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Preparation of soy protein hydrolysates with antioxidant activity by using peptidases from latex of *Maclura pomifera* fruits

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ABSTRACT

A partially purified proteolytic extract prepared from *Maclura pomifera* latex was employed in hydrolyzing a soybean-protein isolate (4.2 mg/mL). The hydrolysis-product formation, monitored by tricine–sodium-dodecyl-sulfate–polyacrylamyde-gel electrophoresis and reverse-phase high-performance liquid chromatography, indicated that after 10 min of reaction the main soybean proteins disappeared. The maximum degree of hydrolysis was 36.2% after a 180-min digestion. The 90-min hydrolysate presented an IC_{50} of 31.6 ± 0.2 μg/mL, and a trolox equivalent antioxidant capacity of 157.6 and 176.9 μmoles TE per g of peptide determined by two different methods. Analysis by matrix-assisted–laser-desorption-ionization–time-of-flight mass spectrometry (MALDI-TOF MS), followed by the application of bioinformatics tools, enabled the deduction of fourteen theoretical peptide sequences containing antioxidant amino acids at > 60%, none of which sequences had been previously reported as antioxidants. Finally, we consider that this 90-min hydrolysate would constitute a promising ingredient in the manufacture of functional foods.

1. Introduction

Reactive-oxygen species (ROSs) are a group of compounds that includes oxygen radicals and several nonradical oxidizing agents, which have tendency to donate oxygen to other substances (Kumar, 2011). Free radicals are species with unpaired electrons that are unstable and rapidly carry out a series of chain reactions with other compounds. ROSs provoke harmful effects on the cell through damage of DNA, oxidations of lipids, oxidations of amino-acid residues in proteins, and inactivation of the cofactors of enzymes (Sarma, Mallick, & Ghosh, 2010). ROSs can be generated in the body either as a result of metabolic reactions or in pathologic states, as well as into the food. In living organisms, ROSs can be combatted by specific enzyme systems and through both endogenous antioxidants such as glutathione and exogenous antioxidant compounds such as vitamin C (Griffiths et al., 2016; Kumar, 2011; Peng et al., 2014). Oxidation reactions furthermore affect the quality of foods by modifying the organoleptic characteristics (De Castro & Sato, 2015). Antioxidants are therefore used to preserve foods by retarding the deterioration, rancidity, or discoloration caused by such oxidation and thus contribute to the field of healthcare through protecting the body against damage by ROSs (Kumar, 2011). The search for effective, nontoxic natural antioxidants has been intensified in recent years since certain frequently used synthetic antioxidants (*e.g.*, butylated hydroxytoluene and butylated hydroxyanisole) have recently been reported to be hazardous to human health (Akbarirad, Gohari Ardabili, Kazemeini, & Mousavi Khaneghah, 2016; Lobo, Patil, Phatak, & Chandra, 2010).

Plant-derived foods—*i. e.*, fruits, vegetables, cereal grains, and seeds—contain natural antioxidants that can confer on the body a significant protection against ROS-induced oxidative stress (Waly et al., 2016). Mediterranean-style diets have been known cause a significant decline in cardiovascular diseases through an intervention in lipoprotein oxidation with a consequent reduction in oxidative stress. Vegetables, fruits, tea, and wine contain flavonoid antioxidants that can reduce coronary disease and even cancer (Griffiths et al., 2016).

Other food-compounds that offer action against oxidation are peptides from either animal or plant sources. Bioactive peptides—fragments encrypted in the primary sequences of proteins that confer different biologic activities including antioxidation—are released through the hydrolysis of peptide bonds by peptidases (Li-Chan, 2015). Antioxidant peptides have been identified in protein hydrolysates of milk, egg, meat, and fish (Abu-Salem, Mahmoud, El-Kalyoub, Gibriel, & Abou-Arab, 2013; Ahmed, El-Bassiony, Elmalt, & Ibrahim, 2015; Erdmann, Cheung, & Schröder, 2008). The plant sources of protein hydrolysates with antioxidant activity are wheat, rice, oats, corn, soybean, peas, and chickpeas. The soybean is one of the most widely

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studied sources of antioxidant peptides (De Castro & Sato, 2015). Puchalska, Marina, and García (2014) reported the presence of at least thirty such peptides in a fraction smaller than 3 kDa of five soybean-based infant commercial formulas. The peptide SGDAL had been previously reported but within the two longer sequences of antioxidant peptides LQ**SGDAL**RVPSGTTYY and LN**SGDAL**RVPSGTTYY from soybean ß-conglycinin.

The peptidases employed in digestion reactions can be commercial enzymes, such as Alcalase™ and trypsin, or ones from nontraditional sources, such as those from plants. The digestion conditions and enzyme-specificities are key parameters in protein hydrolysis for determining the peptide-bonds to be cleaved. The resulting hydrolysates exhibit bioactive properties that are related to changes in protein structure, a reduction in molecular mass, and the surface-exposure of both ionizable and hydrophobic groups (Evangelho et al., 2016). With respect to the mechanism of action of antioxidant peptides, several studies demonstrated an ability to inhibit lipid peroxidation, to remove free radicals, to chelate metal ions, and to eliminate ROSs. The composition of antioxidant peptides is a key consideration: Tyr, Trp, Met, Lys, and Cys have been reported to be able to reduce Fe^{+3} to Fe^{+2}, and chelate Fe^{+2} and Cu^{+2} ions. In addition, Trp, Tyr, and Phe—the aromatic amino acids—may scavenge certain radicals by proton donation from the *pi* orbital of the benzene ring. The basic amino acid His presents a great potential in radical scavenging by chelating, lipid trapping, and decomposition of the imidazole ring. In addition, the sequence of a given peptide also plays a key role in determining the resulting antioxidant activity (De Castro & Sato, 2015). Abu-Salem et al. (2013) compared the antioxidant capability of 28 structurally related peptides to Leu-Leu-Pro-His-His from soybean-protein digests and concluded that Pro-His-His could be an active center to confer antioxidant activity on the entire peptide. *Maclura pomifera* (Raf.) Schneid (Moraceae) is an ornamental species cultivated in Argentina, whose fruit latex contains unusual quantities of serine peptidases that have been used to hydrolyze bovine-whey proteins (Bertucci, Liggieri, Colombo, Vairo Cavalli, & Bruno, 2015) and bovine caseins (Corrons, Liggieri, Trejo, & Bruno, 2017). These hydrolysates were prepared under mild reaction conditions, and both contain angiotensin-converting-enzyme–inhibitory biopeptides. On the basis of the observations described above, we propose as hypothesis of work that peptidases from *M. pomifera* could be capable of hydrolyzing soybean proteins to release the antioxidant peptides present in those food sources. The present study was thus aimed at employing those peptidases to produce hydrolysates of soybean proteins after optimizing the digestion conditions and then monitoring the hydrolysis products to determine the presence of antioxidant activity. The general objective was to prepare a product that can be used as an ingredient in functional foods.

2. Materials and methods

2.1. Chemicals

Bovine-serum albumin, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), casein, 2,4,6-trinitrobenzenesulfonic acid (TNBS), L-cysteine, potassium persulfate, tris(hydroxymethyl)aminomethane (Tris), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) were purchased from Sigma Chemical Company (St. Louis, MO, USA); Coomasie Brilliant Blue G-250, acrylamide, bis-acrylamide, low-range molecular-weight standards (phosphorylase B, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme: 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa, respectively), and tricine from Bio-Rad Hercules, CA, USA); trifluoroacetic acid (TFA) from J.T. Baker (Philipsburg, NJ, USA); L-leucine and trichloroacetic acid from Carlo Erba Reagenti (Rodano, MI, Italy); and Sephadex G-25 fine from GE Healthcare Life Sciences (Uppsala, Sweden). All other chemicals were obtained from local commercial sources and were of the highest purity available.

2.2. Preparation and partial purification of the plant-protease extract

Ten mature fruits (approximately 15 cm in diameter) from *M. pomifera* were collected from a single tree in La Plata city, Province of Buenos Aires, Argentina. Latex (9.6 mL) was extracted from longitudinal incisions through the skin of the fruit and was collected by dripping into a buffer containing 160 mL of 0.1 M phosphate, 5 mM EDTA, and 5 mM cysteine, pH 6.6. This extract—named pomiferin—was centrifuged at 16,000*g* and 4 °C for 30 min to remove insoluble materials and partially purified by precipitation with one volume of cold ethanol at −20 °C (Corrons et al., 2017). The partially purified extract (PPE) was stored in aliquots of 0.5 mL at −20 °C.

2.3. Determination of the caseinolytic activity and the protein concentration

The proteolytic activity of the PPE, determined on bovine casein at 37 °C by the method described by López et al. (2000), was expressed as caseinolytic units per mL (Ucas/mL), an arbitrary value hereafter defined as the amount of enzyme that produces an increase of one absorbance unit per min under the assay conditions. The concentration of protein in the PPE was evaluated by the Bradford method (1976), with bovine-serum albumin as the standard (range: 100–1000 μg/mL). The specific activity was calculated as the ratio between the caseinolytic activity and the protein concentration (*i.e.*, Ucas/mg).

2.4. Preparation of soybean-protein hydrolysate

A soybean-protein isolate was prepared by extracting 5 g of defatted soybean flour (Bunge, Buenos Aires, Argentina) with 50 mL of distilled water (alkalinized to pH 8.0 with 2 N NaOH) at room temperature under constant agitation for 2 h. The suspension was centrifuged at 12,800*g* for 15 min at 20 °C and the precipitate discarded. The supernatant was adjusted to pH 4.5 with 2 N HCl. After a second identical centrifugation, the precipitate was resuspended in distilled water and the pH adjusted to 8.0 with 2 N NaOH (Ortiz & Wagner, 2002).

Soybean-protein hydrolysates were prepared by mixing 4 mL of PPE and 36 mL of soybean-protein isolate (diluted 1/10 in distilled water). The mixtures were incubated at 45 °C for different times (10–180 min) under constant stirring. After the hydrolysis, the enzymes contained in PPE were inactivated by heat shock (7 min at 100 °C). Two controls were carried out by replacing the PPE (substrate blank, SB) and the soybean protein suspension (enzyme blank, EB) with distilled water in the same ratio as was employed in the hydrolysis reaction. The concentration of peptides in the hydrolysates was estimated by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.5. Determination of the degree of hydrolysis

The TNBS method (Adler-Nissen, 1979) was employed to determine the degree of hydrolysis of the hydrolysates. Experimental samples or the standard mixture (40 μl) were added to 320 μl of 0.213 M Na_2HPO_4 plus 1% (w/v) sodium dodecyl sulfate (SDS); pH 8.2 and 320 μl of 0.5% (v/v) aqueous TNBS. The mixtures were incubated for 1 h at 50 °C in the dark and the reaction stopped after 1 h by the addition of 640 μl of 0.1 M HCl at room temperature in the dark. The absorbance was then measured at 340 nm with L-leucine (0–2.25 mM) as a standard. The degree of hydrolysis was expressed as the percent cleavage of peptide bonds with respect to the total number of peptide bonds per protein equivalent (Nielsen, Petersen, & Dambmann, 2001).

2.6. Peptide-profile characterization by sodium-dodecyl-sulfate–polyacrylamide-gel electrophoresis (SDS-PAGE) and reverse-phase high-performance liquid chromatography (RP-HPLC)

The degradation of proteins was visualized by a tricine-SDS-PAGE system (Corrons, Bertucci, Liggieri, López, & Bruno, 2012). The hydrolysate samples and the corresponding blanks (100 μl) were suspended in 100 μl of sample buffer, heated at 100 °C for 5 min, and centrifuged at 16,000*g* for 15 min. Electrophoresis was performed on a vertical slab cell (Mini Protean III, BioRad, Hercules, CA, USA) containing 16% (w/v) polyacrylamide for 3 h at 90 V. The gels were stained with Coomassie brilliant blue G-250 to visualize the protein bands along with the low-range molecular markers (Bio-Rad) employed.

The peptide profile of the hydrolysates was also checked by RP-HPLC. Samples were centrifuged at 16,000*g* for 15 min, and 100 μl of each supernatant obtained was applied to a Bondclone™ 10 C-18 column (3.5 mL, 00H-2117-C0, Phenomenex), operating at room temperature in an ÄKTA-Purifier chromatograph (GE, Uppsala, Sweden) at a flow rate of 0.5 mL/min. After column equilibration with 15 mL of Solution 1 (0.05 mL TFA in 100 mL double-distilled water), each sample was injected on the column, and a linear gradient from 100% Solution 1–100% of Solution 2 (0.025 mL TFA in 100 mL acetonitrile) was applied in 10column volumes (35 mL in total) to obtain a characteristic peptide profile. The elution was monitored by measurement of absorbance at 280 nm.

2.7. Characterization of hydrolysates by size-exclusion chromatography

The soybean-protein hydrolysates were characterized by size-exclusion chromatography on a column containing 37 mL of Sephadex G-25 fine. After equilibration of the column with 150 mL of 0.1 M phosphate buffer pH 6.6, 400 μl of each sample was applied. The elution was carried out at a flow rate of 0.5 mL/min at room temperature with the same phosphate buffer employed in the equilibration step. The eluted peptides were detected at 280 nm.

2.8. Determination of antioxidant activity

2.8.1. ABTS-radical–scavenging capacity

The antioxidant assay was carried out according to Re et al. (1999) with slight modifications. The $ABTS^{\cdot}$ radical solution was prepared by dissolving 29 mg of ABTS plus 5 mg of potassium persulfate in 9.6 mL of distilled water at room temperature in the dark. After 16 h, the solution was diluted with 5 mM phosphate buffer pH 7.4 to a final absorbance at 734 nm of 0.70 ± 0.02 units. The reaction tubes contained 10 μl of sample plus 1 mL $ABTS^{+}$ radical solution; the absorbance at 734 nm (AS_{10min}) was measured after an incubation for 10 min at room temperature. A negative blank, incubated in parallel containing phosphate buffer instead of the sample, was measured at 0 min and after 10 min (AB_{0min} and AB_{10min}, respectively), while the trolox dilutions (stock concentration of 2.5 mg/mL) were likewise incubated as a positive control and a standard for determining the equivalence of the sample. All the measurements were performed in triplicate and reported as the mean value, with the results expressed either as mg of trolox per mL or as the trolox-equivalent antioxidant capacity (TEAC) in micromoles of trolox/g of peptide. Accordingly, the percent inhibition of the radical was calculated from the following equation:

$$\%\,\text{Inhibition}\,ABTS^{+} = [(AB_{10\,min} - AS_{10\,min}) \times 100]/AB_{0\,min}$$

The 50%-inhibitory concentration (IC_{50}) was expressed as the mg/mL of hydrolysate or trolox that scavenged half of the $ABTS^{+}$ radical. This value was calculated on the basis of the final concentration in the cuvette.

The hydrolysate that exhibited the most antioxidant activity was filtered by centrifuging through Amicon Ultra-15 filter units (30-kDa cut-off, Millipore). This step was carried out with the purpose of removing unwanted aggregates. Finally, this sample was fractionated by size-exclusion chromatography according to Section 2.7 above, and 4-mL aliquots were collected and analyzed for antioxidant activity by the ABTS method.

2.8.2. Assay of oxygen-radical–absorbance capacity (ORAC)

The ORAC assay was performed according to the method of Kim, Jang, and Kim (2007) with modifications. Solutions of 53.3 nM fluorescein and 20 mM AAPH, both in 35 mM phosphate buffer (pH 7.8), were first prepared. The reaction mixtures contained 1.5 mL of fluorescein solution, 250 μl of sample, and 250 μl of AAPH. Trolox (1–100 mM) solutions were used as a positive control. The decrease in fluorescence per min (at 485-nm emission and 535-nm excitation frequencies) was followed for 35 min in a spectrofluorometer (Model RF-1501, Shimadzu Corporation, Kyoto, Japan). In the fluorescein-decay curves plotted from the data, the difference in the area under the curves between each sample and the blank were calculated and the results expressed as TEAC values.

2.9. Matrix-assisted–laser-desorption-ionization–time-of-flight mass spectrometry (MALDI-TOF) analysis

The molecular weights of the filtered hydrolysate with the highest antioxidant activity were investigated by MALDI-TOF MS. The analyses were carried out by CEQUIBIEM (Centro de Estudios Químicos y Biológicos de Espectrometría de Masa, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina). The experimental molecular masses were compared with theoretical peptide masses obtained by applying the nonspecific-cleavage tool of the FindPept software (http://web.expasy.org/findpept/) from the database-sequences of major soybean proteins (UniProtKB/Swiss-Prot, protein sequence database; accession numbers: glycinin subunits A_3 and B_4: P04347, β-conglycinin subunits α, α′, and β: P13916, P11827, and P25974, respectively. Δ mass tolerance: ± 0.01 Da).

2.10. Statistical analysis

All the data were reported as the mean ± the standard deviation of three replicates. The results of antioxidant activity were compared by the one-way analysis of variance ANOVA (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA 92037 USA). Significant differences between the means of parameters were determined by Tukey's test ($P < 0.05$). The same software was employed to calculate the area under the curves of the ORAC method.

3. Results and discussion

3.1. Enzymatic extracts and the soybean-protein isolate

Latex from *M. pomifera* containing peptidases was used to prepare an extract referred to as pomiferin that contained a caseinolytic activity of 7.2 ± 0.4 Ucas/mL and a protein content of 1.75 ± 0.08 mg/mL. In previous studies from our laboratory, crude extracts obtained by the same protocol evidenced a higher caseinolytic activity—*i. e.*, 14.1 ± 0.8 Ucas/mL (Corrons et al., 2012), 8.04 ± 0.21 Ucas/ml (Bertucci et al., 2015). The proteolytic activity of plant extracts often exhibits variability since the total enzymatic activity from a given plant would depend on the fruit size, the amount of latex recovered, and the quantity of protease present in the latter along with variations according to both the plant's physiology and developmental stage in addition to the prevailing environmental conditions (Schaller, 2004).

In the present experiments, pomiferin was partially purified by ethanol precipitation since this solvent is less toxic than others habitually employed to precipitate proteins. Because the aim of this work was to employ the peptidases in the preparation of a component for potential use as a functional food, we decided to precipitate with

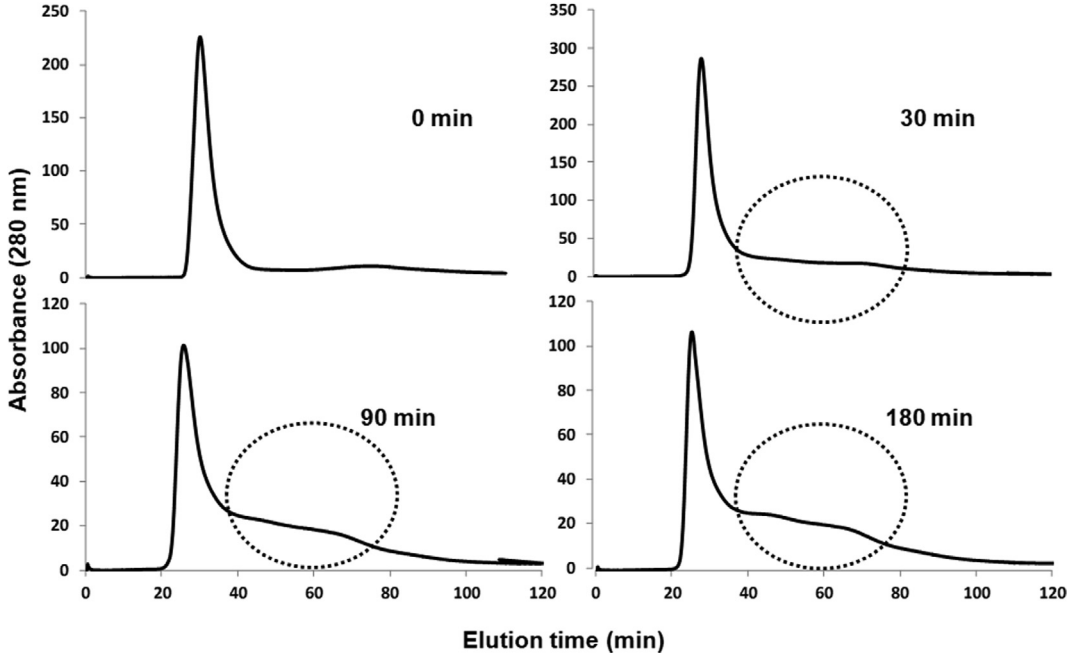

Fig. 1. Size-exclusion chromatography (Sephadex G-25 fine) of soybean-protein hydrolysates. In the panels, the protein absorbance at 280 nm is plotted on the *abscissa* as a function of the elution time in min either before hydrolysis (0 min, Panel A) or after hydrolyses for 30 (Panel B), 90 (Panel C), or 180 (Panel D) min. The dotted circle denotes the fractions with peptides that appeared as hydrolysis products.

ethanol simply in the unlikely eventuality that traces of the solvent might remain in the final product. The specific activity of the PPE was 3.9 ± 0.1 Ucas/mg, a value close to the 5.4 ± 0.4 Ucas/mg reported by Bertucci et al. (2015).

The substrate for the hydrolysis reactions was a soybean-protein isolate prepared from defatted flour at a final protein content of 41.7 ± 26 mg/mL. This isolate was diluted 1/10 with distilled water for the preparation of hydrolysates (about 4.2 mg/mL of protein), and nine volumes of this dilution were mixed with one volume of PPE at 45 °C under constant stirring. Certain authors—*e.g.*, Ruiz et al. (2013) and Benitez, Ibarz, and Pagan (2008)—recommend a prior denaturation of the globular proteins in order to more completely expose the peptide bonds to the peptidase action. We did not choose this option, though, because during denaturation a portion of the proteins usually undergoes an aggregation that could hamper the hydrolysis reactions.

3.2. Characterization of soybean-protein–isolate hydrolysates

The progress of the hydrolysis reaction was followed by different techniques. Fig. 1 depicts the degradation profile as assessed by size-exclusion chromatography. The absorbance measured at 280 nm indicated that fragments of lower molecular mass—eluting between 40 and 80 min—increased upon the progression of hydrolysis (denoted by a dotted circle) that were not detected in the run corresponding to the unhydrolyzed substrate. Fig. 2 illustrates the tricine-SDS-PAGE–gel image of the hydrolysates of the soybean-protein isolate, with the main bands of the nonhydrolyzed soybean-protein visualized in lane 0′. In this lane, two bands corresponding to both the acid (A) and the basic (B) subunits of glycinin are visible (at molecular masses of 36 and 22 kDa, respectively). The bands corresponding to the different β-conglycinin subunits (α, α′, and β) are of molecular masses between 47 and 67 kDa. This profile is in agreement with that obtained in previous studies (Ruiz et al., 2013; Medrano & Del Castillo, 2011; Zhao, Zhu, & Chen, 2015). After 10 min of reaction the main bands of the soybean protein disappeared, and several persistent bands could be observed, one of 18 kDa, two close to 14.4 kDa, and a broad band corresponding to short peptides (< 14.4 kDa). Even though certain authors have found difficulties in hydrolyzing soybean proteins—and particularly the

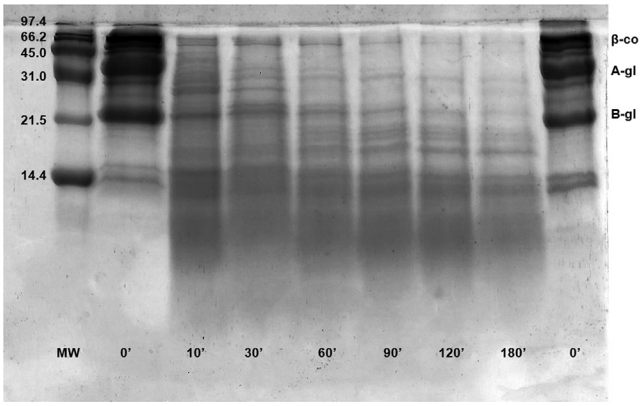

Fig. 2. Tricine SDS-PAGE of the hydrolysates of the soybean-protein isolate. The lanes correspond to the following samples: MW, molecular-weight markers (low range kit, BioRad); 0′, soybean-protein-isolate blank; 10′–180′, hydrolysates of the soybean-protein isolate after 10, 30, 60, 90, 120, and 180 min of digestion. On the right, the repeated lane 0′ depicts the main soybean-protein isolate. β-co, β-conglycinin subunits; A- and B-gl, acid- and basic-glycinin subunits.

subunits of β-conglycinin because of their glycosylation (Gibbs, Zougman, Masse, & Mulligan, 2004)—the peptidases of pomiferin readily hydrolyzed those proteins: indeed, the corresponding bands were not present in the gel after only 10 min. Moreover, a study by Aguirre et al. (2008) of the soybean-protein hydrolysates produced by twelve bacterial proteases demonstrated that β-conglycinin was the fraction preferred by all twelve, but only three exerted an appreciable action against the basic subunit of glycinin. In the present work, glycinin was digested after 90 min of reaction (Fig. 2), thus exemplifying the high capacity of pomiferin proteases to degrade all the principal soybean proteins.

In addition, the generation of new bands that persist upon reaction progression has been reported in hydrolysates obtained with the peptidases from *M. pomifera* (Bertucci et al., 2015; Corrons, Liggieri, Trejo, & Bruno, 2017). Those researches revealed an overall electrophoretic

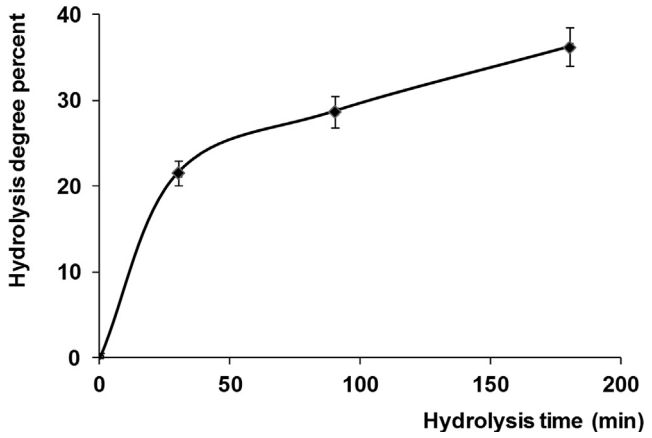

Fig. 3. Degree of hydrolysis of different hydrolysates of the soybean-protein isolate. In the figure, the percent hydrolysis is plotted on the *ordinate* as a function of hydrolysis time in min on the *abscissa*. All the determinations were carried out in triplicate. The vertical bars correspond to the standard deviation.

profile that remained essentially unchanged after short hydrolysis times, and the authors concluded that these proteases possess certain peptide-bond specificities for cleavage.

Because the degradation pattern of proteins visualized by SDS-PAGE (Fig. 2) did not indicate considerable differences between the bands corresponding to the hydrolysis times from 10 through 180 min, we selected 30-, 90-, and 180-min of digestion for our further determinations.

Fig. 3 depicts the plot of the degree of hydrolysis vs. the hydrolysis time. An initial linear segment for the first 30 min reached a value of 21.6 ± 1.5%, followed by a second linear portion with a considerably decreased slope. The degree of hydrolysis obtained at 180 min was 36.2 ± 2.3%, a value close to those reported by Hrčková, Rusňáková, and Zemanovič (2002), for soybean-protein hydrolysates prepared by employing the proteases Alcalase™, Flavourzyme™, and Novozym™ (at respective degrees of hydrolysis of 35.1, 39.5, and 33.3%). In that study, the reaction time was much longer than in the present work (*i. e.*, 8 h). The possible uses for soybean-protein hydrolysates with these values for the degree of hydrolysis could take advantage of the changes in the peptides' functional properties—such as the foaming and gelation capabilities as well as the release of bioactive peptides, as has been reported in other soybean-protein hydrolysates (Sun, 2011; Meinlschmidt, Sussmann, Schweiggert-Weisz, & Eisner, 2016).

Fig. 4 summarizes the peptide profiles obtained after hydrolysis as monitored by RP-HPLC. The persistent presence of two peaks in the substrate blank (0 min) and all the hydrolysates (30, 90, and 180 min) corresponded to the nondegraded soybean peptides (40.6 min, 36.8% Solution 2; 42.9 min, 40.9% Solution 2). The plot of the enzyme blank contained two main peaks at 33.6 and 60.2 min of elution and 27.6 and 65.6% Solution 2, respectively. After 30 min of digestion, peptide profile did not undergo a significant further variation. This profile was characterized by a prominent peak at 47.7 min and 47.7% Solution 2) and also by the replacement the two broad peaks of the substrate blank (30.2 min, 22.8% Solution 2; 50.4 min, 51.6% Solution 2) by two corresponding groups containing seven and three new peaks, respectively. These latter details would provide new evidence apparently confirming that the PPE constitutes an enzyme preparation which cuts at specific sites, since these hydrolases would not digest new peptide bonds and thus manifested an invariable digestion profile at progressive hydrolysis times.

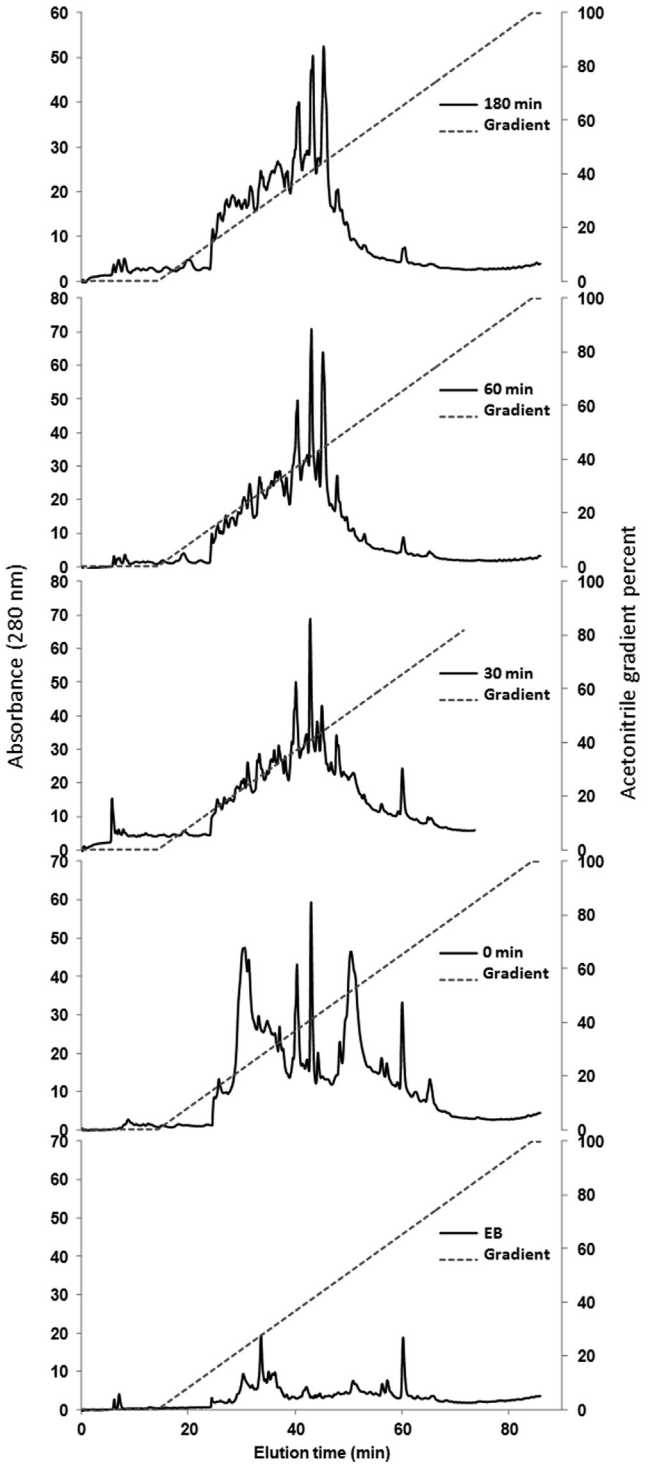

Fig. 4. RP-HPLC of the hydrolysates of the soybean-protein isolate. In the figure, the absorbance at 280 nm on the left *ordinates* and the percent acetonitrile in Solution 2 used in the elution profile on the right *ordinate* pertaining to the ascending dotted lines are plotted as a function of the running time in min on the *abscissas*. EB, enzyme blank; 0 min, substrate blank; 30, 90, and 180 min, hydrolysates at those tme points. Column: Bondclone™ 10 C-18, 00H-2117-C0, Phenomenex. Flow rate, 0.5 mL/min. Elution conditions: linear gradient from 100% Solution 1 (0.05 mL TFA in 100 mL water) to 100% Solution 2 (0.025 mL TFA in 100 mL acetonitrile), in 10 column volumes.

3.3. Antioxidant activity of soybean-protein hydrolysates

The results for the antioxidant activity of the hydrolysates (by the ABTS method) and the corresponding substrate and enzyme blanks were analyzed by the ANOVA. The percentages of antioxidant activity of the 30-, 90-, and 180-min hydrolysates were 66.7 ± 4.1, 68.7 ± 2.4, and 65.2 ± 1.8%, respectively, and were not significantly

different ($p > 0.05$), whereas the corresponding respective substrate- and enzyme-blank values were 26.1 ± 2.3 and 2.1 ± 0.6%, both at significant differences from those obtained with all the hydrolysates ($p < 0.05$). The antioxidant activities of the 30-, 90-, and 180-min hydrolysates were also expressed as the trolox-concentration at values of 0.28 ± 0.02, 0.29 ± 0.01, and 0.28 ± 0.01 mg/mL, respectively.

We selected the 90-min hydrolysate for further study since after only 90 min of digestion no substantial further changes in the digestion profile occurred, thus rendering a longer hydrolysis time, such as 180 min, unnecessary. Furthermore, whereas the SDS-PAGE profiles indicated that the values corresponding to 30 and 90 min of reaction were similar, a complete degradation of the relevant bands had not yet occurred after only 30 min of hydrolysis. Thus, for this reason, the 30-min hydrolysate was not considered.

The TEAC value is an expression of antioxidant activity that enables a comparison of antioxidant capability between different laboratories. The TEAC value for the 90-min hydrolysate was 157.6 μmoles TE/g of peptide, as assesed by the ABTS method, and was 176.9 μmoles TE/g peptide by the ORAC determination. These results are in the same order of magnitude as those reported by Medrano and Del Castillo (2011), who hydrolyzed glycinin using proteases from *Streptomyces griseus* and *Aspergillus oryzae* (*i.e.*, Flavourzyme™) for 3 h, reporting values of 180–220 μmol TE/g. Afterwards, we calculated the IC_{50} for this hydrolysate and for trolox, and we obtained values of 31.6 ± 0.2 and 3.2 ± 0.1 μg/mL, respectively. This latter was almost the same reported by Fitriana, Ersam, Shimizu, and Fatmawati. (2016) whose value obtained was 3.06 μg/mL by using the same method.

The hydrolysate of 90 min was filtered through a 30-kDa–cut-off membrane. The antioxidant activity recovered in this filtrate retained 71% of the original activity, as assessed by the ABTS method and 87% by the ORAC determination. A comparison between data obtained by different methods is usually difficult because of the lack of a single antioxidant standard, a uniform radical concentration, and equivalent experimental conditions.

After the filtration, the sample was analyzed by size-exclusion chromatography and the resulting fractions evaluated by the ABTS method. Fig. 5 depicts the antioxidant activity, expressed in mg/mL of trolox for each of the elution tubes (ten fractions). The highest antioxidant activity was observed in the first two tubes, with the activity decreasing after fraction number 3. The values of antioxidant activity corresponding to the fractions 8–10 were ignored because the ANOVA indicated no significant differences from that of the negative control ($p > 0.05$).

3.4. Analysis by MALDI-TOF MS

The molecular masses of the 90-min hydrolysate filtered through the 30-kDa–cut-off membrane were analyzed by MALDI-TOF MS. The resulting fragments constituted about 40 peaks over the background smaller than 8 kDa, whose intensities were greater than 200 arbitrary units (Supplementary material). These mass values were used to carry out a bioinformatics analysis by means of the FindPept software tool for nonspecific cleavage along with a comparison of those fragments with the sequences of major soybean proteins. Table 1 summarizes the theoretical sequences of peptides present in the hydrolysate, their position in the original soybean protein, and the percentage of antioxidant amino acids according to studies previously reported. In those results, amino acids such as Thr, Met, Cys, Phe, Tyr, His, Gly, Trp, Ala, Val, Leu, and Lys (T, M, C, F, Y, H, G, W, A, V, L, and K in the table, respectively) were considered the ones responsible for the antioxidant capability of the peptides that contained them (Sarmadi & Ismail, 2010; Wang, Zhao, Zhao, & Jiang, 2007).

Likewise, the percentage of antioxidant amino acids was calculated for two peptides found in previous investigations. Thus, the peptides SHCMN (Lin, Liang, Li, Xing, & Yuan, 2016), and FDPAL (Ma et al., 2016) from soybean protein both presented a percentage of antioxidant amino acids of 60%.

On basis of these results, in Table 1 the peptides with a composition of antioxidant amino acids equal to or greater than 60% (denoted with an asterisk in Table 1) were selected as potentially being responsible for the antioxidant activity of the 90-min hydrolysate.

From the analysis of the sequences listed in Table 1, 14 candidates were found, none having been previously reported as antioxidant peptides—namely, 2, 1, 5, 4, and 2 from A3-glycinin, B4-glycinin and the α-, α'-, and β-subunits of β-conglycinin, respectively).

We thus considered that the initial hypothesis of the present work was verified because the peptidases from *M. pomifera* were able to hydrolyze the soybean proteins. This conclusion was verified through the monitoring of the hydrolysis products by different methods. Moreover, antioxidant activity was found in the hydrolysates, which capability was attributed to the presence of bioactive peptides released from the soybean proteins.

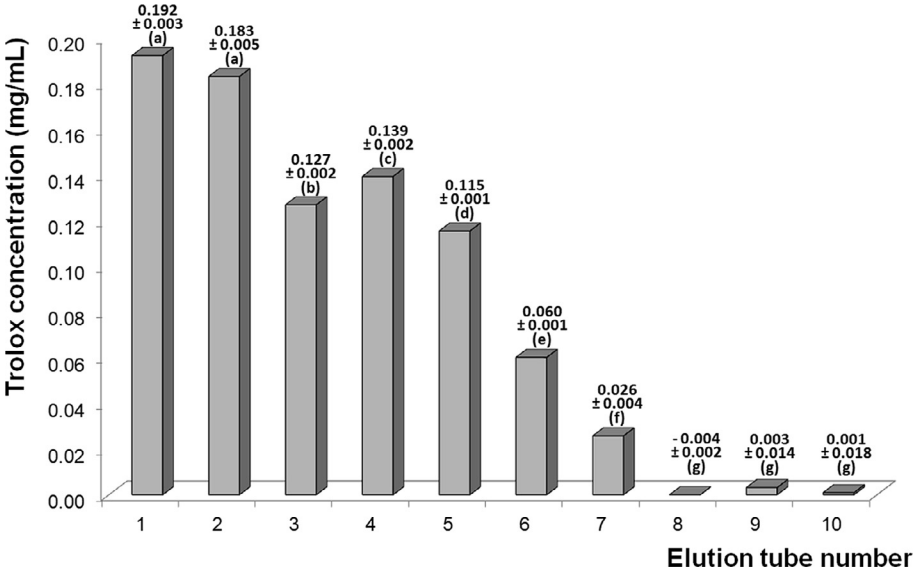

Fig. 5. Antioxidant activity of the fractions of the filtered 90-min hydrolysate obtained by molecular-size chromatograhy. In the figure, the trolox concentration in mg/mL is plotted on the *ordinate* for the sequential elution fractions obtained from the Sephadex G-25 column denoted on the *abscissa* expressed as the tube numbers. Labels over bars: Trolox (mg/mL) value ± SD; same letter indicates values with no significant difference ($P > 0.05$).

Table 1
Theoretical sequences of peptides present in the 90-min filtered hydrolysate within the sequences of A- and B-glycinin and α-, α′- and β-, β-conglycinin. The position of the peptide within the protein of origin corresponding to the number in the amino-acid sequence within that precursor of these proteins is listed in the Position column. The experimental and theoretical mass-value matches were selected with a mass tolerance of ± 0.01 Da. The white letters on the black background correspond to the single-letter amino-acid abbreviations within the sequences that exhibited antioxidant activity. The percentage of antioxidant amino acids was calculated with respect to the total number in each peptide sequence. The asterisk (*) denotes peptides with a composition of antioxidant amino acids equal to or greater than 60% that were selected as potentially being responsible for the antioxidant activity of the 90-min hydrolysate.

Original protein	User mass	Peptide	Position	Percent antioxidant amino acid in peptide sequence
Glycinin; A_3 subunit; e.n.: P04347	1133.616	(N)PRVFYLAGNP(D)	184-193	60.0 (*)
	1164.621	(D)QNPFVFYLAG(N)	182-191	60.0 (*)
	1298.686	(K)IRHRNEGDVLV(I)	138-148	54.5
	1298.686	(I)RFFNEGDVLVI(P)	139-149	54.5
	1458.742	(S)HLPSYLPYPQMI(I)	82-93	50.0
	1458.742	(S)RRGSRSQQQLQQD(S)	122-133	16.6
	1633.837	(Q)LNNLNALEPDHRVE(S)	35-48	42.8
Glycinin; B_4 subunit; e.n.: P04347	1176.607	(L)KYKGNSGPLVN(P)	505-515	54.5
	1176.607	(G)NAVFDGFLRR(G)	432-441	50.0
	1176.607	(A)VFDGFLRRGQ(L)	434-443	50.0
	1384.729	(F)KTHHNAVSSVHK(D)	468-479	66.6 (*)
	1651.899	(S)SMIKVFRVIPSE (L)	476-489	42.8
β-Conglycinin; α subunit; e.n.: P13916	1005.590	(D)YLIVILNGT(A)	255-263	66.6 (*)
	1133.616	(F)NLRSRDPIY(S)	407-415	22.2
	1148.621	(A)DAGYLIVILN(G)	252-261	50.0
	1181.655	(L)FKNQYGRIR(V)	207-215	44.4
	1395.789	(E)TLFKNQYGRIR(V)	205-215	18.2
	1402.721	(N)REETLFKNQYG(R)	202-212	63.6 (*)
	1402.721	(R)EETLFKNQYGR(I)	203-213	63.6 (*)
	1402.721	(R)EKNKNPFLFGSN(R)	190-201	16.7
	1458.742	(I)INGTALSSLVNNDD(R)	260-273	50.0
	1605.834	(D)SVRLQSGDAIRVPSG(T)	276-290	33.3
	1605.834	(P)FLFGSNPEPTLFK(N)	196-208	69.2 (*)
	1651.899	(E)TLPPKNPQLRDLDI(F)	425-438	28.5
	1651.899	(L)SKRAKSSSRKTISSE(D)	388-402	33.3
	1667.903	(N)TLLLPNHADADYLHV(I)	244-258	66.6 (*)
	1780.937	(Q)LQNLRLYRILEENS(K)	227-240	35.7
	1780.937	(F)EITPEKNPQLRDLDI(F)	424-438	26.6
	1793.982	(L)LLPNHADADYLHVILN(G)	246-261	56.2
	1908.991	(P)QLQNLRLYRILEENS(K)	226-240	33.3
	2395.285	(E)DKPENLRSRDPIYSNKLGKE(F)	403-422	45.0
β-Conglycinin; α′ subunit; e.n.: P11827	1005.590	(D)YLIVILNGT(A)	271-279	66.6 (*)
	1148.621	(R)FNKRSQQLQ(N)	236-244	33.3
	1148.621	(A)DAGYLIVILN(G)	268-277	50.0
	1148.621	(Y)LQGFSKNILE(A)	369-378	50.0
	1176.607	(A)FPRSAKDIEN(L)	593-602	30.0
	1298.686	(K)PNTLLLPHHADA(D)	258-269	66.6 (*)
	1347.660	(K)VLFGREEGQQQG(E)	391-402	50.0
	1514.840	(K)LEETLQRNPQLR(D)	457-468	33.3
	1605.834	(Q)DIFVLPAGYPVMVNA(T)	538-552	60.0
	1780.937	(Q)LQNLRLYRILEENS(K)	243-256	35.7
	1908.991	(Y)NLQSGALRVPAGTTFYV(V)	294-311	66.6 (*)
	1908.991	(Q)QLQNLRLYRILEENS(K)	242-256	33.3
	2196.223	(P)QQKEEGNKGRGHLSSILRA(F)	618-637	45.0
	2395.285	(H)KNKNPFHFNSKREKTLFKN(Q)	207-225	57.8
	2395.285	(R)EQTLFKNQYGHVRVLQRFN(K)	219-237	63.1 (*)
β-Conglycinin; β subunit; e.n.: P25947	1005.590	(Y)RIVRQSQK(P)	73-80	37.5
	1164.621	(L)RDLDIFLSSV(D)	268-277	40.0
	1176.607	(N)NQRNFLAGEK(D)	368-377	50.0
	1298.686	(K)PNTHLLPHHADA(D)	81-92	58.3
	1605.834	(L)DIFLSSVDINEGALL(L)	271-285	46.6
	1605.834	(D)LDIFLSSVDINEGAL(L)	270-284	60.0 (*)
	1651.899	(E)TLPPKNPQLRDLDI(F)	259-272	28.6
	1663.947	(Q)LSRRAKSSSRKTISS(E)	221-235	33.3
	1780.937	(Q)LENLRLYRIVQFQS(K)	66-79	35.7
	1780.937	(F)EITPEKNPQLRDLDI(F)	258-272	26.6
	1908.991	(N)NEGKFEEITPEKNPQL(R)	252-267	50.0
	1908.991	(I)PAAYPFVVNATSNLNFLA(F)	343-360	66.6 (*)
	1908.991	(P)QLENLRLYRIVQFQS(K)	65-79	33.3
	2485.274	(F)VLSGRAILTLVNNDDRDS N (P)	98-119	50.0

4. Conclusions

Nowadays, in the food industry, soybean-protein hydrolysates are employed as a high-priced value-added ingredient. Protein hydrolysates possess a higher digestibility than the original proteins. A food of this type has proven to be of interest for consumption by people with difficulties in the intestinal absorption of proteins, with various chronic diseases, or with allergies. In addition, protein hydrolysates are commonly included in sports nutritional regimes.

In the present work, we have prepared hydrolysates of soybean proteins employing peptidases from *M. pomifera* with antioxidant activity. We deduced the theoretical sequences of several peptides that we consider to be most likely responsible for this activity. In order to corroborate these sequences, future work should be directed at analyzing selected hydrolysates by MALDI-TOF/TOF MS/MS so as to obtain the real sequences by fragmentation and thus to acquire additional information about these reaction products. For the present, we consider that this 90-min pomiferin hydrolysate constitutes a promising ingredient for inclusion in the manufacture of functional foods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.05.013.

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