Food Chemistry 198 (2016) 36–44

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Bioactive properties of peptides obtained from Argentinian defatted soy flour protein by Corolase PP hydrolysis ☆

Ezequiel R. Coscueta [a], Maria M. Amorim [a], Glenise B. Voss [a], Bibiana B. Nerli [b], Guillermo A. Picó [b], Manuela E. Pintado [a,*]

[a] CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, Apartado 2511, 4200 Porto, Portugal
[b] IPROBYQ (Institute of Bio-technological and Chemical Processes) – College of Biochemical and Pharmaceutical Sciences, National University of Rosario, Suipacha 570, S2002LRK Rosario, Argentina

ARTICLE INFO

Article history:
Received 4 May 2015
Received in revised form 27 October 2015
Accepted 13 November 2015
Available online 17 November 2015

Keywords:
Soy protein hydrolysis
Corolase PP
Bioactive peptides
ACE inhibitory activity
Antioxidant activity

ABSTRACT

Enzymatic hydrolysis of soybean meal protein isolate (SPI) obtained under two temperature conditions with Corolase PP was studied, assessing the impact of hydrolysis on potential antioxidant and antihypertensive activities. The protein was isolated from soybean meal under controlled conditions of time and temperature (70 °C, 1 h; 90 °C, 30 min). Degree of hydrolysis assessed the progress of hydrolysis at different sampling times. For hydrolysates the antioxidant and angiotensin-converting-enzyme (ACE) inhibitory activities were measured. As observed, the DH was increasing until reaching 20% at 10 h with disappearance of globular proteins and generation of low molecular weight peptides (less than 3 kDa). A significant increase in antioxidant and ACE inhibitory capacities was observed. Five main peptides were identified, which may explain through their sequences the bioactive properties analyzed. Through this study was possible to obtain for the first time with Corolase PP soy hydrolysates with potential antioxidant and ACE inhibitory activities, which can be used to obtain new added value functional ingredients from soy meal.

1. Introduction

The increasing costs and limited supplies of animal proteins has forced the demand for new alternative sources. Soybean is becoming one of the most important alternative proteinaceous sources for human and animal consumption (35–40%) due to its low cost and high abundance. Soybean is the second largest source of vegetable oil worldwide (after palm oil) with a global production of 271 million metric tons per annum (Day, 2013). Since first use encompasses oil extraction, the remaining bulk product, rich in protein and fibre, is usually applied in animal feeding (hence usually known as soybean meal) and only a small portion is further processed into various types of soy protein products for human consumption, e.g. soybean protein concentrates. Therefore, soybean meal as most relevant co-product in the processing of soybean with relevant protein content has been upgraded as low added value co-product until now. So, the valorisation of soybean meal through extraction of proteins (glycinin (11S) and ß-conglycinin (7S) being the most abundant) followed by controlled hydrolysis may generate functional ingredients with high added value, since the resulting hydrolysates frequently exhibit bioactive properties such as antioxidant or antihypertensive properties (Peña-Ramos & Xiong, 2002). Beyond its nutritional benefits, soybean meal also includes different compounds that act as anti-nutritionals, i.e. constrain nutrients availability. Trypsin and chymotrypsin inhibitors are among the most important anti-nutritional factors. They are heat labile compounds that can be deactivated by heat treatments (Olguin et al., 2003).

Few studies have been devoted to assess the antioxidant potential of soy protein fractions as well as to the isolation and structural characterization of the most active peptides (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998; Chiang, Shih, & Chu, 1999; Gibbs, Zougman, Masse, & Mulligan, 2004). As it has been observed for other antioxidant compounds, the activity of peptides depends on the assay considered.

The antioxidant properties of soy protein hydrolysates have been ascribed to the cooperative effect of a number of properties (Chen et al., 1998), including their ability to scavenge free radicals, to act as metal-ion chelator, oxygen quencher or hydrogen donor,

☆ This manuscript has been submitted for publication in *Food Chemistry*. It is not to be reproduced or cited without the written permission of the authors.

* Corresponding author.
E-mail address: mpintado@porto.ucp.pt (M.E. Pintado).

http://dx.doi.org/10.1016/j.foodchem.2015.11.068
0308-8146/

and to the possibility of preventing the penetration of lipid oxidation initiators by forming a membrane around oil droplets (Peña-Ramos & Xiong, 2002). Several biological activities (angiotensin converting enzyme (ACE) inhibition, anti-thrombotic, antihypertensive, antioxidative properties) have been reported for soy peptides (Gibbs et al., 2004), however the number of studies are reduced and the variety of enzymes tested is very restricted. The operational conditions employed in the processing of protein isolates, the type of protease and the degree of hydrolysis affect the antioxidant (Peña-Ramos & Xiong, 2002) and other biological activities (Gibbs et al., 2004). The substitution of synthetic antioxidants by natural ones is gaining interest due to the consumer preferences and health concerns associated to the use of synthetic food additives. Additionally, the importance of several of these natural antioxidants on the prevention or control of certain chronic diseases has been increasing. The use of proteins or their hydrolysates for food and/or cosmetic applications presents additional advantages over other antioxidants, since they also confer nutritional and functional properties.

According to several studies, Corolase PP (food-grade porcine pancreatic proteolytic preparation) is one of the most appropriate enzymes to produce hydrolysates derived from milk proteins possessing antioxidant activity (Pihlanto, 2006) and antihypertensive activity (Hernández-Ledesma, Amigo, Ramos, & Recio, 2004). Although, there are some studies on soybean hydrolysates using different enzymes, no study using Corolase PP on soy protein hydrolysis has been performed until now. So, the goal of this work was to extract the protein fraction from defatted non-deactivated soybean meal by using two different heat treatments. Besides, the hydrolysis of the isolated protein with Corolase PP was studied in order to evaluate the potential antioxidant capacity and antihypertensive activity of the resulting peptide extracts.

2. Materials and methods

2.1. Materials

Defatted non-deactivated soybean meal was obtained from Molinos Río de la Plata (San Lorenzo, Argentina). The commercial digestive-enzyme complex Corolase PP was purchased from AB Enzymes GmbH (Darmstadt, Germany).

2.2. Preparation of soy protein isolate

Soy protein isolate (SPI) was prepared from defatted soybean meal by aqueous extraction under two controlled conditions of time and temperature (70 °C for 1 h and 90 °C for 30 min). These two conditions were used to evaluate the impact of heat treatment on both the elimination of anti-nutritional compounds and the hydrolysis by Corolase, since the level of protein denaturation may facilitate, depending on the enzyme, the access to the active site of hydrolysis. So, solutions of proteins in the ratio of meal to water of 1:10 with an initial pH value of 8.5 (Wolf, 1970) were heated according to the conditions described. After heat treatment the extract was clarified by centrifugation at 2370*g* for 45 min, and then, protein was precipitated by acidification to pH 4.5 with 2 N HCl (Merck, Damdstadt, Germany) and the insoluble fraction was collected by centrifugation at 2370*g* for 30 min. The precipitate was freeze dried. Soybean protein isolates (SPI) were done in duplicate for each condition.

2.3. Determination of trypsin inhibitor activity

The standard method proposed by Kakade, Rackis, Mc Ghee, and Puski (1974) and later modified (AOCS, 2009) is based on the ability of aliquots of soybean extracts to inhibit the activity of trypsin from Sigma–Aldrich (St. Louis, MO) towards the substrate N-benzoyl-DL-arginine p-nitroanilide (BAPNA) from Sigma–Aldrich (St. Louis, MO). The reaction is followed by measuring the absorbance of the released reaction product, the p-nitroaniline, which absorbs at 400 nm (molar absorptivity of 10,500 $M^{-1}\,cm^{-1}$). A modified procedure in which a continuous spectrophotometric determination achieved during 4 min for trypsin activity was used to replace the traditional discontinuous method. The addition of acetic acid is not required to stop the reaction. Final concentrations, used for enzyme, substrate and buffer are the same of the discontinuous method, but the final reaction volume is lowered from 10 to 2.5 mL, thus decreasing the consumption of reagents.

2.4. Total protein determination

Total protein content of dry matter was determined by the Dumas method, using a Leco nitrogen analyser (Model FP-528, Leco Corporation, St. Joseph, USA) using a nitrogen to protein conversion factor of 6.25.

2.5. Enzymatic hydrolysis

For each SPI obtained from 70 and 90 °C extractions, 100 ml of a 1% aqueous dispersion was heated prior to the addition of enzyme to promote total dissolution. Afterwards, proteolysis was carried out using Corolase PP at a ratio of enzyme:substrate (10 mg enzyme/g SPI-1% w/w). Corolase PP possesses chymotrypsin, elastase, dipeptidase, tryptic and aminopeptidase activities, along with carboxypeptidase A1, A2, and B exopeptidase activities, and the optimum pH for overall activity is 8.0 (AB Enzymes). The enzymatic hydrolysis was conducted at optimal conditions previously established for Corolase PP, i.e. 50 °C and pH 8.0 for 24 h. Aliquots (2 ml) were taken after 30 min, 1, 2, 3, 4, 5, 6, 8, 10 and 24 h. The enzyme was inactivated by heat treatment at 80 °C 20 min, and the supernatant was separated from precipitate by centrifugation at 2370*g* for 45 min. Finally, the protein hydrolysates (SPHs) were stored at −80 °C for later analysis. The initial control of non-hydrolysed sample was taken before the enzyme addition. Hydrolysis was done in duplicate for each condition.

2.6. Determinations of degree of hydrolysis

The degree of hydrolysis (DH) of each SPH was determined using the modified OPA method described by Nielsen, Petersen, and Dambmann (2001). The method was modified concerning the volumes used, so the volume of 200 µl standard, blank (distilled water) and sample were added to each respective tube and 1.5 ml OPA reagents were added to all test tubes and let react for 2 min. The absorbance then was read at 340 nm. The %DH was calculated following the equations reported by Adler-Nissen (1984) (Eqs. (1)–(3)).

$$\text{Serine NH}_2 = \frac{OD\ sample - OD\ blank}{OD\ standard - OD\ blank} * 0.9516 * 0.01 * \frac{100}{X * P} \quad (1)$$

$$H = \frac{\text{Serine NH}_2 - \beta}{\alpha} \quad (2)$$

$$\text{DH} = \frac{h}{h_{\text{tot}}} * 100 \quad (3)$$

Where

h = number of hydrolyzed bonds,
h_{tot} = total number of peptide bonds per protein equivalent (7.8 specific to soy protein),
β = 0.342 (specific for soy protein),
α = 0.970 (specific for soy protein),

OD sample = absorbance of sample at 340 nm,
OD blank = absorbance of water at 340 nm,
OD standard = absorbance of L-serine at 340 nm,
X = amount of sample (g),
P = protein (%) in sample.

2.7. Analysis by size exclusion chromatography

The molecular weight distribution of SPHs was studied by gel filtration chromatography using the FPLC system of Pharmacia (Uppsala, Sweden) in a configuration consisting of two P-500 positive displacement pumps, an electrically-powered MV-7 motorized valve, a gel filtration column prepacked with Superose 12 HR 10/30, an UV1 single path spectrophotometer monitor, a REC-102 double channel recorder and a LCC-500 controller. The column was operated at flow rate of 0.4 ml min^{-1} with 0.025 M phosphate buffer (pH 7) containing 0.15 M NaCl and 0.2 g L^{-1} NaN_3. Absorbance of the eluent was monitored at 280 nm. Standard proteins with known molecular weights (IgG (150 kDa); BSA (66 kDa); β-lactoglobulin (36 kDa); α-lactoalbumin (14,178 kDa)) were used to establish the molecular weight standard curve.

2.8. Sodium dodecyl sulfate polyacryalamide gel electrophoresis (SDS–PAGE)

Prior to electrophoresis, samples were mixed with an equal volume of sample buffer, and then heated at 80 °C for 5 min. This buffer was prepared by adding 6 g of glycerol from Merck (Damdstadt, Germany), 1.6 g of sodium dodecyl sulphate from Sigma–Aldrich (St. Louis, MO) and 0.62 g DTT to 4 ml of Tris-SDS solution from Sigma–Aldrich (St. Louis, MO). SDS–PAGE was performed in a Mini-Protean II system from Bio-Rad (Hercules, CA, USA), using 4% stacking gel and 16.5% running gel, as described by Schägger and von Jagow (1987). Protein bands were stained with 0.25% Coomassie brilliant blue solution from Merck (Damdstadt, Germany) and then washed with a distaining solution of 2.5:1 (v/v) acetic acid and methanol from Frilabo (Maia, Portugal), until protein bands became clearly visible in a colourless gel matrix.

2.9. Analysis of total antioxidant activity

The total antioxidant activity was determined by ABTS radical scavenging activity as described by Re et al. (1999). Briefly, ABTS radical cation was produced from the reaction of 7 mM 2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 2.45 mM potassium persulfate (both from Sigma–Aldrich, St. Louis, MO) after incubation at room temperature in dark for 16 h. The ABTS solution was diluted with water to an absorbance of 0.70 ± 0.02 at 734 nm. After the addition of 1.0 mL ABTS solution to 10 μL of sample the mixture absorbance reading was made after 6 min. The % inhibition of the sample was then compared with a standard curve made from the corresponding readings of Trolox (0.01–0.20 mg/mL) and results expressed as μmol of Trolox Equivalent (TE)/mg protein. Trolox and sample solutions were prepared daily in 75 mM phosphate buffer (pH 7.4).

The Oxygen radical absorbance capacity (ORAC-FL) assay was based on that proposed by Contreras et al. (2011). Briefly, the reaction was carried out at 40 °C in 75 mM phosphate buffer (pH 7.4) and the final assay mixture (200 μL) contained fluorescein (70 nM), AAPH (14 mM), and antioxidant (Trolox (9.98×10^{-4}–7.99×10^{-3} μmol/mL) or sample (at different concentrations)). The fluorescence was recorded during 137 min (104 cycles). A FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters was used. The equipment was controlled by the FLUOstar Control software version (1.32 R2) for fluorescence measurement. Black polystyrene 96-well microplates (Nunc, Denmark) were used. AAPH and Trolox solutions were prepared daily and fluorescein was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer (pH 7.4). All reaction mixtures were prepared in duplicate and at least three independent runs were performed for each sample. Final ORAC-FL values were expressed as μmol of TE/mg protein.

2.10. Measurement of ACE-inhibitory effect

The ACE-inhibitory effect was measured using the fluorimetric assay of Sentandreu and Toldrá (2006), as modified by Quirós, Contreras, Ramos, Amigo, and Reci (2009). The ACE (peptidyl-dipeptidase A, EC 3.4.15.1) was purchased from Sigma Chemical (St. Louis, MO, USA). ACE working solution was diluted with 0.15 M Tris buffer (pH 8.3) containing 0.1 mM $ZnCl_2$ with 0.04 U/mL of enzyme in the final reaction solution. A total of 40 mL of distilled water or this working solution were added to each microtiter-plate well, then adjusted to 80 mL by adding distilled water to blank (B), control (C) or samples (S). The enzyme reaction was started by adding 160 mL of 0.45 mM o-Abz-Gly-p-Phe(NO_2)-Pro-OH (Bachem Feinchemikalien, Bubendorf, Switzerland) dissolved in 150 mM Tris-base buffer (pH 8.3), containing 1.125 M NaCl, and the mixture was incubated at 37 °C. The fluorescence generated was measured at 30 min using a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany). Ninety-six-well microplates (Porvair, Leatherhead, UK) were used. Excitation and emission wavelengths were 350 and 420 nm, respectively. The software used to process the data was FLUOstar control (version 1.32 R2, BMG Labtech). The activity of each sample was tested in duplicate. Inhibitory activity was expressed as the peptide concentration required to inhibit the original ACE activity by 50% (IC50). The formula applied to calculate de percentage of ACE-inhibitory activity was: $100 \times (C - S)/(C - B)$. Non-linear fitting to the data was performed to calculate the IC_{50} values, as previously done by Quirós et al. (2007). For this assay, the protein content of the peptide extracts was estimated by the bicinchoninic acid. The albumin was used as standard; the determination of protein concentration of the peptides was based on their actual dry weight.

2.11. Mass spectrometry methods

Sample of SPHs (SPH70 at 4 h and SPH90 at 2 h) were detected and acquired by a MALDI-TOF/TOF mass spectrometer (4800 Plus MALDI TOF/TOF Analyzer, AB SCIEX, Framingham, MA), equipped with a 200-Hz frequency Nd:YAG laser, operating at a wavelength of 355 nm. Pulse ion extraction with a 1300 ns delay time was used for collecting spectra. Measurements were carried out in linear positive mode using an acceleration voltage of 19.4 kV (Grid 1), and a lens 1 voltage of 8 kV. Each spectrum was the accumulated sum of at least 2000 laser shots within the ion range at *m*/*z* 150–1500, due to the good reproducibility of the spectral profile in this interval. The samples were previously dissolved and dispersed in a HCCA matrix (4-hydroxycinnamic-α-cyano acid), spotted onto a 123681 mm stainless steel MALDI sample plate (Opti-TOF 384-Well insert, AB SCIEX, Framingham, MA) and allowed to dry at room temperature. For each sample, two replicates were carried out, and the mean spectra were considered for the analysis. Mass spectra were analysed with the Data Explorer software (v3.7, build 126, AB SCIEX, Framingham, MA) and compared to the peaks in the range of 150–3000 Da. Ion masses were extracted from the raw experimental mass spectra that included all the ion peaks with a relative signal to noise (*S*/*N*) ratio intensity above 2. To enable the calculation of the real masses of the peptides obtained, the results were then treated with online software MASCOT (http://www.matrixscience.com/).

2.12. Statistical analysis

Results were analyzed by the Screening Modelling and the Nonlinear Modelling of the JMP 10 software from SAS institute (Cary, NC). For significant main effects, means were considered to be different at a significance level of 0.05. Data was expressed as means of at least two replicates.

3. Results and Discussion

The protein was isolated from non-deactivated soybean meal under controlled conditions of time and temperature and freeze dried. Heating soy proteins above 70 °C causes dissociation of their quaternary structures, denatures their subunits, and promotes the formation of protein aggregates via electrostatic, hydrophobic and disulphide interchange mechanisms (Barać, Stanojević, Jovanović, & Pešić, 2004). Glycinin has higher thermal transition point (92 °C) than β-conglycinin (72 °C). Based on that, the two conditions of extraction (70 °C, 1 h; 90 °C, 30 min) were chosen. These conditions were close to the temperature of denaturation of each major globulin in soy protein isolate (SPI). Dispersions with protein concentration of 1% were submitted to hydrolysis with Corolase PP at a ratio of enzyme:substrate 1% (weight enzyme/weight SPI) during 24 h at pH 8 and 50 °C without buffering. The final hydrolysates of SPIs were named SPH70 and SPH90 for 70 °C and 90 °C extractions, respectively.

3.1. Isolation of proteins

Extractions performed, showed yields of 89.7% ± 1.2 for SPI70 and 90.6% ± 0.1 for SPI90 of the soluble protein content, and the isolates were obtained with a protein content of 93.4%. The impact of these treatments on the content of trypsin inhibitor in the flour was reduced ca. 35 and 79% on SPH70 and SPH90, respectively.

Olguin et al. (2003) reported decreases of ca. 75% for wet heat treatment at 98 °C during 30 min.

3.2. Hydrolysis

As shown in Fig. 1 the progress of hydrolysis was assessed by determination of degree of hydrolysis (DH) at different sampling times (30 min, 1, 2, 3, 4, 5, 6, 8, 10 and 24 h). The regression models fitted were Exponential Growth and Decay three parameters, and showed a significant difference between both SPH tested ($p < 0.0001$; $R^2 = 0.9074$). As observed, the DH reached 10.0% ± 0.6 and 13.2% ± 1.4 for SPH70 and SPH90 respectively, at 6 h. These values are similar to those showed by Chiang, Tsou, Tsai, and Tsai (2006) for pepsin and trypsin. However, they were lower than those produced by alcalase and flavourzyme at the same time of hydrolysis of 3% (w/v) SPI (from heated soy meal) dispersion and 1% (w/w of SPI) protease carried out at the optimum reaction conditions of temperature and pH suggested by suppliers. Although the dispersion is a third of SPI, the corolase was able to break a similar number of peptide bonds to pepsin and trypsin (enzymes with high proteolytic activity), thus evidencing a higher efficiency relative to the amount of hydrolyzed protein than pepsin and trypsin. Nevertheless, despite this good performance, it did not achieve the high proteolytic power of alcalase or flavourzyme. DH was increasing until reaching a maximum value of 18.9% ± 1.9 for SPH70 and 20.4% ± 1.4 for SPH90 at 10 h, showing no significant difference after of 24 h in both cases. According to Lee (2011), SPI hydrolysis above a certain limit (more than 8%) results in the release of bitter peptides. Therefore, the use of SPH in food applications is limiting because of adverse effects on sensory quality bitter taste caused by hydrolysis. Consequently it is important to maintain a controlled and limited hydrolysis to achieve desired functional properties and bioactivity with reduced production of bitterness (Lee, 2011).

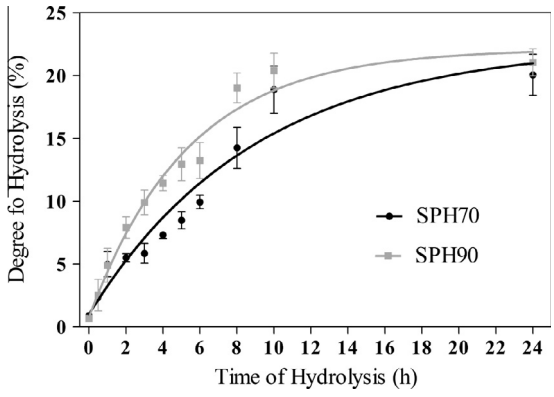

Fig. 1. Degree of Hydrolysis (DH) within Corolase PP hydrolysates (SPH70 and SPH90) as a function of hydrolysis time expressed in hours. Error bars are expressed as SEM.

3.2.1. Hydrolysate peptide profiles

3.2.1.1. Gel filtration chromatography. Fig. 2 shows the molecular weight distribution of SPI extracted at 70 °C (SPI70) (Fig. 2a–c) and at 90 °C (SPI90) (Fig. 2d–f), respectively. It is observed the major globulins glycinin (peak 1 at 18 min retention time (RT); hexameric structure with a mean molar mass of 360 kDa) and β-conglycinin (peak 2 at 24 min retention time (RT); trimeric structure with a mean molar mass of 180 kDa). For SPI70 the effect of temperature up to the transition point of β-conglycinin, is observed by a decrease in the content of the quaternary structure of this globulin (peak 2) and the appearance of its dissociated subunits (peak 3). In SPI90, the influence of temperature above the transition point of β-conglycinin was observed by the absence of a defined peak 2, demonstrating the dissociation of the quaternary structures almost completely. As shown in Fig. 2(b, c, e and f), the FPLC profile of SPHs showed a disappearance of compact quaternary structure and subunits of globular proteins, represented by reduction of peaks 1 and 2 and the release of low molecular weight peptides (peaks 4, 5 and 6) as time elapsed, but the globulins tended to disappear after 4 h for SPH70 and 2 h for SPH90. After 24 h, it could be observed that the peak 4 was also hydrolysed and consequently the signal of peaks 5 and 6 increased. Hydrolysis mainly generated peptides with molecular mass below 3 kDa. Consistent with that observed in our work, the hydrolysis of a 3% SPI dispersion with a solution of Flavourzyme: Alcalase enzyme mixture (3:2/substrate (w/w)) produced hydrolysates primarily composed of amino acids and small peptides with molecular weight <4 kDa (Chiang et al., 1999).

3.2.1.2. Tricine–SDS-PAGE. As alternative method to characterize molecular weight profile of soluble samples, tricine–SDS-PAGE was carried out. The protein subunit bands of β-conglycinin and glycinin were present in both non-hydrolysed control samples (Fig. 3, lanes 10 and 11 for SPI70 and SPI90 respectively), in agreement with the bands showed in other works (Hrčková, Rusňáková, & Zemanovič, 2002; Lamsal, Jung, & Johnson, 2007). Glycinin subunits were practically intact in SPH70 at 30 min, 2 and 4 h, but β-conglycinin bands were hydrolysed from 30 min to almost disappear at 4 h (Fig. 3 lanes 2, 4 and 6). For SPH90, glycinin subunits were maintained intact at 30 min, but almost disappeared at 4 h of hydrolysis (Fig. 3 lanes 3, 5 and 7). β-conglycinin bands from SPH90 began disappearing at 30 min, and completely disappeared after 2 h (Fig. 3 lanes 3, 5 and 7). According to previous reports (Hrčková et al., 2002), the basic subunit of the SPH90 glycinin

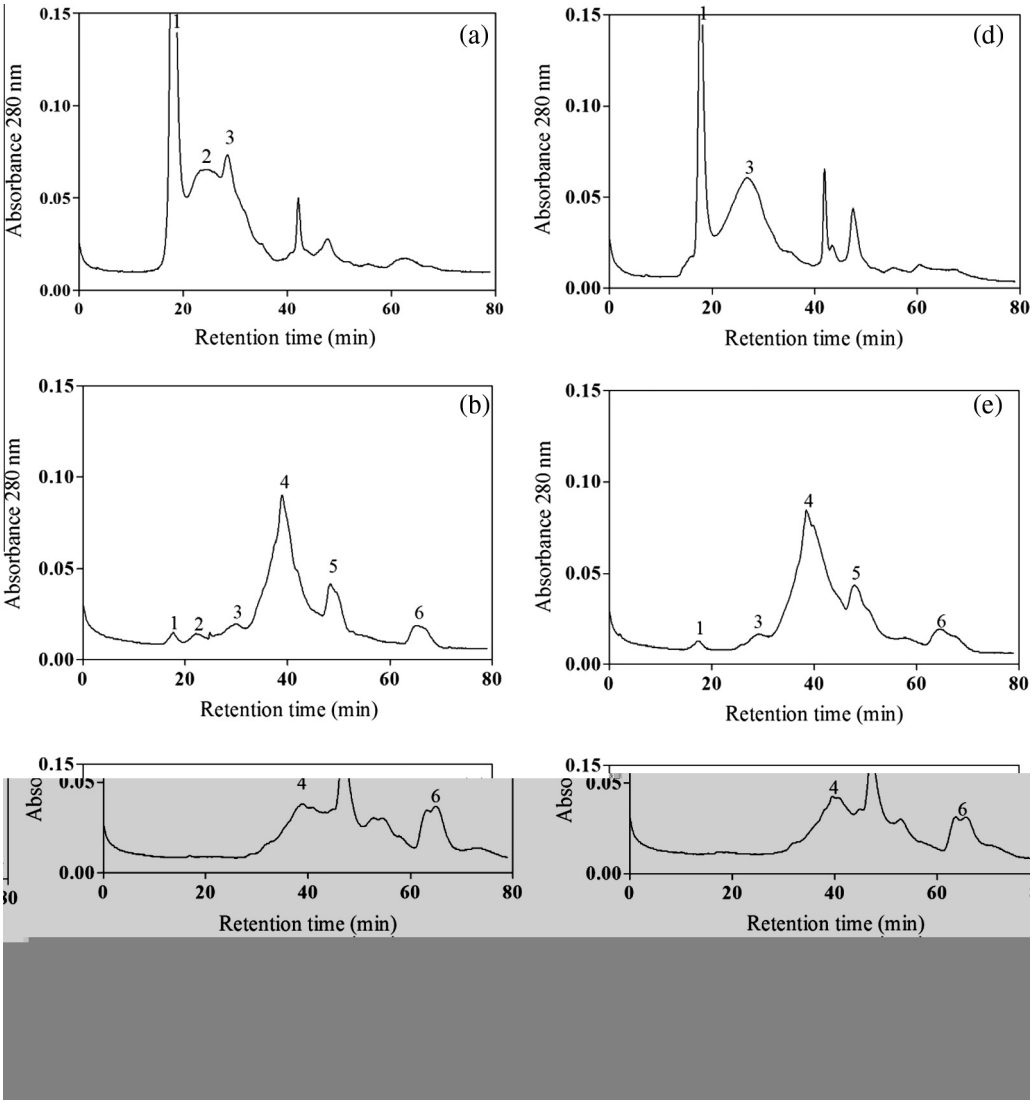

Fig. 2. Typical size exclusion chromatograms of (a) SPI70, (b) SPH70 at 4 h, (c) SPH70 at 24 h, (d) SPI90, (e) SPH90 at 2 h, (f) SPH90 at 24 h, at 280 nm. 1: quaternary structure of glycinin; 2: quaternary structure of β-conglycinin; 3: globulins (glycinin and β-conglycinin) subunits; 4–6: peptides area generated by hydrolysis.

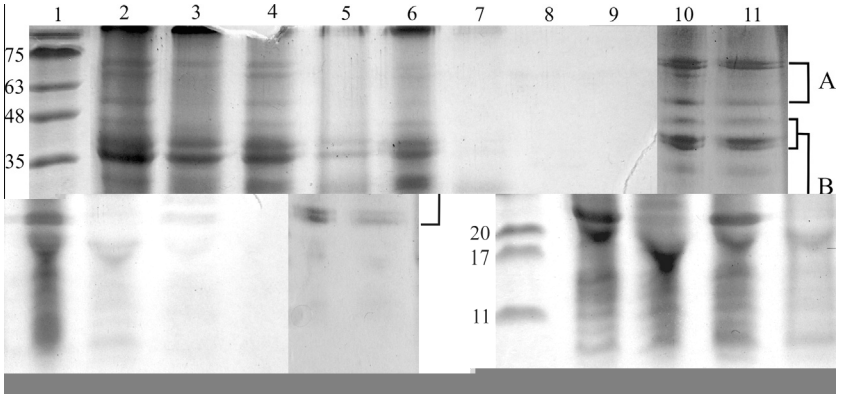

Fig. 3. Tricine–SDS-PAGE patterns of SPIs and hydrolysates at different times (30 min, 2 h, 4 h and 24 h). Lanes are as follows: 1: molecular weight marker (kDa); 2: SPH70 at 30 min; 3: SPH90 at 30 min; 4: SPH70 at 2 h; 5: SPH90 at 2 h; 6: SPH70 at 4 h; 7: SPH90 at 4 h; 8: SPH70 at 24 h; 9: SPH90 at 24 h; 10: SPI70; 11: SPI90. A: β-conglycinin subunits; B: glycinin subunits.

appears to be more susceptible to hydrolysis than the acidic subunits since, these subunits reside in the interior of the undenatured glycinin complex, thus being lesser exposed to enzymatic attack. However, this protector behaviour only is present up to the limit of their denaturation point. SPH70 and SPH90 hydrolysates showed new bands at 25 kDa and below 20 kDa up to small peptides at the bottom of the gel (Fig. 3 lanes 2–9). This was in contrast with the behaviour observed for bromelain hydrolysis (Lamsal et al., 2007), that generated a degradation without defined bands at low DH about 2 and 4%, but in agreement with the pattern generated by a bacterial neutral protease preparation having mainly endo-peptidase activities at DH about 2 and 4% (Jung, Murphy, & Johnson, 2005). The smallest peptides could not be resolved by the gel for SPHs at 24 h (Fig. 3 lanes 8 and 9). The results presented for electrophoresis, showed a clear increased susceptibility of SPH90 to be hydrolysed compared with SPH70, which agrees with the role played by substrate denaturation in the enzymatic hydrolysis, reported by others (Jung et al., 2005; Lamsal et al., 2007).

3.2.2. Antioxidant activity of hydrolysates

According to Neuzil, Gebicki, and Stocker (1993) the ability of proteins to act as antioxidant compounds was attributed to their ability to trap free radicals. It is known that enzyme hydrolysis improves nutritional and functional properties of proteins. Protease specificity determines amino acid sequence of the hydrolysates, which influence their antioxidant activity. In the present study, we evaluated the protective effect of SPHs against two sources of free radicals including the ABTS radicals and the peroxyl radicals. The antioxidant activity was measured by the ABTS and ORAC methods. The results were expressed relative to the protein content of the initial suspension of SPI and relative to soy flour mass.

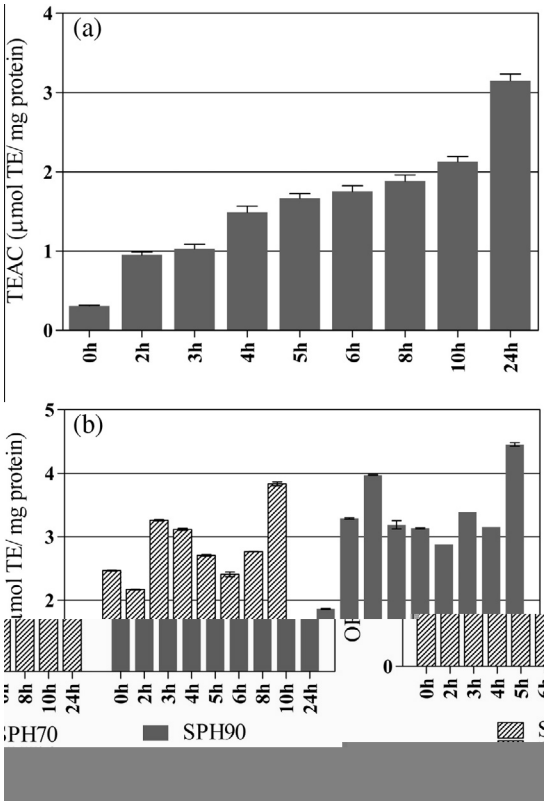

Fig. 4. (a) Antioxidant activities of SPH by ABTS scavenging as a function of hydrolysis time expressed in hours. (b) Antioxidant activities of SPH70 and SPH90 by ORAC as a function of hydrolysis time expressed in hours. Error bars are expressed as SEM.

3.2.2.1. ABTS scavenging assay. This method measures the discolouration of the ABTS radical due to the presence of antioxidant compounds (Re et al., 1999). Statistical analysis indicated no significant differences between the two extraction conditions, which mean that final SPH70 and SPH90 exhibited similar antioxidant activity. As shown in Fig. 4(a), under the study conditions, SPIs presented a low ABTS scavenging activity, but the Trolox Equivalent Antioxidant Capacity (TEAC) values increased significantly in a linear mode with time of hydrolysis, indicated by the regression model obtained ($p < 0.0001$, $R^2 = 0.9055$). These results were in line with those reported for hydrolysates of SPI by de-Oliveira et al. (2014) with a microbial protease; and ovine casein by Gómez-Ruiz, López-Expósito, Pihlanto, Ramos, and Recio (2008) with pepsin, trypsin and chymotrypsin. In fact, under study conditions with corolase it was observed a significant positive relationship between DH and ABTS scavenging ($p < 0.0001$, $R^2 = 0.8015$), corroborating Corrêa et al. (2011), who showed that the degree of hydrolysis affects the ABTS quenching ability. For non-hydrolyzed SPI minimum value of 46.41 µmol TE/g soy flour (0.31 µmol TE/mg protein) was observed, comparable with results reported by Slavin, Kenworthy, and Yu (2009) for non-hydrolysed black soybean (genotypes MD 06-5440-2, Peking, Pi 88788 and Pi 90763), which showed values between 20 and 102 µmol TE/g flour using ABTS assay. Gómez-Ruiz et al. (2008) reported for a concentration of 1 mg/mL of non-hydrolysed ovine κ-casein a value of 0.53 mg TE/mL (2.12 µmol TE/mg protein), the same mean value observed in this study for both SPHs at 10 h of hydrolysis. Cys was the most active amino acid on the antioxidant activity assessed by the ABTS assay, followed by Trp, Tyr and His. Probably peptides derived from glycinin are mainly responsible of antioxidant activity assessed by the ABTS assay, followed by the peptides generated from the β-conglycinin chains α and α′, because their rich content in Cys, being that 11s fraction shows 3–4 times more Cys than 7s.

3.2.2.2. ORAC assay. This method allows evaluating the scavenging capacity due to a hydrogen-atom transfer mechanism. The sample was exposed to a peroxyl radical generator (AAPH) and the oxidative degradation of fluorescein was measured as the area under the decay curve (AUC) (Ou, Hampsch-Woodill, & Prior, 2001). A Trolox calibration curve was made with different concentrations, and Trolox equivalent (TE) values (expressed as µmol TE/mg protein) were calculated from this curve using the AUC values for each sample assayed. Fig. 4(b) shows that TE values increased after hydrolysis for both conditions, in concordance with results obtained by the ABTS assay but with significant difference ($p = 0.0022$) between the two extraction conditions. According with Amigo-Benavent, Silván, Moreno, Villamiel, and del Castillo (2008) the peroxyl radical scavenging activity (determined by ORAC FL assay) can be affected as a function of the intensity of the thermal processing. TE values for hydrolysates, under the conditions of the study, were increased compared with the corresponding control, but according to the results, the model obtained ($R^2 = 0.44$) could not explain the dependence of ORAC on the degree of hydrolysis in the range assayed. Both intact SPIs presented ORAC values (1.42 µmol TE/mg protein for SPI70 and 1.87 µmol TE/mg protein for SPI90) significantly superior ($p = 0.039$ and $p = 0.0041$, respectively) with respect to the values presented by Tavares et al. (2011) for antioxidant activity of intact whey protein concentrate, α-lactalbumin and caseinomacropeptide (0.195, 0.499 and 0.105 µmol TE/mg protein, respectively), even greater than the maximum antioxidant activity obtained with cardoon extract, that it was with α-

lactalbumin hydrolysates (1.061 μmol TE/mg protein). In other work, Ranamukhaarachchi, Meissner, and Moresoli (2013) presented a study of SPI heated (95 °C for 5 min) and non-heated with a 90 min sequential hydrolysis with pepsin and pancreatin, showing values of 1.71 and 1.79 μmol TE/mg respectively, and also showed values of 2.37 and 3.07 μmol TE/mg for respective ultrafiltration permeates (<10 kDa). In comparison with that work, total corolase SPHs at 2 h (120 min) showed comparable values (2.47 μmol TE/mg protein for SPH70 and 3.23 μmol TE/mg protein for SPH90) with that of the permeates of heated and non-heated SPI hydrolysates. In fact, our hydrolysates at 4, 5 and 24 h for SPH70 and 2, 3, 4, 5, 8, 10 and 24 h for SPH90 presented superior values (3.26, 3.12 and 3.83 μmol TE/mg protein for SPH70, respectively; 3.29, 3.97, 3.19, 3.13, 3.39, 3.16 and 4.45 μmol TE/mg protein for SPH90, respectively) in comparison with α-lactalbumin hydrolysate obtained with Corolase PP for 24 h at 37 °C, previously reported (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005), with an ORAC value of 2.95 μmol TE/mg protein.

3.2.3. ACE inhibitory activity

Many studies have shown that consumption of soy protein has several positive effects on health, being one of those features the antihypertensive activity (De Leo, Panarese, Gallerani, & Ceci, 2009). Antihypertensive activity is fundamentally based on inhibiting the activity of a crucial regulator of blood pressure, angiotensin-converting enzyme (ACE), which could raise the blood pressure too high when is abnormally active, causing hypertension (Mondorf et al., 1998). In other works with soy protein hydrolysates antihypertensive peptides were identified (Cha & Park, 2005; Chiang et al., 2006).

For further characterization of ACE inhibition by SPI and SPHs, IC_{50} was determined. As shows Table 1, the SPI showed no significant inhibition (IC_{50} > 1000 μg protein/mL), but a significant positive decrease in ACE activity was observed when both SPH were analysed ($p < 0.001$), indicating a potential for ACE inhibition. Higher IC_{50} value indicates lower ACE inhibitory activity since more protein is required to achieve 50% inhibition. DH increased showed no significant effect on the ACE inhibitory activity for SPH70, but showed a significant effect between 2 and 4 h of hydrolysis for SPH90. The IC_{50} of SPH70 and SPH90 were 274–237 μg protein/mL and 178–403 μg protein/mL, respectively for 2–10 h of hydrolysis (Table 1). In previous work it was reported potential antihypertensive activity of different peptides, from milk and soy derived protein hydrolysates, with values for ACE inhibitory activitya0753159TD(to20031719.8865Tm44438306d)TJ

Table 2
Predominant peptide sequences identified by MALDI-TOF/TOF in SPH70 and SPH90.[a]

Putative sequence	Expt mass (Da)	Calc mass (Da)	Protein source
IRHFNEGDVLVIPPGVPY	1744.96	1744.93	Glycinin G4
IRHFNEGDVLVIPPGVPYW	1908.02	1907.99	Glycinin G4
YNFREGDLIAVPTG	1330.72	1330.69	Glycinin G1 and G2
IYNFREGDLIAVPTG	1493.78	1493.75	Glycinin G1 and G2
VSIIDTNSLENQLDQMPRR	1972.99	1972.95	Glycinin G1 and G2
YRAELSEQDIFVIPAG	1586.87	1586.83	β-conglycinin α and α′
FEITPEKNPQLRDLDIFLSI	2127.15	2127.12	β-conglycinin α and α′
INAENNQRNFLAGSQDNVISQIPSQV	2742.35	2742.33	β-conglycinin α and α′
FAIGINAENNQRNFLAGSQDNVISQIPSQV	3096.58	3096.56	β-conglycinin α and α′

Sequences with intensity of peaks higher than 20%.
[a] Peptide sequences listed were found predominant, with identity confirmed at a confidence of at least 95% ($p < 0.05$).

4. Conclusions

The results obtained in the present study suggest that extraction temperatures assayed did not affect the extraction yield, but affected the degree of protein denaturation and reduced the presence of anti-nutritionals such trypsin inhibitors, with different efficiency; however no impact on the final pattern of the generated peptides fractions was observed.

The hydrolysis of SPI with Corolase PP strongly enhanced ABTS and ORAC antioxidant capacity up to values comparable or higher than values reported in other works with different enzymes and different substrates. This positive effect is also observed for ACE inhibitory activity, showing inhibition values that suggest a high potential activity. According with the criteria of avoidance of higher DH than 8% but pursuing the maximum values of potential ACE inhibitory activity and antioxidant capacity, we decided to select for further samples within the analysed conditions 4 h for SPI70 (221.0 μg protein/mL, 3.26 μmol TE/mg protein, respectively) and 2 h for SPI90 (402.8 μg protein/mL, 3.29 μmol TE/mg protein, respectively). For these conditions, the identified peptides were the same in both cases and showed sequences that could explain the observed bioactive properties for those peptide extracts. However, the length of each peptide chain suggested that under physiological conditions, may be changed by gastric proteases, thereby conferring properties different to those detected in vitro, conditions to be tested in future. These results suggest that it was possible to obtain for the first time, with this enzymatic food grade preparation, soy peptide hydrolysates with potential antioxidant and ACE inhibitory activities to develop new functional ingredients. However, further studies will be performed in future using other models to reinforce and confirm the results obtained in this study.

Acknowledgments

Author E.R. Coscueta acknowledges BiValBi – Biotechnologies to Valorise the regional Biodiversity in Latin America (Ref[a] PIRSES-GA-2013-611493 BI_1) – for the research fellowship administered for funding this work, as well as Hugo Osório and IPATIMUP (Instituto de Patologia e Imunologia Molecular da Universidade do Porto), for their contribution to the analysis of MALDI-TOF/TOF.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.11.068.

References

Adler-Nissen, J. (1984). Control of the proteolytic reaction and of the level of bitterness in protein hydrolysis processes. *Journal of Chemical Technology and Biotechnology, 34*(3), 215–222.
Amigo-Benavent, M., Silván, J. M., Moreno, F. J., Villamiel, M., & del Castillo, M. D. (2008). Protein quality, antigenicity, and antioxidant activity of soy-based foodstuffs. *Journal of Agricultural and Food Chemistry, 56*(15), 6498–6505. http://dx.doi.org/10.1021/jf800697n.
Barać, M. B., Stanojević, S. P., Jovanović, S. T., & Pešić, M. B. (2004). Soy protein modification – A review. *APTEFF, 35*, 3–16.
Contreras, M. D. M., Hernández-Ledesma, B., Amigo, L., Martín-Álvarez, P. J., & Recio, I. (2011). Production of antioxidant hydrolyzates from a whey protein concentrate with thermolysin: Optimization by response surface methodology. *LWT – Food Science and Technology, 44*(1), 9–15. http://dx.doi.org/10.1016/j.lwt.2010.06.017.
Corrêa, A. P. F., Daroit, D. J., Coelho, J., Meira, S. M. M., Lopes, F. C., Segalin, J., & Brandelli, A. (2011). Antioxidant, antihypertensive and antimicrobial properties of ovine milk caseinate hydrolyzed with a microbial protease. *Journal of the Science of Food and Agriculture, 91*(12), 2247–2254. http://dx.doi.org/10.1002/jsfa.4446.
Cha, M., & Park, J. R. (2005). Production and characterization of a soy protein-derived angiotensin I-converting enzyme inhibitory hydrolysate. *Journal of Medicinal Food, 8*(3), 305–310. http://dx.doi.org/10.1089/jmf.2005.8.305.
Chen, H.-M., Muramoto, K., Yamauchi, F., Fujimoto, K., & Nokihara, K. (1998). Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. *Journal of Agricultural and Food Chemistry, 46*, 49–53.
Chiang, W.-D., Shih, C.-J., & Chu, Y.-H. (1999). Functional properties of soy protein hydrolysate produced from a continuous membrane reactor system. *Food Chemistry, 65*(2), 189–194. http://dx.doi.org/10.1016/S0308-8146(98)00193-9.
Chiang, W.-D., Tsou, M.-J., Tsai, Z.-Y., & Tsai, T.-C. (2006). Angiotensin I-converting enzyme inhibitor derived from soy protein hydrolysate and produced by using membrane reactor. *Food Chemistry, 98*(4), 725–732. http://dx.doi.org/10.1016/j.foodchem.2005.06.038.
Day, L. (2013). Proteins from land plants – Potential resources for human nutrition and food security. *Trends in Food Science & Technology, 32*(1), 25–42. http://dx.doi.org/10.1016/j.tifs.2013.05.005.
de-Oliveira, C. F., Corrêa, A. P. F., Coletto, D., Daroit, D. J., Cladera-Olivera,, F., & Brandelli, A. (2014). Soy protein hydrolysis with microbial protease to improve antioxidant and functional properties. *Journal of Food Science and Technology*, 1–11. http://dx.doi.org/10.1007/s13197-014-1317-7.
De Leo, F., Panarese, S., Gallerani, R., & Ceci, L. R. (2009). Angiotensin Converting Enzyme (ACE) inhibitory peptides: Production and implementation of functional food. *Current Pharmaceutical Design, 15*(31), 3622–3643. http://dx.doi.org/10.2174/138161209789271834.
Garcia-Redondo, A. B., Roque, F. R., Miguel, M., López-Fandiño, R., & Salaices, M. (2010). Vascular effects of egg white-derived peptides in resistance arteries from rats. Structure–activity relationships. *Journal of the Science of Food and Agriculture, 90*(12), 1988–1993.
Gibbs, B. F., Zougman, A., Masse, R., & Mulligan, C. (2004). Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. *Food Research International, 37*(2), 123–131. http://dx.doi.org/10.1016/j.foodres.2003.09.010.
Gómez-Ruiz, J., López-Expósito, I., Pihlanto, A., Ramos, M., & Recio, I. (2008). Antioxidant activity of ovine casein hydrolysates: Identification of active peptides by HPLC–MS/MS. *European Food Research and Technology, 227*(4), 1061–1067. http://dx.doi.org/10.1007/s00217-008-0820-3.
Hernández-Ledesma, B., Amigo, L., Ramos, M., & Recio, I. (2004). Angiotensin converting enzyme inhibitory activity in commercial fermented products. Formation of peptides under simulated gastrointestinal digestion. *Journal of Agricultural and Food Chemistry, 52*(6), 1504–1510. http://dx.doi.org/10.1021/jf034997b.

Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., & Amigo, L. (2005). Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry, 53*(3), 588–593. http://dx.doi.org/10.1021/jf048626m.

Hideaki, K., Kunio, D., Shigeru, S., Hideyo, U., Ryuji, S., Umeji, M., & Shizume, T. (1990). Antihypertensive effect of tryptic hydrolysate of milk casein in spontaneously hypertensive rats. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 96*(2), 367–371. http://dx.doi.org/10.1016/0742-8413(90)90023-3.

Hrčková, M., Rusňáková, M., & Zemanovič, J. (2002). Enzymatic hydrolysis of defatted soy flour by three different proteases and their effect on the functional properties of resulting protein hydrolysates. *Czech Journal Food Science, 20*(1), 7–14.

Jung, S., Murphy, P. A., & Johnson, L. A. (2005). Physicochemical and functional properties of soy protein substrates modified by low levels of protease hydrolysis. *Journal of Food Science, 70*(2), C180–C187.

Kakade, M., Rackis, J., Mc Ghee, J., & Puski, G. (1974). Determination of trypsin inhibitor activity of soy products: A collaborative analysis of an improved procedure. *Cereal Chemistry, 51*, 376–382.

Lamsal, B. P., Jung, S., & Johnson, L. A. (2007). Rheological properties of soy protein hydrolysates obtained from limited enzymatic hydrolysis. *LWT – Food Science and Technology, 40*, 1215–1223.

Lee, J. (2011). Soy protein hydrolysate; solubility, thermal stability, bioactivity, and sensory acceptability in a tea beverage. (M. Sc. thesis), University of Minnesota.

Mondorf, U. F., Russ, A., Wiesemann, A., Herrero, M., Oremek, G., & Lenz, T. (1998). Contribution of angiotensin I converting enzyme gene polymorphism and angiotensinogen gene polymorphism to blood pressure regulation in essential hypertension. *American Journal of Hypertension, 11*(2), 174–183. http://dx.doi.org/10.1016/S0895-7061(97)00402-0.

Neuzil, J., Gebicki, J. M., & Stocker, R. (1993). Radical-induced chain oxidation of proteins and its inhibition by chain-breaking antioxidants. *Biochemical Journal, 293*, 601–606.

Nielsen, P. M., Petersen, D., & Dambmann, C. (2001). Improved method for determining food protein degree of hydrolysis. *Food Chemistry and Toxicology, 66*(5), 642–646.

Olguin, M. A. C., Hisano, N., D'Ottavio, A. E., Zingale,, M. A. I., Revelant, G. C., & Calderari, S. A. (2003). Nutritional and antinutritional aspects of an Argentinian soy flour assessed on weanling rats. *Journal of Food Composition and Analysis, 16*(4), 441–449. http://dx.doi.org/10.1016/S0889-1575(03)00005-X.

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry, 49*(10), 4619–4626. http://dx.doi.org/10.1021/jf010586o.

Peña-Ramos, E. A., & Xiong, Y. L. (2002). Antioxidant activity of soy protein hydrolysates in a liposomal system. *Journal of Food Science, 67*(8), 2952–2956.

Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. *International Dairy Journal, 16*, 1306–1314.

Pihlanto, A., Virtanen, T., & Korhonen, H. (2010). Angiotensin I converting enzyme (ACE) inhibitory activity and antihypertensive effect of fermented milk. *International Dairy Journal, 20*(1), 3–10. http://dx.doi.org/10.1016/j.idairyj.2009.07.003.

Quirós, A., Contreras, M. D. M., Ramos, M., Amigo, L., & Reci, I. (2009). Stability to gastrointestinal enzymes and structure-activity relationship of β-casein-peptides with antihypertensive properties. *Peptides, 30*(10), 1848–1853. http://dx.doi.org/10.1016/j.peptides.2009.06.031.

Quirós, A., Ramos, M., Muguerza, B., Delgado, M. A., Miguel, M., Aleixandre, A., & Recio, I. (2007). Identification of novel antihypertensive peptides in milk fermented with *Enterococcus faecalis*. *International Dairy Journal, 17*(1), 33–41. http://dx.doi.org/10.1016/j.idairyj.2005.12.011.

Ranamukhaarachchi, S., Meissner, L., & Moresoli, C. (2013). Production of antioxidant soy protein hydrolysates by sequential ultrafiltration and nanofiltration. *Journal of Membrane Science, 429*, 81–87. http://dx.doi.org/10.1016/j.memsci.2012.10.040.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*(9–10), 1231–1237. http://dx.doi.org/10.1016/S0891-5849(98)00315-3.

Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: A review. *Peptides, 31*(10), 1949–1956. http://dx.doi.org/10.1016/j.peptides.2010.06.020.

Schägger, H., & von Jagow, G. (1987). Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry, 166*, 368–379.

Sentandreu, M. Á., & Toldrá, F. (2006). A rapid, simple and sensitive fluorescence method for the assay of angiotensin-I converting enzyme. *Food Chemistry, 97*(3), 546–554. http://dx.doi.org/10.1016/j.foodchem.2005.06.006.

Slavin, M., Kenworthy, W., & Yu, L. (2009). antioxidant properties, phytochemical composition, and antiproliferative activity of Maryland-grown soybeans with colored seed coats. *Journal of Agricultural and Food Chemistry, 57*(23), 11174–11185. http://dx.doi.org/10.1021/jf902609n.

Tavares, T., Contreras, M. D. M., Amorim, M., Martín-Álvarez, P. J., Pintado, M. E., Recio, I., & Malcata, F. X. (2011). Optimisation, by response surface methodology, of degree of hydrolysis and antioxidant and ACE-inhibitory activities of whey protein hydrolysates obtained with cardoon extract. *International Dairy Journal, 21*, 926–933.

Wolf, W. J. (1970). Soybean proteins: Their functional, chemical, and physical properties. *Journal of Agricultural & Food Chemistry, 18*(6), 969–976.

Wu, J., Aluko, R. E., & Nakai, S. (2006). Structural requirements of angiotensin I-converting enzyme inhibitory peptides: Quantitative structure-activity relationship study of di-and tripeptides. *Journal of Agricultural and Food Chemistry, 54*(3), 732–738.