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PRODUCTION OF PEPTIDES WITH RADICAL SCAVENGING ACTIVITY AND RECOVERY OF TOTAL CAROTENOIDS USING ENZYMATIC PROTEIN HYDROLYSIS OF SHRIMP WASTE

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ABSTRACT

The enzymatic hydrolysis of shrimp waste by the commercial protease Alcalase was evaluated as an alternative for recovery of valuable components, protein and astaxanthin. The influence of temperature (40–70C) and enzyme : substrate ratio (2.0–6.0%, g enzyme/100 g protein) on degree of hydrolysis, recovery of protein and antioxidant activity of hydrolysate was studied. The total carotenoids content in each insoluble fraction obtained after centrifugation of raw hydrolysate were also determined. All variables had significant effect on the responses. To obtain maximum antioxidant activity of hydrolysate and recovery of protein and total carotenoids in sediment, the following optimum conditions are suggested, 55C and 4.0%. Under these conditions, degree of hydrolysis, antioxidant activity and recovery of protein and total carotenoids were determined, obtaining values of 3.63%, 66.8%, 39.53 μmol Trolox equivalents/g protein and 72.67%, respectively.

PRACTICAL APPLICATIONS

Shrimp wastes, removed during processing, represent up to 50% of the weight of raw material. These discards are normally used to obtaining low-value products, such as animal feed and fertilizers. Since they contain valuable components such as protein, chitin and astaxanthin carotenoid, significant amounts of nutrients are underused. Thus, better utilization of these discards through the manufacture of value-added products can result in expansion of aquaculture industry, maximization of economical benefits and reduction of environmental pollution. Enzymatic hydrolysis of shrimp waste represents a potencial process for recovery of total carotenoids in the insoluble fraction and obtaining a hydrolysate that can be used as protein supplementation and/or antioxidant component in food systems.

INTRODUCTION

According to the Food and Agriculture Organization of the United Nations (FAO 2014), the shrimp global production in 2012 was over 7.5 million tons. In the last 10 years (2002–2012), Brazilian shrimp production has increased approximately 30%, reaching almost 115.4 thousand tons. Shrimp meat is very appreciated by local consumers and is commercialized fresh and frozen. Generally, cephalothorax and exoskeleton of shrimps are removed during processing, representing up to 50% of the weight of raw material. These discards contain valuable components such as protein, chitin and astaxanthin carotenoid (Heu *et al.* 2003), and they are normally used to obtaining low-value products, such as animal feed and fertilizers. Thus, these by-products could be transformed into feed ingredients for the expanding aquaculture industry, thereby maximizing economical benefits and reducing of environmental pollution. Several proposals for recovery of protein and amino acids, astaxanthin and chitin from shrimp have been undertaken, in which protein enzymatic hydrolysis was effective, such as reported by Sila *et al.* (2014), Dey and Dora (2014), Babu *et al.* (2008), Holanda and Netto (2006) and Gildeberg and Stenberg (2001). However, little information is available on the enzymatic hydrolysis of shrimp meat, considering yield from the

process, compounds recovered and product quality to optimize this process.

Therefore, the protein hydrolysis of shrimp waste could be an alternative solution to obtain value-added products, such as protein hydrolysate and astaxanthin.

Protein hydrolysis can be accomplished using enzymes, acids or alkali, but enzymatic hydrolysis is strongly preferred over chemical methods for the production of hydrolysates for food applications. The proteolytic enzymes are used to dissolve or break down the meat protein, resulting in two distinguishable fractions, the soluble and the insoluble fractions. The soluble fraction contains the hydrolyzed protein with well-defined peptide profiles and a low fat content. The protein hydrolysates may be used as flavor enhancers, functional ingredients or simply as nutritional additives to foods of low protein quality. Moreover, enzymatic hydrolysis can release biologically active peptides, which can be 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). Protein hydrolysates from various sources have strong antioxidant activity, such as shrimp (Cao *et al.* 2009; Sila *et al.* 2014), brownstripe red snapper (Khantaphant *et al.* 2011) and loach (You *et al.* 2009).

After enzymatic hydrolysis, a large fraction of the astaxanthin in the shrimp waste can be recovered in the sediment after centrifugation of the crude protein hydrolysate (Gildeberg and Stenberg 2001). Astaxanthin, the main carotenoid found in crustaceans, is a red fat-soluble pigment which has been used as food colorant in animal and fish feed. This compound can prevent or reduce risk of various disorders in humans and animals and exerts physiological benefits when consumed such as anti-inflammation, antidiabetic activity, cardiovascular disease prevention, anticancer activity and immuno-modulation (Ambati *et al.* 2014). Moreover, astaxanthin has higher antioxidant activity when compared to several carotenoids such as lutein, lycopene, α-carotene and β-carotene as reported by Naguib *et al.* (2000). The use of astaxanthin as a nutritional supplement has been rapidly growing in foods, feeds, nutraceuticals and pharmaceuticals. Thus, major efforts to improve astaxanthin production from biological sources instead of synthetic have been promoted.

The objective of the present work was to optimize the reaction conditions (temperature and enzyme : substrate ratio) on the enzymatic hydrolysis of shrimp cephalothorax. Specifics objectives were: (1) to obtain the protein enzymatic hydrolysis kinetic curves at several reaction conditions, and (2) to model the influence of the reaction conditions on the degree of hydrolysis, recovery of protein, antioxidant activity of hydrolysate and recovery of total carotenoids in insoluble fraction.

MATERIAL AND METHODS

Material

Fresh shrimp *Farfantepenaeus subtilis* cephalothorax was purchased from a fish market located at Angra dos Reis, RJ, Brazil, on July 2011. The sample was stored in a cold chamber at −18C and thawed according to the quantity required to produce the hydrolysate. The composition of the shrimp cephalothorax, obtained according to AOAC (1995), was: moisture content of 77.7 ± 0.1% (wet basis), protein content of 44.4 ± 4.0% (dry basis), fat content of 43.1 ± 5.1% (dry basis) and ash content of 12.5 ± 1.0% (dry basis). Despite these data, chemical composition of shrimp could be affected by some factors, such as seasonal and size variability (Donaldson 1976; Hopkins *et al.* 1993; Rødde *et al.* 2008).

For the enzymatic hydrolysis, the commercial protease Alcalase 2.4L (Novozymes, Bagsvaerd, Denmark) kindly supplied by Tovani Benzaquen Ingredientes (Campinas, Brazil) was used. This enzyme, a serine endopeptidase obtained from *Bacillus licheniformis* with a declared activity of 2.4 AU/g, was used.

Enzymatic Hydrolysis

Hydrolysis experiments were carried out according to the pH-stat procedure, as described by Adler-Nissen (1986) with some modifications. Samples (75 g) were defrosted overnight, ground in a food processor and homogenized with 150 g of distilled water. The mixture was then kept under agitation and heated by a magnetic stirrer until desired temperature, with pH adjusted to 8.0 using NaOH 2 N. Alcalase was added to the mixture and the reaction pH maintained constant by the continuous addition of NaOH 2 N. The amount of alkali consumed was recorded at regular intervals until no pH variation was observed. All tests were performed under different conditions regarding temperature (T, 40–70C) and enzyme : substrate ratio (E/S, 2.0–6.0%, g enzyme/100 g protein), according to Table 1. Total time for hydrolysis varied from 50 to 100 min, depending on experimental run conditions. The resulting slurry was centrifuged at 3,500 rpm for 15 min to separate the total carotenoids, lipid and nonhydrolyzed protein (precipitate) from protein hydrolysate (supernatant). Mass of precipitate and supernatant were taken to calculate recovery of protein and total carotenoids.

Experimental Design

A central composite rotatable design (CCRD) with two variables was used to evaluate the influence of the independent variables temperature and enzyme : substrate ratio on the degree of hydrolysis (DH), antioxidant activity of protein hydrolysate (AA) and recovery of protein in hydrolysate

TABLE 1. EXPERIMENTAL DESIGN FOR THE DEGREE OF HYDROLYSIS (*DH*, %), RECOVERY OF PROTEIN (*PR*, %), ANTIOXIDANT ACTIVITY OF PROTEIN HYDROLYSATE (*AA*, μMOL TROLOX EQUIVALENTS/G PROTEIN) AND RECOVERY OF TOTAL CAROTENOIDS IN PRECIPITATE (*CAR*, %) FROM SHRIMP WASTE PROTEOLYSIS USING ALCALASE

	Independent variables				Dependent variables			
	Real		Coded					
Design point	*T* (C)	*E/S* (%)	*T*	*E/S*	*DH*	*PR*	*AA*	*CAR*
1	44	2.6	−1	−1	3.0	57.0 ± 1.5	24.7 ± 0.4	94.1 ± 8.7
2	66	2.6	1	−1	2.9	59.9 ± 2.3	39.1 ± 0.9	38.2 ± 3.7
3	44	5.4	−1	1	3.6	53.8 ± 0.6	40.2 ± 0.9	74.5 ± 8.2
4	66	5.4	1	1	3.1	60.4 ± 2.8	33.3 ± 0.8	79.2 ± 9.8
5	40	4.0	−1.41	0	4.3	52.2 ± 0.6	32.8 ± 0.8	–
6	70	4.0	1.41	0	3.0	51.0 ± 2.1	28.4 ± 1.0	–
7	55	2.0	0	−1.41	3.2	65.4 ± 1.1	36.8 ± 1.7	–
8	55	6.0	0	1.41	2.4	68.4 ± 1.5	39.2 ± 1.5	–
9	55	4.0	0	0	3.5	62.7 ± 1.0	37.3 ± 1.7	69.8 ± 12.6
10	55	4.0	0	0	3.7	69.0 ± 1.6	41.8 ± 1.5	71.3 ± 7.1
11	55	4.0	0	0	3.7	68.7 ± 1.3	39.5 ± 0.7	76.9 ± 18.4

T is temperature and *E/S* is enzyme : substrate ratio.

(*PR*) and total carotenoids in precipitate (*CAR*) as shown in Table 1.

It was assumed that a mathematical function φ existed for the response variable Y in terms of the independent variables:

$$Y=\varphi(T,E/S)=\beta_0+\beta_1\times T+\beta_2\times E/S+\beta_{11}\times T^2+\beta_{22}\times E/S^2+\beta_{12}\times T\times E/S \quad (1)$$

where β_0, β_1, β_2, β_{11}, β_{22} and β_{12} are the constant, linear, quadratic and cross product regression coefficients of the model, and T and E/S represent the coded independent variables.

For *CAR* response, the analysis of the results indicated that the model is linear and it is not necessary to expand the screening design for CCRD, with addition of axial experiments (points 5 to 8).

Degree of Hydrolysis

The *DH*, defined as the percent ratio between the number of peptide bonds cleaved (h) and the total number of bonds available for proteolytic hydrolysis (h_{total}) (Adler-Nissen 1986), was obtained by the pH-stat method and calculated according to Eq. (2).

$$DH(\%)=\frac{h}{h_{total}}\times 100=\frac{B\times N_b}{M_{prot}\times\alpha\times h_{total}}\times 100 \quad (2)$$

where B is the NaOH consumption (mL) to keep the pH constant during the reaction; N_b is the normality of the base; M_{prot} is the mass of the protein (g, $N\times 6.5$); h_{total} is the total number of peptide bonds in the protein substrate (7.7 mEq/g protein, according to Holanda and Netto (2006) for shrimp *Xiphopenaus kroyeri* waste) and α is the degree of dissociation of the α-NH_2 groups expressed as:

$$\alpha=\frac{1}{1+10^{pK-pH}} \quad (3)$$

The pK value varies significantly with temperature T (K) and can be estimated according to Eq. (4).

$$pK=7.8+\frac{298-T}{298\times T}\times 2400 \quad (4)$$

Recovery of Protein in Hydrolysate

The *PR*, or the ratio of the mass of protein in the hydrolysate to that in the original substrate, was used as an index of protein dissolution and calculated by Eq. (5).

$$PR\,(\%)=\frac{MP_s}{MP}=\frac{MP_s}{MP_s+MP_p}=\frac{x_sM_s}{x_sM_s+x_pM_p}\times 100 \quad (5)$$

where MP is the mass of protein present in the original substrate (g); MP_s is the mass of protein present in the supernatant (g); MP_p is the mass of protein present in the precipitate (g); x_s is the protein content in the supernatant (g protein/100 g supernatant); x_p is the protein content in the precipitate (g protein/100 g precipitate); M_s is the mass of supernatant (g); M_p is the mass of precipitate (g).

The protein contents in the supernatant and substrate were determined using the Kjeldahl method (AOAC 1995).

2,2-Diphenyl-1-picryl hydrazyl Radical Scavenging Activity Assay

2,2-Diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity was determined in triplicate by the method of

Brand-Williams *et al.* (1995) with modification. Aliquots of 0.1 mL of protein hydrolysate were mixed with 3.9 mL of 6×10^{-5} M ethanolic solution of DPPH (Sigma Chemical Co., St. Louis, MO), followed by vortex agitation. The reaction was carried out in the dark, at room temperature for 60 min and filtered using a Millipore (Billerica, MA) 0.22 μm syringe filter. The absorbance of the solution was recorded at 517 nm using an UV spectrophotometer (Model NEW 2000, São Paulo, Brazil). A standard curve was constructed using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma Chemical Co., St. Louis, MO) ethanolic solutions in dilutions varying from 0 to 2,000 μM. The activity was expressed as μmol Trolox equivalents (TE)/g protein.

Recovery of Total Carotenoid in Precipitate

The ratio of the mass of carotenoids in the precipitate to that in the original substrate (24.0 ± 2.2 μg/g original substrate) was used as an index of recovery of total carotenoids (*CAR*) in the insoluble fraction. Total carotenoids content was determined according to Rodriguez-Amaya (2001) in triplicate.

Statistical Analysis

To obtain the regression coefficients, an analysis of variance (ANOVA) was carried out using Statistica 5.0 (Statsoft, Tulsa, OK) software package. Only variables with a confidence level above 95% ($P < 0.05$) were considered as significant.

RESULTS AND DISCUSSION

Enzymatic Hydrolysis Kinetics

From a practical point of view, it is interesting to control the hydrolysis process, analyzing the overall rate of hydrolysis in terms of degree of hydrolysis (*DH*). From these results, it is possible to design and optimize batch bioreactors and to predict the extension of protein bond cleavage (Camacho *et al.* 1998).

Figure 1 shows the enzymatic hydrolysis kinetics curves of shrimp cephalothorax at several reaction conditions. The curves were characterized by high initial reaction rates followed by decreases in the reaction rate up to the stationary phase. In Fig. 1a, the curves related to *E/S* of 2.6% exhibited a rapid cleavage of peptide bonds in the first 10–15 min of hydrolysis, followed by stabilization at a *DH* of 3%. Conversely, for protein hydrolysis carried out at *E/S* of 5.4%, the initial reaction rates extended until 30–40 min, followed by their decrease until *DH* constant of 3.6 and 3.1% at 44 and 66C, respectively. In Fig. 1b, total time of protein hydrolysis, except for hydrolysis at 55C and *E/S* of 4.0%, were higher than 60 min, reaching until 100 min. At 55C, higher *DH* values were observed for *E/S* of 4%, in which maximum *DH* reached was 3.5%. At *E/S* of 4%, the influence of temperature on protein hydrolysis kinetics is clearly observed in Fig. 1b, in which lower temperatures resulted in higher *DH* values.

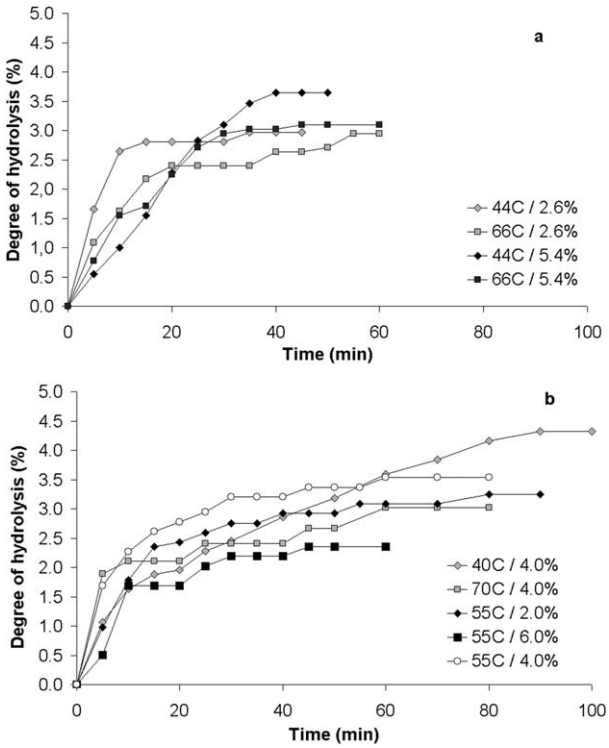

FIG. 1. ENZYMATIC PROTEIN HYDROLYSIS KINETICS OF SHRIMP CEPHALOTHORAX AT: (A) TEMPERATURES OF 44 AND 66C AND ENZYME : SUBSTRATE RATIOS OF 2.6 AND 5.4% RELATED TO FATORIAL EXPERIMENTS, (B) TEMPERATURE OF 40, 55 AND 70C AND ENZYME : SUBSTRATE RATIO OF 2, 4 AND 6% RELATED TO AXIAL AND CENTRAL EXPERIMENTS
The lines are provided as a visual guide only.

The curves shapes are in accordance with the ones given for the hydrolysis of protein from different substrates, including shrimp waste (Holanda and Netto 2006; Sila *et al.* 2014), chicken breast (Kurozawa *et al.* 2008) and tuna waste (Guerard *et al.* 2001, 2002). According to Camacho *et al.* (1998), this decrease in the overall hydrolysis rate may be caused by several factors, such as: (i) scarcity of peptides bonds capable of being cleaved, (ii) enzyme inhibition by hydrolysis product and (iii) inactivation of proteases. These mechanisms were evaluated in several works (Guerard *et al.* 2001, 2002; Demirhan *et al.* 2011) by adding fresh substrate or hydrolyzed protein to the hydrolysis reactor after a determined reaction time to verify the availability of peptides bonds and competitive inhibition by hydrolysis products, respectively. To identify the mechanism of the enzymatic hydrolysis of proteins, Valencia *et al.* (2014) studied the

TABLE 2. ANALYSIS OF VARIANCE FOR THE RESPONSES

	Source	*SS*	*DF*	*MS*	F_c	F_t
Degree of hydrolysis	Regression	1.75	2	0.88	9.91	4.46
	Residual:	0.71	8	0.09		
	Lack of fit	0.68	6	0.11		
	Pure error	0.03	2	0.01		
	Total	2.46	10		$R^2 = 0.712$	
Protein recovery	Regression	383.10	1	383.10	54.91	5.12
	Residual:	62.79	9	6.98		
	Lack of fit	37.62	7	5.37		
	Pure error	25.18	2	12.59		
	Total	445.90	10		$R^2 = 0.859$	
Antioxidant activity	Regression	225.49	2	112.75	14.35	4.46
	Residual:	62.85	8	7.86		
	Lack of fit	53.04	6	8.84		
	Pure error	9.81	2	4.91		
	Total	288.34	10		$R^2 = 0.782$	
Recovery of total carotenoids	Regression	1687.94	3	562.65	55.63	9.28
	Residual:	30.34	3	10.11		
	Lack of fit	2.33	1	2.33		
	Pure error	28.01	2	14.00		
	Total	1718.28	6		$R^2 = 0.982$	

SS, sum of squares; *DF*, degrees of freedom; *MS*, mean square; F_c, calculated *F* distribution value; F_t, tabulated *F* distribution value ($P \leq 0.05$).

effect of substrate, product and thermal inactivation using the enzyme Alcalase and salmon muscle protein as the substrate. The authors verified that substrate exhaustion did not explain the progressive decrease in the reaction rate, while the thermal inactivation of enzyme had an insignificant effect on the hydrolysis. Conversely, a strong product inhibition was observed as low values of the inhibition constants were found.

Central Composite Rotatable Design

The experimental data were obtained using 11 combinations of the independent variables temperature and enzyme : substrate ratio, as shown in Table 1. The results from the experimental design were fitted to a second-order regression model at function of *T* and *E*/*S* (Eq. 1). The models were tested for adequacy and goodness-of-fit by the ANOVA without considering the non-significant terms ($P \geq 0.05$), as shown in Table 2. When F_c value is bigger than F_t value, the variation is explained by the regression and not by the residue. Thus, the regression is significant and the model can be considered predictive.

Second-order polynomial models, valid for *F. subtilis* shrimp cephalothorax, were proposed to explain the degree of hydrolysis (*DH*), antioxidant activity of hydrolysate (*AA*) and recovery of protein (*PR*) and total carotenoids (*CAR*) in terms of the encoded variables, as shown in Eqs. 6–9:

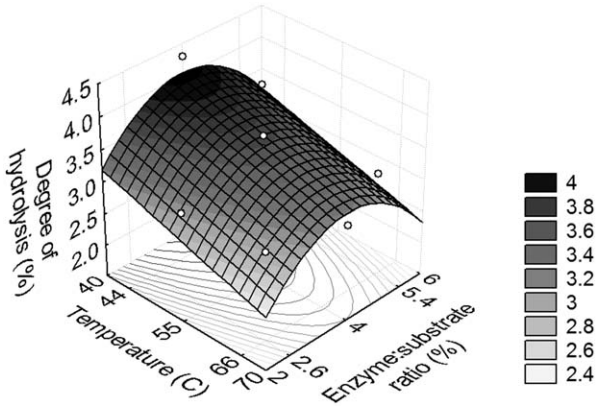

FIG. 2. INFLUENCE OF THE TEMPERATURE AND ENZYME : SUBSTRATE RATIO ON THE DEGREE OF HYDROLYSIS

$$DH = 3.61 - 0.29 \times T - 0.42 \times E/S^2 \quad (6)$$

$$PR = 66.52 - 7.91 \times E/S \quad (7)$$

$$AA = 38.84 - 4.26 \times T^2 - 5.34T \times E/S \quad (8)$$

$$CAR = 72.00 - 12.80T + 5.35E/S + 15.15T \times E/S \quad (9)$$

where *T* is the temperature and *E/S* is the enzyme : substrate ratio (codified values).

The coefficients of determination R^2 of the adjusted model were 0.712, 0.859, 0.782 and 0.982 for the degree of hydrolysis, antioxidant activity and recovery of protein and total carotenoids, respectively, indicating that the models explain 71.2, 78.2, 85.9 and 98.2% of the total variation.

Figures 2, 3, 5 show the response surfaces generated by the proposed models. These figures express the interaction between the two independent variables.

Degree of Hydrolysis. According to Table 1, *DH* varied from 2.4 to 4.3%. These values were inferior to results obtained for protein shrimp hydrolyzed with Alcalase and reported by other authors, such as Sila *et al.* (2014) (13%), Cao *et al.* (2009) (6.11–27.09%) and Holanda and Netto (2006) (15.5%). This fact could be due to the differences in shrimp species, reaction conditions and presence of inhibitory substances. Dey and Dora (2014) evaluated the effect of temperature (50, 55 and 60C), pH (7, 8 and 9), enzyme/substrate ratio (0.1, 1.05 and 2.0%) and time (30, 60 and 90 min) on *DH*. The high range of *DH* (2.3–32.9%) found by the authors was due to different combinations of reaction conditions.

In Fig. 2, a negative linear effect between temperature and *DH* was observed, probably due to thermal denaturation of the protease structure, reducing its enzymatic activity. In respect to the influence of enzyme : substrate ratio, a quadratic trend can be seen between this variable and the *DH*, with an increase in the response up to approximately 4 g

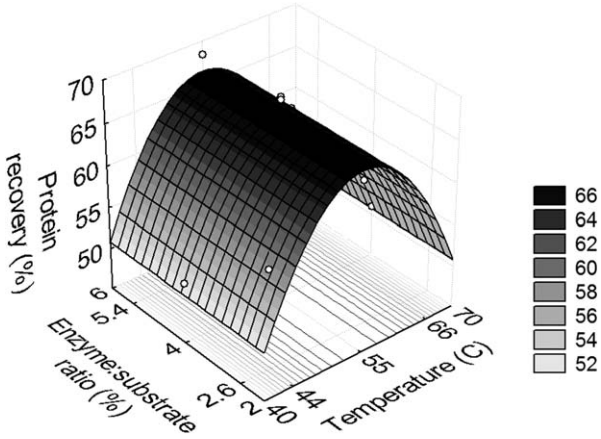

FIG. 3. INFLUENCE OF THE TEMPERATURE AND ENZYME : SUBSTRATE RATIO ON THE PROTEIN RECOVERY

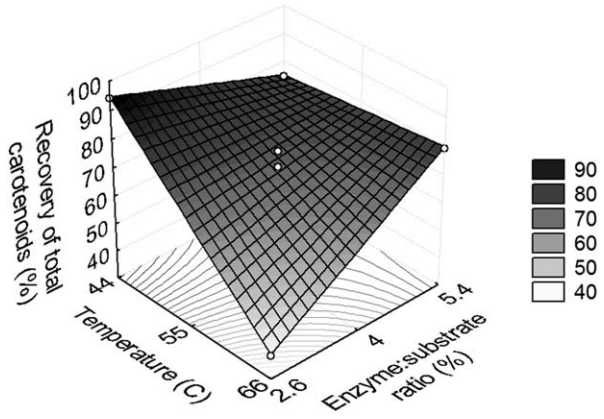

FIG. 5. INFLUENCE OF THE TEMPERATURE AND ENZYME : SUBSTRATE RATIO ON THE RECOVERY OF TOTAL CAROTENOIDS

enzyme/100 g protein. At higher enzyme concentrations, there are more molecules of enzymes available per molecules of substrates, resulting in greater cleavage of the peptide bonds. However, at enzyme concentration above 4%, this independent variable had a negative effect on *DH*. Bhaskar *et al.* (2007) verified that the *DH* at an enzyme concentration of 1.0 and 1.5% was significantly higher than 0.5%; however, between 1.0 and 1.5%, there was no significant difference on *DH*. The authors concluded that an enzyme substrate ratio of 1.0% was the optimum concentration for hydrolysis of sheep viscera mass. According to Diniz and Martin (1996), probably this fact occurred due to enzyme inhibition and the possibility that the enzyme hydrolyzes itself.

Recovery of Protein in Hydrolysate. Recovery of protein varied from 52.2 to 68.7% (Table 1), demonstrating that enzymatic hydrolysis is effective in recovering protein from raw material, with little loss of this substances to the insoluble fraction. These values were similar to the ones obtained for shrimp protein sources hydrolyzed with Alcalase as

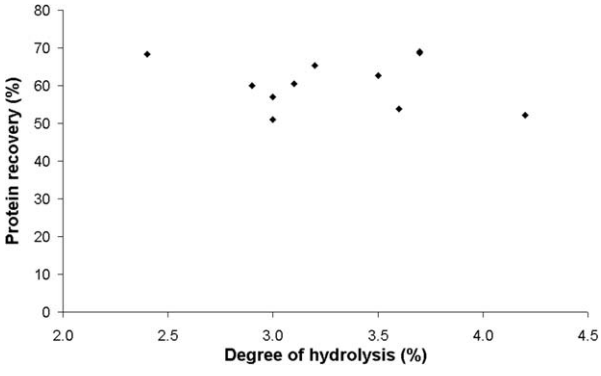

FIG. 4. RELATIONSHIP BETWEEN THE RESPONSES FOR DEGREE OF HYDROLYSIS AND PROTEIN RECOVERY

reported by Dey and Dora (2014) (60%), Holanda and Netto (2006) (49.53–59.50%) and Gildeberg and Stenberg (2001) (68.5%). Analyzing Fig. 3, only temperature had a significant effect on recovery of protein: increasing temperature up to 55C resulted in higher *PR* values.

Some authors reported a relationship between *DH* and *PR*, in which an increase in protein recovery is achieved by increasing the degree of hydrolysis. Dey and Dora (2014) evaluated several proteases on recovery of protein, which was higher for shrimp waste hydrolyzed by protease used in the current work. According to these authors, this result was due to the highest *DH* value obtained by Alcalase when compared with other proteases. Studying enzymatic hydrolysis of chicken breast meat, Kurozawa *et al.* (2009) verified an increase in *PR* was achieved by increasing the *DH* up to 30%. According to these authors, enzymatic hydrolysis is characterized by high initial reaction rates, when there are more active sites available for hydrolysis and lower concentration of soluble peptides in competition with the substrate. As a consequence, a great amount of bonds are broken (high *DH* values), resulting in a greater protein dissolution (high *PR* values). In this same work, these authors verified that *DH* values above 30% did not result in further dissolution of the protein in the meat and greater cleavage of the soluble peptide bonds, thus the *PR* value stabilized. Similar results were found by Holanda and Netto (2006), who studied the enzymatic hydrolysis of shrimp processing waste using two types of enzyme : Alcalase and pancreatin. Analyzing Fig. 4, in the present study this behavior was not observed, indicating no dissolution of the protein in the supernatant, only cleavage of the soluble peptide bonds.

Recovery of Total Carotenoids in Precipitate. In this study, carotenoids were recovered from shrimp waste after enzymatic hydrolysis and centrifugation. The carotenoids content in precipitate varied from 29.3 to 54.1 μg/g

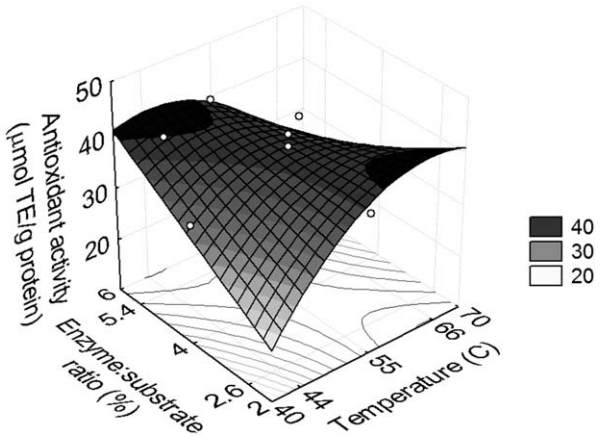

FIG. 6. INFLUENCE OF THE TEMPERATURE AND ENZYME : SUBSTRATE RATIO ON THE ANTIOXIDANT ACTIVITY

precipitate, according to the screening design. In respect to the recovery of total carotenoids in precipitate, the values varied between 38.2 and 94.1% (Table 1), these were similar to those reported by Babu *et al.* (2008).

Analyzing Fig. 5, the effect of temperature on recovery of total carotenoids in insoluble fraction was significant. Lower temperature caused higher yield of this component. This behavior can be related to degree of hydrolysis. According to Fig. 2, decreases in temperature resulted in increases on *DH*. The high carotenoids extraction observed with increase in hydrolysis extension could be due to a desestabilization of the complex between carotenoids and high density lipoprotein denoted carotenoprotein (Lakshman and Okoh 1993). Similar behavior was observed by Holanda and Netto (2006), who obtained astaxanthin concentrations in insoluble fraction of 51.5 and 56.7 μm/g from shrimp hydrolyzed with *DH* of 6 and 12%, respectively. Moreover, as this compound is highly thermo-sensitive, high temperature could favor its degradation.

Armenta and Guerrero-Legarreta (2009) evaluated the enzymatic hydrolysis of fermented shrimp and reported that the most efficient proteolysis using commercial enzyme Savinase was obtained with 15 proteolytic units, 24 h and pH 8.0, producing up to 48 mg/g total carotenoids. An interesting result obtained by these authors showed that a combination of protease with lipase was used for hydrolyzing the carotenoprotein complex. This treatment increased up to 38 and 200% total carotenoid content, as compared to the treatment with protease solely and to the control (no enzyme added). According to the authors, as a red-orange color increases as it is separated from the protein moiety, a relative protein-free carotenoids produced by hydrolysis of carotenoproteins may be of interest for commercial applications such as salmon culture and as ingredient of natural origin.

Antioxidant Activity. Antioxidant capacity of protein hydrolysate was evaluated by DPPH radical scavenging activity assay. DPPH is a stable free radical, which can be reduced by a proton-donating compound such as an antioxidant. The antioxidant activity varied from 24.7 to 41.8 μmol TE/g (Table 2). These values are in agreement with those reported for peptides from brownstripe red snapper (Khantaphant *et al.* 2011). Figure 6 shows the influence of enzyme : substrate ratio and temperature on the antioxidant activity of shrimp protein hydrolysate. A quadratic trend can be seen between the temperature and the antioxidant activity, with an increase in the response up to approximately 64 and 44C (at 2 and 6% enzyme concentration, respectively). At lower temperature (below 55C), antioxidant activity increased with enzyme : substrate ratio. However, for temperatures above 55C, an opposite behavior was observed. This fact occurred due to the significance of the interaction factor temperature × enzyme concentration.

Some authors found a relationship between *DH* and *AA*. Khantaphant *et al.* (2011) reported the increase in DPPH radical scavenging activity as the *DH* of hydrolysate from muscle of brownstripe red snapper using Flavourzyme, Alcalase and pyloric caeca protease increased from 20 to 40%. Similar behavior was obtained by Batista *et al.* (2010), evaluating black scabbardfich by-products hydrolysates using Protamex enzyme. As *DH* is defined as the percent ratio of the number of peptides bonds cleaved during the hydrolysis reaction to the total number of bonds (Adler-Nissen 1986), the hydrolysate with high *DH* is believed to contain larger amount of low molecular weight peptides than the hydrolysate with low *DH*. Therefore, this behavior could indicate that *DH* can greatly affect the *AA* of peptides, and that smaller peptides have higher *AA* values than larger peptides.

Nevertheless, comparing Figs. 2 and 6, it is not possible to establish a relationship between them, indicating that the *DH* did not appear as the major criterion in the production of hydrolysate with antioxidant activity. Klompong *et al.* (2007) reported that protein hydrolysate prepared from yellow stripe using Alcalase showed decreasing DPPH radical scavenging activity as *DH* increased, but no differences were observed for Flavourzyme hydrolysates. According to Chabeaud *et al.* (2009), the intrinsic characteristics of peptides in the expression of antioxidant capacity also play a significant role. Byun *et al.* (2009) speculated that the Leu-Gly and Gly-Pro sequence, obtained from hydrolysis of rotifer, may play an important role in radical scavenging activity. Rajapakse *et al.* (2005) reported that 67% of the *AA* of giant squid muscle hydrolysate was attributed to cationic and hydrophobic peptides. Byun *et al.* (2009) verified that amino acid composition, sequence and positioning of hydrophobic amino acid leucine at the N-terminus of peptide sequences induced by protein source and enzyme seem to be important for antioxidant activity.

Moreover, in this study, AA can be also due to the carotenoids present in hydrolysate, which presents antioxidant capacity. Antioxidant activity of astaxanthin, one of the dominant carotenoid in crustaceans, has been reported to be 10-fold stronger than that of other carotenoids, namely, zeaxanthin, lutein, canthaxanthin and β-carotene (Miki, 1991). After enzymatic reaction, the resulting slurry was centrifuged to separate the carotenoids, lipid and nonhydrolyzed protein (precipitate) from protein hydrolysate (supernatant). Despite the affinity between carotenoids and the lipid phase (precipitate), a fraction could be leached to the aqueous phase (hydrolysate).

Optimization of Protein Enzymatic Hydrolysis and Validation of Models

Optimization of the protein enzymatic hydrolysis of shrimp waste was carried out using Response Surface Methodology for the maximum values of *PR*, *AA* and *CAR*. Combining all the optimal regions of the Figs. (3 and 5) and 6, a temperature of 55C and enzyme : substrate ratio of 4.0 g enzyme/100 g protein was selected as the optimal condition for the responses. Under this condition, the predicted results for *DH*, *PR*, *AA* and *CAR*, obtained by polynomial models (Eqs. (6–9)), are 3.61, 66.52, 38.84 and 72.00, respectively. Because the optimum condition did not differ from assays 9 to 11 (Table 1), these tests were used to validate and to verify the adequacy of the polynomial models. Thus, analyzing Table 1, the experimental mean values of *DH*, *PR*, *AA* and *CAR* were 3.63, $66.80 \pm 0.30\%$, 39.53 ± 0.53 μmol TE/g protein and $72.67 \pm 5.65\%$, respectively. These values were close to the predicted responses. Therefore, there was a good fit between the predicted results and the experimental responses, with an average relative error of 0.6%, 0.4%, 1.7% and 0.9% for the *DH*, *PR*, *AA* and *CAR*, respectively.

CONCLUSIONS

The enzymatic hydrolysis of shrimp cephalothorax showed the influence of the process variables temperature and enzyme : substrate ratio on the responses of degree of hydrolysis (*DH*), recovery of protein (*PR*), antioxidant activity of hydrolysate (*AA*) and recovery of total carotenoids on sediment (*CAR*). To obtain maximum *PR*, *AA* and *CAR*, the following optimum condition is suggested: 55C and 4.0 g enzyme/100 g protein. At this condition, 66.8 and 72.7% protein and carotenoids present in raw material are recovered in the hydrolysate and precipitate, respectively. Under this condition, the hydrolysate presents antioxidant activity of 39.5 μmol TE/g protein. Enzymatic hydrolysis of shrimp waste was presented as a potencial process to recovery carotenoids in the insoluble fraction and obtain a hydrolysate that can be sources for protein supplementation and/or antioxidant component in food systems.

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