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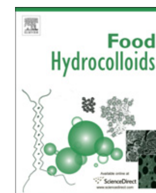

Addition of bovine plasma hydrolysates improves the antioxidant properties of soybean and sunflower protein-based films

Pablo R. Salgado [a,c], Graciela B. Fernández [b], Silvina R. Drago [b,c], Adriana N. Mauri [a,c,*]

[a] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA, CCT La Plata CONICET and Facultad de Ciencias Exactas, Universidad Nacional de La Plata), Calle 47 y 116 S/N°, B1900AJJ La Plata, Buenos Aires, Argentina
[b] Instituto de Tecnología de Alimentos (ITA), Facultad de Ingeniería Química (FIQ), Universidad Nacional del Litoral (UNL), Calle 1° de Mayo 3250, 3000 Santa Fe, Argentina
[c] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

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ABSTRACT

The effect of adding different amounts of a bovine plasma hydrolysate (BPH) with high antioxidant capacity on the functional properties of protein films based on soybean and sunflower protein was studied. BPH caused a decrease in tensile strength, elastic modulus and glass transition temperature of the films, as well as an increase in their elongation at break and water vapor permeability, without noticeably affect their appearance. These results suggest that this hydrolysate had a plasticizing effect on film properties despite its wide distribution of molecular weights. BPH also conferred important antioxidant properties to protein films. It is noteworthy that the antioxidant capacity of the protein-based films activated with bovine plasma hydrolysate, proved to be the result of the sum of the characteristics of the protein matrix, plus of the characteristics of the hydrolysate, no synergistic or antagonistic effects were observed in this property. The use of vegetable proteins (soybean or sunflower) as a biopolymer for film preparation and its activation with bovine plasma hydrolysate with antioxidant capacity should be an approach for maximization the use of these underused proteins.

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1. Introduction

Soybean and sunflower crops are very important in Argentina's economy and are used mainly for production of edible oils (Argentinean National Food Direction, 2010). By-products generated in this activity (oilcakes) are important sources of low-cost proteins with good functionality than can be used in food and packaging industry (Moure, Sineiro, Domínguez, & Parajó, 2006). Regarding food packaging applications, protein-based films can act as barriers to the transfer of oxygen, carbon dioxide, oils, fats and volatile compounds, thus preventing quality loss and increasing the shelf-life of food products. One great advantage of these films is that they can be used as vehicles of additives, like antioxidant and antimicrobial agents, vitamins, flavors and colorants, thus acting as compound-releasing packagings that help to improve the quality and preservation of food (Gómez Estaca, Giménez, Gómez-Guillén, & Montero, 2009; Han & Krochta, 2007).

Synthetic antioxidants (BHA, BHT and n-propyl gallate) exhibit strong activity against several oxidant systems. However, the current trend is to use natural products whose antioxidant activity takes place not only in packaged food but also once the food has been ingested. Such products include, among others, vegetable extracts (Gómez Estaca, Giménez, et al., 2009; Mayachiew, Devahastin, Mackey, & Niranjan, 2010; Norajit, Kim, & Ryu, 2010; Siripatrawan & Harte, 2010), essential oils (Atarés, Bonilla, & Chiralt, 2010; Gómez Estaca, López de Lacey, Gómez-Guillén, López-Caballero, & Montero, 2009; Oussalah, Caillet, Salmiéri, Saucier, & Lacroix, 2004) and isolated phenolic compounds (Gemili, Yemenicioğlu, & Altinkaya, 2010; Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2008). Another feasible and economic way to produce this type of antioxidants is protein hydrolysis (Chang, Wu, & Chiang, 2007). In the last decade, numerous studies have shown the nutraceutical activities of peptides generated by the enzymatic hydrolysis of diverse vegetal and animal proteins, including an inhibitory activity on angiotensin I converting enzyme (ACE), immunological regulatory activity, and antioxidant activity (Pihlanto-Leppälä, 2000; Sarmadi & Ismail, 2010; Vioque et al., 2000). To our knowledge, there is only one study about the improvement of the antioxidant activity of protein films by the addition of protein hydrolysates. Giménez, Gómez-Estaca, Alemán,

* Corresponding author. Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA, CCT La Plata CONICET and Facultad de Ciencias Exactas, Universidad Nacional de La Plata), Calle 47 y 116 S/N°, B1900AJJ La Plata, Buenos Aires, Argentina. Tel./fax: +54 221 4890741.
E-mail address: anmauri@quimica.unlp.edu.ar (A.N. Mauri).

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Gómez-Guillén, and Montero (2009) reported that the incorporation of increasing percentages of gelatin hydrolysate to squid skin gelatin films resulted in an increasing antioxidant activity of the films, measured by FRAP and ABTS assays, although in detriment of the mechanical properties and the water vapor permeability.

Animal blood produced during slaughtering is also a valuable protein source. Blood can be collected hygienically in tubes containing an anticoagulant, usually 2% sodium citrate, and centrifuged to separate the plasma (52–70% w/v) from erythrocytes (Hyun & Shin, 2000; Wanasundara, Amarowicz, Pegg, & Shand, 2006). Proteins are the main solutes in plasma (≈7–7.5 g/dl). Although approximately 150 different proteins with physiological significance have been identified in plasma, the most important are fibrinogen, prothrombin, albumins, and globulins (Wanasundara et al., 2006). Several bioactive peptides (opioids, bacterial growth stimulants, ACE inhibitors, and antioxidants) were obtained from blood proteins, mainly haemoglobin (Chang et al., 2007; Cian, Drago, De Greef, Torres, & González, 2010).

Against this background, the aim of this work was to study whether the addition of bovine plasma hydrolysates to soybean and sunflower protein dispersions affects the functionality of films obtained from such dispersions.

2. Materials and methods

2.1. Materials

Soybean protein isolate (**SPI**) and sunflower oilcake was kindly provided by The Solae Company (Supro 500E, Brazil) and Santa Clara oil factory (Molinos Río de La Plata, Argentine), respectively. Bovine plasma was supplied by YERUVÁ SA (Esperanza, Argentine). The acid range enzyme was provided by Genencor SA (Arroyito, Córdoba, Argentine). All the other reagents used in this study were analytical grade.

2.2. Preparation of sunflower protein isolate (*SFPI*)

Proteins present in sunflower oilcake were extracted in alkaline medium (oilcake:water ratio 1:10 w/v, pH 9) and then they were subjected to isoelectric precipitation by adjusting the pH to 4.5. The obtained protein pellet was recovered by centrifugation (Westfalia SAADH 205 model, Germany), resuspended in water (10% w/v, pH 9) in a Manton-Gaulin two-stage homogenizer (Gaulin Corp., USA) and finally dehydrated in Niro Atomiser spray drier (**SFPI**).

2.3. Preparation of bovine plasma hydrolysates (*BPH*)

Hydrolysates were obtained from an aqueous suspension of the bovine plasma (5% w/w) using a 800 ml batch thermostatized reactor. Working conditions for the enzyme were T: 45 °C, pH: 4.3, enzyme/substrate (E/S) ratio: 0.5%. The reaction pH was continuously measured using an IQ Scientific Instruments pH-meter, and adjusted by adding acid (HCl) with a burette. Samples were taken at different times (1, 2 or 3 h), the enzyme was inactivated by thermal treatment following the manufacturer guidelines. The samples were centrifuged and the supernatants obtained were analyzed for degree of hydrolysis and antioxidant properties.

In another batch, the hydrolysis was performed as described before during 2 h. Once the hydrolysis was finished, the hydrolysate was neutralized and the enzyme was inactivated by thermal treatment following the manufacturer guidelines. Finally, the whole hydrolysate was frozen and lyophilized (**BPH**).

2.4. Degree of hydrolysis (DH)

Free amino groups of the hydrolysates and the bovine plasma were measured using o-phthaldialdehyde (OPA, Sigma Chemical Co. St. Louis, USA) according to Nielsen, Petersen, and Dambmann (2001), and the degree of hydrolysis (DH) was calculated as:

$$DH = \frac{(h - h_0)}{h_{tot}} \times 100\% \quad (1)$$

where: h_{tot} is the total number of peptide bonds in the protein substrate (7.77 mEq/g protein); h is the number of peptide bonds cleaved during hydrolysis; h_0 is the content of free amino groups of substrate.

2.5. Chemical composition

Chemical composition of the protein isolates (**SPI** and **SFPI**) and hydrolysate (**BPH**) were determined using AOAC (1995) procedures. All determinations were performed at least in duplicate.

2.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The polypeptide composition of the protein isolates (**SPI** and **SFPI**) and hydrolysate (**BPH**) were analyzed by SDS-PAGE using a separating gel (12% w/v in polyacrylamide) with a stacking gel (4% w/v in polyacrylamide) in a minislabs system (Bio-Rad Mini-Protean II Model) (Laemmli, 1970). Protein molecular weights were estimated using low MW markers (Pharmacia, Amersham, England) that included phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400). Gel images were analyzed with the Image J software (Bethesda, MD: US National Institute of Health).

2.7. Preparation of films

Films were prepared by casting, dispersing **SPI** or **SFPI** (5% w/v) and glycerol (1.5% w/v, Anedra, Argentine) in distilled water with the addition of variables contents of **BPH** (10, 20, 40 g **BPH**/100 g of isolate) at pH 11. Ten ml of each film-forming dispersion were poured on polystyrene Petri dishes (64 cm^2) and then dehydrated at 60 °C for 5 h in an oven with air flow circulation (Yamato, DKN600, USA). The dry films were conditioned 48 h at 20 °C and 58% relative humidity (RH) in desiccators with saturated solutions of NaBr before being peeled from the casting surface for characterization.

2.8. Film thickness

Film thickness was measured by a digital coating thickness gauge (Check Line DCN-900, USA). Measurements were done at five positions along the rectangular strips for the tensile test, and at the center and at eight positions round the perimeter for the water vapor permeability (WVP) determinations. The mechanical properties and WVP were calculated using the average thickness for each film replicate.

2.9. Moisture content (MC)

MC was determined after drying in an oven at 105 °C for 24 h. Small specimens of films collected after conditioning were cut and placed on Petri dishes that were weighed before and after oven drying. MC values were determined in triplicate for each film, and

calculated as the percentage of weight loss relative to the original weight (ASTM D644-94, 1994).

2.10. Color

Film colors were determined using a Minolta Chroma meter (CR 300, Minolta Chroma Co., Osaka, Japan). A CIE Lab color scale was used to measure the degree of lightness (L), redness ($+a$) or greenness ($-a$), and yellowness ($+b$) or blueness ($-b$) of the films. The instrument was standardized using a set of three Minolta calibration plates. Films were measured on the surface of the white standard plate with color coordinates of $L_{standard} = 97.3$, $a_{standard} = 0.14$ and $b_{standard} = 1.71$. Total color difference (ΔE) was calculated from:

$$\Delta E = \sqrt{\left(L_{film} - L_{standard}\right)^2 + \left(a_{film} - a_{standard}\right)^2 + \left(b_{film} - b_{standard}\right)^2} \quad (2)$$

Values were expressed as the means of nine measurements on different areas of each film.

2.11. Opacity

Each film specimen was cut into a rectangular piece and placed directly in a spectrophotometer test cell, and measurements were performed using air as the reference. A spectrum of each film was obtained in an UV–Vis spectrophotometer (Beckman DU650, Germany). The area under the absorption curve from 400 to 800 nm was recorded, and the opacity of the film (UA/mm) was calculated by dividing the absorbance at 500 nm by the film thickness (mm) (Cao, Fu, & He, 2007). All determinations were performed in quadruplicate.

2.12. Water vapor permeability (WVP)

Water vapor permeability tests were conducted using ASTM (1989) method E 96-80 with some modifications (Gennadios, McHugh, Weller, & Krochta, 1994). Each film sample was sealed over a circular opening of 0.00185 m^2 in a permeation cell that was stored at 20 °C in a desiccator. To maintain a 75% relative humidity (RH) gradient across the film, anhydrous silica (0% RH_c) was placed inside the cell and a saturated NaCl solution (75% RH_d) was used in the desiccator. The RH inside the cell was always lower than outside, and water vapor transport was determined from the weight gain of the permeation cell. When steady-state conditions were reached (about 1 h), eight weight measurements were made over 5 h. Changes in the weight of the cell were recorded and plotted as a function of time. The slope of each line was calculated by linear regression (Microsoft® Office Excel 2007) and the water vapor transmission rate (WVTR) was calculated from the slope (g H_2O/s) divided by the cell area (m^2). WVP (g H_2O/Pa s m) was calculated as:

$$WVP = \frac{WVTR}{P_v^{H_2O} \cdot (RH_d - RH_c) \cdot A} \cdot d \quad (3)$$

where $P_v^{H_2O}$ = vapor pressure of water at saturation (1753.35 Pa) at the test temperature (20 °C), HR_d = RH in the desiccator, HR_c = RH in the permeation cell, A = permeation area and d = film thickness (m). Each WVP value represents the mean value of at least three samples taken from different films.

2.13. Mechanical properties

The tensile strength, Young's modulus and elongation at break of the films were determined following the procedures outlined in the ASTM methods D882-91 (ASTM, 1991), taking an average of six measurements for each film and using at least two films per formulation. The films were cut into 6 mm wide and 80 mm long strips, and mounted between the grips of the texture analyzer TA.XT2i (Stable Micro Systems, Surrey, England). The initial grip separation was set at 50 mm and the crosshead speed at 0.5 mm/s. The tensile strength (σ = force/initial cross-sectional area) and elongation at break (ε) were determined directly from the stress–strain curves using Texture Expert V.1.15 software (Stable Micro Systems, Surrey, England), and the Young's modulus (E) was calculated as the slope of the initial linear portion of this curve.

2.14. Differential scanning calorimetry (DSC)

The glass transition temperature (Tg) of films and the denaturation degree of protein products were determined by differential scanning calorimetry, using a DSC TA 2010 calorimeter Q100 V9.8 Build 296 (TA Instrument, New Castle, Del., USA) controlled by a TA 5000 module with a quench cooling accessory. Temperature and heat flow calibration of the equipment were carried out according to ASTM Standards, using lauric and stearic acids and indium as standards. Hermetically sealed aluminum pans containing 10 mg of films or 10–15 mg of protein products dispersions (20% w/v) were prepared and scanned at 10 °C/min over the range −100 to 220 °C in order to determine Tg or between 20 and 120 °C to determine the degree of protein denaturation. Glass transition temperature (Tg) was considered to be the inflexion point of the base line, caused by the discontinuity of the specific heat of the sample. Denaturation enthalpies (ΔH) and peak temperatures (Td) were taken from the corresponding thermograms. Enthalpy values (ΔH) were expressed as J/g protein, taking into account the dry weight (determined by perforating the pans and heating overnight at 105 °C) and the protein content of sample. All parameters were calculated with the help of the Universal Analysis V4.2E software (TA Instruments, New Castle, Del., USA). All the assays were performed at least in duplicate.

2.15. Antioxidant capacity

The $ABTS^{\bullet+}$ radical (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) scavenging capacity of the samples was determined according to a modified version of the method of Re et al. (1999). The stock solution of $ABTS^{\bullet+}$ radical consisted of 7 mM ABTS (Sigma Chemical Co. St. Louis, MO) in 2.45 mM potassium persulfate (Anedra, Argentine), kept in the dark at room temperature for 12–16 h. An aliquot of the stock solution was diluted with distilled water in order to prepare the working solution of $ABTS^{\bullet+}$ radical with absorbance of 0.70 ± 0.03 at 734 nm. *Samples of protein isolates* (10 μl of 1 mg/ml of **SPI** or **SFPI** in PBS: 0.01 M sodium phosphate buffer, pH 7.4) or *hydrolysates* (6 mg of **BPH**), and PBS (10 μl) were added to 990 μl of the solution containing the diluted $ABTS^{\bullet+}$. The mixture was vortexed for 2 min and its absorbance at 734 nm (Abs_s) was measured 6 min after the addition of the $ABTS^{\bullet+}$ solution. *Samples of each protein film* (5 mg) were added with 500 μl of PBS and were stirred by 20 h. The supernatant obtained was used to evaluate the antioxidant activity. Samples (25 μl) were added to 950 μl of the solution containing the diluted $ABTS^{\bullet+}$ radical. The mixture was vortexed for 2 min and its absorbance at 734 nm (Abs_s) was measured 6 min after the addition of the $ABTS^{\bullet+}$ solution. To obtain a reaction blank (Abs_{rb}) in each assay, the same procedure was carried out but the sample replaced with PBS. The antioxidant capacity (AC), as measured by the ability to scavenge the $ABTS^{\bullet+}$ radical, was calculated with Equation (4). All determinations were performed at least in triplicate.

$$AC = \frac{Abs_{rb} - Abs_s}{Abs_{rb}} \times 100 \quad (4)$$

To estimate the Trolox equivalent antioxidant capacity (TEAC) a concentration–response curve for the absorbance at 734 nm for $ABTS^{\bullet +}$ as a function of concentration of standard Trolox solution (0–2.5 mM, 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid, Aldrich Chemical Co., UK) in PBS was carried out.

2.16. Statistical analysis

Results were expressed as mean ± standard deviation and were analyzed by analysis of variance (ANOVA). Means were tested with the Tukey's HSD (honestly significant difference) test for paired comparison, with a significance level $\alpha = 0.05$, using the Statgraphics Plus version 5.1 software (Statgraphics, USA).

3. Results and discussion

3.1. Characterization of soybean and sunflower protein isolates

Table 1 shows protein content, denaturation enthalpy and antioxidant capacity of soybean protein isolates (**SPI**) and sunflower protein isolate (**SFPI**). Both isolates differ in their protein content, close to 85% and 70% for **SPI** and **SFPI** respectively. Fig. 1A shows the SDS-PAGE patterns of both isolates. Lane 2 shows the typical electrophoretic patterns for **SPI** under non-reducing conditions. Bands corresponding to the α (≈72 kDa), α′ (≈68 kDa) and β (≈52 kDa) subunits of β-conglycinin (a trimeric glycoprotein of 150–200 kDa), the AB (≈50–60 kDa) subunits, and the acidic (A, ≈30–40 kDa) and the basic (B, ≈18–22 kDa) polypeptides of glycinin (a hexameric protein of 300–380 kDa), and high molecular mass aggregates (>94 kDa, some of them did not enter the gel) were observed, in agreement with a previous reported by Denavi et al. (2009). Under reduced conditions, **SPI** pattern (lane 5) only shows the bands of the corresponding polypeptides α, α′, β, A and B, suggesting that soluble aggregates seen in lane 2 were stabilized by disulfide bonds. On the other hand, a typical profile of **SFPI** under non-reducing conditions is shown in lane 3 (Fig. 1A). It can be observed the AB (≈45–65 kDa) subunits of helianthinin (a hexameric protein of 300–350 kDa) and high molecular weight aggregates (>94 kDa, which could not be resolved in the gel). Under reducing conditions (lane 4) the AB subunits were dissociated into acidic (A, ≈30–40 kDa) and basic (B, ≈20–30 kDa) polypeptides, in agreement with a previous reported by Molina, Petruccelli, and Añón (2004).

These protein isolates also had different degrees of denaturation (Table 1). Thermograms obtained by DSC for commercial **SPI** did not show the two characteristics endotherms at 75 °C and 90 °C for β-conglycinin fractions and conglycinin respectively (Denavi et al., 2009), indicating that proteins are completely denatured. In **SFPI** thermogram a single denaturation endotherm of 5.4 J/g of protein at 100 °C was observed. It corresponds to 60% of protein

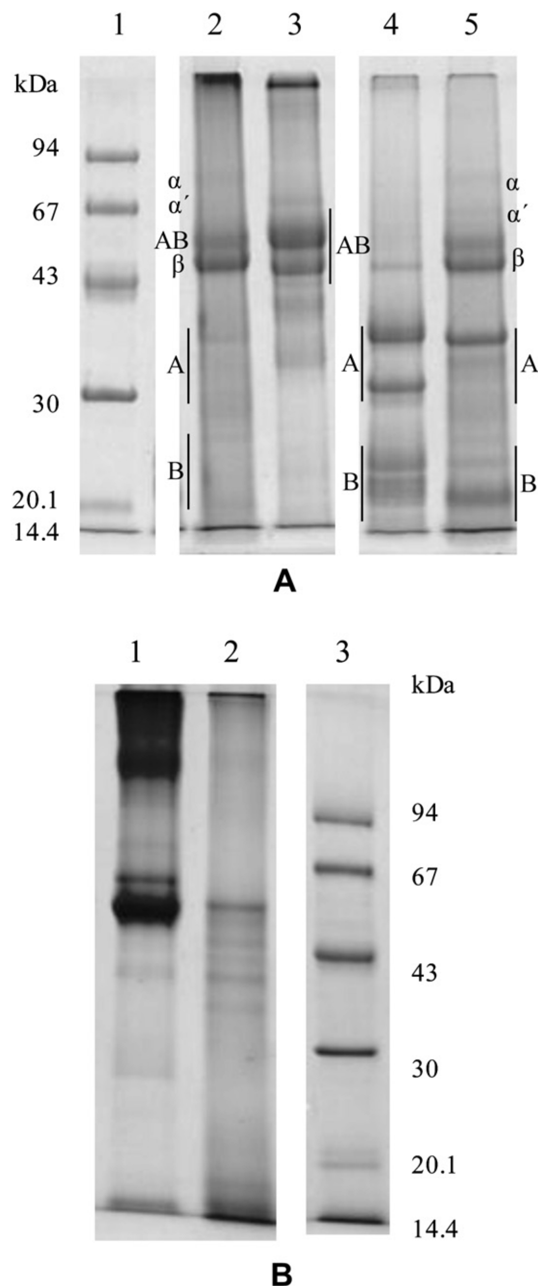

Fig. 1. **A**) SDS-PAGE electrophoretic patterns under non-reducing (lanes 2 and 3) or reducing conditions (lanes 4 and 5, β-mercaptoethanol added) of **SPI** (lanes 2 and 5) and **SFPI** (lanes 3 and 4). Low molecular weight markers are shown in lane 1. **B**) SDS-PAGE electrophoretic patterns under reducing conditions of bovine plasma proteins (lane 1), **BPH** (lane 2) and low molecular weight markers (lane 3).

denaturation when compared with the enthalpy value reported by González-Pérez and Vereijken (2007) for a native sunflower protein isolate.

Both protein isolates showed antioxidant activity, being **SFPI** more effective than **SPI**. The residual content of phenolic compounds, mainly chlorogenic and caffeic acids, allowed antioxidant properties to **SFPI** (Salgado, Molina Ortiz, Petruccelli, & Mauri, 2009), while the presence of isoflavones (daidzein and genistein) and/or minor amounts of phenolic compounds (such as chlorogenic, gallic, ferulic and syringic acids) would be responsible for the antioxidant activity presented by **SPI** (Tyug, Prasad, & Ismail, 2010).

Table 1
Protein content, denaturation enthalpy and antioxidant capacity of raw materials (**SPI**, **SFPI** and **BPH**) used for preparing biodegradable films.

	SPI	SFPI	BPH
Protein content (% dry basis)	85.0 ± 1.2c	70.4 ± 0.8b	65.0 ± 0.1a
Denaturation enthalpy (J/g)	–	5.44 ± 0.30	–
Antioxidant capacity (%)	9.5 ± 0.2a[a]	28.4 ± 0.7b[a]	97.2 ± 0.2c[b]

Results are expressed as mean value ± standard deviation.
[a] AC corresponding to 1 mg/ml of **SPI** or **SFPI** in PBS.
[b] AC corresponding to 6 mg of **BPH**.

3.2. Selection and characterization of bovine plasma hydrolysate (**BPH**)

The bovine plasma proteins used in this work could be classified in albumins (66 kDa), globulins (41–850 kDa), fibrinogen (340 kDa) and a slight concentration of polypeptides of lower molecular mass (<43 kDa) (Fig. 1B, lane 1), according to Chang et al. (2007). These proteins were hydrolysed with an acid protease in order to produce bioactive peptides with antioxidant activity. Fig. 2 shows the degree of hydrolysis (**panel 2A**) and the antioxidant capacity of soluble polypeptides (**panel 2B**) obtained at different times of hydrolysis. From these results, hydrolysis time of 2 h was selected because the resulting hydrolysate (**BPH**) had the highest $ABTS^{•+}$ radical scavenging activity. Even though the increase of the antioxidant capacity from 1 to 2 h hydrolysis is not too high, the handle of the 2-h hydrolysate is easier than 1-h hydrolysate, since a sample with low degree of hydrolysis (or the sample without hydrolysis) coagulate during enzyme inactivation thermal treatment. Longer times of hydrolysis (3 h) decreased the antioxidant capacity.

The neutral whole **BPH** presented a protein content of 65% with a DH of 19.4% (see Table 1 and Fig. 2). During the hydrolysis process, bovine plasma proteins lose their native structure. The resulting **BPH** showed no denaturation endotherm by DSC studies. Electrophoretic pattern of **BPH** (Fig. 1B, lane 2) shows a significant decrease in the concentration of polypeptides of high molecular weight (>43 kDa) – albumin, globulin and fibrinogen – and an increased concentration of lower molecular weight species (<43 kDa). The antioxidant capacity of the selected **BPH** was 97.2%, measured as percentage of $ABTS^{•+}$ inhibition.

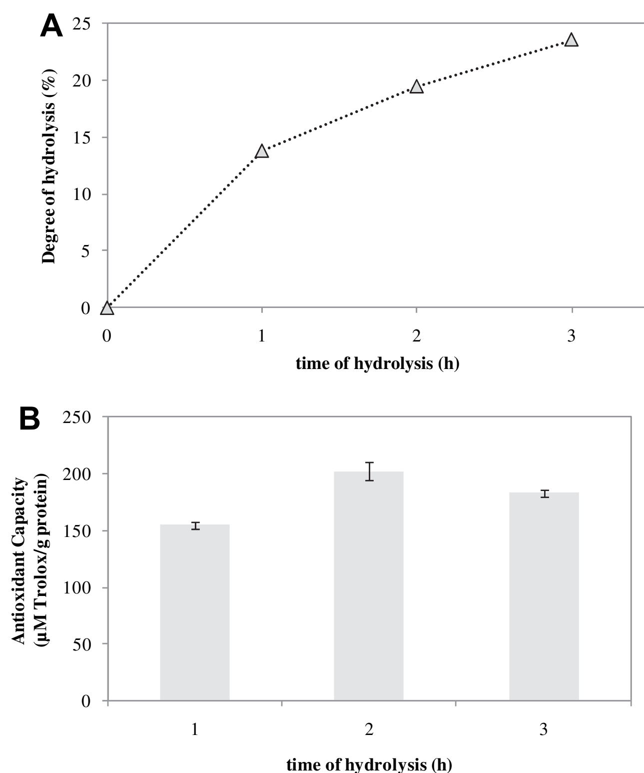

Fig. 2. **A**) Degree of hydrolysis (%) vs. time of hydrolysis of bovine plasma with acidase at *T*: 45 °C, pH: 4.3, enzyme/substrate (*E*/*S*) ratio: 0.5%. **B**) Antioxidant capacity (TEAC: µM Trolox/g protein) from neutral supernatants from different hydrolysis times. The values are significantly different ($p < 0.05$).

3.3. Thickness and moisture content of protein films

All protein-based films (**SPI** and **SFPI**), with or without the addition of **BPH** were found to be homogeneous and flexible. Their thickness and moisture content are shown in Table 2. It is noteworthy that among protein films without **BPH** addition, those of soybean proteins exhibited significantly higher thickness than that of sunflower proteins although similar water content, indicating a lower degree of compaction in the film network when soybean proteins are present, probably due to their higher degree of molecular unfolding as was observed by DSC (Section 3.1).

For both types of formulations, films obtained with addition of **BPH** were thicker than their respective controls (**SPI** or **SFPI** films) ($p > 0.05$). These increases were independent with the **BPH** concentration for **SFPI** and progressive with the concentration of peptides for **SPI**. These results indicate that the addition of **BPH** on **SPI** or **SFPI** films produces a less compact protein network, which in principle would suggest that the peptides would not be promoting the cross-linking of the matrix. No significant differences ($p \leq 0.05$) were observed in the moisture content of the activated films compared with their corresponding controls.

3.4. Appearance of protein films

Hunter-Lab color parameters and opacity of the studied protein films are shown in Table 3. These results give an idea of the appearance of the developed materials. The appearance of **SPI** and **SFPI** films was very different. The first ones showed a yellowish coloration ($a-$ and $b+$) and a high transparency, while the latter have a dark green color ($a-$ and $b \approx 0$), which can be attributed to the oxidation of the present phenolic compounds to o-quinones during the alkaline extraction of proteins in the isolates preparation process. The opacity of sunflower films was greater than those of soybean.

The addition of **BPH** to both protein systems did not modify the films opacity and slightly affected their coloration, as expected due to the chemical similarity between the isolates proteins and the hydrolysate. Its addition to **SPI** films caused a slight increase in the *b* parameter (improving the yellow coloration) and therefore in ΔE, without significantly affecting the *L* and *a* parameters. While its addition on **SFPI** films only showed a slight decrease of the *L* and *a* parameters (softening the green coloration), which did not affect ΔE.

3.5. Water vapor permeability of protein films

Fig. 3 shows WVP values of the studied protein films. No significant differences were found among the vapor barrier

Table 2
Thickness, moisture content, and glass transition temperature of films prepared from **SPI** or **SFPI** with different concentrations of **BPH** (0, 10, 20 and 40 g **BPH**/100 g protein isolate).

	BPH	Thickness (µm)	Moisture content (%)	Tg (°C)
SPI	0	$67 \pm 9^{b,c}$	19.3 ± 1.8^{a}	-41.9 ± 0.3^{b}
	10	76 ± 17^{d}	19.9 ± 1.2^{a}	-46.0 ± 1.1^{a}
	20	$74 \pm 17^{c,d}$	$20.9 \pm 2.0^{a,b}$	-44.9 ± 0.3^{a}
	40	84 ± 16^{e}	19.6 ± 0.7^{a}	-45.5 ± 0.1^{a}
SFPI	0	51 ± 8^{a}	$21.7 \pm 2.3^{a,b}$	-32.3 ± 0.3^{d}
	10	61 ± 8^{b}	$23.1 \pm 1.7^{a,b}$	-35.3 ± 0.5^{c}
	20	62 ± 9^{b}	25.1 ± 1.0^{b}	-34.6 ± 0.3^{c}
	40	61 ± 8^{b}	$21.4 \pm 0.7^{a,b}$	-34.6 ± 0.2^{c}

Results are expressed as mean value ± standard deviation. Values means followed by the same letter are not significantly ($p \leq 0.05$) different according to Tukey's HSD test.

Table 3
Hunter color parameters (L, a and b), total color difference (ΔE) and opacity of films prepared from **SPI** or **SFPI** with different concentrations of **BPH** (0, 10, 20 and 40 g **BPH**/100 g protein isolate).

	BPH	Hunter-Lab color parameters				Opacity (UA/mm)
		L	a	b	ΔE	
SPI	0	91.4 ± 1.0^{c}	$-3.1 \pm 0.1^{a,b}$	20.1 ± 2.0^{b}	19.6 ± 2.2^{a}	1.07 ± 0.66^{a}
	10	89.4 ± 1.9^{c}	$-3.2 \pm 0.7^{a,b}$	24.1 ± 3.3^{c}	24.0 ± 3.6^{b}	0.99 ± 0.05^{a}
	20	89.9 ± 2.0^{c}	$-3.4 \pm 0.1^{a,b}$	24.0 ± 4.3^{c}	23.8 ± 4.7^{b}	0.99 ± 0.20^{a}
	40	89.1 ± 1.6^{c}	$-3.5 \pm 0.2^{a,b}$	25.5 ± 3.3^{c}	25.4 ± 3.5^{b}	0.73 ± 0.21^{a}
SFPI	0	29.9 ± 2.2^{b}	-3.9 ± 1.2^{a}	6.0 ± 2.1^{a}	67.7 ± 2.0^{c}	13.11 ± 1.92^{b}
	10	$28.0 \pm 1.1^{a,b}$	$-2.8 \pm 1.2^{a,b}$	4.3 ± 2.1^{a}	69.5 ± 1.0^{c}	10.41 ± 1.97^{b}
	20	$28.0 \pm 1.7^{a,b}$	$-3.4 \pm 1.7^{a,b}$	4.4 ± 1.0^{a}	69.5 ± 1.8^{c}	10.35 ± 1.96^{b}
	40	27.2 ± 1.7^{a}	-2.3 ± 0.9^{b}	4.5 ± 1.8^{a}	70.2 ± 1.6^{c}	10.20 ± 0.52^{b}

Results are expressed as mean value ± standard deviation. Values means followed by the same letter are not significantly ($p \leq 0.05$) different according to according to Tukey's HSD test.

properties of **SPI** and **SFPI** films, as it was previously reported (Salgado et al., 2009), being these values in the order of those reported for other proteins networks (Cuq, Gontard, & Guilbert, 1998; Gennadios, 2002; Mauri & Añón, 2008). The addition of **BPH** in both formulations produced an increase in the WVP of the resulting films, being this increase statistically significant ($p > 0.05$) when adding 40 g **BPH**/100 g protein isolate. Specifically, WVP values of these films were 40 and 50% higher than those of sunflower and soybean controls, respectively. This effect could be attributed to the hydrophilic nature of peptides, to the increased thickness of films activated with **BPH**, as has been previously reported McHugh, Avena-Bustillos, and Krochta (1993) and Mauri and Añón (2008), and finally to a plasticizing effect of these peptides. This latter reason is the one used by Giménez et al. (2009) to explain the increase in WVP of gelatin films activated with peptides obtained by enzymatic hydrolysis of gelatin, suggesting that the peptides may lead to an increase in the free volume of the film, making the protein network less dense and more permeable.

3.6. Mechanical properties of protein films

Fig. 4 shows the mechanical properties (measured in tensile test) of the studied films. **SPI** films had better mechanical properties than those of **SFPI** – higher tensile strength and elongation at break. These differences can be attributed to the different degree of denaturation of the proteins present in each isolate (see Table 1). Proteins in **SPI** were totally denatured, their unfolded structures

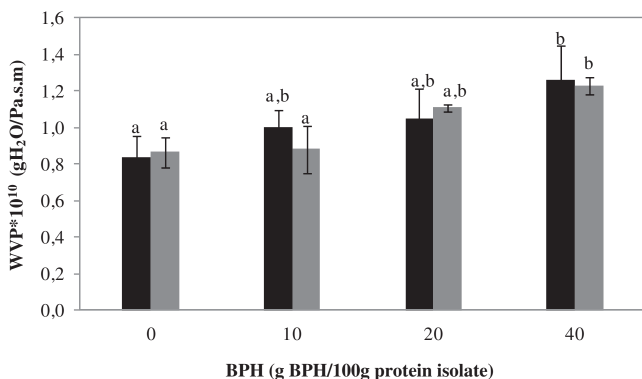

Fig. 3. Water vapor permeability of films prepared from **SPI** (■) or **SFPI** (■) with different concentrations of **BPH** (0, 10, 20 and 40 g **BPH**/100 g protein isolate). Results are expressed as mean value ± standard deviation. Values means followed by the same letters are not significantly ($p \leq 0.05$) different according to Tukey's HSD test.

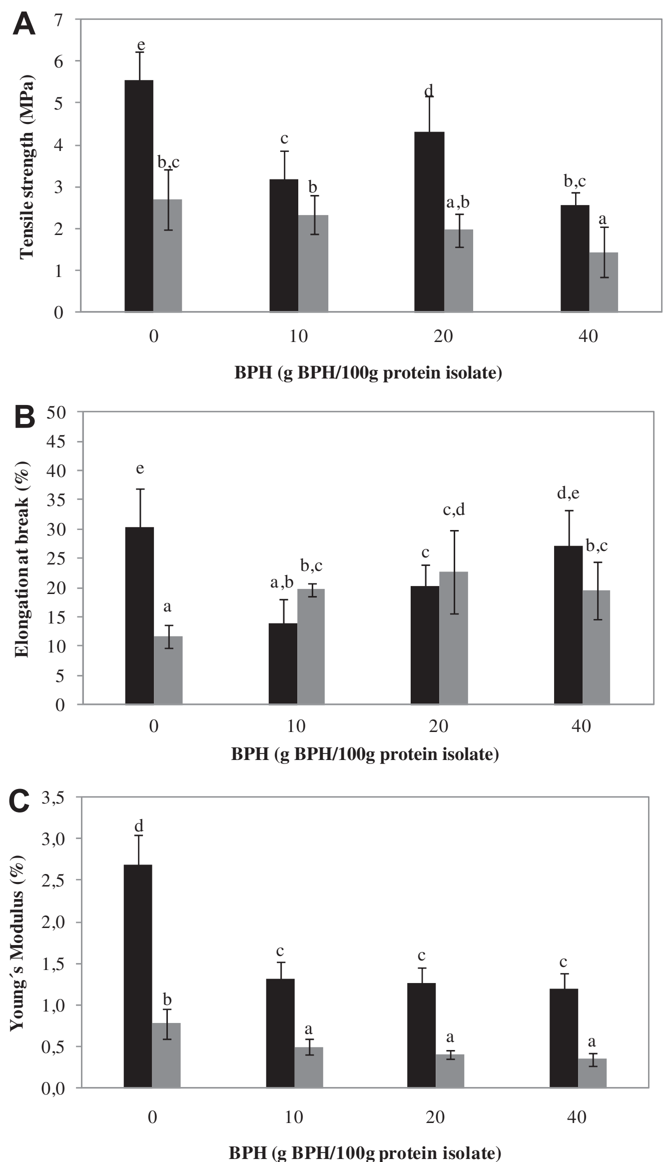

Fig. 4. Mechanical properties – measured in tensile test – of films prepared from SPI (■) or SFPI (■) with different concentrations of **BPH** (0, 10, 20 and 40 g **BPH**/100 g protein isolate). **Panel A**. Tensile strength. **Panel B**. Elongation at break. **Panel C**. Young's modulus. Results are expressed as mean value ± standard deviation. Values means followed by the same letters are not significantly ($p \leq 0.05$) different according to Tukey's HSD test.

allowed them to easily interact among protein chains. This largest cross-linking is one of the causes of the increased resistance of the films. In a previous work we observed that **SPI** films had higher elongation but similar tensile strength, than those of **SFPI**, and after testing the films solubility in buffers with different chemical action, we suggested that it could be attribute to the most significant role of disulphide bonds in stabilizing soybean protein networks (Salgado et al., 2009). As it has been reported, materials with higher capacity to establish covalent interactions through disulfide bonds form more resistant and elongable networks (Pérez-Gago & Krochta, 2001).

For both types of formulations, as the content of **BPH** in the formulation increased, films showed a decreased in their strain at break and elastic modulus, and an increased in their elongation at break (Fig. 3). This behavior, which also agrees with that observed by Giménez et al. (2009) suggest that the peptides would be acting

as plasticizers interfering in the cross-linking of protein network. This effect confirmed the suggestions done in the previous section (Section 3.5).

According to Sothornvit and Krochta (2000) there are two main types of plasticizers: 1. Molecules capable of forming hydrogen bonds, thus interacting with polymers by interrupting polymer–polymer bonding and maintaining the furthers distance between polymers chains, 2. Molecules capable of interact with large amounts of water to retain more water molecules, thus resulting in higher moisture content and larger hydrodynamic radius. Usually, owing to the hydrophilic nature of water, biopolymers and plasticizer, and due to the abundantly existing hydrogen bonds in their structures it is very difficult to separate these mechanisms (Han & Gennadios, 2005). In this case, since there was no increased in water content with increasing concentration of peptides (see Table 2), the plasticizing effect of **BPH** could be attributed to the interference generated by these peptides in the cross-linking among isolates protein chains.

It is noteworthy that the elongation of control **SPI** films was found to be higher than those of **SPI** films with **BPH**. It is feasible that the presence of the hydrolysates interfere in the formation of interactions among protein chains, including disulfide bonds, so their presence or absence would lead to the formation of different protein networks, so that the trend would not be continuous.

3.7. Glass transition temperature

The plasticizing effect of **BPH** was also observed when studying the glass transition of the films (Table 2). Films with **BPH** in the formulation had lower Tg than those of their respective control films, although no effect concentration was observed ($p \leq 0.05$). It is conceivable that these peptides of lower molecular mass can be able to position themselves between protein chains and interfere with the protein–protein interaction, increasing the free volume between the polymer chains (films with less density and higher thickness), and favoring the mobility of molecules (higher WVP). These properties imply that this hydrolysate decreased the materials Tg.

When comparing the control protein films, it is curious to note that those formed by soybean protein, which showed higher thickness for the same solids content, including plasticizers (water and glycerol), had lower Tg than those of sunflower. It is clear that **SPI** and **SFPI** protein chains interacted differently during films formation according to the characteristics of each protein – e.g. amino acid composition, degree of distortion, chain stiffness, etc. (Cherian, Gennadios, Weller, & Chinachoti, 1995), which were verified when analyzing their different mechanical properties.

3.8. Antioxidant capacity

Fig. 5 shows the antioxidant capacity (measured by ABTS) of studied protein films. The two control films showed antioxidant capacity (Fig. 5A), being most important by **SFPI** films than those of **SPI**, as was also observed when analyzing this property in the respective isolates (see Table 1). These protein-based films could be considered as materials naturally activated with antioxidant properties (Salgado et al., 2009). For **SFPI** films, as explained previously, this activation was produced by residual content of phenolic compounds present in seed remaining in the protein isolation, whereas in the case of **SPI** films, this result could be attributed to presence of flavonoid compounds, which according Tyug et al. (2010) possess antioxidant activity.

The antioxidant capacity of the protein films was significantly increased ($p > 0.05$) when added **BPH** to the formulations. In all cases, the **SFPI** films had higher antioxidant capacity than their respective films obtained with **SPI**. Fig. 5B shows the relative increase in antioxidant capacity compared to the initial value of the film and its relation to the percentage of **BPH** incorporated. The increase in antioxidant capacity of the films with the addition of **BHP** is the same regardless of the origin of the protein matrix film, indicating an additive effect that each film had initially. It was also noted an increase around 64% of the antioxidant capacity of protein films by adding 40 g **BPH**/100 g protein isolate. Is important to highlight that the increase in antioxidant activity was correlated with the **BPH** concentration added to the formulation using a second order polynomial ($r^2 = 0.997$), being observed a saturation effect for concentrations above 20 g **BPH**/100 g protein isolate.

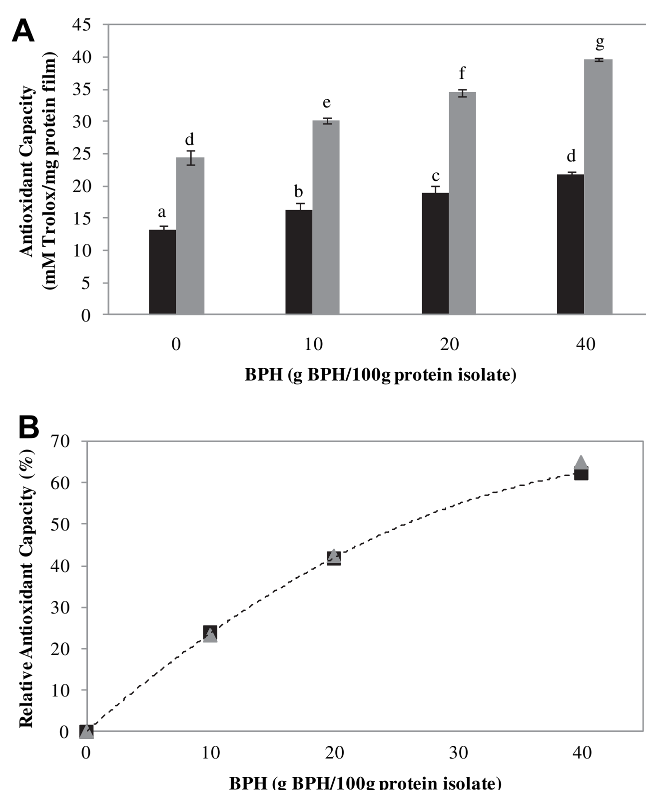

Fig. 5. **A**) Antioxidant capacity (ABTS$^{\cdot+}$ scavenging activity) of films prepared from **SPI** (■) or **SFPI** (■) with different concentrations of **BPH** (0, 10, 20 and 40 g **BPH**/100 g protein isolate). **B**) Correlation between antioxidant capacity and content of **BPH** added in protein-based films (**SPI** ■ and **SFPI** ▲). Results are expressed as mean value ± standard deviation. Values means followed by the same letter are not significantly ($p \leq 0.05$) different according to Tukey's HSD test.

4. Conclusions

It was possible to significantly increase the antioxidant capacity of soybean and sunflower protein-based films by adding a bovine plasma protein hydrolysate with high antioxidant capacity to the formulations. The presence of this hydrolysate in the protein film also affected the physicochemical properties of these resulting materials, as its presence plasticized the protein network, without significantly altering their appearance.

The properties of the developed materials suggest that they could have applications in packaging for food susceptible to oxidation, although it should be evaluated in particular. Additionally, this potential application could increase the use of agro-industrial proteins that are currently underutilized and, particularly in the case of plasma, can become an environment contaminant.

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