem to contribute to reducing power of the peptides. The same authors informed later that the peptide fraction with the least cationic property had the strongest scavenging activity against 1,1-diphenyl-2-picrylhydrazyl radical, hydrogen peroxide, and superoxide radicals, although, regardless of cationic property, all peptide fractions were effective to inhibit lipid oxidation (Pownall, Udenigwe, & Aluko, 2011). In other work, alcalase hydrolysates from pea protein isolate pretreated with high temperature showed no ORAC, superoxide, or hydroxyl scavenging activity; these activities were improved by pretreatment of protein isolates with high pressure (Girgih et al., 2015). Also, we cannot discard the presence of other kinds of nonprotein components, which the content would be differential between flours and protein isolates and/or could become active after digestion, such as polyphenolic compounds. The presence of this type of compound in free, esterified, and/or linked to protein fractions has been demonstrated in diverse legumes (Fratianni et al., 2014; Vaz Patto et al., 2015) and will be later studied in our pea varieties.

4 | CONCLUSIONS

Literature about antioxidant activity of peptides derived from pea proteins is scarce and, as far as we know, the effect of gastrointestinal digestion on flours and protein isolates have not been studied until now. In the present work, a first evaluation of yellow peas flours and protein isolates as antioxidant functional ingredients was performed. Two pea varieties were studied, and there were no substantial differences between them in molecular composition of flours and protein isolates, before and after simulated gastrointestinal digestion. The proteolysis degree achieved during gastrointestinal digestion was independent on the variety of pea as well as on the pea ingredient (flour or protein isolate). Gastrointestinal digestion was able to produce an increment in the capacity of peroxyl radicals scavenging in all cases and in the inhibition in the hydroxyl radical's formation in the case of protein isolates but not of flours. These facts could be associated with some differences in the molecular composition registered between flour and protein isolate digests. The differences in the peptide profiles could be related to the presence of other nonprotein components or/and the initial state of proteins that could affect the proteolytic attack of digestive enzymes. These results position flours and protein isolates from both pea varieties as promissory functional ingredients with antioxidant properties. in vitro and in vivo assays will be performed in order to confirm the bioactivity.

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CONFLICT OF INTEREST

There are no conflicts to declare.

AUTHORS CONTRIBUTION

Conceptualization, *Methodology*, *Supervision*, *Resources*, *Funding acquisition*, *Project administration*, *Writing - original draft*, *Writing - review & editing*: Valeria Tironi. *Investigation*, *Methodology*, *Formal analysis*, *Visualization*, *Writing - original draft*: María Agustina Cipollone.

ETHICS STATEMENT

This research did not include any human subjects and animal experiments.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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