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Full Length Research Paper

Optimization of peptide production by enzymatic hydrolysis of tuna dark muscle by-product using commercial proteases

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A protein hydrolysate was prepared from proteins of tuna dark muscle by-product. The hydrolysis conditions (time, temperature, pH and enzyme concentration) using Alcalase and Neutrase were optimized by response surface methodology (RSM). The regression coefficient close to 1.0, observed during experimental and validation runs, indicated the validity of the model. The hydrolysate produced under the optimum conditions determined by RSM using Alcalase, had a low rate of peptide fraction of molecular weight of 1-4 kDa. Meanwhile, the results obtained by hydrolysis under optimal conditions determined by a complementary study (temperature 55°C, time 60 min, 1% enzyme concentration and pH 8.5) show that the hydrolysate produced had a high rate of the peptide fraction of molecular weight of 1-4 kDa. The amino acid composition of the protein hydrolysate prepared proved to have the potential for application as an ingredient in balanced fish diets and as a source of nitrogen in microbial growth media.

Key words: Alcalase, neutrase, tuna dark muscle, RSM, optimization, protein hydrolysate, peptides, degree of hydrolysis.

INTRODUCTION

Each year, more than 132 million tones of fish are caught which 29.5% are processed into fishmeal (FAO, 2006) and almost 50% of the amounts converted was dismissed without any attempt to recovery or valorization. The by-products are considered as waste valueless despite their intrinsic characteristics (Kristinsson and Rasco, 2000). Although some co-products of the fish industry such as shrimp waste are used in various applications, an enormous amount of these by-products was still released into the environment without proper treatment causing pollution problems (Bhaskar et al., 2008a).

The total amounts of global tuna catch were about 3 billion tons per year (FAO, 2006). Processing industry and canned tuna generates large amount of solid waste that can reach 50 to 70% of the raw material. Considering the limited biological resources and increasing environmental pollution, there is a great need to find a solution for better management and use of by-products generated.

Traditionally, a great amount of by-products was converted into fish meal by combined methods of cooking, separation of insoluble compounds and concentration followed by dehydration. Traditional methods use often the endogenous enzymes for the production of fish protein hydrolysate. However, such methods have the limitation of requiring more time for the completion of the process and depending on the temperature, the degree of hydrolysis varies between 20 and 70% (Espe and Lied, 1999). Thus, taking into account several factors such as fish species, seasonality, the type and amount of enzymes,

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the control of autolysis by the endogenous enzymes is quite difficult (Sikorski and Naczk, 1981). In this context, the controlled enzymatic hydrolysis is an alternative approach for recovery of the biomass of marine origin. The choice of type of enzymes, the source of proteins and study of the parameters of enzymatic hydrolysis process (temperature, pH, duration of hydrolysis, enzyme concentration) will provide the ability to control the process of hydrolysis and the characteristics of the product obtained. The soluble fish protein hydrolysate (FPH) obtained can be subjected to dehydration in view to obtain a stable powder with high protein content (Bhaskar and Mahendrakar, 2008b; Liaset et al., 2000)

In particular, the enzymatic hydrolysis can be used to obtain hydrolysates enriched in peptides and amino acids (Vidotti et al., 2003). Thus, the hydrolysate obtained can be used in many applications such as the search for bioactive peptides. Recent studies have shown that the hydrolysis of by-products by alkali or neutral proteases under controlled conditions produces hydrolysates containing peptides similar to hormones and growth factors (Guérard et al., 2001; Nilsang et al., 2005; Rousseau et al., 2001; Vidotti et al., 2003). However, other studies show that protein hydrolysates of many fish such as capelin (Shahidi et al., 1995), Pacific whiting (Benjakul and Morrissey, 1997) and cuttlefish (Balti et al., 2010) have nutritional and functional properties.

Proteolytic enzymes from plants and microorganisms have been considered most suitable for producing protein hydrolysate of fish (Benjakul and Morrissey, 1997; Guérard et al., 2001; Nilsang et al., 2005; Shahidi et al., 1995). For a technical and economic aspect, the microbial enzymes such as Alcalase were reported to be the most effective for the production of fish protein hydrolysate (FPH). Many studies have used Alcalase to produce FPH (Guérard et al., 2001; Kristinsson and Rasco, 2000). Using this enzyme under optimal conditions gives high degree of hydrolysis in a relatively short time compared to other enzymes acting in neutral or acidic conditions (Benjakul and Morrissey, 1997; Diniz and Martin, 1996). In addition, it was reported that fish protein hydrolysates obtained has less bitter taste compared to those prepared by papain (Hoyle and Merrltt, 1994).

The classical method of optimization is tedious, incomplete and requires a lot of time to complete experiences. The response surfaces methodology (RSM) seems to be a very popular method and inventive for optimization of enzymatic hydrolysis (Bhaskar et al., 2008a; Bhaskar and Mahendrakar, 2008b; Cao et al., 2008; Guérard et al., 2007; Xia et al., 2007). This method uses quantitative data to simultaneously determine and solve multivariable equations, graphically represented as response surfaces (Montgomery, 2004). Based on this background, the objectives of this study were to optimize the hydrolysis reaction conditions (time, temperature, pH and enzyme concentration) using a commercial alkali and neutral proteases in order to produce a tuna by-products protein hydrolysate enriched in peptide fractions of hydrolysate enriched in peptide fractions of molecular weight of 1-4 KDa and to evaluate the protein hydrolysate prepared under optimized conditions for its amino acid composition and molecular weight distribution of the obtained population of peptides.

MATERIALS AND METHODS

Substrate

The tuna dark muscle by-product was supplied by TUNA EL SULTON Foods Industry Ltd. (Sfax, Tunisia). The raw material was vacuum-sealed in 400-ml polyethylene bags, transferred on ice to our laboratory and it was stored at -20°C until used. The protein, lipid, ash and moisture contents of the dark muscle by product were 26, 2.4, 1.35 and 70.5%, respectively as determined according to the methods described hereafter.

Enzyme

Alcalase PROLYVE 1000, provided by Lyven France, is produced by fermentation using a strain selected from the bacterium *Bacillus licheniformis*. The optimal conditions of use specified by the producer are: a temperature between 50 and 60°C and a pH between 9.0 and 10.5. Minimal activity confirmed was 500,000 DU/g or 2.2 Anson. Alcalase can be inactivated by heating at 90°C for 10 to 15 min.

Neutrase PROLYVE BS, provided by Lyven France, was obtained from a selected strain of *Bacillus subtilis*. According to the supplier, the optimal conditions for BS PROLYVE activity were: a temperature of around 50°C and a pH ranging from 6.5 to 7.5. The minimal activity was 70,000 $PC.g^{-1}$, which corresponds to 0.5 Anson. PROLYVE BS can be inactivated by heating at 85°C for 10 min.

Chemical composition / proximate composition

Moisture content was determined by placing approximately 2 g of sample into a pre-weighed aluminum dish. Samples were then dried in an oven at 105°C until a constant weight (AOAC, 2005). The total crude protein (Nx6.25) in raw material was determined using the Kjeldahl method (AOAC, 2005). Total lipid content was determined by Soxhlet extraction (AOAC, 2005). Ash content was estimated by charring in a predried sample in a crucible at 600°C until a white ash was formed (AOAC, 2005).

Protein content in the fish hydrolysates were measured in the supernatant following centrifugation by the Biuret method (Layne, 1957), using bovine serum albumin as a standard protein. Absorbance was measured at 540 nm with a UV/VIS spectrophotometer.

Optimization experiments

The optimization of the hydrolysis conditions was accomplished by employing the response surface methodology (RSM) with a completely randomized factorial design (CRFD) (Box and Wilson, 1951). The CRFD model based on four factors (pH, temperature, time and enzyme/substrate ratio) was used as experimental design model. The factor levels were coded as −1 (low), 0 (central point) and +1 (high). The different factors and their levels are presented in Table 1. Thus, temperature (40 to 60°C), time (40-160 min) enzyme concentration (E/S) (0.5 to 1.5%) and pH (7 to 9) were taken as input variables. A total of 27 experiments were performed. In

Table 1. Independent factors and their coded and reels levels used in RSM studies for optimizing hydrolysis conditions using Neutrase (X1. X2 and X3) and Alcalase (X1. X2. X3 and X4) (Experiment domain and level distribution of the variables used for optimization of DH).

Parameter	Factor	Unit	Level of factor		
			-1	0	1
X1	Temperature (°C)		40	50.00	60.00
X2	Time (min)		40	100.00	160.00
X3	Enzyme (%)		0.5	1.00	1.50
X4	pH		7	8	9

addition, three central replicates were added to the experimental design to calculate pure experimental error. Degree of hydrolysis (DH;%) was determined as the response variable (Y). Tables 2 and 3 show the CRFD used for optimization of enzymatic hydrolysis and the various factors and their levels and response values. The experimental data obtained from CRFD model experiments can be indicated by the following Equation (Y1):

$$Y1 = \alpha_0 + \sum_{i=1}^{n} \alpha_i X_i + \sum_{i=1}^{n-1} \sum_{j=2}^{n} \alpha_{ij} X_i X_j + \sum_{i=1}^{n} \beta_i X_i^2 + \varepsilon$$

Where, α_0 is the constant, α_i is the linear effect of the input factor X_i, α_{ij} is the linear effect by linear interaction effect between the input factors X_i and X_j, β_i is the quadratic effect of input factor X_i and **ε** is the error (Benyounis et al., 2005).

The optimized design was further validated through different combinations of parameters, with DH as the response variable, to evaluate the usefulness of the design.

Preparation of the hydrolysate

Protein hydrolysate from tuna dark muscle was prepared, as per the scheme shown in Figure 1 and according to the experimental plan shown in Tables 2 and 3. For each test, 50 g of dark muscle were homogenized with 100 ml of deionized water and incubated at a specified temperature and pH. After homogenization and incubation, the enzyme was added. The pH of the mixture was adjusted to the desired levels with 2 N sodium hydroxide and maintained constant during hydrolysis reaction using pH-stat (Titroline Alpha Plus). After hydrolysis, the reaction was terminated by heating the solution to 90°C for 20 min to inactivate the enzymes. Afterward, the hydrolysates were centrifuged (Centrifuge SIGMA) at 9500 rpm for 30 min. A portion of the supernatant was taken to determine the protein content and the other part was freeze–dried and stored in a desiccator for further analysis.

For validation of results, the tuna protein hydrolysate was produced at pilot-scale in a 5L batch reactor using Alcalase. Indeed, the dark muscle by-product (1.5 kg) was suspended in 3v L of deionised water and the pH was adjusted at 8.5 with a 2vN NaOH solution and 30 $g.L^{-1}$ of Alcalase was added and the reaction was carried out at 55°C for 1 h.

Determination of the degree of hydrolysis (DH)

Degree of hydrolysis (DH) was generally used as a proteolysis monitoring parameter when the pH-stat method was employed. The pH-stat reaction allowed the estimation of DH based on the consumption of alkali to maintain a constant pH at the desired value. The values for DH could be determined using the following equation described by Adler-Nissen (1982).

$$DH = \frac{V.N}{\alpha.Mp.h_{tot}}.100$$

Where, DH is the percent ratio between the number of peptide bonds cleaved (htot) and the total number of peptide bonds in the substrate studied (htot). The variable V is the amount of alkali consumed to keep the pH constant during the reaction, N is the normality of the alkali, Mp is the mass of the substrate (protein, determined as N × 6.25) in the reaction and α was the average degree of dissociation of α-NH2 groups released during hydrolysis.

Size exclusion chromatography

The molecular weight distribution of native hydrolysate fractions was obtained by size exclusion chromatography using gel permeation chromatography in FPLC mode using a Superdex Peptide® GL 10/300 column (Amersham, fractionation range: 7000–100 Da) according to the method described by Guérard et al. (2001). The liquid chromatographic system consists in a Water 600 automated gradient controller pump and a Water 996 photodiode array detector. Data acquisition and chromatographic analysis were performed with Empower® software. The mobile phase isocratic elution consisted of water with TFA 0.1% and acetonitrile (70:30) at a flow rate of 0.5 mL/min, 20 µL of hydrolysate (30 mg/mL in MilliQ water), sterile filtered, injected into the column, and elution was carried out isocratically in MilliQ water with mobile phase.

Standards used for the calibration were Cytochrome C (12384 Da), Aprotinin (6512 Da), Neurotensin (1678.9 Da), Insulin chain B (3495 Da), Substance P fragment 6-11 (764 Da), and Leupeptine (463 Da). The total area of the chromatogram was integrated and separated into fractions of five molecular weight (MW) ranges (>7000, 7000–4000, 4000–1000, 1000–300 and <300 Da, respectively), expressed as the per cent of the total area.

Amino acid analysis and chemical score of protein hydrolysates

Amino acid composition was determined using a Biochrom amino acid analyzer equipped with a Na^+ column. Indeed, proteins are hydrolyzed using 4N methane sulfoxide at 150°C for 2 h. The hydrolysates obtained were analyzed by HPLC using ion exchange resin and the detection was realised at 570 nm and 440 nm after reaction with ninhydrin. Considering the contents of essential amino acids (EAA) in the protein hydrolysate and in the standard protein as described by FAO/WHO (1999), the chemical score of the protein hydrolysates was calculated using the following relation (Vidotti et al., 2003).

Chemical score = EAA in test protein ($g.100g^{-1}$) / EAA in Standard Protein ($g.100g^{-1}$).

Table 2. Actual levels of independent variables used in optimizing the hydrolysis conditions using Neutrase along with the observed and predicted values for the response variable (DH, Y).

N°Exp	X1	X2	X3	Y^a	Y^b
1	-1	-1	-1	3.250	2.742
2	-1	-1	0	6.500	7.161
3	-1	-1	1	5.960	6.492
4	-1	0	-1	3.250	3.448
5	-1	0	0	8.130	7.779
6	-1	0	1	7.580	7.022
7	-1	1	-1	4.330	4.864
8	-1	1	0	9.750	9.108
9	-1	1	1	8.130	8.263
10	0	-1	-1	4.470	3.843
11	0	-1	0	8.530	8.344
12	0	-1	1	7.720	7.756
13	0	0	-1	4.060	4.268
14	0	0	0	8.530	8.681
15	0	0	1	8.130	8.006
16	0	1	-1	4.870	5.405
17	0	1	0	10.160	9.730
18	0	1	1	8.530	8.967
19	1	-1	-1	3.510	3.971
20	1	-1	0	8.350	8.554
21	1	-1	1	8.620	8.047
22	1	0	-1	4.150	4.116
23	1	0	0	7.820	8.611
24	1	0	1	8.300	8.018
25	1	1	-1	5.740	4.973
26	1	1	0	9.580	9.380
27	1	1	1	8.300	8.699

[a]Observed response (DH); [b] predicted response (DH).

Statistical analysis

The optimization experiments of the enzymatic hydrolysis of tuna by-product were carried out through response surface method (RSM) by generating the CRFD (for or three factors, three levels and single block of 27 runs) generated using experimental design module of the STATISTICA software (Statsoft, 2007). The results of the experimental design, regression and graphical analysis of the data obtained through RSM were analyzed and interpreted to obtain the optimized conditions using NemrodW version 2000-D statistical software.

RESULTS AND DISCUSSION

Optimization of hydrolysis conditions

Statistical analysis

CRFD and Response Surface Methodology (RSM) were applied to investigate the effect of the parameters of enzymatic hydrolysis of tuna dark muscle on the hydrolysis degree and molecular weight of the different fractions obtained. The statistical analysis was achieved using Fisher 'F' test and Student's 't' test. Analysis of the variance (NemrodW) regarding the hydrolysis degree shows that the second order response surface model was highly significant with $F_{statistics}$ (223.9) > $F_{Fischer}$ (4.31) ($p < 0.01$) for Alcalase and $F_{statistics}$ (165.3) > $F_{Fischer}$ (4.76) ($p< 0.01$) for Neutrase as shown in Table 4. The Student's 't' test was used to determine the significance of the variable regression coefficients. The value of "p" is the indicator of the importance of the test. The comparison between student test "t" statistic and calculated values indicates that the parameter test was significant at the significance level of 1, 2 or 5%. In general, the larger magnitude of 't' and the smaller value of 'p', the more significant is the corresponding coefficient term (Montgomery, 1991).

Model fitting

While using the RSM approach, experiments were conducted using a model factorial design of experiments to visualize the effects of independent factors on the response under different experimental conditions.

According to the sequential model sum of squares, the

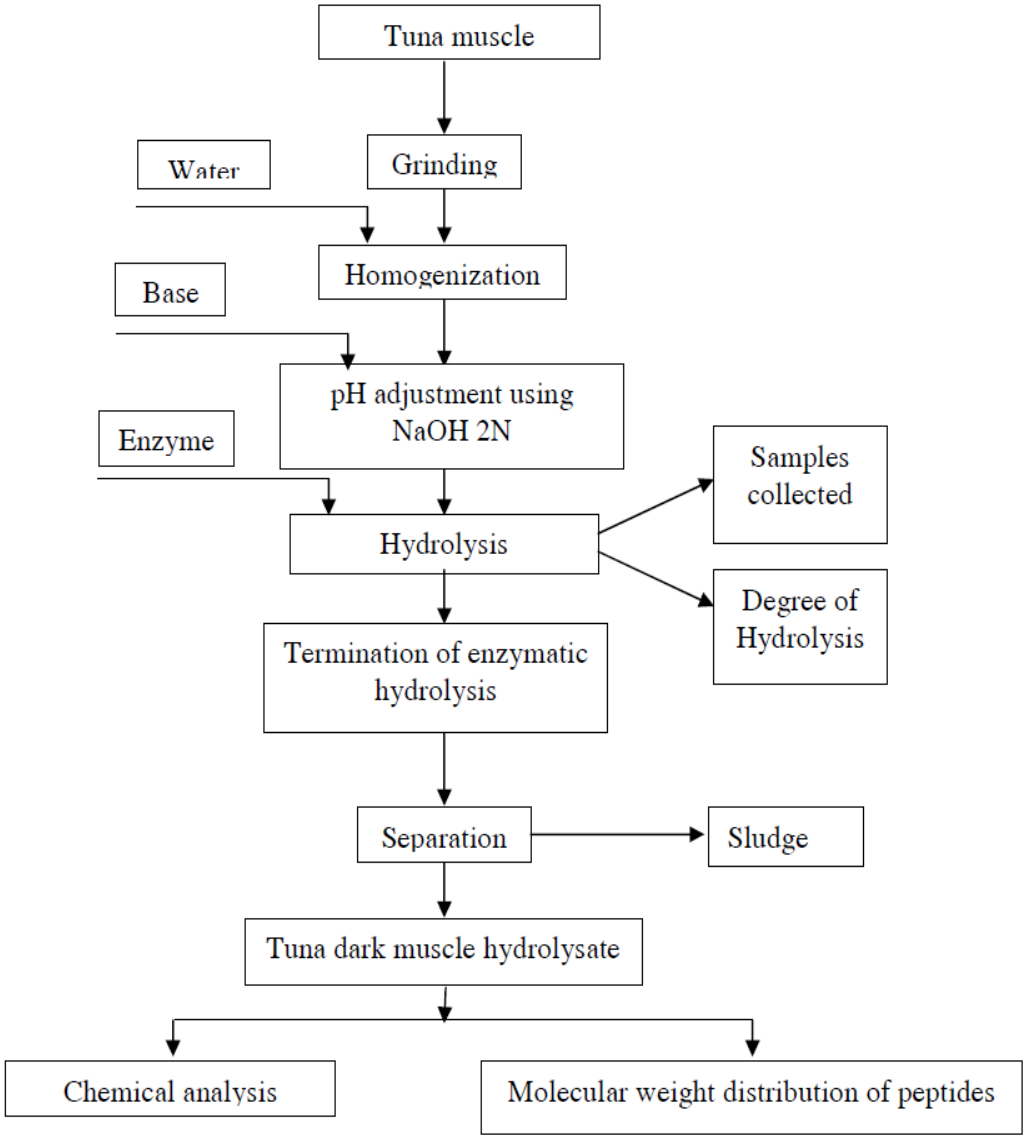

Figure 1. Flow sheet for the production of protein hydrolysate from dark muscle tuna using Alcalase and Neutrase under optimized conditions.

appropriate models were selected based on highest-order polynomials where the additional terms were significant. The experimental results were evaluated and approximating function of the model (Y, DH) was obtained in the form of the following equations equation (Y2) and (Y3) for Neutrase and Alcalase, respectively [The regression equations (Y2) and (Y3) represent an empirical relationship between the response and the tested variables (in coded units)].

Y (2) = 8,681 + 0,416*X1 + 0,693*X2 + 1,869*X3 - 0,486*(X1*X1) + 0,356* (X2*X2) -2,544*(X3*X3) - 0,280*(X1*X2) + 0,082*(X1*X3) - 0,087*(X2*X3) Y (3) = 19.121+ 1.161* X1 + 1.105* X2 + 1.081* X3 + 0.777* X4 - 1.133* (X1*X1) - 0.893*(X2*X2) - 0.043 * (X3*X3) - 1.594* (X4*X4) - 0.077* (X1*X2) - 0.264* (X1*X3) - 0.942* (X2*X3)-0.380* (X1*X4) - 0.596*(X2*X4) - 0.326 * (X3*X4)

Positive sign in front of the terms indicates synergistic effect, whereas negative sign indicates antagonistic effect. The quality of the model developed was evaluated based on the correlation coefficient value.

Effect of interactive variables on the degree of hydrolysis of tuna dark muscle

The influence of temperature, time, enzyme concentration and pH was determined using factorial design as mentioned in the previous section. The observed and theoretical values for degree of hydrolysis (DH%) obtained at different combinations of independent variables are pre-

Table 3. Actual levels of independent variables used in optimizing the hydrolysis conditions using Alcalase along with the observed and predicted values for the response variable (DH, Y).

N°Exp	X1	X2	X3	X4	Y^a	Y^b
1	-1	-1	-1	-1	8.75	8.750
2	-1	-1	0	1	14.96	14.907
3	-1	-1	1	0	16.67	16.992
4	-1	0	-1	1	15.06	15.325
5	-1	0	0	0	17.31	16.828
6	-1	0	1	-1	15.80	15.700
7	-1	1	-1	0	16.94	16.690
8	-1	1	0	-1	14.63	14.980
9	-1	1	1	1	16.15	16.100
10	0	-1	-1	1	14.91	15.180
11	0	-1	0	0	17.25	17.146
12	0	-1	1	-1	17.07	16.480
13	0	0	-1	0	18.06	17.976
14	0	0	0	-1	16.26	16.728
15	0	0	1	1	18.97	18.997
16	0	1	-1	-1	17.06	17.002
17	0	1	0	1	18.25	17.946
18	0	1	1	0	19.08	19.456
19	1	-1	-1	0	15.76	15.396
20	1	-1	0	-1	14.05	14.611
21	1	-1	1	1	18.07	18.029
22	1	0	-1	-1	16.20	15.853
23	1	0	0	1	18.43	17.946
24	1	0	1	0	19.38	19.918
25	1	1	-1	1	17.52	17.889
26	1	1	0	0	19.23	19.279
27	1	1	1	-1	18.52	18.038

[a]Observed response (DH); [b]predicted response (DH).

Table 4. Analysis of variance (ANOVA) and comparison $F_{Fischer}$ and $F_{statistics}$.

Parameter	Source of variation	Sum of square	Degree of freedom	$F_{statistics}$ value	F_{Fisfer} value
Neutrase	Regression	116.7731	9	165.3022	4.765
	Residues	5.4159	17		
	R^2	0.956			
Alcalase	Regression	121.7292	14	223.991	4.313
	Residues	2.8672	12		
	R^2	0.977			

presented in Tables 2 and 3. The equations (Y2) and (Y3) were used to visualize the effects of experimental factors on responses under optimized conditions in two and three-dimensional graphs (Figures 2 and 3). The analysis of variance (ANOVA) for degree of hydrolysis using Neutrase and Alcalase shown in Table3, demonstrate that the R^2 values for equation (Y2) was 0.95 and 0.976 for equation (Y3). The R^2-value were considered relatively high, indicating that there was a good agreement between the experimental and the predicted hydrolysis degree (DH) uptake from this model.

The significance of each coefficient was determined using the regression analysis. The regression coefficients and the interaction between each independent factor can be considered statistically significant for p-values below 0.05, with 95% of confidence interval listed in Table 4.

Thus, the regression coefficients showed that temperature and time had a linear effect on the DH values while the enzyme concentration had a linear and quadratic effect for Neutrase and the interaction effects on the values of DH were not observed between all factors (Table 5). In the case of Alcalase, the regression coefficients showed that temperature, time, enzyme concentration and pH have a linear effect on the DH values while the temperature, time and pH have quadratic effect (Table 5). The interaction effect on the values of DH were observed between time and enzyme concentration ($p < 0.01$), temperature and pH ($p > 0.05$), time and pH ($p < 0, 05$) and the enzyme concentration and pH ($p > 0.05$).

The largest value of estimated regression coefficient for enzyme concentration (b1 = 1,869) for Neutrase and the coefficient for temperature (b1= 1.161) for Alcalase indicates that they were the most important linear variables influencing the DH values, respectively. In the case of Neutrase, the concentration enzyme was the variable that has the greatest effect on the values of DH, followed by the hydrolysis time and even in the case of Alcalase, it was noticed that the temperature was the variable that has the greatest effect on the values of DH, followed by the hydrolysis time. The positive value of coefficients implies that the values of DH increase with the concentration enzyme and temperature and time of hydrolysis.

The best explanatory model equation for the DH values obtained from Neutrase and Alcalase hydrolysis are given by equations (Y4) and (Y5) respectively:

Y (4) = 8,681+ 0,416* X1 + 0,693* X2 + 1,869* X3 - 2,544* (X3*X3)

Y (5) = 19.121+ 1.161* X1 + 1.105* X2 + 1.081* X3 + 0.777* X4 - 1.133* (X1*X1) - 0.893* (X2*X2) - 1.594* (X4*X4) - 0.942* (X2*X3)-0.380* (X1*X4) - 0.596*(X2*X4) - 0.326 * (X3*X4)

The model has shown a good fit with the experimental data since the coefficient of determination R^2 had a value of 0.956 for Neutrase and 0.976 for Alcalase. This means that the fitted model could explain 95.6 and 97.6%, respectively of the total variability within the range studied. These results indicate that the accuracy of the polynomial models was well adapted. The regression coefficients and the interaction between each independent factor can be considered statistically significant.

To investigate the effect of temperature, time, concentration of enzyme and pH on the DH. The response (DH) was represented in graph surface response. The response surface presents the degree of hydrolysis (DH) based on two factors, while other factors are kept at mean levels.

Figure 2 shows the two-dimensional and three-dimensional response surface regarding the effect of the three variables (time, temperature and enzyme concentration) on the degree of hydrolysis for Neutrase. When the concentration of enzyme was fixed at mean level (1%), as can be seen from the two-dimensional surface plots in Figure 2a, the DH increased with the increase of temperature. The highest DH was obtained at a temperature of 52°C, above this value, the DH was decreased when the temperature was increased.

Figure 2b shows the effect of enzyme concentration and temperature. The time was fixed at 100 min. The DH increased with the increase of temperature between 45 and 65°C. The highest DH was obtained for a temperature of 52°C and enzyme concentration of 1.25% in the concentration range studied. When the temperature was fixed at mean level (50°C), as seen in Figure 2c, the DH increased with the increase of both enzyme concentration and time of reaction. For Alcalase, it can be seen from Figure 3 that the DH increased to 19% at a temperature of 55°C and then decreased (Figure 3a). Similarly, the DH increased with increasing pH from 7 to 8.5, beyond which it decreased (Figure 3c). This shows the effect of temperature and pH on enzyme activity (the enzyme concentration and time were maintained at their mean levels). Figure 3d shows that the degree of hydrolysis increased with increasing enzyme concentration. These results show that the operating parameters namely, temperature, enzyme concentration and pH affected the hydrolysis with Alcalase and Neutrase except the pH for Neutrase. A similar result was reported earlier using the same commercial multifect-neutrase and Alcalase for hydrolysis of visceral waste proteins of Indian freshwater major carp (Bhaskar et al., 2008a; Bhaskar and Mahendrakar, 2008b). The coordinates of the stationary point of the iso-response curves corresponding to a maximum of degree hydrolysis were determined by software design of the experiments in coded values. Thus, the optimal conditions were determined for Neutrase: temperature of 50°C, enzyme concentration of 1.25% and time of 132 min and for Alcalase: temperature of 55°C, time of 136 min, enzyme concentration of 1.5% and pH value of 8.5.

In order to confirm these results, the usefulness of the model was further validated using different random combination (other than used for optimization) of the different factors. The data from validation experiment are presented in Table 6. The observed and predicted values were compared to evaluate the validity of the above model. These results seem to confirm the validity of the model. For the following experiments regarding the production of the protein hydrolysate, the Alcalase was chosen since it gave the higher degree of hydrolysis.

The tuna dark muscle hydrolysate produced under optimal conditions using Alcalase, was analyzed using size exclusion chromatography (Figure 4). The samples were collected during the enzymatic hydrolysis at different time intervals to analyze the evolution of molecular weight distribution of peptide fractions with time in the fish protein hydrolysate.

The enzymatic treatment by Alcalase of the tuna muscle proteins produced the peptide fractions with molecular

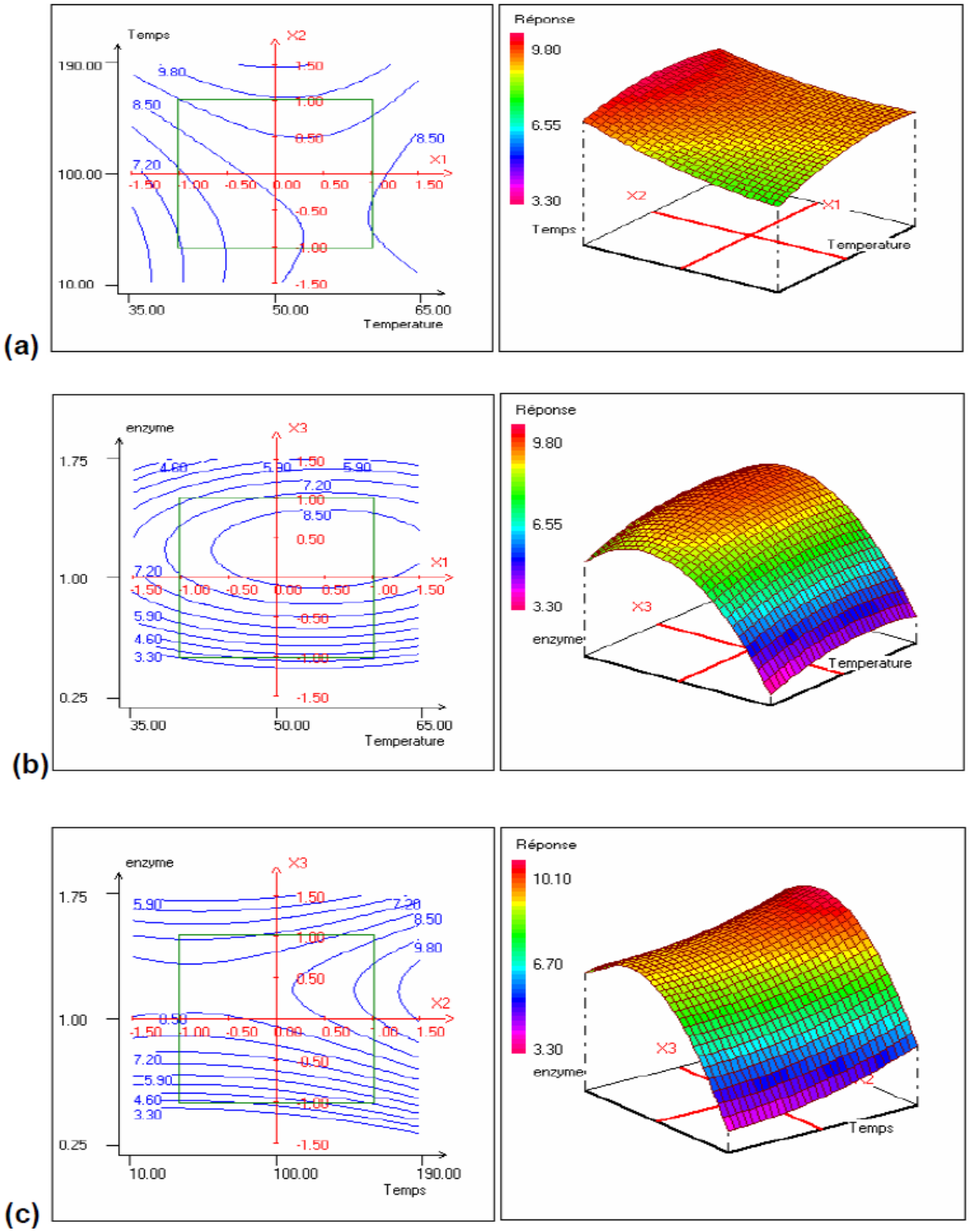

Figure 2. Response surface graphs for DH as a function of the different parameters during hydrolysis of tuna by-product with Neutrase. **a)** effect of temperature and time. **b)** Effect of temperature and enzyme concentration. **c)** Effect of time and enzyme concentration.

weight ranging from 7000 Da to free amino acids. The chromatographic data show that the hydrolysates were composed of low molecular weight peptides. Indeed, the hydrolysate at 19.3% DH was characterized by a low content of peptides with molecular weight above 5000 Da. However, the hydrolysate with 7.3% DH was characterized by a high content of peptides with molecular weight above 5000 Da. Also, the quantity of small peptides

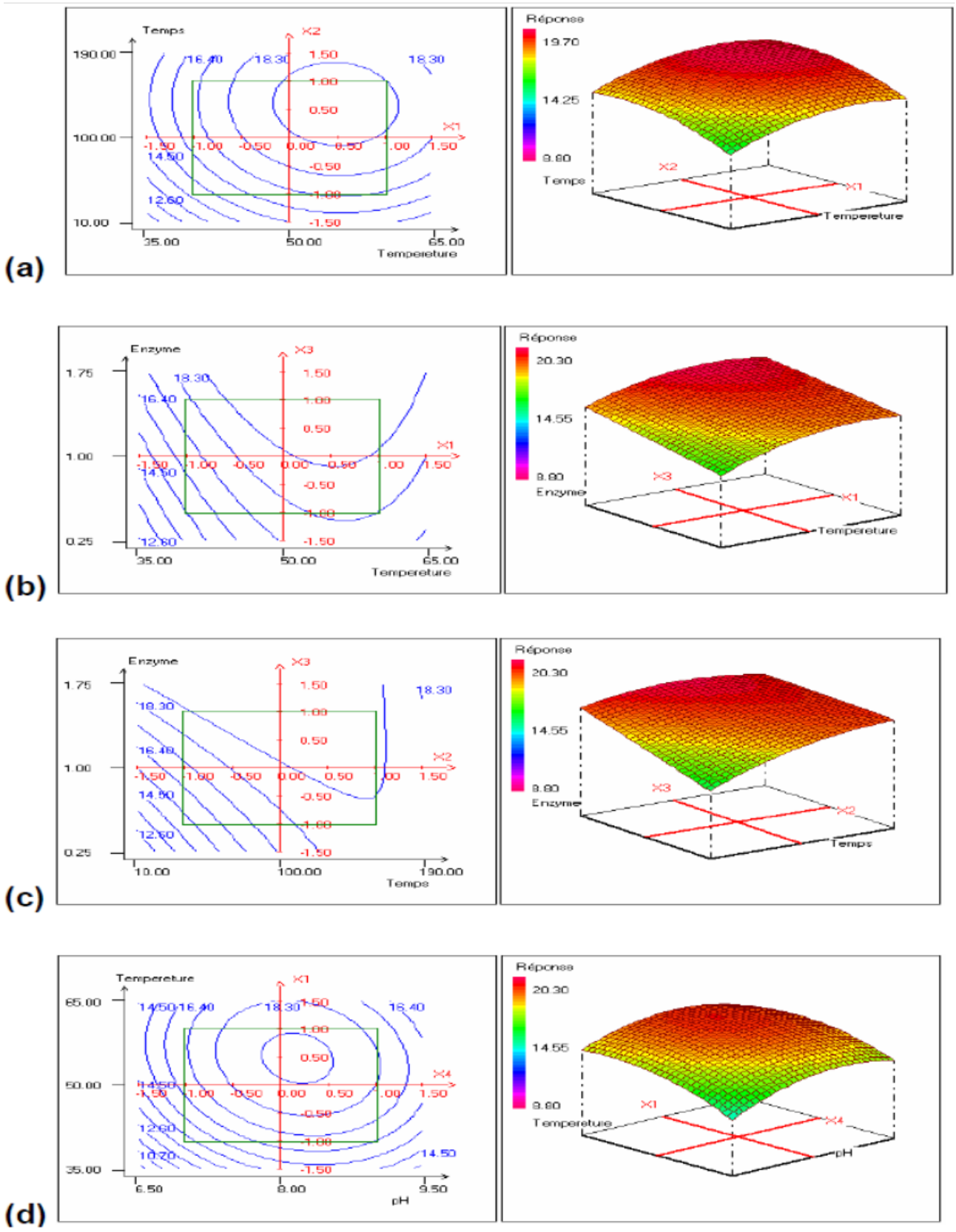

Figure 3. Response surface graphs for DH as a function of the different parameters during hydrolysis of tuna co-product with Alcalase. **(a)** effect of time and temperature**. (b)** Effect of temperature and enzyme concentration. **(c)** Effect of time and enzyme concentration. **(d)** Effect of temperature and pH.

Table 5. Regression coefficients for main factors and their interactions during optimisation experiments using Neutrase and Alcalase.

Factor	Coefficient (Neutrase)	Coefficient (Alcalase)
Constant	8.681***	19.121***
Linear		
Temperature (X1)	0.416***	1.149***
Time (X2)	0.693***	1.105***
Enzyme level (X3)	1.869***	1.092***
pH (X4)		0.788***
Quadratic		
X11	-0.486*	-1.144***
X22	0.356	-0.871 ***
X33	-2.544***	-0.054
X44		-1.606***
Interactions		
X1 X2	-0.280	-0.082
X1 X3	0.082	-0.246
X2 X3	-0.087	-0.937***
X1 X4		-0.363*
X2 X4		-0.592***
X3 X4		-0.344*

*** Significative 1% (t=2.81) ; ** significative 2% (t=2.508) ; * significative 5% (t=2.07)

Table 6. Degree of hydrolysis (DH %) observed during validation experiments and the corresponding predicted values for DH using Neutrase and Alcalase.

N°Exp	Alcalase / Neutrase			X4	Neutrase		Alcalase	
	X1	X2	X3		Y^a	Y^b	Y^a	Y^b
1	0	0	-1	-1	4.47	4.268	15.44	15.343
2	0	-1	1	-1	9.35	7.988	16.26	16.505
3	-1	1	-1	1	5.96	4.545	15.87	16.193
4	-1	0	1	1	9.21	7.59	17.7	17.145
5	1	1	0	0	10.22	9.79	19.8	19.361
6	0	1	0	0	9.75	9.374	18.67	19.333
7	-1	1	0	0	9.75	8.958	18.1	17.039
8	1	0	1	-1	9.26	8.422	17.34	18.565

[a] Observed response (DH); [b] predicted response (DH).

below 500 Da was increased from 20 (T = 10 min; DH = 7.3%) to 30% (T = 136 min; DH = 19.3%).

Therefore, a decrease of the high molecular weight fractions was noticed as the hydrolysis time of enzymatic hydrolysis increased. So, this technique was useful for comparing peptide profiles from different hydrolysis durations and for checking the profile adequacy to determine the best time of hydrolysis for recovery of interesting peptide fractions. This approach may lead to the estimation of small peptides and free amino acids and could allow us to deduce the hydrolysis time to recover the suitable molecular weight peptide fractions for a desired application. Thus, Size Exclusion Chromatography (SEC) technique represents an adequate tool for the analysis of the evolution of molecular weight distribution as a function of time during enzymatic hydrolysis.

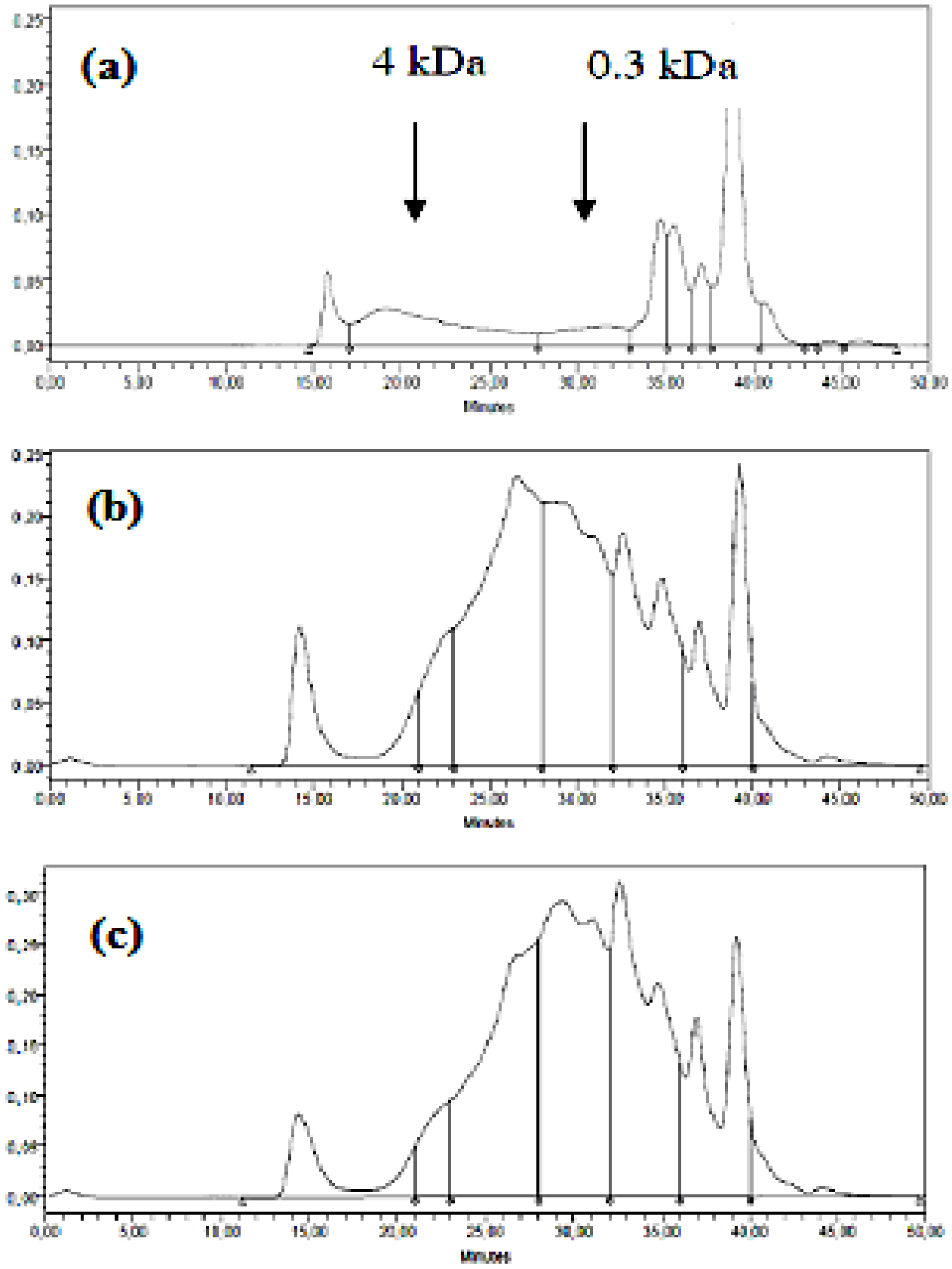

Figure 4. Size exclusion chromatography of hydrolysates on SUPERDEX PEPTIDES GL 10/300 column. Samples collected during enzymatic hydrolysis of tuna dark muscle using Alcalase under optimized conditions.

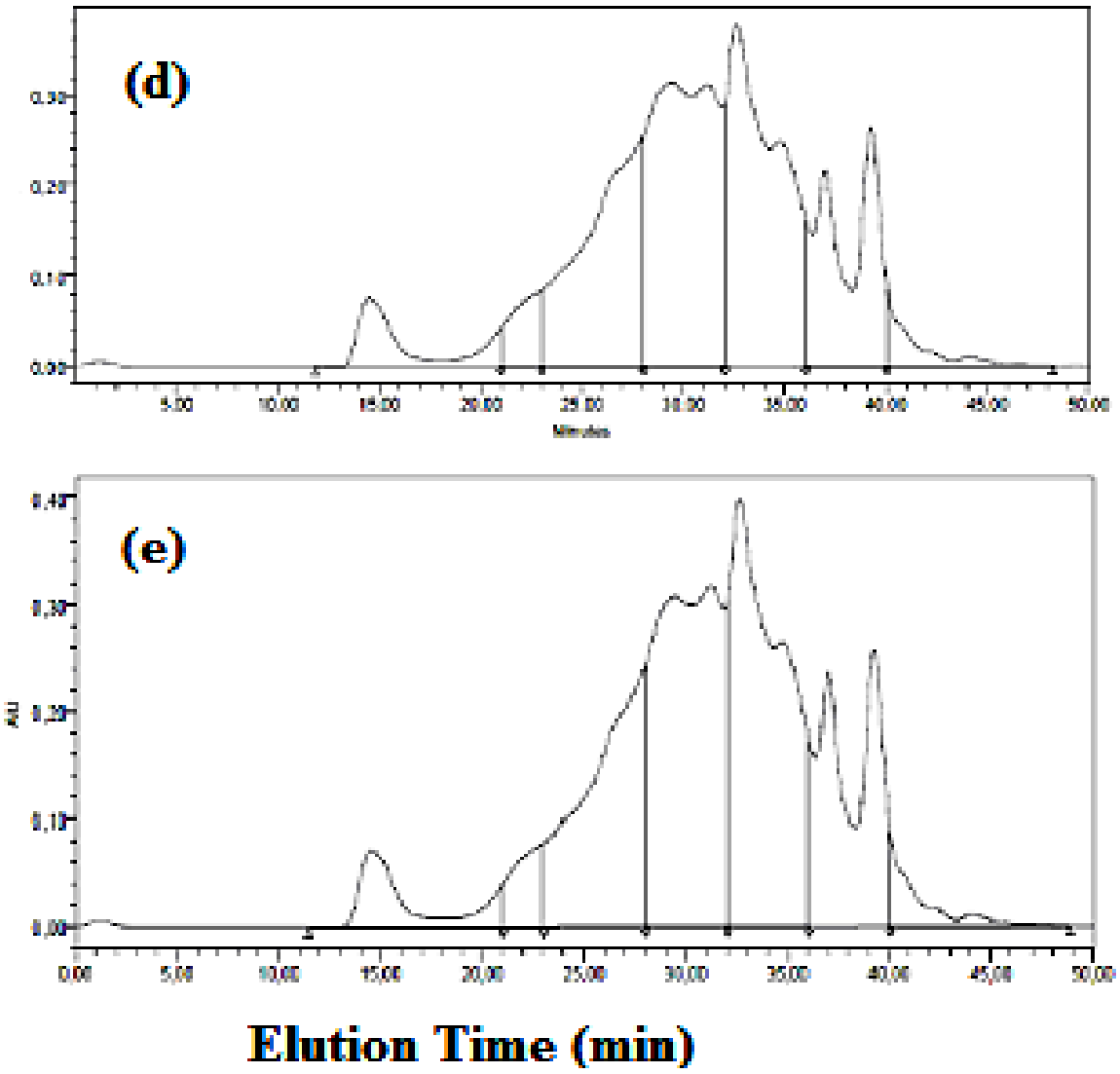

Figure 4. Contd.

Determination of the conditions of interesting peptide fraction production

The results obtained using the optimum conditions determined by response surface methodology associated with the factorial design show that the rate of interest peptide fractions was affected by the time of hydrolysis and also by the enzyme concentration. Indeed, it was found that even if these hydrolysis conditions give a high rate of soluble proteins and a degree of hydrolysis of about 20%, the rate of peptide fractions of molecular weights obtained was low. Therefore, it is necessary to complete the study by determination of the effect of time and enzyme concentration on the variation of this interesting fraction during the hydrolysis reaction.

Figure 5 shows the effect of time and enzyme concentration respectively on the rate of peptide fractions (1-4 kDa) and on the amount of soluble protein at different enzyme concentration. Related to the rate of peptide fractions, it can be observed that the influence of the enzyme concentration is important. Thus, the rate of 1-4 kDa fraction decreased with the increase of the enzyme concentration. After 60 min of hydrolysis, the rate decreased from 25 to nearly 15% depending on Alcalase concentration (respectively for 1 and 4.5%). Regarding the effect of the duration of hydrolysis, the rate of peptide fractions was higher at the first hour of hydrolysis and then became constant.

However, the production of the maximum amount of soluble protein was not significant to obtain a higher amount of interesting peptide fractions of 1-4 kDa. This could be explained by the action of enzyme during the reaction. Indeed, after the enzyme hydrolysis of the protein chains, the reaction continued by hydrolyzing the small peptide fractions generating a decrease of 1-4 kDa fractions. The best rate of interesting peptide fractions was obtained at

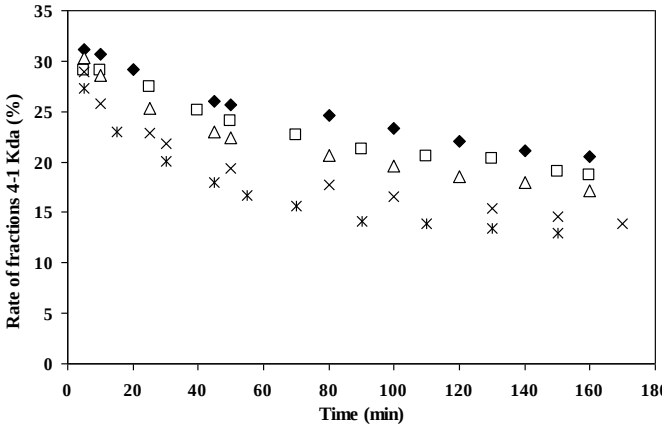

Figure 5. Variation of the percentage of peptide fractions 4-1KDa in the hydrolysate with time and at different enzyme/substrate ratios (E/S).

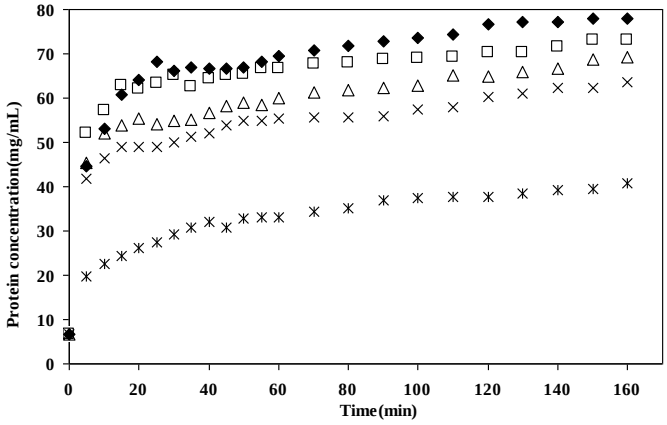

Figure 6. Variation of the soluble protein concentration with time and at different enzyme/substrate ratios (E/S).

1% of enzyme concentration and at 60 min of duration of hydrolysis reaction.

Figure 6 shows that the amount of the soluble fraction increased sharply during the first few minutes of hydrolysis and then stabilized at a value dependent on the enzyme concentration. The higher value was obtained at the highest enzyme concentration and the amount decreased considerably by using an enzyme concentration of 0.75%.

The analysis of the variation of soluble extract shows that the rate of soluble extract increased with increasing time during the first 60 min. After that no significant variation was observed. On the contrary, the rate of insoluble extract decreased over time and stabilized after about 60 min of hydrolysis. From this behavior, we concluded that it is not advisory to continue hydrolysis beyond 60 min, because there was not a significant increase in rates of soluble proteins or soluble solids. So, it is not profitable to go to the end of the reaction to have an increase of 7% during 90 min more.

So, we can deduce the optimum conditions for enzymatic hydrolysis to produce the most interesting peptide fractions as: temperature of 55°C, duration of 60 min, an enzyme concentration of 1% and a pH of 8.5 and the yield of interesting peptide fractions has a value of almost 22% of total fractions.

Validation of the process

The production of hydrolysate using Alcalase was made under optimal conditions already obtained at two different scales (laboratory and pilot scale) in order to validate the process of production of interesting peptide fractions. The results obtained at laboratory scale regarding the rate of different peptide fractions produced under the optimum conditions (temperature= 55°C; time = 60 min, pH = 8.5 and 1% of enzyme concentration) using Alcalase were very similar with those obtained at pilot scale under the same conditions when a volume of 4.5 L of hydrolysate was used. The variation of the quantity of soluble protein presented similar profiles in the two cases. Thus, the quantity of soluble protein increased sharply during the first 10 min and then became almost stable at a value of 69 mg/ml for pilot scale and 66 mg/ml for lab scale.

The yield of solubilisation of tuna protein obtained after 1 h of hydrolysis using Alcalase was 80% and 79 for laboratory scale and pilot scale, respectively. This result was better than obtained by others study of hydrolysis of different fish by-products using Alcalase (Benjakul and Morrissey, 1997; Liaset et al., 2000; Shahidi et al., 1995). Similar molecular weight distribution was observed when hydrolysis reaction was performed at laboratory or pilot scale. The rate of peptide fractions obtained in the hydrolysate was 19.84% for lab scale and 19.50% for pilot scale.

Nutritional aspects

The yield of solubilisation of protein in the hydrolysate was 80% showing that the hydrolysate was enriched in protein. The nutritive value of any ingredient depends on the proteins capacity to fulfill the needs of organisms with respect to essential amino acids. Chemical score has been used to evaluate the nutritive value of a protein. This parameter compares levels of essential amino acids between the test and standard proteins (Vidotti et al., 2003). In the current study, the chemical scores were calculated on the basis of reference proteins of the FAO / WHO (FAO/WHO, 1999) and amino acid requirements of juvenile common carp as indicated by the NRC (NRC, 1993).

The results indicate tryptophan to be the most limiting amino acid as compared to both reference proteins, followed by phenylalanine as compared to reference pro-

Table 7. Amino acid composition of the fish protein hydrolysate and its chemical score in comparison with FAO/WHO and NRC reference protein.

Amino acid	Quantity (g/100g)			Chemical score	
	TPH	RP1[a]	RP2[b]	RP-1	RP-2
Threonine*	2.995	0.9	3.9	3.327	0.768
Valine*	3.200	1.3	3.6	2.462	0.889
Methionine*	1.739	1.7	3.1	1.023	0.561
Isoleucine*	4.082	1.3	2.5	3.140	1.633
Leucine*	4.932	1.9	3.3	2.596	1.494
Tyrosine	1.835	-	-	-	-
Phenylalanine*	2.413	1.9	6.5	1.270	0.371
Histidine*	2.401	1.6	2.1	1.501	1.144
Tryptophan	0.343	0.5	0.8	0.685	0.428
Lysine*	4.710	1.6	5.7	2.944	0.826
Arginine	2.378	-	1.31	-	1.816
Aspartic acid	5.351				
Ornitine	0.050				
Glutamique acid	8.523				
Ammonium Cl	2.657				
Proline	2.222				
Serine	2.339				
Glycine	3.620				
Alanine	4.561				
Cystine	0.388				

TPH, Tuna protein hydrolysate; RP1, reference protein 1; RP2, reference protein 2; RP-1, Chemical scores calculated with FAO/WHO reference protein as the base; RP-2, Chemical scores calculated with amino acid requirements as per NRC (1993); [a] essential amino acids of reference protein according to FAO/WHO (1999); [b] essential amino acid requirements of common carp according to NRC (1993); (*) essential amino acids (EAA).

tein of NRC standard. All other amino acids were present in sufficient or excess quantity as required for juvenile common carp and for an adult (Table 7). However, it can be noted that for an amino acid to be considered limiting in fish diet, it has to be available in quantities less than 30% of minimum requirement (Tacon, 1994). By this standard, the amino acid composition of the tuna protein hydrolysate fulfills this requirement for fish diets (Vidotti et al., 2003). Further, for many fish species, growth rates produced by diets with large amounts of free amino acids are inferior to diets of similar amino acid composition in which the nitrogen component is protein (Walton et al., 1986). Thus, the amino acid composition of the protein hydrolysate prepared under optimized conditions using Alcalase (Table 7) showed that the hydrolysate has a composition almost similar to the FAO/WHO reference protein and the hydrolysate waste of Pacific whiting reported by Benjakul et al. (1997) with amino acid concentration higher than the protein reference and the hydrolysate. It can be concluded that in spite of minor deficiencies in certain essential amino acids, the protein hydrolysate of tuna by-products does not lose its nutritional value and thus, this product can be considered as an ingredient in balanced fish diets and can be for example used as nitrogen source in microbial growth media (Guérard et al., 2001).

Conclusion

Tuna protein hydrolysate produced under optimum conditions (temperature of 55°C, pH of 8.5, enzyme concentration 1%, time of 60 min) using Alcalase had a high proportion of peptides having various molecular weights from 7000 Da to free amino acids. The concentration of available hydrolysable bonds was the factor controlling the hydrolysis rate while the time of hydrolysis was the main factor controlling the molecular weight of peptide fractions and the characteristic of the fish protein hydrolysate. The hydrolysate prepared from tuna dark muscle has potential for applications in aquaculture diets and as source of nitrogen in microbial growth media.

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