Soy protein hydrolysis with microbial protease to improve antioxidant and functional properties

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Abstract Soybean proteins are widely used as nutritional and functional food ingredients. This investigation evaluated through a 2^3 central composite design the effect of three variables (pH, temperature and enzyme/substrate (E/S) ratio) on the production of soy protein isolate (SPI) hydrolysates with a microbial protease. Soluble peptides, antioxidant activity, and foaming and emulsifying capabilities of the hydrolysates were analyzed. All variables, as well as their interactions, were significant for the soluble peptides content of SPI hydrolysates. Optimal conditions for obtaining soluble peptides were around 30–35 °C, pH 6.5–9.5, and E/S ratios of 1,650–6,300 U g⁻¹. SPI hydrolysates produced at 30–45 °C, pH 8.0–9.5, and E/S ratios of 4,000–8,000 U g⁻¹ showed higher capacity to scavenge the 2,2'-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical. Models for soluble peptides and ABTS activity of hydrolysates were obtained. In the range studied, the variables had not significant influence on the ability of hydrolysates to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. SPI hydrolysates also presented reducing power and ability to chelate iron. Hydrolysis temperature was significant for the Fe²⁺-chelating ability of hydrolysates. Temperature of hydrolysis was significant for the foaming capacity of hydrolysates, with higher values observed at 45 °C and 8,000 U g⁻¹. For emulsifying capacity, only E/S ratio presented a significant effect. Temperature and E/S ratio appeared to be more significant variables influencing

the properties of the SPI hydrolysates. The results of this study indicate that specific hydrolysis conditions should be selected to obtain SPI hydrolysates with preferred characteristics.

Keywords Soy protein · Protease · Hydrolysis · Antioxidant · Functional properties · Central composite design

Introduction

Proteins of plant and animal origin are important ingredients in diverse food formulations. Besides its nutritional value as a source of energy and amino acids, proteins are also employed to modulate the physicochemical and sensory properties of foods (Moure et al. 2006). The functional properties of proteins can be modified by enzymatic hydrolysis under controlled conditions, since it affects the molecular size, hydrophobicity and exposition of polar groups of the protein. Enzymatic hydrolysis is shown, for instance, to influence the emulsifying and foaming properties of proteins, thus affecting their utilization as food ingredients (Adler-Nissen 1986). Also, many bioactive peptides are inactive within the sequence of parent protein and can be released during enzymatic hydrolysis (Korhonen 2009). Such peptides might be employed to retard or inhibit the lipid peroxidation in foods, and to develop food supplements and nutraceuticals. Hence, bioactive peptides find applications in food science, technology, and nutrition (Sarmadi and Ismail 2010).

It is important to emphasize that the incorporation of antioxidants into food products has been widely practiced with the purpose of enhancing food quality and the shelf life. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been used in numerous products. However, there is growing concern about their safety, particularly their potential carcinogenicity and genotoxicity (Gharavi et al. 2007). This fact along with consumer

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preference of natural occurring bioactive components has prompted the researchers to identify and develop novel natural and cost-effective antioxidants. Food proteins hold promise as a potential dietary source of natural antioxidants, but information on the utilization of microbial enzymes for producing functional peptides from soy protein is relatively scarce.

Commercial proteases, such as the neutral protease from *Bacillus subtilis* and validase from *Aspergillus oryzae* (Zhang et al. 2010), or Alcalase from *Bacillus licheniformis* (Vernaza et al. 2012), can be useful to generate soy protein hydrolysates with improved antioxidant properties. However, novel microbial enzymes can be useful, since their distinct specificities could be applied to generate peptides and hydrolysates with unique characteristics. In this context, the keratinolytic bacterium *Chryseobacterium* sp. kr6 produces extracellular proteases during submerged cultivations on media containing feathers as the only nutrient source. The hydrolysis of keratin, casein, albumin, among other proteins by these proteases suggests their potential application for the production of protein hydrolysates (Brandelli 2005; Silveira et al. 2009). The strain kr6 produces an unusual Zn-protease of 64 kDa with optimum pH 8.5 that belongs to the M14 metalloprotease family (Riffel et al. 2007).

Temperature, pH, and enzyme/substrate (E/S) ratio are among the important parameters to be controlled in the production of protein hydrolysates by microbial proteases (Surówka et al. 2004). In this perspective, the design of experiments such as factorial design and response surface techniques are robust tools to evaluate the effects of multiple variables and their interactions on process performance, also reducing the number experiments when compared to traditional approaches that usually modify one variable per trial (Myers and Montgomery 2002). Such approaches were successfully applied to optimize hydrolysis conditions aiming to obtain protein hydrolysates with improved functional properties (Surówka et al. 2004; Contreras et al. 2011; Tavares et al. 2011).

The current investigation evaluates, through an experimental design approach, the effect of three variables (pH, temperature and E/S ratio) on the production of soy protein hydrolysates with a protease preparation from *Chryseobacterium* sp. kr6. The amount of soluble peptides, antioxidant activities, emulsifying and foaming capacities of the resulting hydrolysates were then assessed.

Materials and methods

Microorganism and cultivation conditions

The bacterium *Chryseobacterium* sp. kr6 was kept in feather meal agar plates (Riffel et al. 2003). For protease production, this strain was inoculated in feather meal broth containing the following components (g L⁻¹): NaCl (0.5), KH₂PO₄ (0.4),

CaCl₂ (0.015) and feather meal (10.0). The initial medium pH was adjusted to 8.0. Cultures were performed in 250 mL Erlenmeyer flasks (working volume of 50 mL) at 30 °C for 48 h in a rotary shaker (150 rpm) and, after this period, the cultures were centrifuged (10,000×g for 20 min, at 4 °C) and the supernatants were collected as enzyme source (Silveira et al. 2009).

Enzyme purification

Solid ammonium sulfate was added to culture supernatants to reach 50 % saturation, in an ice bath, under constant stirring. The mixture was centrifuged (10,000×g for 20 min, at 4 °C), the resulting pellet was dissolved in 50 mmol L⁻¹ Tris–HCl buffer (pH 8.0) and centrifuged again to remove any insoluble material. The concentrated sample was applied to a Sephadex G-100 gel-permeation column (30×0.8 cm) equilibrated and eluted with 50 mmol L⁻¹ Tris–HCl buffer (pH 8.0). Fractions with proteolytic activity were pooled and used as protease preparation in experiments of soy protein hydrolysis.

Protease activity was determined using azocasein as substrate, essentially as described by Thys et al. (2004). One unit of enzyme activity was defined as the amount of enzyme required to produce a change in absorbance of 0.01 at 420 nm under the assay conditions (40 min at 45 °C, pH 8.0).

Experimental design

A central composite design (CCD) was employed to obtain different soy protein isolate (SPI) hydrolysates by varying three hydrolysis parameters, namely temperature, initial pH and E/S ratio. These parameters exert essential influence on enzyme activity (Purich 2010). The full 2³ CCD was constructed with its eight points, augmented with three replications at the central points (all factors at level 0), plus six star points, that is, points having one factor at an axial distance to the center of ± α, whereas the other two factors are at level 0. The axial distance α was chosen to be 1.68 to make this design orthogonal. According to previous studies (Silveira et al. 2009; Riffel et al. 2007), the central values (level 0) for the experimental design were chosen to be: temperature at 45 °C; initial pH of 8.0, and an E/S ratio of 4,000 U g⁻¹. Real and codified values are shown in Table 1. From this design, a set of 17 hydrolysis experiments was carried out (Table 2).

For a three factors system, the model equation is:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (1)$$

where Y is the predicted response, b_0 is the intercept, b_1 , b_2 , b_3 are the linear coefficients, b_{11} , b_{22} , b_{33} are the quadratic

Table 1 Real values of independent variables at different coded levels of the 2^3 factorial design

Independent variable	Variable symbol	Coded levels				
		−1.68	−1	0	+1	+1.68
Temperature (°C)	x_1	28.2	35.0	45.0	55.0	61.8
Initial pH	x_2	5.48	6.50	8.00	9.50	10.52
E/S ratio (U g^{-1})	x_3	100	1,679	4,000	6,321	7,900

coefficients, and b_{12} , b_{13} , b_{23} are interaction coefficients. Therefore, the model permitted evaluation of the effects of linear, quadratic and interactive terms of the independent variables on the dependent variables. Results were analyzed by the Experimental Design Module of the Statistica 7.0 software (Statsoft, USA). Response surface plots and Pareto charts were drawn to illustrate the main and interactive effects of the independent variables on the dependent variables evaluated (Myers and Montgomery 2002).

Soy protein hydrolysis

Hydrolysates were prepared from SPI (Bunge S/A, Esteio, Brazil) using the partially purified protease preparation. The protein was dissolved in 50 mmol L^{-1} phosphate buffer at

different pH values (ratio 1:10 w/v), and the hydrolysis was performed at distinct temperatures, with different E/S ratios, as presented in Table 2. Hydrolyses were carried out with constant shaking (150 rpm) for 6 h and, after this period, the reaction was stopped by boiling in a water bath (100°C , 5 min). Hydrolysates were then centrifuged ($10,000\times g$ for 20 min) to remove insoluble materials, and the supernatants were frozen and stored at -18°C (Rossini et al. 2009). The amount of soluble peptides was determined and the concentration was adjusted to 10 mg mL^{-1} with 50 mmol L^{-1} phosphate buffer pH 7.0, before evaluation of antioxidant activities, emulsifying and foaming capacities, as described in the sections below. These analyses represent the dependent variables (responses) evaluated in the experimental design. A control (without added enzyme) was carried for each response in the best condition, for example, higher value for soluble peptides was obtained in run 7 (35°C , pH 9.5, $6,321 \text{ U g}^{-1}$), thus the control for soluble peptides has been made under these conditions without enzyme addition.

Determination of soluble peptides concentration

The concentration of soluble peptides in the hydrolysates was determined by the Folin phenol reagent method (Lowry et al. 1951). Bovine serum albumin (BSA) was used as standard.

Table 2 Experimental design for SPI hydrolysates production and the responses evaluated for the hydrolysates

Test	Independent variables			Evaluated responses						
	T (°C)(x_1)	pH (x_2)	E/S (U g^{-1})(x_3)	Soluble peptides (mg mL^{-1})	ABTS (%)	DPPH (%)	Reducing power (Abs 700 nm)	Fe^{2+} -chelating ability (%)	Foaming activity (%)	Emulsifying activity (%)
1	35	6.5	1,649	67.93	24.68	72.43	0.140	39.75	51.09	4.67
2	55	6.5	1,649	14.73	12.16	75.78	0.094	10.84	18.42	5.73
3	35	9.5	1,649	65.64	35.62	74.26	0.309	79.22	41.95	8.38
4	55	9.5	1,649	16.68	14.92	73.29	0.093	18.02	21.44	5.29
5	35	6.5	6,321	51.94	28.95	74.45	0.191	46.11	54.17	7.40
6	55	6.5	6,321	55.58	31.13	74.99	0.248	36.77	28.96	4.55
7	35	9.5	6,321	73.74	32.43	74.93	0.464	60.30	51.10	5.17
8	55	9.5	6,321	59.69	33.19	73.28	0.294	18.75	37.09	4.58
9	28.2	8.0	4,000	66.74	40.40	72.03	0.265	84.58	52.66	4.87
10	61.8	8.0	4,000	22.49	18.66	70.65	0.271	86.12	19.79	10.53
11	45	5.5	4,000	10.53	13.99	72.53	0.088	3.69	14.88	2.94
12	45	10.5	4,000	30.59	23.12	77.44	0.247	12.57	18.41	5.41
13	45	8.0	100	26.09	20.37	71.04	0.290	46.40	35.84	12.01
14	45	8.0	8,000	46.04	42.83	64.87	0.465	10.46	65.49	5.13
15	45	8.0	4,000	21.93	21.92	74.53	0.378	29.35	31.25	6.25
16	45	8.0	4,000	22.04	23.32	71.58	0.262	37.34	33.28	5.28
17	45	8.0	4,000	23.99	18.67	74.57	0.210	21.62	18.41	5.13
C ^a				14.09 (run 7)	10.20 (run 14)	68.62 (run 12)	0.88 (run 14)	45.30 (run 10)	9.10 (run 14)	5.13 (run 13)

^a C=control (without enzyme), determined at the condition of maximum response

The measurements were performed using a Shimadzu UV mini-1240 spectrophotometer (Shimadzu do Brasil, Agua Branca, SP, Brazil).

Determination of radical scavenging activities

The ability of SPI hydrolysates to scavenge radicals was evaluated by the 2,2'-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) cation radical method, essentially as described by Re et al. (1999). Also, the method based on the capture of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was employed (Brand-Williams et al 1995).

Metal chelating activity

The chelating activity of Fe^{2+} was measured using the method described by Chang et al. (2007) with slight modifications. One milliliter of sample was mixed with 3.7 mL distilled water and then the mixture was reacted with 0.1 mL of 2 mmol L^{-1} FeSO_4 (Fe^{2+}) and 0.2 mL of 5 mmol L^{-1} ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine). After 10 min the absorbance was read at 562 nm. One milliliter of distilled water, instead of sample, was used as a control. The results were expressed as: Chelating activity (%) = $[1 - (A/A_0)] \times 100$, where A is the absorbance of the test and A_0 is the absorbance of the control.

Determination of reducing power

Reducing power was measured by mixing the hydrolysates with 2.5 mL phosphate buffer (0.2 mol L^{-1} , pH 6.6) and 2.5 mL of potassium ferricyanide (10 mg mL^{-1}), and then the mixture was incubated at 50°C for 20 min. Then, 2.5 mL trichloroacetic acid ($10\% \text{ v v}^{-1}$) was added and the mixture was centrifuged ($3,000 \times g$ for 10 min). Supernatant (1 mL) was mixed with 2.5 mL of distilled water and 0.2 mL of ferric chloride (1 mg mL^{-1}), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated greater reducing power (Corrêa et al. 2011).

Determination of emulsifying and foaming capacities

The foaming capacity of the hydrolysates was determined by the method of Fernandez and Macarulla (1997) and Coffman and Garcia (1977), with minor modifications. Aliquots (500 μL) of each hydrolysate were mixed with 10 mL of distilled water and homogenized for 2 min in a mixer at maximum speed. Foaming was calculated according to the percentage increase in volume as: $\% \text{ foaming} = [(V_2 - V_1)/V_1] \times 100$, where V_1 is the volume after homogenization and V_2 is the volume before homogenization.

The capacity of the hydrolysates to emulsify hydrophobic substances was tested. Each hydrolysate was mixed with 2 mL

of soybean oil and allowed to stand for 24 h at room temperature (Willumsen and Karlson 1997). After this time the emulsifying index (E24) was determined as: $E24 = (\text{height of the layer} / \text{height of the total}) \times 100$.

Results and discussion

Hydrolysis of soy protein

A microbial protease preparation was employed to obtain SPI hydrolysates. Hydrolysis was carried out for 6 h at varying conditions of temperature, pH and E/S ratio, according to a 2^3 CCD, and fifteen distinct hydrolysates were obtained from a total of 17 experiments. Different dependent variables (responses) were evaluated for each SPI hydrolysate, and the results are summarized in Table 2.

The amount of soluble peptides was among the responses evaluated for the SPI hydrolysates (Table 2). In the range studied, the variables pH, temperature and E/S, as well as the interaction between pH and E/S, and T and E/S had significant effects ($P < 0.05$) on the amount of soluble peptides (Fig. 1a). E/S ratio was previously reported to significantly affect ($P < 0.1$) the degree of hydrolysis of whey proteins by cardosins (Tavares et al. 2011). The results of the second-order response surface models were examined by analysis of variance (ANOVA) and Fischer's *F*-test (Table 3). Calculated value of $F_{c(9,7)}$ was equal to 5.75, which was greater than the tabulated value ($F_{t(9,7)} = 3.68$), demonstrating the significance of the regression model (Myers and Montgomery 2002). Regressions obtained indicated a R^2 of 0.761 for soluble peptides, indicating that the model could explain 76.1 % of the variability for the response (soluble peptides). A value of $R^2 > 0.75$ indicates the aptness of the model. The following regression equation was obtained:

$$Y = 21.560 - 13.697x_1 + 4.341x_2 + 8.023x_3 + 11.417x_1^2 + 2.896x_2^2 + 8.388x_3^2 - 1.681x_1x_2 + 11.469x_1x_3 + 3.281x_2x_3. \quad (2)$$

The response surface graphs were then plotted (Fig. 1b–d). From Fig. 1b, it could be observed that, in the ranges tested, lower temperatures and higher pH values resulted in an increment of soluble peptides. Hydrolysis experiment 7 (35°C , pH 9.5 and E/S ratio of $6,321 \text{ U g}^{-1}$) resulted in the highest soluble peptides concentration (73 mg mL^{-1} ; Table 2). These results are in partial agreement with the optimal conditions for purified proteases produced by *Chryseobacterium* sp. kr6 (Riffel et al. 2007; Silveira et al. 2009). However, it should be considered that a partially purified protease preparation was employed in the current investigation. At constant

Fig. 1 **a** Pareto chart of standardized effects of the different hydrolysis variables on soluble peptides concentration of SPI hydrolysates; tested variables were temperature (T), pH and the enzyme/substrate (E/S) ratio; the point at which the effect estimates were statistically significant ($P=0.05$) is indicated by the vertical line. **b** Response surface for soluble peptides

concentration, depending on the temperature and pH of hydrolysis (E/S ratio at level 0). **c** Response surface for soluble peptides concentration, depending on the E/S ratio and pH of hydrolysis (temperature at level 0). **d** Response surface for soluble peptides concentration, depending on the E/S ratio and temperature of hydrolysis (pH at level 0)

temperature (level 0; 45 °C), a higher concentration of soluble peptides was obtained with increasing pH and E/S ratio (Fig. 1c). When the pH remains constant (at level 0; pH 8), higher concentrations of soluble peptides are obtained at lower temperature and E/S ratios (Fig. 1d). The same effect is observed at higher values of temperature and E/S ratio (Fig. 1d); nevertheless, these conditions may not be economically interesting, since higher energy inputs and enzyme amounts are needed. These results clearly indicate the soluble peptides content of SPI hydrolysates is related to the independent variables, which directly affect, for instance, enzyme performance.

Antioxidant activity

The antioxidant activity of protein hydrolysates is related to the radical quenching capacity, chelation of metal ions, and

reducing power of the peptides released from the precursor protein (Sarmadi and Ismail 2010). For this reason, the antioxidant potential of SPI hydrolysates was evaluated through different methods based on these distinct capabilities. Regarding the ABTS^{•+} method, control hydrolysate (without enzyme addition) showed to scavenge 10 % of this radical. From Table 2, it could be observed that hydrolysis of SPI presented a general beneficial effect on ABTS^{•+} quenching, as reported for hydrolysates of whey protein Dryáková et al. (2010) and ovine casein (Gómez-Ruiz et al. 2008). Maximum ABTS scavenging (43 %) was achieved in hydrolysates obtained at 45 °C, pH 8 and an E/S ratio of 8,000 U g⁻¹ (Table 2). Statistical analysis showed that temperature, E/S, and interaction between them, in the range studied, presented a significant effect on the production of hydrolysates with the ability to scavenge the ABTS cation radical (Fig. 2a). In a previous work, the antioxidant activity of whey protein

Table 3 Analysis of variance of the regression model for the soluble peptides concentration of SPI hydrolysates

Sources of variation	Sum of squares	Degrees of freedom	Mean square	F-value	P-value
x_1	2559.80	1	2559.79	1916.25	0.0005 ^a
x_1^2	1465.39	1	1465.39	1096.99	0.0009 ^a
x_2	257.14	1	257.14	192.50	0.0052 ^a
x_2^2	94.27	1	94.27	70.57	0.0139 ^a
x_3	878.20	1	878.20	657.41	0.0015 ^a
x_3^2	790.92	1	790.92	592.08	0.0017 ^a
$x_1^2 x_2$	22.61	1	22.61	16.93	0.0543
$x_1^2 x_3$	1052.26	1	1052.26	787.72	0.0013 ^a
$x_2^2 x_3$	86.13	1	86.13	64.48	0.0152 ^a
Lack of Fit	974.69	5	194.94	145.93	0.0068 ^a
Pure Error	2.67	2	1.34		
Total	7643.51	16			

^a Statistically significant at 95 % of confidence level

hydrolysates obtained with thermolysin, evaluated by a oxygen radical-scavenging assay, was reported to be significantly affected ($P < 0.1$) by the E/S ratio (linear and quadratic values), and the linear values of time and temperature of hydrolysis (Contreras et al. 2011).

The results of the second-order response surface model were examined by analysis of variance (ANOVA) and Fischer's F -test (Table 4). The calculated F -value ($F_{c(9,7)} = 18.75$) was greater than the tabulated F -value ($F_{t(9,7)} = 3.68$), which demonstrate significance for the regression model. Regressions obtained indicated an R^2 -value of 0.922 for ABTS^{•+} scavenging, indicating that the model could explain 92.2 % of the variability for response. The following regression equation was obtained

$$Y = 21.313 - 4.908x_1 + 2.536x_2 + 5.578x_3 + 2.849x_1^2 - 1.032x_2^2 + 3.592x_3^2 - 1.193x_1x_2 + 4.530x_1x_3 - 1.025x_2x_3. \quad (3)$$

The contour plot for the effects of temperature and E/S ratio on the quenching of ABTS^{•+} by the different hydrolysates is presented in Fig. 2b. Maximal activity was observed at low values of temperature and E/S ratio; however, the same was observed at higher temperatures and higher E/S ratio. A similar behavior was observed for soluble peptides (Fig. 1d), suggesting that a higher level of hydrolysis results in superior antioxidant capacity of the hydrolysates obtained at pH 8.0. Nevertheless, the higher ABTS scavenging activity (43 %) was detected in SPI hydrolysates with intermediate values for soluble peptides (46 mg mL⁻¹; run 14, Table 2). In fact, a correlation analysis of these variables shows $R = 0.813$ ($P < 0.05$) indicating strong positive correlation (Cohen

1988). The degree of hydrolysis is showed to affect the ABTS quenching ability of protein hydrolysates (Corrêa et al. 2011; Nalinanon et al. 2011).

The ability of the SPI hydrolysates to capture the DPPH radical was also investigated. In the range studied, the evaluated variables did not show a significant effect for this response. The hydrolysates showed a high antioxidant capacity by the DPPH radical method (around 70–77 %) regardless of the conditions tested (Table 2). Nevertheless, the control (without enzyme added) also showed an elevated value, which can be associated to the ability of intact SPI to quench DPPH radicals. In agreement, Chen et al. (2013) showed that untreated SPI caused 50 % DDPH inhibition. Previous studies have shown that protein hydrolysis improves the ability to capture the DPPH radical (Peng et al. 2010; Zhang et al. 2010), but in this work, no significant correlation was observed between these responses. Differences in the results from ABTS and DPPH assays might relate to the distinct stereoselectivity of the radicals, different peptides able to quench these radicals, and solubility of ABTS (water-soluble) and DPPH (oil-soluble) radicals in aqueous environments (Zhu et al. 2008).

Iron acts as a catalyst for the generation of hydroxyl radicals through the Fenton reaction, which may contribute to diseases related to oxidative stress. In addition, transition metals can stimulate lipid peroxidation in foods, resulting in rancidity. Consequently, the chelation of metal ions can contribute to the antioxidant activity of protein hydrolysates (Pownall et al. 2010). Therefore the Fe²⁺ chelating ability of SPI hydrolysates was investigated. For the iron chelating activity of SPI hydrolysates, only the temperature had a significant effect (Fig. 3). As the R^2 value was inferior to 0.75, and the calculated F -value was smaller than the tabulated value, the model was not considered adequate. The iron chelating activity of soy protein hydrolysates was maximum when the temperature was 61.8 °C or 28.2 °C (86 % and 84 %, respectively), the highest and lowest temperatures tested (Table 2). Sheep caseinate subjected to hydrolysis with protease from *Bacillus* sp. P7 at 45 °C, pH 8 for 30 min, was reported to chelate 83 % of iron (Corrêa et al. 2011). Pownall et al. (2010) studied hydrolysates of pea protein isolate, and found a high ability to chelate iron ions (around 95 %), and silver carp protein hydrolyzed for 6 h with Alcalase showed 95 % of metal-chelating activity (Dong et al. 2008). The control (without enzyme) demonstrated an iron chelating ability around 45.3 %, indicating that, depending on the process conditions, hydrolysis might negatively affect the iron-chelating ability of native SPI (Table 2), possibly through the cleavage of the peptides responsible for this activity. However, a correlation analysis of these variables shows $R = 0.486$ ($P < 0.05$), indicating a moderate positive correlation (Cohen 1988). In fractions of SPI hydrolysates obtained through ultrafiltration, high-molecular mass fractions showed higher chelating abilities than that of low-

Fig. 2 **a** Pareto chart of standardized effects of the different hydrolysis variables on the ability of SPI hydrolysates to capture the radical ABTS; the variables tested were temperature (T), pH and the enzyme/substrate (E/S) ratio; the point at which the effect estimates were statistically significant (at $P=0.05$) is indicated by the vertical line. **b** Response surface for the capacity of hydrolysates to scavenge the ABTS radical, depending on the E/S ratio and temperature of hydrolysis (pH at level 0)

molecular mass fractions (Zhang et al. 2010). However, increasing degree of hydrolysis (DH) resulted in higher Fe^{2+} chelating ability of whey protein hydrolyzed with Alcalase (Peng et al. 2010), and similar results were obtained for yellow stripe trevally meat hydrolysates obtained with Alcalase (Klompong et al. 2007).

In the range studied, the tested variables were not significant for increasing the reducing power of SPI hydrolysates. However, the reducing power reached 0.465 absorbance units at 700 nm (run 14, Table 2). The control had a reducing power of 0.088 absorbance units, which was equal to that observed for test 11 (Table 2), indicating that the reducing power of the majority of SPI hydrolysates was enhanced by hydrolysis. However, the correlation between these responses was not significant ($P>0.05$). In this sense, hydrolysis conditions of

35–45 °C, pH values of 8.0–9.5, and E/S ratio at 4,000–8,000 U g^{-1} tended to increase the reducing power of SPI hydrolysates (Table 2). As this assay is based on the ability of a compound to reduce Fe^{3+} to Fe^{2+} , some SPI hydrolysates could act as electron donors, which might contribute to the antioxidant activity. Such potential could be exploited, for instance, in the reduction of oxidized intermediates that act on the lipid peroxidation processes in foods. Wheat germ proteins treated with Alcalase were also capable of reducing Fe^{3+} (Zhu et al. 2006), as well as sheep milk caseinate hydrolyzed with a *Bacillus* sp. P7 protease preparation (Corrêa et al. 2011). Klompong et al. (2007) observed that higher DH of yellow stripe trevally meat resulted in less reducing power, whereas Peng et al. (2010) reported the opposite pattern for hydrolysates of whey protein.

Table 4 Analysis of variance of the regression model for ABTS scavenging ability of SPI hydrolysates

Sources of variation	Sum of squares	Degrees of freedom	Mean square	F-value	P-value
x_1	328.64	1	328.63	58.01	0.0168 ^a
x_1*x_1	91.30	1	91.29	16.11	0.0568
x_2	87.76	1	87.76	15.49	0.0589
x_2*x_2	11.97	1	11.96	2.11	0.2832
x_3	424.53	1	424.53	74.96	0.0131 ^a
x_3*x_3	145.05	1	145.05	25.61	0.0369 ^a
x_1*x_2	11.38	1	11.38	2.01	0.2921
x_1*x_3	164.17	1	164.17	28.98	0.0328 ^a
x_2*x_3	8.41	1	8.41	1.48	0.3474
Lack of Fit	42.05	5	8.41	1.48	0.4492
Pure Error	11.33	2	5.66		
Total	1343.76	16			

^a Statistically significant at 95 % confidence level

From the results presented in Table 2, the relationship between soluble peptides content (indicating the proteolysis level) and the antioxidant activities (radical-scavenging, iron chelation, reducing power) is not always clearly observed. In this context, as pointed out by Alemán et al. (2011), the presence of specific peptide sequences is more important than the whole hydrolysate molecular mass distribution for the hydrolysate bioactivities. Additionally, *Chryseobacterium* sp. kr6 produces diverse extracellular proteases with different features (Riffel et al. 2011). Therefore, the protein substrate and protease specificity directly affect the characteristic of the generated peptides which, in turn, determines the antioxidant activities of the SPI hydrolysates. Thus, aiming to obtain a

hydrolysate with maximum antioxidant activity is advisable to work under conditions of the experiment 14 (45 °C, pH 8.0 and E/S 8,000 U g⁻¹).

Foaming and emulsifying properties

Soy proteins are largely employed in the food industry, but they have limited utilization as foaming and emulsifying agents (Lusas and Riaz 1995). Therefore, protein modification by chemical or enzymatic processes is employed to improve these functional properties (Nir et al. 1994; Moure et al. 2006; Martínez et al. 2009). Enzymes act hydrolyzing peptide bonds in the protein, producing peptides of desired size, charge and surface properties (Moure et al. 2006). The enzymatic modification is preferred over chemical treatments, since enzyme reactions are specific, occur at mild process conditions and minimally affect the nutritional quality (Were et al. 1997).

In this sense, the hydrolysates were evaluated for their foaming and emulsifying capacities. The statistical analysis showed that only the temperature (linear effect), in the range tested, had a significant effect in obtaining hydrolysates with foaming properties (Fig. 4a). The effect of increasing temperatures was negative (data not shown), indicating that hydrolysates obtained at lower temperatures (up to 45 °C) show an increased capacity to foam. The foaming capacity of the hydrolysates ranged from 14.9 to 65.5 % (Table 2), with the maximum value obtained for the run 14. As for control (non-hydrolyzed SPI) the foaming capacity was 9.1 %, the results indicate that SPI hydrolysis was beneficial for foaming capacity. A trend could be observed from Table 2, where higher amounts of soluble peptides tended to be associated with a higher foaming capacity of SPI hydrolysates. In fact, a

Fig. 3 Pareto chart of standardized effects of the different hydrolysis variables tested on the ability of SPI hydrolysates to chelate iron; the variables tested were temperature (T), pH and the enzyme/substrate (E/S) ratio; the point at which the effect estimates were statistically significant (at $P=0.05$) is indicated by the vertical line

Fig. 4 Pareto chart of standardized effects of the different hydrolysis variables tested on the foaming (a) and emulsifying (b) capacities of SPI hydrolysates; the variables tested were temperature (T), pH and the enzyme/substrate (E/S) ratio; the point at which the effect estimates were statistically significant (at $P=0.05$) is indicated by the vertical line

correlation analysis of these responses shows $R=0.754$ ($P<0.05$) indicating strong positive correlation (Cohen 1988). Foam production involves the generation of a protein film surrounding a gas bubble, and the packing of gas bubbles into an overall structure. The spontaneous behavior of proteins in aqueous solutions to adsorb to the air-water interface, causing the lowering of the interfacial (surface) tension, is of central importance for foaming properties (Foegeding et al. 2006). Protein hydrolysis usually presents a positive effect on foaming capacity (Were et al. 1997; Tsumura et al. 2005; Sinha et al. 2007; Jamdar et al. 2010), since the generated peptides usually present an increased adsorption rate due to their faster diffusion to the interface when compared to the

non-hydrolyzed proteins (Foegeding et al. 2006; Martínez et al. 2009).

Emulsions, similarly to foams, are two-phase systems with one of the phases dispersed in an aqueous continuous one. Peptides might decrease the interfacial tension and facilitate formation of stable oil–water interfaces by adsorbing on the surface of oil droplets formed during homogenization, creating a protective membrane that inhibits the coalescence of the oil droplets. Therefore, emulsifying capacity and emulsion stability depend on the amphiphilic character and charges of peptides (Moure et al. 2006; Klompong et al. 2007). For the emulsifying capacity, only the E/S ratio of hydrolysates had a significant effect in the range studied (Fig. 4b). Surówka et al.

(2004) found an increased emulsifying capacity for soy protein hydrolysates obtained with a high enzyme (Neutrase) to protein ratio; however, the use of higher Protamex to protein ratio resulted in lower ability to form emulsions. In this work the correlation between these responses was negative ($R=-0.118$) but not significant ($P>0.05$). Were et al. (1997) observed that the emulsifying activity of papain-modified soy protein was not different from that of unmodified protein. The selective hydrolysis of β -conglycinin (by papain) or glycinin (by pepsin) from SPI resulted in poorer emulsifying ability at pH 5.5 and 7.0 when compared to control SPI (Tsumura et al. 2005). The increased hydrolysis of peanut protein tended to decrease its emulsifying capacity (Jamdar et al. 2010), and the same profile was observed for hemp protein isolate (Yin et al. 2008), whey protein concentrates (Sinha et al. 2007) and for ornate threadfin bream muscle (Nalinanon et al. 2011). An extensive hydrolysis results in smaller peptide chain lengths, which improves the migration and adsorption to the interface; however, short peptides might negatively affect the emulsion activity due to its lower efficiency in reducing interfacial tension (Klompong et al. 2007; Yin et al. 2008; Jamdar et al. 2010).

Although the protease form *Chryseobacterium* sp. kr6 is promising to improve antioxidant and functional properties of soy protein, toxicology studies must be warranted before its safe use in food. In this regard, the related bacterium *Chryseobacterium proteolyticum* is used for safe production of glutaminase (Scheuplein et al. 2007). Other Gram-negative bacteria often considered pathogenic, such as *Pseudomonas fluorescens*, fulfill recognized safety criteria pertinent to microbial production strains used in the manufacture of food enzyme preparations (Halich et al. 2012).

Conclusion

In this work, the effect of three variables (temperature, pH, and E/S ratio) on the production of SPI hydrolysates was studied. The obtained data showed that enzymatic hydrolysis by a protease preparation from *Chryseobacterium* sp. kr6 resulted in increased soluble peptides content, and in generally positive effects on the antioxidant and functional properties of SPI. Such process might be a promising alternative in obtaining natural antioxidants for use in foods, in addition to providing interesting functional properties. Models for soluble peptides and ABTS were obtained. Given the factors that affect protein hydrolysis (such as enzyme specificity, E/S ratio, temperature, pH), the variety of peptides derived from this process, and also the complexity and diversity of their mechanisms of action, optimization of both functionality and bioactivity on a single hydrolysis process is an intricate endeavor. Depending on the desired features of hydrolysates is

necessary to use a specific temperature, pH and E/S ratio. The robustness of enzymatic hydrolysis to modify proteins functional properties is undeniable, and the efficient control of hydrolysis conditions might be employed to obtain hydrolysates with satisfactory features that match the increasingly rigorous needs of the food industry, which are ultimately guided by consumer demands.

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