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Action of multi-enzyme complex on protein extraction to obtain a protein concentrate from okara

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Abstract The objective of this study was to optimize the extraction of protein by applying a multi-enzymatic pretreatment to okara, a byproduct from soymilk processing. The multi-enzyme complex Viscozyme, containing a variety of carbohydrases, was used to hydrolyze the okara cell walls and facilitate extraction of proteins. Enzyme-assisted extraction was carried out under different temperatures (37–53 °C), enzyme concentrations (1.5–4%) and pH values (5.5–6.5) according to a central composite rotatable design. After extraction, the protein was concentrated by isoelectric precipitation. The optimal conditions for maximum protein content and recovery in protein concentrate were 53 °C, pH 6.2 and 4% of enzyme concentration. Under these conditions, protein content of 56% (dry weight basis) and a recovery of 28% were obtained, representing an increase of 17 and 86%, respectively, compared to the sample with no enzymatic pretreatment. The multi-enzyme complex Viscozyme hydrolyzed the structural cell wall polysaccharides, improving extraction and obtaining protein concentrate from the okara. An electrophoretic profile of the protein concentrate showed two distinct bands, corresponding to the acidic and basic subunits of the protein glycinin. There were no limiting amino acids in the protein concentrate, which had a greater content of arginine.

Keywords Soy pulp · Enzymatic hydrolysis · Amino acid composition · Fluorescence microscopy · SDS-Page

Abbreviations

E	Enzyme concentration (%)
m	Mass (g)
PC	Protein content (%, dry weight basis)
PR	Protein recovery (%)
SR	Total solids recovery (%)
T	Temperature (°C)
X	Mass fraction (g/g)

Subscript

conc	Protein concentrate
okara	Defatted okara flour
prot	Protein
solids	Total solids

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Introduction

During the production of soymilk and *tofu*, an insoluble byproduct called okara, or soy pulp, is generated. Since about 1 kg of wet okara result from every kilogram of soybeans (Khare et al. 1995), substantial amounts of this by-product are underutilized or discarded, representing an industrial problem due to the expensive treatment and destination. Despite being rich in protein (27–38%, dry weight basis), okara has little market value; and is thus usually used as animal feed (Surel and Couplet 2005).

Soy proteins have been widely used in the food industry because of their ability to improve the functional properties while contributing to the nutritive value. The main protein fractions present in okara are the basic 7S globulin and 11S proteins, as reported by Stanojevic et al. (2012). A high content of basic 7S globulin is desirable due to its nutritive value, since it is a cysteine-rich glycoprotein. The 11S protein fraction contains a high methionine content and has been related to the gelation property (Liu 1997). In addition, unlike other vegetable proteins, okara proteins contain all of the essential amino acids necessary for good health (Waliszewski et al. 2002). Therefore, the extraction of protein from okara could be of interest to the food industry in order to obtain a protein concentrate/isolate for application in nutritional supplementation or to improve the functional properties of food products.

However, most of the soluble protein is extracted in the soymilk, meaning that the residue okara contains predominantly non-extractable proteins. Vishwanathan et al. (2011) reported that the solubility of okara protein was lower than that of soybean protein in the pH range from 2 to 11. Prior to the water extraction of the soluble components, the soybeans are ground, causing cell wall rupture. This exposes the soluble compounds inside the cell, facilitating the percolation of water into which the substances can diffuse. Nevertheless, a proportion of the cells do not suffer physical damage, and as a consequence, a significant amount of protein, representing about 25–40% of the soy proteins, remains in the okara (Liu 2008). In their study of the microstructure of soybeans and okara by confocal microscopy, Preece et al. (2015) identified the location of protein inside the intact plant cells, and suggested that some treatment of the okara to disrupt the cell wall could improve protein extraction. In addition, the non-starch polysaccharides of the plant cell wall can impair the extraction of intracellular protein. According to Stanojevic et al. (2012), basic 7S globulin, one of the main proteins in okara, is tightly bound to the plasma membranes and cell walls, and is thus not easily extractable. Vishwanathan et al. (2011) determined the nitrogen solubility index of okara, and found it was only about half that of soybean,

indicating that the solubility of okara protein was less than that of soy protein.

An enzymatic pretreatment to reduce the cell wall rigidity and improve the protein extraction yield could be a promising method. Okara cells have advanced and complex structures, in which they are attached to each other. Kasai et al. (2003, 2004) published interesting studies demonstrating that the primary cell wall was easily hydrolyzed by the action of celluloses; however, only pectinases could digest the secondary cell wall. Thus the use of multi-enzyme complexes could be capable of disintegrating the okara cell wall by hydrolysis of the linkages between the structural polysaccharides, releasing the intracellular proteins. Viscozyme contains cellulases (activity of 33 µg/ml/min, expressed as the reducing sugar equivalents released), hemicellulases, including xylanase (191.1 µg/ml/min) and endoglucanase (263.6 µg/ml/min). In addition, differently from other carbohydrase complexes, it has higher pectinase activity (1177.3 µg/ml/min) than, for example, the Celluclast complex enzyme (180.6 µg/ml/min) (Gama et al. 2015), which is necessary to hydrolyze the resistant secondary cell wall, as suggested by Kasai et al. (2004).

To enhance the process yield, carbohydrase-assisted protein extraction was applied to pressed rapeseed cake (Rommi et al. 2015), oat bran (Jodayree et al. 2012; Guan and Yao 2008), defatted soybean flour (Rosset et al. 2014), soybeans (Rosenthal et al. 2001) and olive leaves (Vergara-Barberán et al. 2015). However, a study of the enzymatic pretreatment of okara to improve protein extraction has not been previously reported.

Since the enzyme-assisted protein extraction from several vegetable materials has demonstrated promising results, this study aimed to optimize the extraction and obtaining of a protein concentrate by applying a multi-enzymatic complex pretreatment to okara.

Materials and methods

Material and sample preparation

Wet okara, obtained from soymilk processing, was donated by Cocamar Cooperativa Agroindustrial (Maringá, PR, Brazil). According to the manufacturer, for soymilk extraction soybean was cooked in hot water at 80–90 °C, ground to obtain a slurry and the okara was separated from the soymilk using a decanter centrifuge. During processing of the soymilk, the okara was collected and forwarded to the city of Londrina, PR, Brazil at room temperature. As soon as the material arrived in the laboratory, it was dehydrated in a forced-air oven (Fabbe, model 170, São Paulo, Brazil) at 40 °C for 24 h, until it reached a moisture content of about 15% (wet weight basis). The dried okara

was milled in a knife mill; defatted with n-hexane (1:10, w/v) under orbital agitation (shaker MA-140 model, Marconi, Piracicaba, Brazil) at 300 rpm and room temperature for 30 min; and vacuum filtered using 80 g qualitative filter paper. The defatted okara flour was stored in a refrigerator at 7 °C until analyzed.

A multi-enzyme complex Viscozyme (Novozymes, Bagsvaerd, Denmark), which consists of cellulase, hemicellulase, arabanase, beta-glucanase and xylanase, was used for the enzymatic pretreatment of the defatted okara flour. This enzyme, derived from Aspergillus aculeatus, has a declared activity of 100 FBG/g. Each FBG is the amount of enzyme required to hydrolyze β-glucans under the standard condition (30 °C, pH 5.0).

Enzymatic pretreatment of okara to extract and obtain a protein concentrate

The enzymatic pretreatment of okara was carried out according to a 2^3 central composite rotatable design (CCRD) (Table 1), in order to evaluate the effect of the independent variables of temperature, pH and enzyme concentration (%, w/w) on the protein content PC (%, dry basis) and protein recovery PR (%) in the protein concentrate. The ranges of temperature, pH and enzyme concentration studied in the current study were based on the literature and preliminary tests.

The experimental data were fitted to the following polynomial equation:

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 \tag{1}$$

where y is the response (dependent variable PC or PR); b_0 is the constant regression coefficient; b_1, b_2 and b_3 are the linear regression coefficients; b_{11}, b_{22} and b_{33} are the quadratic regression coefficients; b_{12}, b_{13} and b_{23} are the cross-product regression coefficients; and x_1, x_2 and x_3 represent the coded values of the independent variables (temperature, pH and enzyme concentration, respectively).

For the enzymatic pretreatment experiments, about 5.0 g of defatted okara flour were added to 95 mL phosphate citrate buffer at different pH values (5.5, 5.7, 6.0, 6.3 or 6.5). After the desired temperature was reached (37, 40, 45, 50 or 53 °C), the enzyme was added (1.5, 2.0, 2.75, 3.5 or 4%, w/w) and the mixture was maintained under orbital

Table 1 Experimental design for the enzymatic pretreatment of okara

Design trial	Independent variables[a]			Dependent variables			
	T (°C)	PH	E (%)	PC (%)[b]	PC (%) predicted[c]	MDE (%)[d]	PR (%)[e]
1	40 (−1)	5.7 (−1)	2 (−1)	53.8	56.1	4.3	24.0
2	50 (+1)	5.7 (−1)	2 (−1)	46.9	45.5	3.0	27.2
3	40 (−1)	6.3 (+1)	2 (−1)	55.3	53.5	3.3	22.1
4	50 (+1)	6.3 (+1)	2 (−1)	49.7	54.1	8.9	28.6
5	40 (−1)	5.7 (−1)	3.5 (+1)	59.3	54.1	8.8	31.6
6	50 (+1)	5.7 (−1)	3.5 (+1)	52.7	53.5	1.5	35.3
7	40 (−1)	6.3 (+1)	3.5 (+1)	45.0	45.5	1.1	35.4
8	50 (+1)	6.3 (+1)	3.5 (+1)	59.0	56.1	4.9	31.3
9	37 (−1.68)	6 (0)	2.75 (0)	54.3	58.6	7.9	24.5
10	53 (+1.68)	6 (0)	2.75 (0)	61.7	58.6	5.0	30.5
11	45 (0)	5.5 (−1.68)	2.75 (0)	43.1	47.6	10.4	19.4
12	45 (0)	6.5 (+1.68)	2.75 (0)	51.0	47.6	6.7	24.8
13	45 (0)	6 (0)	1.5 (−1.68)	54.0	53.2	1.4	27.0
14	45 (0)	6 (0)	4 (+1.68)	51.4	53.2	3.6	25.7
15	45 (0)	6 (0)	2.75 (0)	67.2	66.5	1.0	31.4
16	45 (0)	6 (0)	2.75 (0)	66.1	66.5	0.6	28.8
17	45 (0)	6 (0)	2.75 (0)	66.7	66.5	0.3	33.4

[a]The independent variables correspond to the real values. Values in parentheses correspond to the coded values. T is the temperature (°C) and E is enzyme concentration (%)

[b]PC is the protein content on a dry weight basis (%)

[c]PC values predicted by the polynomial model (Eq. 1 and Table 2)

[d]MDE (%) is the mean deviation error between the experimental and predicted values for PC (Eq. 3)

[e]PR is the protein recovery (%), for which the polynomial model PR was not considered predictive

agitation in a controlled temperature water bath (Dubnoff shaker, model T-53, TECNAL, Piracicaba, Brazil) for 2 h. After pretreatment of the sample, the okara proteins were extracted by adjusting the pH of the mixture to 9.0 with 1 M NaOH. The mixture was maintained under orbital agitation in the Dubnoff shaker for 30 min at 60 °C, and then centrifuged (model R-5804, Eppendorf, Hamburg, Germany) at a relative centrifugal force of $14,204 \times g$ for 30 min at 4 °C. The precipitate was discarded and the pH of the supernatant adjusted to 4.5 with 0.5 M HCl to precipitate the proteins. The proteins were recovered in the precipitate by centrifugation at $14,204 \times g$ for 30 min at 4 °C, and the supernatant discarded. The protein concentrate was evaluated with respect to its PC and PR. A control procedure without the addition of the enzyme was also carried out, and the PC and PR of this protein concentrate also determined. This procedure was carried out in the same way as described above, including the pretreatment of the sample, except that no enzyme was added.

The PC was determined by the Kjeldahl method (AOAC 1995). The PR, or ratio of the protein mass in the concentrate to that in the okara flour, was used as an index of the protein extraction efficiency (Eq. 2).

$$PR(\%) = \frac{m_{prot, \, conc}}{m_{prot, \, okara}} \times 100 = \frac{X_{prot, \, conc} \times m_{conc}}{X_{prot, \, okara} \times m_{okara}} \times 100 \tag{2}$$

where $X_{prot, \, conc}$ and $X_{prot, \, okara}$ are the protein contents in the concentrate and defatted okara flour, respectively (g/g); and m_{conc} and m_{okara} are the masses of the concentrate and defatted okara flour, respectively (g).

Analytical procedures

Microstructure of the okara after the enzymatic pretreatment

The microstructures of the okara with enzymatic pretreatment and the control were evaluated by optical fluorescence microscopy. The samples were first poured onto microscope slides with 25 μL aliquot of a solution prepared from 40 μL of an aqueous DABCO solution [1,4-diazabicyclo (2.2.2) octane (2.3%), 20 mM Tris HCl pH 8.0 (2%) and glycerol (90%)], 40μL of distilled water and 8 μL of calcofluor. All the images were obtained using a fluorescence light microscope (DM 4500 B, Leica, Wetzlar, Germany) equipped with a DFC 300FX camera and Leica IM50 4.0 software. The software iGrafx image was used to optimize and improve the contrast and brightness.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic profiles of the okara protein and the protein concentrate obtained under optimum conditions were obtained according to Laemmli (1970). A separating gel of 20% acrylamide was used with a 4% stacking gel. The protein concentrate was diluted in a 0.5 M Tris–HCl pH 6.8 buffer containing 5% β-mercaptoethanol to obtain a protein concentration of 4%, and was heated at 95 °C for 5 min. After cooling to room temperature, 12 μL aliquots of the sample were applied to the gel with 2% SDS and 20% glycerol. The analyses were carried out in a Mini-Protein II system (Electrophoresis Power Supply Loccus Biotechnology, São Paulo, Brazil) at 200 V and 60 mA for 1 h and 20 min. The apparent molecular weight of each protein band was estimated using molecular weight markers (Precision Plus Protein Standard, Bio-Rad Laboratories, Hercules, USA).

Total amino acid contents

The amino acid composition of the protein concentrate obtained under optimum conditions was determined by digestion in 6 N HCl/0.1% phenol at 110 °C for 20 h, according to the Pico-Tag method as described by White et al. (1986). After acid hydrolysis, sample derivatization was initiated by adding an ethanol:water:triethylamine: phenylisothiocyanate solution (7:1:1:1, v/v), which was mixed using a vortex mixer and allowed to stand at room temperature for 20 min. Identification of the amino acids was carried out by high-performance liquid chromatography (Thermo Fisher Scientific Inc., Waltham, MA, USA) using a LUNA C18 column (Phenomenex, Torrance, CA, USA).

Statistical analysis

Regression coefficients of the predictive model (Eq. 1) were obtained using the Statistica 10 software (Statsoft, Tulsa, USA). Due to the large inherent variability in bioprocesses which involve enzymes, regression coefficients for PC and PR in a confidence level above 90% were considered significant ($p < 0.1$). Non-significant terms were eliminated and the model was tested for adequacy and goodness of fit by an analysis of variance (ANOVA), evaluating the coefficient of determination (R^2) and the F-test. When the calculated F value is greater than the tabulated F value, the variation is explained by the regression and not by the residues, in which case the regression is significant and the model can be considered predictive. In addition, the degree of fit of the polynomial

model was evaluated from the mean deviation error (MDE) (Eq. 3).

$$MDE(\%) = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(V_E - V_P)^2} \qquad (3)$$

where V_E is the experimental value, V_P is the predicted value and N is the population of experimental data.

Results and discussion

Characterization of defatted okara flour

Defatted okara flour showed moisture content of $16.5 \pm 0.1\%$ (wet weight basis), protein content of $31.7 \pm 0.8\%$ (dry weight basis, dwb), fat content of $3.0 \pm 0.2\%$ (dwb), ash content of $5.3 \pm 0.3\%$ (dwb), total fiber content of $60.2 \pm 1.4\%$ (dwb, in which the insoluble and soluble fiber contents were 58.9 ± 1.6 and $1.4 \pm 0.1\%$, respectively). The particle size distribution of the defatted okara flour was determined by passing the material through sieves of different mesh sizes (28, 48, 60, 80, 100 and 150 mesh). The mass fractions retained between > 0.6, 0.3–0.6, 0.25–0.3, 0.18–0.25, 0.15–0.18, 0.106–0.15 and < 0.106 μm were 31.0, 22.9, 7.2, 4.4, 6.4, 11.8 and 16.4%, respectively.

Effect of the enzymatic pretreatment conditions of the okara on the extraction and obtaining of a protein concentrate

The experimental data for PC and PR were obtained using 11 combinations of the independent variables, as shown in Table 1. The PC and PR values ranged from 43.1 to 67.2 and 19.4 to 35.4%, respectively. Second order polynomial models were proposed to explain the PC and PR in terms of the encoded variables, as shown in Eqs. 4 and 5:

$$PC = 66.5 - 2.8\,T^2 - 6.7\,pH^2 - 4.7\,E^2 + 2.8\,T$$
$$\times pH + 2.5\,T \times E - 1.5\,pH \times E \qquad (4)$$

$$PR = 29.1 + 1.4\,T - 1.7\,pH^2 + 2.2\,E \qquad (5)$$

where T is the temperature (codified value) and E is the enzyme concentration (codified value).

The response for PC obtained a R^2 of 0.85 and the calculated F value F_c (7.23) was higher than the tabulated F value F_t (2.50). Moreover, for all the assays, the mean deviations between the predicted and observed PC values presented values below 10% (0.3–8.9%), with the exception of assay 11 (Table 1). Thus the model (Eq. 4) can be

considered predictive for the response of PC. On the other hand, for the response of PR, the coefficient of determination R^2 was only 0.63 and hence Eq. 5 could not be considered predictive and the response surface could not be obtained.

The response surfaces (Fig. 1) generated by the predictive model for the PC response (Eq. 4) express the interaction between two independent variables, with the third variable kept constant at the central point.

Figure 1a, c showed a quadratic trend, where the maximum PC was achieved at around 45 °C. Temperature is one of the factors that significantly affect the catalytic activity of enzymes. High temperatures increase the kinetic energy of the molecules in the system, resulting in a faster reaction. There is thus greater hydrolysis of the cell wall polysaccharides, and, as a consequence, a greater amount of protein is extracted. On the other hand, at temperatures above 45 °C, a significant decrease in PC was observed due to thermal denaturation of the enzyme. According to Lehninger and Cox (2006), high temperatures increase the potential chemical energy of enzymes, but higher values lead to denaturation and inactivation of the enzymes. This fact is due to the breakage of weak bonds that determine the enzyme structure. Gama et al. (2015) reported that Viscozyme retained a residual activity above 90% after 24 h incubation at up to 37 °C; however, the residual activity decreased to about 60% at 50 °C. Thus, at temperatures above 45 °C, due to reduced enzymatic hydrolysis of the okara cell wall, the release of proteins located inside the cells was impaired, decreasing the PC in the concentrate. Similar results were reported by Guan and Yao (2008) and by Rosset et al. (2014) in their studies with protein extraction from oat bran and defatted soybean flour, respectively. The enzymatic hydrolysis of cellobiose at different temperatures (40–70 °C) using β-1,4-glucosidase was inactivated at 70 °C in a short process time, as reported by Bravo et al. (2000).

The enzyme concentration (Fig. 1a, b) presented a negative quadratic influence on the response for PC, with an increase in PC up to around 65% at an enzyme concentration of 2.75%. By increasing the enzyme concentration, more molecules of enzyme become available to cleave the polysaccharide matrix of the cell wall, releasing more proteins. However, at enzyme concentrations above 2.75%, a decrease in PC was observed. This trend was also verified by Zhou et al. (2009), studying the enzymatic hydrolysis of corn stover by a mixture of cellulases, when all the enzyme concentrations were above certain values. The authors supposed that this result was due to competition for binding sites between the individual enzymes and the saturation of each site. On the other hand, Vergara-Barberán et al. (2015) stated that such behavior was probably due to competition-inhibition, in which the

Fig. 1 Influence of the independent variables on protein content: a enzyme concentration and temperature; b pH and enzyme concentration; c pH and temperature
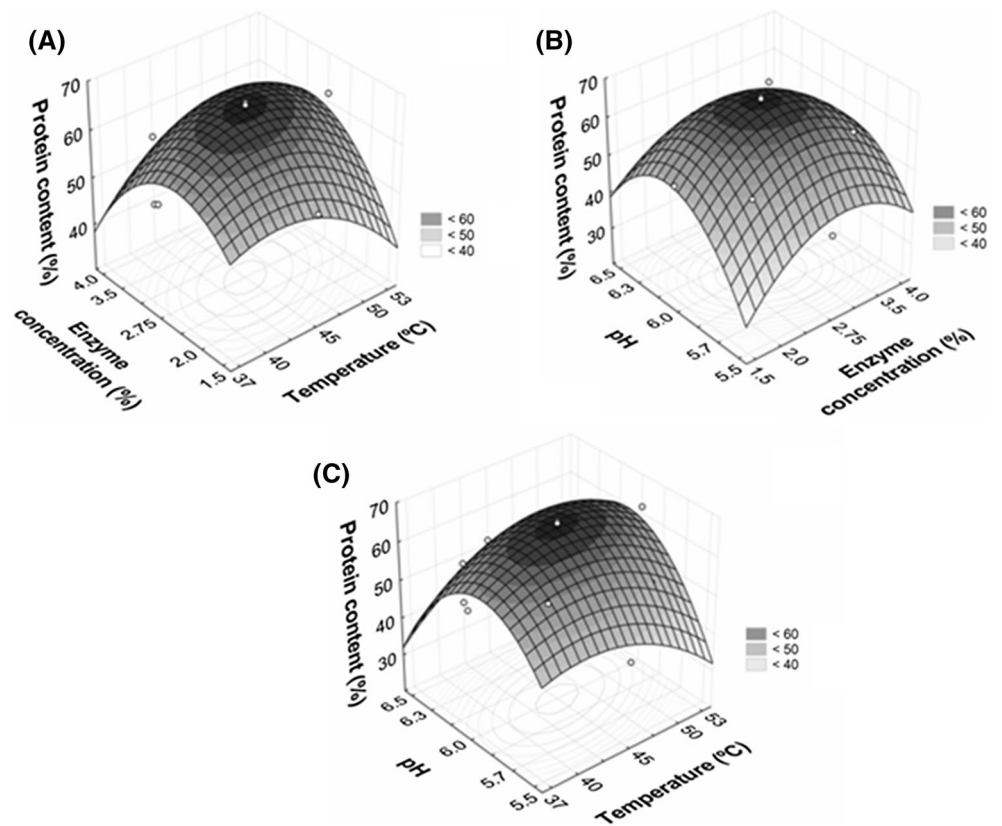
proteins extracted can act as inhibitors, thus reducing the enzyme activity.

The variable pH value also had a negative quadratic influence on the PC, indicating a maximum region close to the central point (Fig. 1b, c). This trend was expected, since it is well known that enzymes have an optimum pH range in which their activity is maximal. According to Lehninger and Cox (2006), the ionized protein side chains may play an essential role in the interactions that maintain the enzyme structure. Thus, pH changes can affect enzyme stability, causing an irreversible denaturation of their conformational structure (Whitaker 1994), and, as a consequence, there is a continuous loss of enzyme activity. The same behavior was reported by Guan and Yao (2008), studying the Viscozyme-assisted extraction of oat bran protein, in which a higher PR was reached at pH 4.6.

Since the coefficient of determination of the fitted model (Eq. 5) for the response of PR was low, the response surface cannot be obtained. However, it is possible to evaluate the influence of temperature, pH and enzyme concentration on this response, since such factors had a significant effect ($p < 0.1$). Analyzing Eq. 5, the effect of pH on PR showed the same behavior observed for the response of PC. Temperature and enzyme concentration both showed linear positive effects, indicating that the increase in these variables resulted in greater values for PR.

Optimization and model validation

The optimum conditions for the enzymatic pretreatment of okara were determined using the desirability function (Fig. 2), in order to achieve higher PC and PR values. The desirability parameters indicated that higher PR and PC values could be obtained at higher temperatures and enzyme concentrations, and with pH values between 6.0
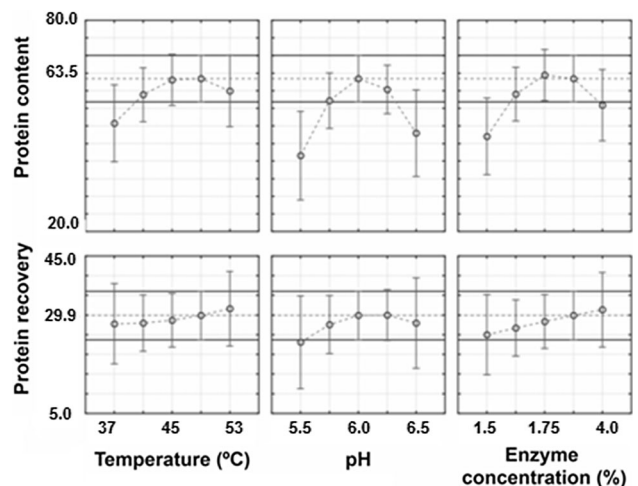

Fig. 2 Desirable parameters estimated for the variables of temperature T, pH and enzyme concentration for the enzymatic pretreatment of okara

and 6.3. Thus, combining all the optimum regions for both responses, a temperature of 53 °C, pH 6.2 and enzyme concentration of 4% can be recommended as the optimized condition. The temperature was consistent with the product manufacture sheet (Novozymes, Bagsvaerd, Denmark), in which the optimum temperature range is 25–55 °C. However, the optimum pH range for Viscozyme (3.3–5.5) differs from the value found in the present study. Similar behavior was reported by Rosenthal et al. (2001), evaluating protein extraction from soybean when the cell wall was degraded with cellulases. According to the authors, the lower protein extraction yield was probably due to the fact that the optimum pH value for the enzyme was close to the isoelectric point of the okara protein. Although the pH value was increased to a value of high protein solubility (pH 9) after the enzymatic pretreatment, it seems that part of the protein still remained insoluble at that pH, decreasing the PR (Rosenthal et al. 2001).

Validation tests were carried out under the optimum conditions to verify the adequacy of the predictive model (Eq. 4). According to this model, the predicted PC (51.4%) was close to the experimental response (56.0%). Therefore, the model was suitable for predicting the PC response, with an average relative error of 8.2%. Regarding the response for PR, an experimental value of 29.8% was observed. Rosset et al. (2014) investigated the effect of Viscozyme on protein extraction from defatted soy flour and the results indicated that the optimal temperature was higher (60 °C) than that found in the present study, whereas the optimum enzyme concentration (3%) was lower. This condition resulted in a protein concentrate with 59.6% of protein, which was close to the value obtained in the present study. On the other hand, on studying protein extraction from defatted rice bran using amylase, Celluclast and Viscozyme, Tang et al. (2003) obtained a product with only 28.5% of PC.

Comparing the results obtained for PR and PC under the optimum conditions and for the sample with no enzymatic pretreatment (control), the control sample presented a PC of 47.8% and PR of 15.1%. These values were lower than those found for the enzymatically pretreated sample, where the concentrate presented 56.0% of PC and recovered 28% of the okara proteins. Thus these results indicate that the use of the multi-enzyme complex Viscozyme is efficient and appropriate to improve protein extraction, increasing the PC and PR values by 17 and 86%, respectively, when compared with the control sample.

Most of the soybean proteins are found in the form of discrete cellular organelles called protein bodies in the cotyledon cells (Liu 1997; Rosenthal et al. 1996). In the production of soymilk, the aqueous soluble compounds were lixiviated to the soymilk, but a significant amount of protein remained in the residue (okara). Using confocal microscopy, Preece et al. (2015) verified that a certain proportion of the okara cells remained intact and protein bodies were also located within the cells. Since the wall which surrounds the cotyledon cells is constituted of cellulose, hemicellulose and lignin in addition to pectin (Liu 1997), hydrolytic enzymes such as cellulases, hemicellulases and pectinases play an important role in breaking the structure of the okara cell walls, making the structure more permeable.

In the present study, the okara presented a large amount of fiber (60.2% on a dry weight basis), of which 1.4 and 58.9% corresponded to the soluble and insoluble fractions, respectively. According to Redondo-Cuenca et al. (2008), the structures of fibers in soybeans and in okara are diverse and not well understood. However, by analyzing the monomeric compositions found in their study, the authors suggested the presence of the polysaccharides cellulose, xylan, galactan, arabinan and galacturonan. In addition, according to Rommi et al. (2015), the enzymatic hydrolysis of carbohydrate could reduce the water holding capacity of the samples, thereby facilitating protein recovery in the extract by solid–liquid separation.

Therefore, the multi-enzyme nature of Viscozyme, which contains cellulases and hemicellulases, including xylanase and endoglucanase, and also pectinases, such as arabanase and polygalacturonase, seemed to be advantageous in order to cleave the linkages of this complex and diverse polysaccharide matrix, and hence liberate more intercellular proteins.

Optical fluorescence microscopy

To verify the efficiency of the multi-enzyme complex Viscozyme on disrupting the okara cell wall, the presence of cellulose was detected using calcofluor and observed by epifluorescence microscopy. Calcofluor is a fluorochrome that marks the polysaccharides with β 1–3 and β 1–4 linkages, present in the whole cell wall (Hageage and Harrington 1984).

In the Online Resource 1A, before enzymatic pretreatment, plant tissue agglomerates can be seen with a magnification of 40×. In this case, the cell wall contour could not be observed, only the intense blue fluorescence corresponding to the calcofluor (360–400 nm). On the other hand, with a magnification of 100× (Online Resource 1B), the cell wall contour was highlighted by a blue fluorescence signal, showing polysaccharides present in the cell wall. This cell microstructure can be seen with autofluorescence using a red filter (560–595 nm) in Online Resource 1C.

After the enzymatic pretreatment of the okara, the cell walls were damaged and disrupted, but some areas highlighted by a blue fluorescence signal can still be seen,

showing partial enzymatic hydrolysis (Online Resource 1D). Two large agglomerates with intense blue fluorescence (indicated by arrowheads in Online Resource 1D) show the presence of intact cell wall. In this same enzymatically pretreated microstructure, another two areas (indicated by arrows) suffered little hydrolysis, and still retained their β 1,3–1,4 linkages. When the enzymatically pretreated okara was observed with autofluorescence (Online Resource 1E) differences in the intensity of the fluorescence in the degraded areas could be verified, but without the specificity of the calcofluor. Comparing the images obtained using calcofluor and autofluorescence, degradation of the cell wall by the action of the multienzyme complex was confirmed. Thus the enzymatic pretreatment was effective in disrupting the okara cell wall, enhancing protein extraction.

Evaluation of the protein concentrate

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Figure 3 shows the electrophoretic profiles of the okara protein and the protein concentrate. For both samples, two distinct bands with molecular weights of 20 and 37 kDa can be seen, corresponding to glycinin (11S globulin),
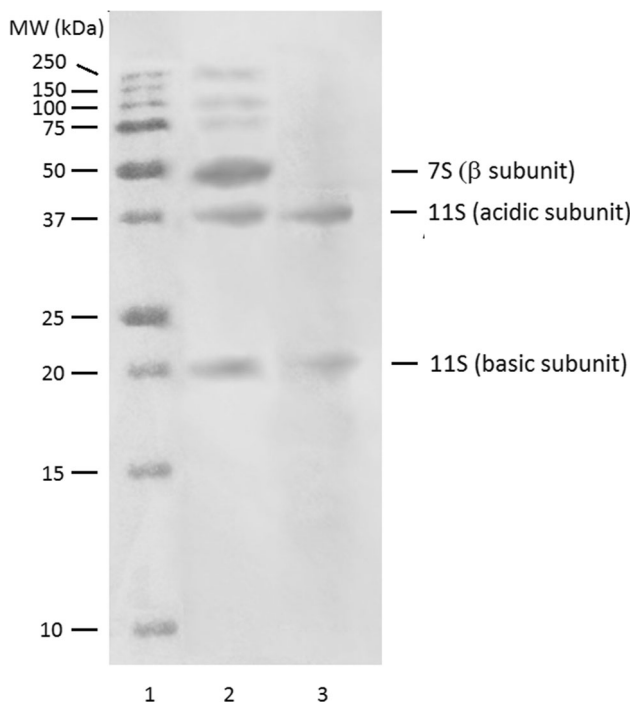

Fig. 3 SDS-PAGE profile of the proteins from the defatted okara flour (column 2) and protein concentrate (column 3) obtained under the optimum conditions and compared with the molecular weight (MW) markers (column 1)

which is composed of acidic and basic subunits with molecular weights (MW) between 36–40 and 18–20 kDa, respectively (Liu et al. 2007). β-conglycinin (7S globulin) and glycinin (11S globulin) are the main soybean proteins but the β-subunits of the 7S fraction (MW ∼ 50 kDa) were only found in the defatted okara flour. These results indicated that the 7S fraction was not extracted from the okara flour into the protein concentrate. Since the β-conglycinins are more soluble in salt solutions than the glycinins (Liu 1997) this fraction probably could not be extracted by the aqueous solution used in the current work. Moreover, some non-starch polysaccharides in the plant cell wall may not have been hydrolyzed by Viscozyme. Since the β-conglycinin fraction found in okara (Fig. 3) is tightly bound to the plasma membranes and cell walls (Stanojevic et al. 2012), the extraction of this protein fraction was impaired.

Total amino acids composition

As shown in Table 2, the protein concentrate had high concentrations of glutamic and aspartic acids. This result could be related to the SDS-Page profile (Fig. 3), which showed that the main protein found in the protein concentrate was glycinin. Yuan et al. (2009) reported that the isolated acidic and basic polypeptides of soy glycinin were rich in these amino acids.

The nutritional quality of a protein source can be evaluated from its essential amino acid score (AS). AS compares the levels of essential amino acids in the sample with those of a protein standard recommended by FAO/WHO (1991). Analyzing Table 2, there were no limiting amino acids (AS > 1, for all the amino acids) in the samples. In addition, the protein concentrate had a greater arginine content than that of whey protein isolate (Peña-Ramos et al. 2004). Since arginine is essential for the health of low birth weight infants (Wang et al. 1999), the protein concentrate of okara could be used as nutritional supplements. Kaushik et al. (2016) evaluated the nutritional quality of flaxseed protein by calculating the lysine to arginine ratio, in which lower values indicate that a protein has little atherogenic and cholesterolemic effects (Czarnecki and Kritchevsky 1992). The protein concentrate of okara exhibited a lysine to arginine ratio of 0.8 which is lower than to that reported by Peña-Ramos et al. (2004) for whey protein isolate (5.4). This result indicates that protein concentrate of okara may be a better protein for cardiac health than whey protein. Thus, the protein from okara could be used as ingredient for infant formula and formulations to enhance cardiac health.

Table 2 Total amino acid composition (g/100 g protein) of the protein concentrate obtained from okara under optimum conditions

Amino acid	Amino acid content (g/100 g protein)	FAO/WHO (1991)[1]	AS$_{concentrate}$
Non-essential			
Alanine	4.12 ± 0.01		
Arginine	7.06 ± 0.00		
Aspartic acid	11.50 ± 0.01		
Cysteine	0.84 ± 0.01		
Glutamic acid	21.24 ± 0.04		
Glycine	4.18 ± 0.00		
Proline	5.09 ± 0.01		
Serine	5.11 ± 0.00		
Tyrosine	3.87 ± 0.01		
Essential			
Histidine	2.29 ± 0.01	1.9	1.21
Isoleucine	5.42 ± 0.00	2.8	1.94
Leucine	7.60 ± 0.00	6.6	1.15
Lysine	5.81 ± 0.00	5.8	1.00
Methionine	1.77 ± 0.01	2.5[2]	1.04
Phenylalanine	5.52 ± 0.00	6.3[3]	1.49
Threonine	3.39 ± 0.00	3.4	1.00
Tryptophan	Nd		
Valine	5.17 ± 0.01	3.5	1.48
AAE	36.97		

The values represent the means of two determinations ± standard deviations. Where: Nd = not determined; AAE = total essential amino acid content and AS = the essential amino acid score = amino acid in test sample/reference FAO/WHO (1991)

[1]Reference FAO/WHO (1991) standard (essential amino acid for child of 2–5 years)

[2]Methionine + cystine

[3]Phenylalanine + tyrosine

Conclusion

The enzymatic pretreatment of okara with a multi-enzyme complex using the central composite rotatable design improved the extraction and obtaining the protein concentrate. The optimum condition was: 53 °C, pH 6.2 and 4.0% of enzyme. Under this condition, a protein concentrate with 56% of protein was obtained and 29.8% of the proteins were recovered from okara. An increases of 17 and 86% were found for the PC and PR, respectively, when compared with non-pretreated okara. Thus the enzymatic pretreatment with the multi-enzyme carbohydrase complex, Viscozyme, could be of interest to the food industry in order to enhance protein extraction from okara. In contrast to the chemical catalysis, the enzymatic process can also take place under mild conditions, resulting in lower energy consumption, without generating degradation products. Despite the high cost of the enzyme, the pretreatment using immobilized Viscozyme may be economically feasible for industrial usage, since the enzyme complex can be recovered for posterior reuse. This process is little reported in the literature and this is the first time that the hydrolysis conditions of this raw material were optimized.

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