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Optimization of enzymatic protein hydrolysis conditions of okara with endopeptidase Alcalase

[1]Montilha, M. S., [1]Sbroggio, M. F., [1]Figueiredo, V. R. G., [1]Ida, E. I. and [1,2*]Kurozawa, L. E.

[1]Department of Food Science and Technology, State University of Londrina, Rod. Celso Garcia Cid, PR 445, km 380, 86057-970, Londrina, PR, Brazil
[2]Department of Food Engineering, University of Campinas, R. Monteiro Lobato, 80, 13083-862, Campinas, SP, Brazil

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Abstract

The production of soymilk and tofu results in an insoluble residue called okara that present 23% of soybean protein. The objective of this study was to optimize the protein hydrolysis conditions of okara with Alcalase@ endopeptidase. A central composite rotational design was carried out to evaluate the influence of enzymatic reaction temperature (40 - 70°C), enzyme:substrate ratio (1.0 - 10.0%, g enzyme/100 g protein) and pH (7.0 - 9.0) on the degree of hydrolysis (DH). Kinetic of reaction curves (DH versus time) were characterized by high initial reaction rates followed by decreases in the reaction rate up to the stationary phase. By the response surface methodology, the process of hydrolysis was optimized in order to get higher values of DH. The results showed a quadratic dependence of DH in respect to all independent variables. The optimum condition of enzymatic hydrolysis was 55°C, enzyme:substrate ratio of 8.8% and pH 9.0. Under this condition, a experimental DH of 37.3% was obtained and the predicted model was validated. In addition, the pretreatment of okara using ultrasound was evaluated, aiming to increase the DH. There was significant difference ($p < 0.1$) on DH value (38.8%) obtained at optimum condition, increasing 4%.

Introduction

Soybean *(Glycine max)* is an oilseed, whose Brazilian production has been growing continuously in recent decades, reaching 86.7 million ton in 2014 (FAO, 2016), due to the demand for human consumption, production of oil and biodiesel, and animal feed. Cultivated especially in the Midwest and South regions of Brazil, soybean is an important crop of national agriculture because its sales have important participation on Brazilian trade balance, with annual negotiations above US$ 20 billion (IBGE, 2011; MAPA, 2013). In addition to economic importance, soybean has great nutritional value, because it is an important source of vegetable protein and contains functional components, such as phospholipids, antioxidants, oligosaccharides, phytosterols and isoflavones (Liu, 1997).

From the soybean, various derivative products have been developed such as misso, shoyu, tempeh, natto, soymilk, tofu, soy flour, toasted flour soybean and soybean sprouts. The production of soymilk and tofu results in an insoluble byproduct called okara with little market value and usually used as animal feed. In this process, about one kilogram of okara is generated from every kilogram of soybeans (Khare *et al*., 1995). Thus, substantial quantities of this by-product that contains about 23% of soybean proteins (O'Toole, 1999; Liu, 2008), are underutilized or discarded, being an industrial problem due to expensive treatment and destination.

In order to utilize the nutrients normally discarded in okara, to reduce treatment of residues and to be competitive, the soymilk and tofu industries must develop new products and/or ingredients. Thus, the protein hydrolysis of okara with enzymes could be an alternative to obtain value-added products. Proteins hydrolysis is used to improve or modify the chemical, functional and sensory properties of protein without affecting its nutritional value (González-Tello *et a*l., 1994). Protein hydrolysates, especially di- and tripeptides, have greater nutritional value and more efficient gastrointestinal absorption compared to intact protein and free amino acids (Bhaskar *et al.*, 2007). In addition, the protein hydrolysis can release biologically active peptides, defined as short sequences of amino acids (from 2 to 20 units) that exert physiological benefits on the organism. The bioactivity of peptides can be described by their antimicrobial, anticancer, immuno-modulating, antithrombotic, antioxidant, or antihypertensive properties (Clare and Swaisgood, 2000).

*Corresponding author.
Email: *louisek@unicamp.br*

Table 1. Experimental design for optimization of the protein enzymatic hydrolysis conditions of okara

Assay	Independent variables			Dependent variable		
	T (°C) X_1 (x_1)	E/S (%) X_2 (x_2)	pH X_3 (x_3)	DH (%) Experimental	DH (%) Predicted**	E (%)***
1	46 (-1)	2.8 (-1)	7.4 (-1)	16.05	15.73	2.0
2	64 (+1)	2.8 (-1)	7.4 (-1)	13.68	15.73	15.0
3	46 (-1)	8.2 (+1)	7.4 (-1)	22.1	22.85	3.4
4	64 (+1)	8.2 (+1)	7.4 (-1)	22.8	22.85	0.2
5	46 (-1)	2.8 (-1)	8.6 (+1)	19.56	19.47	0.5
6	64 (+1)	2.8 (-1)	8.6 (+1)	20.87	19.47	6.7
7	46 (-1)	8.2 (+1)	8.6 (+1)	30.89	32.99	6.8
8	64 (+1)	8.2 (+1)	8.6 (+1)	37.5	32.99	12.0
9	40 (-1.68)	5.5 (0)	8.0 (0)	21.21	21.02	0.9
10	70 (+1.68)	5.5 (0)	8.0 (0)	20.33	21.02	3.4
11	55 (0)	1.0 (-1.68)	8.0 (0)	7.77	7.47	3.9
12	55 (0)	10.0 (+1.68)	8.0 (0)	24.01	24.80	3.3
13	55 (0)	5.5 (0)	7.0 (-1.68)	27.41	25.74	6.1
14	55 (0)	5.5 (0)	9.0 (+1.68)	35.24	37.40	6.1
15	55 (0)	5.5 (0)	8.0 (0)	23.32	25.27	8.4
16	55 (0)	5.5 (0)	8.0 (0)	28.81	25.27	12.3
17	55 (0)	5.5 (0)	8.0 (0)	23.78	25.27	6.3

*The independent variables correspond to the real values (X). Values enclosed in parentheses correspond to the coded values (x). T is temperature (°C) and E/S is enzyme:substrate ratio (%).
**DH values predicted by polynomial model (Eq. 6).
***E (%) is root mean square error (Eq. 5) between experimental and predicted values.

According to Adler-Nissen (1985), it is necessary to control the reaction hydrolysis variables, such as the concentration and specificity of protease, temperature, pH, time and nature of substrate. Knowledge of the critical factors and kinetic of the processes is important, because the optimization of process parameters is essential to develop an economical and optimal process. The effect of variables and the type of enzyme on protein hydrolysis has been studied for several protein sources, such as chickpeas, protein concentrate fish, milk whey, chicken breast, soybeans, and seeds of Jatropha curcas (Clement *et al.*, 1999; Nilsang *et al.*, 2005; Cheison *et al.*, 2007; Kurozawa *et al.*, 2008; Hu *et al.*, 2010; Selanon *et al.*, 2014). Currently, several commercial proteases are available for the production of protein hydrolysates. In food protein hydrolysis, the endopeptidase Alcalase@ is generally used. However, no information is available for the protein hydrolysis of okara using this enzyme.

In addition, several studies have reported that soybean proteins are generally resistant to hydrolysis due to compact tertiary and quaternary structures that protect the peptide bonds (Govindaraju and Srinivas, 2007; Tsumura *et al.,* 2004). Moreover, the protein bodies are located within cells of okara (Preece *et al.*, 2015). Thus, pretreatment of samples is necessary to change the structural characteristics of soybean protein and to disrupt the cell wall matrix, increasing the accessibility of enzyme to peptides bonds. Ultrasound is a technology that has been applied to modify the structure of proteins by the effects of cavitation and shear. The use of ultrasound-assisted pretreatment to increase the efficiency of protein hydrolysis was reported by Chen *et al.* (2011) and Uluko *et al.* (2014).

The objective of this study was to optimize the protein hydrolysis conditions of okara with endopeptidase Alcalase@ applying a central composite design followed by ultrasound pre-treatment of sample in order to increase the efficiency of hydrolysis carried out at optimum condition.

Materials and Methods

Material

Okara was acquired from Cocamar Cooperativa Agroindustrial industry (Maringá, Brazil) and stored in a freezing chamber at -18°C. The main characteristics of okara were obtained according to AOAC (2006). Alcalase@ 2.4 L (Novozymes, Bagsvaerd, Denmark), which is a serine endopeptidase obtained from Bacillus licheniformis, with a declared activity of 2.4 AU/g, was used for enzymatic hydrolysis. The other reagents used were of analytical grade.

Experimental design to evaluate and optimize the protein enzymatic hydrolysis conditions of okara

To evaluate and optimize the protein hydrolysis conditions of okara by endopeptidase, a rotatable central composite design 23 was carried out. Three independent variables were investigated: temperature (°C, X_1), enzyme:substract ratio (X_2) and pH (X_3). Table 1 shows the experimental design with coded and real independent variables. Five levels of

each variable were chosen for the trials, including the central point and two axial points, giving 17 experiments that were conducted randomly. The degree of hydrolysis (DH) of okara protein was selected as dependent variables and calculated according to Adler-Nissen (1986). Thus, for each experiment, 75 g of okara were thawed overnight and homogenized in a beaker with 150 mL of distilled water. The mixture was then kept under agitation by a magnetic stirrer, heated by a thermostatic circulator bath (TE-2005 model, Tecnal, Piracicaba, Brazil) until desired temperature (X1) and the pH (X_3) was adjusted with NaOH 1N. The enzyme was added to the mixture at a given concentration (X_2). The hydrolysis was monitored by titrating NaOH 1N continuously in order to maintain constant pH. The volume of NaOH consumed was recorded at regular intervals of time until the end of protein hydrolysis (no change in pH) and used to calculate the degree of hydrolysis (DH). Total time of experiments varied from 1 to 3 h, depending on experimental design assay. To verify the effect of independent variables, the maximum DH values reached for each experiment were used in experimental design. Resulting slurry was cooled in an ice bath and centrifuged 5,228xg (Centrifuge 5804R, Eppendorf, Hamburg, Germany) at 4°C for 20 minutes to separate the lipid and non-hydrolyzed protein fraction (precipitate) from protein hydrolysate (supernatant).

Measurement of degree of hydrolysis

Degree of hydrolysis, defined as the percent ratio between the numbers of peptide bonds cleaved (h) and the total number of bonds available for proteolytic hydrolysis (h_{total}), was calculated according to Adler-Nissen (1986) (Eq. 1).

$$DH(\%) = \frac{h}{h_{total}} \times 100 = \frac{B \times N_b}{M_{prot} \times \alpha \times h_{total}} \times 100 \quad (1)$$

Where: B is consumption of NaOH (mL) to keep the pH constant during the hydrolysis; N_b is normality of NaOH titrant; M_{prot} is total mass of protein (g) in the mixture; h_{total} is total number of peptide bonds in the protein substrate (8 moles equiv/kg) and α is average degree of dissociation of the α-NH_2 groups expressed as:

$$\alpha = \frac{1}{1+10^{pK-pH}} \quad (2)$$

The pK value varies significantly with temperature T (K):

$$pK = 7.8 + \frac{298 - T}{298 \times T} \times 2400 \quad (3)$$

Ultrasound pretreatment of okara

After establishing and optimizing the protein hydrolysis condition of okara, the ultrasound pretreatment of okara was performed in order to evaluate the effect on the DH. Thus, 75 g of defrosted okara were dispersed with 150 mL of distilled water. An ultrasonic water bath (40 kHz frequency, power 135W, USC 1400, Unique, Indaiatuba, Brazil) was employed to sonicate the dispersion during 15 minutes. The subsequent enzymatic hydrolysis under optimum condition of hydrolysis was carried out immediately.

Statistical analysis

Experimental data were fitted to a polynomial equation (Eq. 4).

$$DH = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (4)$$

Where DH is the dependent variable; β_0, is the constant regression coefficient; β_1, β_2, and β_3 are the linear regression coefficients; β_{11}, β_{22}, and β_{33} are the quadratic regression coefficients; β_{12}, β_{13} and β_{23} are the cross-product regression coefficients; x_1, x_2, and x_3 represent the coded values of the independent variables (temperature, enzyme:substrate ratio and pH, respectively).

To obtain the regression coefficients, an analysis of variance (ANOVA) was carried out using the Statistica 9.0 (Statsoft, Tulsa, USA) software package. Only variables with a confidence level above 90% ($p \leq 0.10$) were considered significant. The degree of fitness of polynomial model was evaluated by the coefficient of determination (R^2) and root mean square error (E):

$$E(\%) = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(V_E - V_P)^2} \quad (5)$$

Where V_E is the experimental value, V_P is the predicted value and N is the population of experimental data. Response surface methodology (RSM) was used to obtain the best optimum hydrolysis condition assessed by maximum DH. At optimum condition, validation test was performed to verify the adequacy of the polynomial model (Eq. 4). The effect of ultrasound pretreatment on DH was analyzed by the ANOVA and Tukey test with a confidence level above 90% ($p \leq 0.1$), using the Statistica 9.0.

Results and Discussion

Chemical composition of okara

The main characteristics of okara, obtained according to AOAC (1995), were: moisture content of 76.7±0.7% (wet basis), carbohydrate content

Table 2. Analysis of variance for degree of hydrolysis

Source	*SS*	*DF*	*MS*	F_{calc}	F_{tab}
Regression	812.0	6	135.3	23.02	2.46
Residual:	58.8	10	5.9		
- Lack of fit	40.3	8	5.0		
- Pure error	18.5	2	9.3		
Total	870.8	16			

SS is sum of squares, DF is degree of freedom, MS is mean square, Fcal is calculated F distribution value, Ftab is tabulated F distribution value ($p \leq 0.1$)

of 62.2±3.8% (dry basis, d.b.), protein content of 26.2±1.9% (d.b.), fat content of 6.4±0.5% (d.b.) and ash content of 5.2±0.9% (d.b.).

Kinetics of protein enzymatic hydrolysis of the okara

The overall rate of protein hydrolysis of okara by DH was evaluated, because the control of hydrolysis is necessary to design and optimize the batch bioreactors and to predict the extension of protein bond cleavage (Camacho *et al.*, 1998). Kinetics curves of protein hydrolysis of okara at several reaction conditions (17 assays) are represented in Figure 1. The curves presented a high initial reaction rates followed by decreases up to the stationary phase. This kinetic profile curve can be related by several factors, such as, scarcity of peptides bonds capable of being cleaved, competition between original substrate and hydrolysis products and enzyme denaturation that decrease its activity (Adler-Nissen, 1985; González-Tello *et al*., 1994; Guerard *et al*., 2002). Guerard *et al*. (2002) and Demirhan *et al.* (2011) verified the availability of peptides bonds and competitive inhibition by hydrolysis products by adding fresh substrate or hydrolyzed protein to the hydrolysis reactor after a determined reaction time, respectively. The mechanisms of protein hydrolysis using Alcalase@ were also evaluated by Valencia *et al.* (2014). These authors reported a strong product inhibition, decreasing the reaction rate during hydrolysis; however, substrate exhaustion did not explain this behavior, and thermal inactivation of enzyme had an insignificant effect on the hydrolysis. Similar hydrolysis curves were reported for several protein substrates, such as Jatropha curcas seed cake (Selanon *et al*., 2014), milk protein concentrate (Uluko *et al.*, 2014), peanut (Jamdar *et al*., 2010), chicken breast (Kurozawa *et al*., 2008 and haemoglobine (Chang *et al*., 2007).

Optimization of the protein enzymatic hydrolysis condition of okara

The experimental degree of hydrolysis (DH) of okara were obtained using 17 combinations of the independent variables X_1 (temperature), X_2 (enzyme:substrate ratio) and X_3 (pH), as shown in Table 1. After exclusion of non-significant terms ($p > 0.1$), the results from the experimental design were fitted to Equation 4. Second-order polynomial model was proposed to predict DH in terms of the encoded variables:

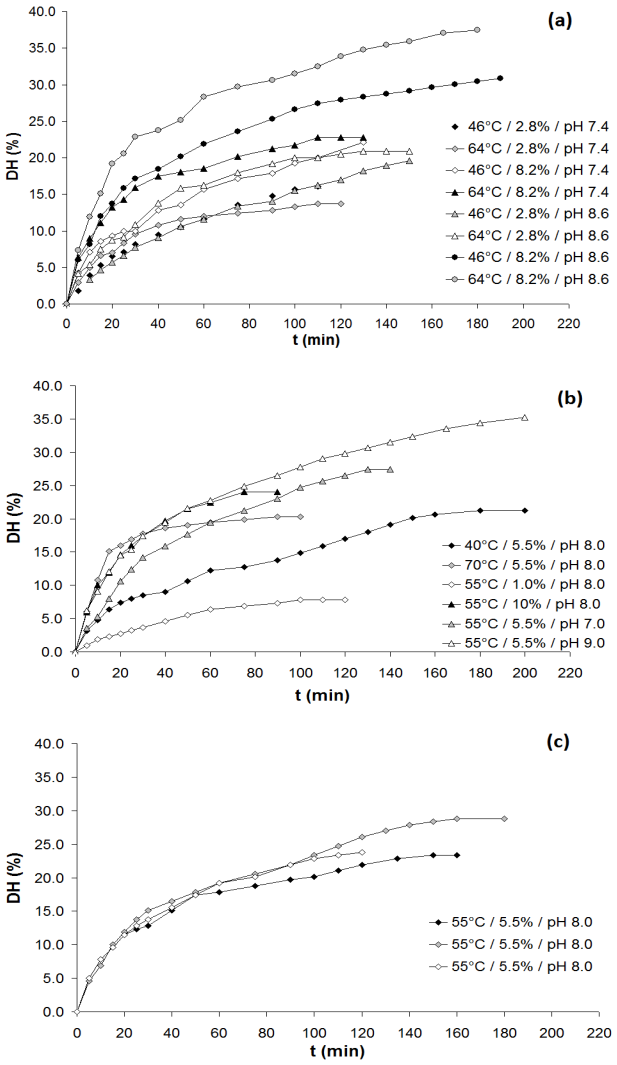

Figure 1. Protein hydrolysis kinetics of okara: (a) fatorial experiments (assays 1 – 8), (b) axial experiments (assays 9 – 14). (c) central experiments (assays 15 – 17). The lines are provided as a visual guide only

$$DH = 25.3 - 1.5x_1^2 + 5.2x_2 - 3.2x_2^2 + 3.5x_3 + 2.2x_3^2 + 1.6x_2 \times x_3 \quad (6)$$

Where DH is the degree of hydrolysis (%), x1, x2 and

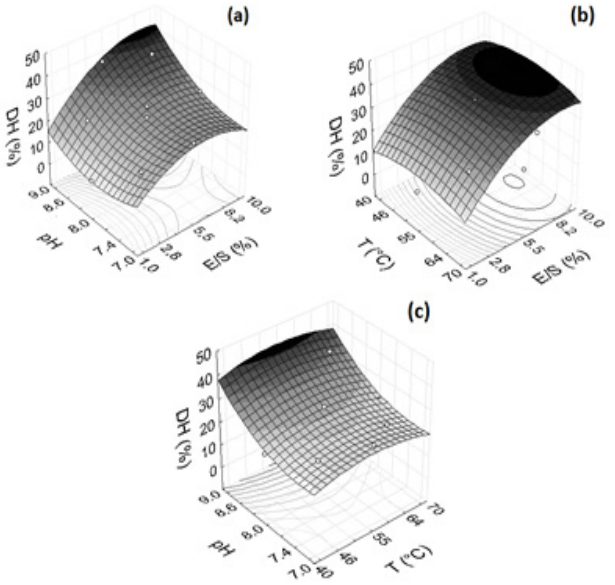

Figure 2. Influence of independent variables on degree of hydrolysis: (a) enzyme:substrate ratio E/S versus pH, at 55°C; (b) temperature T versus enzyme:substrate ratio E/S, at pH 9.0; (c) temperature versus pH, at enzyme:substrate ratio of 8.8%

x3 are the coded temperature, enzyme:substrate ratio and pH, respectively.

This model was tested for adequacy and goodness of fit by analysis of variance ANOVA (Table 2). Calculated F value (23.02) was greater than tabulated F value (2.46). The coefficient of determination R^2 of the adjusted model was 0.932, showing that the model explains 93.2% of the total deviation of experimental data. Therefore, equation 6 can be considered predicted DH for protein enzymatic hydrolysis of okara.

Optimization of the protein hydrolysis of okara was carried out using Response Surface Methodology for the maximum DH. Figure 2 shows the response surfaces generated by the proposed model (Eq. 6). Figure 2(a) express the interaction between X_3 (pH) and X_2 (enzyme:substrate ratio), in which the variable X_1 (temperature) was maintained constant at the central point (55°C). The greater DH values were found at pH 9.0 and higher enzyme concentrations. Thus, Figure 2(b), which shows the effect of X_1 (temperature) and X_2 (enzyme:substrate ratio) on DH, was obtained at constant pH 9.0. Analyzing this figure, the maximum values of DH were achieved at 55°C and 8.8% of enzyme:substrate ratio. Based on this result, Figure 2(c) shows the interaction between X_1 (temperature) and X3 (pH) and the X_2 (enzyme:substrate ratio) was constant at 8.8%. Therefore, combining all the optimal regions for the three situations, a temperature of 55°C, enzyme:substrate ratio of 8.8% and pH of 9.0 can be recommended as the optimized of protein enzymatic hydrolysis condition of okara.

A quadratic and negative effect can be observed between temperature and DH (Figures 2b and 2c), with an increase on the response up to around 40% at 55°C, followed by its decreasing. At higher temperatures, a thermal denaturation of the enzyme occurs, reducing its activity and, as consequence, the DH. Several authors reported this same behavior (Ng *et al*., 2013; Hu *et al*., 2010; Vaštag *et al*., 2010). Camacho *et al.* (1998) evaluated the positive effect of temperature (50, 60 and 70°C) on kinetic of hydrolysis kh and kinetic denaturation kd constants for whey protein. These findings indicated that both enzymatic hydrolysis and enzyme denaturation enhanced with temperature. However, by determining the overall rate of hydrolysis kh/kd ratio, the authors concluded that the maximum value occurred at 60°C instead of 70°C.

The enzyme:substrate ratio showed a positive effect on hydrolysis, that is, the increase on this parameter resulted in higher DH (Figures 2a and 2b). By increasing enzyme concentration, there are more active available enzyme to cleavage peptides bonds. However, for enzyme concentration superior 8.8%, a stationary area of DH could be noticed. According to Diniz and Martin (1996), probably this fact occurred due to enzyme inhibition and the possibility that the protease hydrolyzes itself. Peričin *et al*. (2009) investigated the hydrolysis with enzymes of protein isolate from hull-less pumpkin oil cake. The authors verified that DH increased with enzyme concentration, but for concentration above 0.13% (v/v), DH decreased. Same profile was observed by Bhaskar *et al*. (2007) working with sheep viscera mass. The authors reported that enzyme concentrations of 1.0% and 1.5% were significantly higher than 0.5%; however, between 1.0 % and 1.5%, there was no significant difference on DH.

Concerning pH variable, higher DH values were obtained at higher pH values when a great enzyme concentration was used. The stability of an enzyme is affected by alterations in pH, causing irreversible denaturation of its conformational structure and loss of enzyme activity (Whitaker, 1994).

Model validation and effect of ultrasound pretreatment of okara on protein hydrolysis at optimum condition

Validation tests (Figure 3) were performed under optimum condition to determine the adequacy of the polynomial model (Eq. 6). According to this model, the predicted result for DH (42.1%) obtained under the optimum condition was close to the experimental response observed (37.3%). Therefore, considering

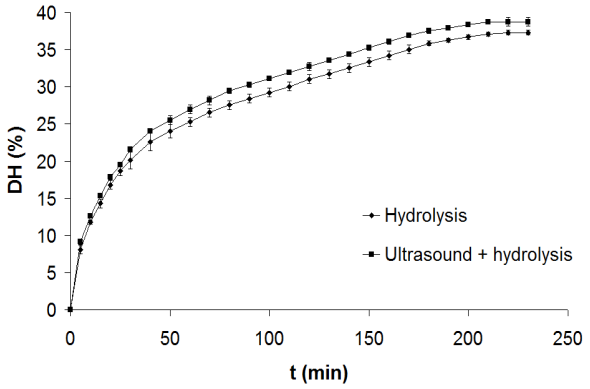

Figure 3. Validation tests (degree of hydrolysis DH versus time t) at optimal condition of protein enzymatic hydrolysis of okara

the hydrolysis process nature, a relative error between the experimental mean and model predict values, in the range of 10% to 15%, is reasonable for optimization purposes. In this case, the relative error was 13%, indicating that the obtained model was adequate to predict the DH of protein hydrolysates of okara.

In order to increase the efficiency of protein hydrolysis of okara, an ultrasound-assisted pretreatment of okara was carried out. The effect of ultrasound on DH is showed in Figure 3. It should be noted that a little enhanced of hydrolysis was observed for okara pretreated compared with sample without pretreatment. The final DH of okara pretreated with ultrasound was 38.8 ± 0.3%. A significant difference ($p < 0.1$) was observed when compared with DH for sample without ultrasound pretreatment (DH of 37.3 ± 0.6%). Other authors reported the same behavior observed in this work. Chen *et al.* (2011) verified an enhancement of hydrolysis for soy protein isolate (SPI) pretreated with ultrasound (200 - 600 W) when compared with SPI without pretreatment. In addition, in this same work, SDS-PAGE showed that ultrasound pretreatment could significantly improve the accessibility of some subunits (R-7S and A-11S) in soy proteins to papain hydrolysis, resulting in changes in DH.

Due to the effects of cavitation and shear, high intensity ultrasound treatment can change protein conformation by affecting hydrogen bonds and hydrophobic interactions, and disrupting the quaternary and/or tertiary structure of globular proteins. As results, there is an increasing on accessibility of protease to peptides bonds, because more hydrolysis sites are then exposed (Chen *et al*., 2011; Jambrak *et al*., 2009). Thus, the use of ultrasound as a pretreatment of sample might be a promising way to increase enzymatic protein hydrolysis. However, in this work, a slight increase of 4% on DH value was obtained. Thus, more studies are necessary aiming to improve more this objective. The influence of type of ultrasound (direct - with ultrasound probe, or indirect - with ultrasound water bath), power, time, and temperature, could be studied more detailed to obtain higher DH values for protein okara.

Conclusion

Temperature of 55°C, enzyme:substrate ratio of 8.8% and pH of 9.0 were considered as the optimum enzymatic protein hydrolysis condition, on the basis of degree of hydrolysis DH. Under this condition, the DH was 37.3%. The polynomial model obtained in this work was adequate to predict the DH of protein hydrolysates of okara by endopetidase Alcalase@. Ultrasound pretreatment of okara before enzymatic hydrolysis enhanced the DH. Optimization of process parameters is essential to develop an economical and optimal process. Protein hydrolysis of okara by protease presented as a potential process to recovery protein as hydrolysate form. Protein hydrolysate can be source for protein supplementation, antioxidant component in food systems and diets due to to high biological value peptides.

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