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Enzymatic hydrolysis of shrimp meat

B. K. Simpson,[a]* G. Nayeri,[a] V. Yaylayan[a] & I. N. A. Ashie[b]

[a]*Department of Food Science and Agricultural Chemistry, McGill University (Macdonald Campus), 21,111 Lakeshore Road, Ste Anne de Bellevue, PQ., Canada H9X 3V9*
[b]*Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624, USA*

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Fresh and frozen shrimp were hydrolyzed with chymotrypsin or trypsin, and their proximate composition, pH, amino acid content, and sensory properties were evaluated. The hydrolysates prepared with both enzymes had high levels of glycine, proline, arginine, and valine, and there were no significant differences in the sensory properties of the frozen and fresh products. The optimum conditions for aroma quality using chymotrypsin were obtained with an enzyme to substrate (E:S) ratio of 0.25–0.3% for 2.5 h at 35°C, while the optimum conditions with trypsin were 0.25–0.3% E:S ratio for 2.5–3.0 h at 40°C. Statistical analysis showed that second order polynomial models could be used to predict the content of specific amino acids to a reasonable degree of accuracy. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Crustacean species such as shrimp, crab, lobster, etc., are a rich source of amino acids, peptides, protein and other useful biochemicals which may be recovered for utilization as ingredients in various food applications. In the last two decades, more than 6000 volatile flavour compounds comprising of aldehydes, ketones, alcohols, N- and S-containing compounds, furans, etc, have been isolated from crustaceans and several other species and evaluated for their potential application as food flavourants (Cha *et al*., 1993; Kim *et al*., 1994). This trend is due to consumer preferences for 'natural' products, vs their synthetic counterparts that are perceived to exhibit some form of toxicity or health risks (Dziezak 1986). Recent advances in biotechnology have demonstrated the capacity of enzymes to produce novel food products, modify foodstuffs, and improve waste management (Armstrong and Yamazaki, 1986). The application of proteolytic enzymes to extract seafood flavour was recently suggested as a way of putting digestive enzymes from fish offal to better economic use (Knorr and Sinskey, 1985; Haard, 1992; Haard *et al*., 1994). The advantages conferred by the use of proteolytic enzymes for flavour extraction include: (i) having the status of a 'natural' substance; (ii) defined product stereochemistry due to the high substrate and reaction specificity of biocatalysis; (iii) mild reaction conditions; and (iv) reduction in waste product formation. These have resulted in various proteases being utilized to produce peptones and protein hydrolysates, fish sauce, removal of fish skins and scales, and membranes from seafoods, and isolation of flavouring agents and pigments (Haard, 1992; Haard *et al*., 1994).

According to Venugopal and Shahidi (1995), the potential of seafood protein hydrolysates as food flavourants and protein supplements has not been fully exploited. Fish protein hydrolysates have been shown to be very good sources of bacterial peptones, and are currently used for production of milk substitutes for calves and weaning pigs, fish feed, pet food, and as a flavouring agent in countries like Japan, France, Norway, and the United States (Gildberg, 1993; Vecht-Lifshitz *et al*., 1990). Studies on the effect of commercial fish protein hydrolysates on mouse lymphocytes also revealed that certain compounds in the hydrolysate promoted immunostimulation, while feeding of domestic animals with fish silage hydrolysate reduced their disease frequency (Vinot *et al*., 1989; Gildberg, 1993). Several proteases have been used for the production of these hydrolysates. These include papain, ficin, bromelin, trypsin, pancreatin, alcalase, and pronase (Venugopal and Shahidi, 1995; Gildberg, 1993). However, the product quality varies with the nature of the enzyme and processing conditions employed. For instance, while pepsin proved to be more effective at solubilizing proteins in threadfin bream, pronase formed a product with less bitterness (Hevia *et al*., 1976; Venugopal and Lewis, 1981). The use of other enzymes capable of degrading such bitter peptides, would eliminate this problem (Sugiyama *et al*., 1991). Other studies with fish hydrolysates indicated that hydrolysates prepared with

*To whom correspondence should be addressed.

Streptomyces proteases had better nutritional value for rats than casein, whereas hydrolysates formulated with *Bacillus subtilis* protease were inferior to casein probably due to an imbalance of the essential amino acids (Higashi *et al.*, 1965). Furthermore, the nature of the free amino acids and peptides formed also influence the taste and other properties of the hydrolysate (Hujita *et al.*, 1972; Konosu and Yamaguchi, 1982; Matsumoto and Yamanaka, 1990). The objectives of this study were to optimize reaction conditions for the hydrolysis of fresh and frozen shrimp by trypsin and chymotrypsin; and to determine the influence of the composition of the hydrolysate on its quality.

MATERIALS AND METHODS

Frozen and fresh shrimp (*Pandalus borealis*) were obtained from a local seafood processor (Les Fruits de Mers de l'Est du Québec, Matane, Québec). The frozen samples, stored at −80°C, were thawed and exoskeletal material removed by manual peeling. The meat/flesh obtained were cut into small pieces and homogenized using a polytron (model PT 3000, Brinkman Instruments, Littau Switzerland) at a speed setting of 6000 rpm, at room temperature (~25°C). The fresh samples were transported on ice to the laboratory where the flesh was subjected to the same treatments as the frozen samples. Bovine pancreatic trypsin type III (activity 10 000–13 000 BAEE units per mg protein) and chymotrypsin type II (activity: 40–60 units mg^{-1} protein) were purchased from Sigma Chemical Co. (St Louis, Missouri). Chemicals used for protein determination were purchased from Fisher Scientific Co (Montreal, Canada).

Proximate analysis

Initial moisture contents of frozen and dried samples were determined by the method of Heaton *et al.* (1975). Triplicate samples, 5 g each, were dried under vacuum in pre-weighed aluminum dishes in a vacuum oven (Precision Scientific Inc., model 19) at 70°C for 12 h. These were cooled in a desiccator and re-weighed. The moisture content was estimated as the difference in the two weight measurements. Protein content was determined using standard A. O. A. C. (1980) procedures with a rapid Kjeldahl system (Labconco Rapidstill III, model 65300, Chicago, IL) from total nitrogen content (%N×6.25). Crude fat content was also determined using the standard A. O. A. C. (1980) procedures. Ten grams of samples in pre-weighed flasks were extracted with petroleum ether (250 ml) using a Soxhlet fat extractor, and the crude fat dried in a vacuum oven at 55°C for 16 h and re-weighed. Ash content was determined by burning 2.0 g samples in pre-weighed porcelain crucibles at 600°C for 16 h in a muffler furnace according to standard A. O. A. C. (1980) methods.

Experimental design for optimization

A 3×3 factorial central composite design (CCD) was employed to optimize hydrolysis of shrimp for recovery of amino acids. The factors and levels used are shown in Tables 1 and 2. The selection of these factors was based on preliminary studies on enzyme hydrolysis conducted in our laboratory. The shrimp flesh was ground and mixed with buffer containing trypsin or chymotrypsin to commence hydrolysis. For chymotrypsin, the buffer was 0.8 M Tris–HCl (pH 7.8, containing 0.1 M $CaCl_2$), while the buffer for trypsin assay was 0.05 M Tris–HCl (pH 8.2, containing 0.02 M $CaCl_2$). The shrimp meat:buffer ratio was kept at 1:10 (w/v) for all the experiments which were run in duplicate. Hydrolysis with chymotrypsin was terminated by incubation of hydrolysates for 3 min at 80°C, while soybean trypsin inhibitor was used to terminate trypsin hydrolysis (because of the thermal stability of trypsin). The hydrolysates obtained were analyzed for pH, degree of hydrolysis, volatile nitrogen, amino acid content and sensory properties.

pH

The pH of shrimp hydrolysate was measured by dipping the electrode of a Corning model 220 pH meter into 5 ml of hydrolysate.

Table 1. Values of coded levels used in the optimization experiment

	Coded levels		
Process variable	−1	0	+1
Temperature, °C(X_1)	25	35	45
Time, h(X_2)	1	2	3
Enzyme concentration, % (X_3)	0	0.15	0.3

Table 2. Coded level combinations for a three variable central composite rotatable design for enzymatic hydrolysis of shrimp meat

	Coded levels		
Runs	X_1	X_2	X_3
1	−1	−1	−1
2	1	−1	−1
3	−1	1	−1
4	1	1	−1
5	−1	−1	1
6	1	−1	1
7	−1	1	1
8	1	1	1
9	−1	0	0
10	1	0	0
11	0	−1	0
12	0	1	0
13	0	0	−1
14	0	0	1
15	0	0	0
15	0	0	0
15	0	0	0

Degree of hydrolysis

Formol nitrogen was determined by the formol titration method of Beddows *et al*. (1976) and used as an index of hydrolysis. One millilitre of the hydrolysate was mixed with 40 ml of deionized water and titrated to pH 7.0 with 0.1 m NaOH. Ten millilitres of formalin solution (38% v/v) were then added and the titration continued further to pH 8.5 with 0.1 m NaOH. The results were expressed as mg N g^{-1} of sample.

Volatile nitrogen

The volatile N content was determined by the method of Chayovan *et al*. (1983). About 0.7 ml of hydrolysate was placed in the Kjeldahl steam distillation apparatus and 4 ml of 0.2% $Mg(OH)_2$ added. This was distilled to release volatile nitrogen into 50 ml of saturated boric acid solution containing bromocresol green and methyl red as indicators. The solution was titrated against a control solution using 0.05 m HCl until the colour changed to pink-red. The results were expressed as mg N ml^{-1} of hydrolysate.

Amino acid analysis

Free amino acid content was determined using a modified form of the method by Jones (1958). It involved extraction of 20 ml of hydrolysate with absolute ethanol, followed by filtration of the resulting homogenate. The residue was re-extracted twice with 80% ethanol, centrifuged at 16 000 ***g*** for 15 min, and the filtrates pooled. An aliquot of the pooled filtrates was further extracted with chloroform (1:3 v/v), and the resulting emulsion shaken for 5 min and left to separate overnight. The organic layer was discarded and residual chloroform removed by vacuum evaporation using a rotary evaporator (Buchi, model RE 21). The aqueous layer was freeze-dried for dabsylation.

Twenty microlitres of the dabsylation reagent buffer (0.05 M $NaHCO_3$, pH 8.1) were added to each of the freeze-dried samples and the amino acid standards; and 40 μl of the re-constituted dabsylation reagent (1 mg of dabsyl chloride in 1 ml acetonitrile) was added to each sample tube. These were sealed with stoppers and heated at 70°C in a heating block for 12 min with periodic shaking in a vortex. The tubes were then cooled and 440 μl of dilution buffer ((50 mM sodium phosphate, pH 7.0 / ethanol 1:1 (v/v)) added and mixed gently. The derivatized samples were stored at 4°C until needed for further analysis by HPLC using a reversed phase column. The HPLC analysis was performed with a Beckman HPLC Gold System using an ultrasphere C-18 column (4.6×250 mm). There were two components in the mobile phase. Mobile phase A comprised of 100 ml of 0.1 M sodium citrate, 860 ml of HPLC grade water, and 40 ml of dimethylformamide (DMF); while the composition of mobile phase B was 300 ml of mobile phase A and 700 ml of 4% DMF in acetonitrile. The elution gradient is shown in Table 3. Integration of peak areas from the chromatograms obtained at 436 nm was performed using the System Gold program. Results were expressed as μmol g^{-1} of sample.

Sensory evaluation

Sensory analysis was carried out by a panel of five co-workers who were familiar with shrimp aroma. The samples were heated for 2 min before they were presented to the panel, who were asked to rank them for aroma quality (i.e., acceptability based on the 'freshness' of the aroma and lack of rancid odors) and aroma intensity. The results were coded and subjected to statistical analysis.

Statistical analysis

Statistical analysis was done using the general linear model (GLM) of the Statistical Analysis System (SAS, 1982). All three dimensional graphs were plotted using the SAS/graphics program on the McGill University mainframe.

RESULTS AND DISCUSSION

Table 4 shows results of the proximate analysis of fresh and frozen shrimp. The moisture contents of 75.13 and 76.69% for the frozen and fresh samples, respectively, were similar to those reported by Watt and Merill (1963), and Kinsella, 1988. The hydrolysates from both the frozen and fresh samples also had very high protein contents. On a wet weight basis, this was 17.23% for fresh shrimp and 21.43% for the frozen samples. These values had been reported to be 19 and 18.15% in fish tissues by other workers (Kinsella, 1988; Watt and Merill, 1963). Fat content was also found, based on wet

Table 3. The elution gradient used for the reversed phase system

Time (min)	Flow rate (ml min^{-1})	%A	%B
Initial	1.4	75	25
0.00	1.4	44	56
17.20	1.4	14	86
23.20	1.4	0	100
29.20	1.4	75	25
39.90	1.4	75	25

Table 4. Proximate composition of fresh and frozen shrimp

Component	Frozen	Fresh
Moisture	75.13 ± 0.75	79.69 ± 0.40
Protein[a]	86.41 ± 1.65	85.81 ± 1.62
Fat[a]	4.33 ± 0.78	6.15 ± 0.91
Ash[a]	8.22 ± 0.06	n.d.

[a]Expressed as dry wt basis; n.d., not determined.

weight, to be 0.88 and 1.25% for frozen and fresh samples, respectively. The fat levels were lower than that (2.43%) reported by Ackman (1988) for shrimp. It should be noted, however, that factors such as species type, age, season, nutritional status, and environmental conditions all affect the composition of fish and shellfish (Gordon and Roberts, 1977; Konosu, 1979). The ash content of frozen shrimp was found to be 2.04% on a wet wt basis, which was similar to the values of 1.85 and 2.49% reported by Gordon and Roberts, 1977 and Amer *et al*. (1991), respectively.

The pH values of the frozen and fresh shrimp hydrolysates ranged between 6.5–7.2 for the various hydrolysis conditions with either chymotrypsin or trypsin (Table 5). Similarly, the levels of ammonia N were not significantly ($p < 0.01$) different, ranging between 0.12–0.2 mg N ml^{-1} sample. According to Cobb and Vanderzant (1971), microbial breakdown of free amino acids is a major source of ammonia. The low ammonia content thus indicates relatively lower levels of microbial presence and/or activity.

The hydrolysates were evaluated on a five-point scale for both aroma intensity and aroma quality. Statistical analysis of the results showed that both enzyme concentration and duration of hydrolysis had significant ($p < 0.01$) effects on the two parameters. With chymotrypsin hydrolysis of frozen shrimp, the optimum conditions for the most desirable aroma quality were an E:S ratio of 0.25–0.3% for 2–2.5 h at 35°C. The optimum conditions for trypsin were an E:S ratio of 0.25–0.3% for 2.5–3 h at 40°C. For both enzymes, increasing enzyme concentration and duration of hydrolysis resulted in increased aroma quality and intensity (Figs 1(a) and (b)). The higher aroma quality of the enzyme-treated samples vs the fresh shrimp extract is probably due to increased levels of free amino acids from the enzymatic hydrolysis. This is because heat treatment of the extracts (as was done prior to sensory analyses by the panel), would generate higher levels of volatile compounds from the free amino acids through Strecker and Maillard reactions, and also via thermal decarboxylation and deamination reactions. Similar trends were obtained for the fresh samples, except that the temperatures required for optimum quality were lower (data not shown). For example, the optimum temperature for producing a quality of trypsin hydrolysate aroma was reduced from 40 to 30°C. This may be due to the relative tenderness of the fresh samples rendering them more readily susceptible to hydrolysis than the frozen samples. Furthermore, freezing of the shrimp may cause freeze-denaturation of some endogenous proteases and other proteins in the flesh resulting in reduced proteolytic activity (Sikorski and Kolakowska, 1994). The two enzymes also showed slight differences in the degree of hydrolysis. (Figs 2(a) and (b)) show that the degree of hydrolysis continued to increase with chymotrypsin concentration. However, with trypsin, it

Table 5. pH of fresh and frozen shrimp hydrolysates

	Chymotrypsin		Trypsin	
Runs	Fresh	Frozen	Fresh	Frozen
1	7.23	7.07	7.20	6.96
2	7.13	6.93	7.18	7.04
3	7.02	6.99	6.70	7.12
4	7.11	6.91	6.72	7.19
5	6.64	6.75	6.71	7.07
6	6.60	6.62	6.78	7.07
7	6.65	6.52	6.81	7.03
8	6.58	6.53	6.86	7.12
9	6.80	6.72	6.83	6.99
10	6.65	6.61	6.82	7.02
11	6.60	6.53	6.88	6.98
12	6.65	6.55	6.72	7.02
13	6.60	6.63	6.94	7.06
14	6.58	6.52	6.92	6.92
15	6.65	6.47	6.88	6.85

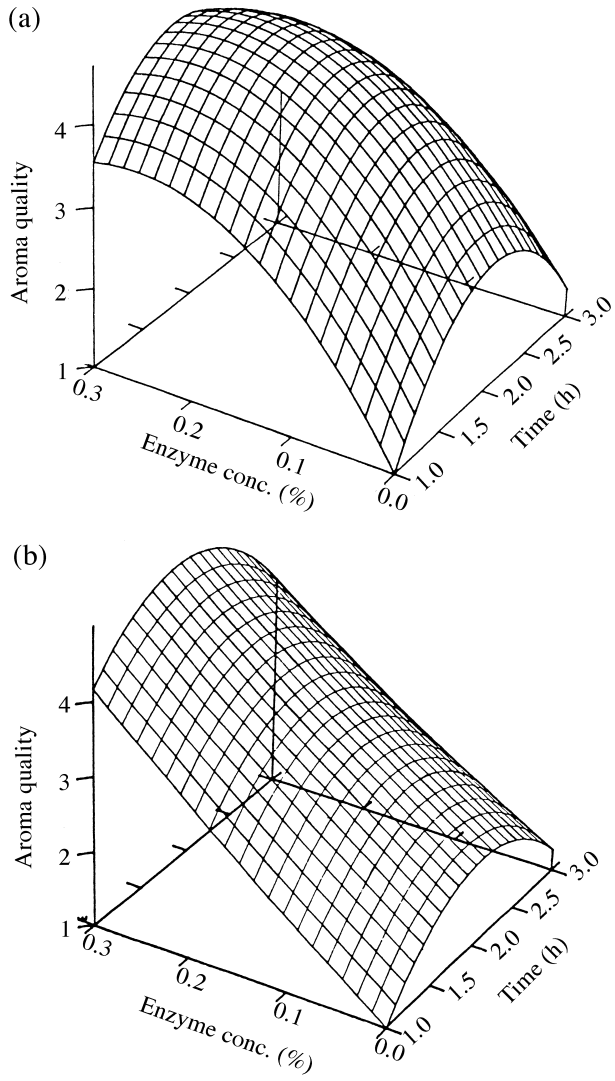

Fig. 1. Three dimensional response surface plot of the effects of enzyme (chymotrypsin) concentration and duration of hydrolysis on (a) aroma quality and (b) aroma intensity of frozen shrimp hydrolysate at a constant temperature.

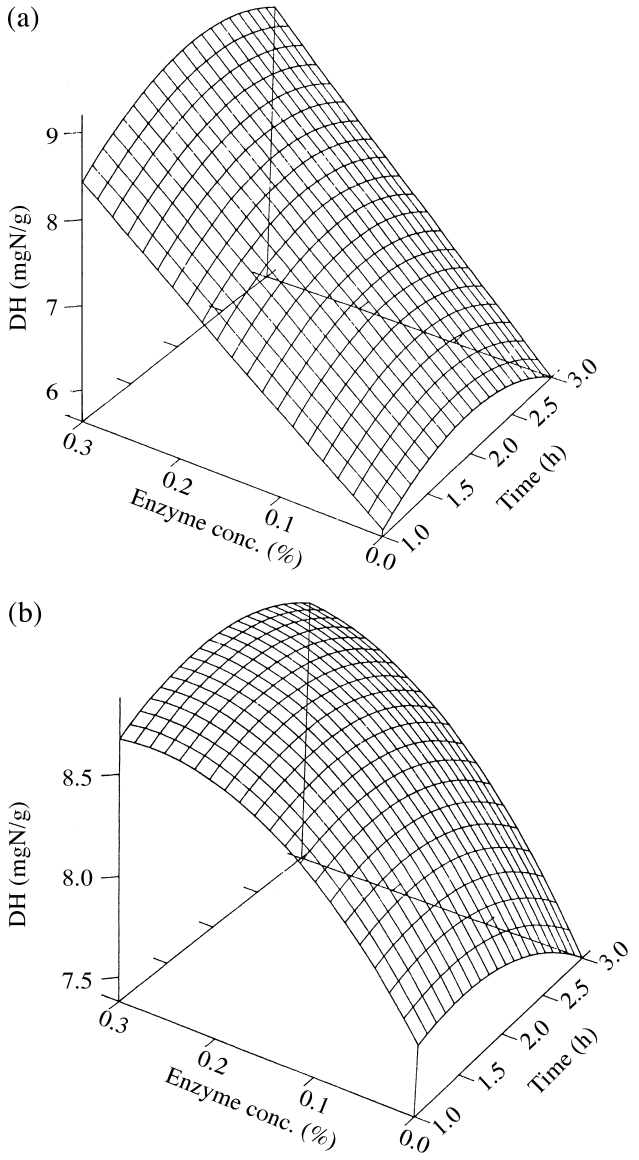

Fig. 2. Three dimensional response surface plot of the effects of enzyme concentration and duration of hydrolysis on degree of hydrolysis of fresh shrimp. (a) and (b) represent hydrolysis by chymotrypsin and trypsin, respectively.

increased to a maximum at a concentration of 0.27% and tapered off thereafter. This effect could be attributed to the relatively narrow specificity of trypsin for peptide bonds on the carboxyl side of arginine and lysine unlike chymotrypsin which has a much broader specificity for bonds between aromatic as well as leucyl, methionyl, asparaginyl, and glutamyl residues. As for the aroma quality and intensity, similar trends were observed with the fresh shrimp.

The free amino acid content of the hydrolysates were measured and used as an index for the flavour changes, since various researchers have shown a correlation between some amino acids and flavour. According to Konosu and Yamaguchi (1982); Hayashi *et al.* (1981), and Matsumoto and Yamanaka (1990), the major amino acids influencing shrimp flavour include alanine, arginine, glycine, glutamic acid, and proline. Komata (1964) also found glycine, valine, alanine, glutamic acid, and particularly methionine, to be important determinants of 'Uni' (the unripe gonad of the sea urchin) flavour. A summary of the free amino acid content shown in Table 6 indicates high levels of glycine, alanine, arginine, proline, and valine were present in the hydrolysates. On the whole, there were no significant ($p > 0.05$) differences between the levels of these amino acids produced by chymotrypsin and trypsin, but their levels appeared to be higher, particularly with chymotrypsin, in hydrolysates from frozen than fresh shrimp. The higher levels of amino acids obtained with chymotrypsin and frozen shrimp could be due to a combination of factors, including protein denaturation during frozen storage of shrimp, thus making them more amenable to proteolysis. It is worth noting, however, that the levels of these amino acids can also vary with environmental conditions, season, spawning, and genetic make-up of the species. For example, Hujita *et al.* (1972) studied free amino acid contents in different species of shrimp (*P. borealis, P. japonicus, P. orientalis*), and found the levels of serine, alanine, proline, and glycine to range between 26 and 230, 30 and 129, 126 and 493 mg $100\,g^{-1}$, respectively. In other studies, Tsai and Pan (1988) reported the content of glycine, alanine, arginine, and proline in *P. monodon*, as 721, 139, 327, and 293 mg $100\,g^{-1}$, respectively. The levels of the same amino acids reported by Hujita *et al.* (1972) for this specie were 1145, 26, 922, and 188 mg $100\,g^{-1}$, respectively. Thus any comparison of such data will have to take the aforementioned factors into consideration.

Our data also shows that high levels of essential amino acids like valine, leucine, isoleucine, and phenylalanine were produced by both enzymes even though the levels of arginine and lysine derived from trypsin hydrolysis were about 40% higher than those produced by chymotrypsin. The total essential amino acid content from frozen shrimp was also significantly ($p < 0.05$) higher than those derived from fresh shrimp similar to the observation made with chymotrypsin hydrolysis of frozen versus fresh shrimp. However, there were no significant ($p > 0.05$) differences between the two enzymes in terms of the total amount of essential amino acids in the hydrolysate. The high content of essential amino acids is noteworthy in terms of the potential use of the product. For instance, nutritional studies with calves have shown that up to two-thirds of the protein in skimmed milk-based substitutes could be replaced by fish protein hydrolysates without adverse effects on the growth or feed efficiency of the animals (Diaz-Castaneda and Brisson, 1987). In another report, feeding of experimental animals with fish (*Mugil cephalus*) protein hydrolysate promoted higher body weight ($p < 0.05$) and improved ($p < 0.01$) feeding efficiency, protein efficiency ratio, and net protein ratio (Rebeca *et al.*, 1991). Yanase and Murayama (1965) also demonstrated that hydrolysates made from mackerel waste contained an unknown

Table 6. Free amino acid composition of hydrolysates from fresh and frozen shrimp using trypsin and chymotrypsin under optimum conditions

Amino acid (mg 100 g^{-1})	Control[a] (no hydrolysis)	Chymotrypsin treated		Trypsin treated	
		Fresh	Frozen	Fresh	Frozen
Asp	7.68	138.29	68.68	17.30	26.86
Glu	26.58	21.48	n.d.	6.76	15.30
Ser	7.77	9.87	25.54	24.38	29.25
Thr	12.16	15.09	33.10	17.86	13.10
Gly	15.87	370.09	420.39	624.13	306.28
Ala	101.09	75.54	142.54	158.13	195.98
Arg	372.53	235.69	483.19	298.40	671.17
Pro	929.34	396.73	546.50	353.67	368.42
Val	16.39	201.84	138.00	137.88	91.37
Met	22.46	26.26	57.60	28.20	45.80
Leu	24.16	31.34	79.62	54.82	106.24
Ile	20.97	55.87	92.08	49.18	125.92
Phe	105.82	54.30	124.39	46.74	59.46
Cys	0.00	20.42	24.99	9.37	15.85
Lys	0.00	26.16	37.28	67.39	80.40
His	2.76	12.56	29.95	25.29	31.96
Tyr	3.08	9.42	n.d.	13.22	14.48

[a]Data from Mandeville *et al.* (1992).
n.d., not detected; data presented in Table 6 are average values of duplicate determinations.

growth factor which improved growth of rats when 10% of a casein-based protein diet was substituted by the fish hydrolysate.

Second order polynomial models (eqn (1)) generated from multiple regression analysis of the individual amino acid contents (frozen and fresh) had r^2 values ranging from 0.80 to 0.96 indicating that 80–96% of total variation may be explained by the models. The linear, quadratic and interaction effects were found to be significant ($p < 0.01$–0.05) for all the parameters studied.

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

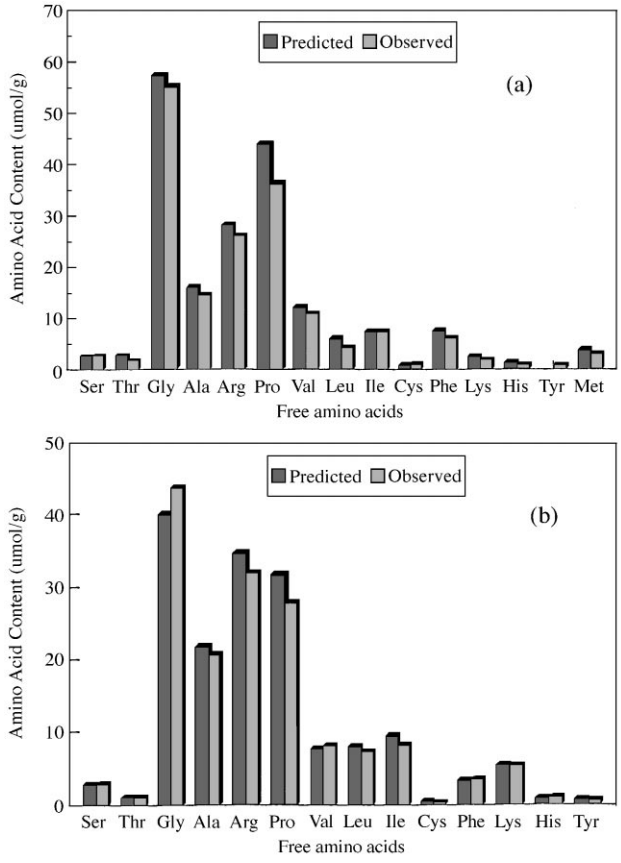

Fig. 3. Comparison of observed and predicted values of free amino acid content of (a) chymotrypsin and (b) trypsin-treated frozen shrimp

Comparison of predicted and observed data for the frozen shrimp showed close agreement between these values (Fig. 3). The difference between total amino acid content with chymotrypsin hydrolysis was approx. 14 μmol g^{-1} giving about 90% agreement between model predictions and observed values. For trypsin, the difference was about 13 μmol g^{-1}. Thus the models developed may be considered to be very reliable for predicting amino acid content under the conditions used in this experiment.

CONCLUSION

Trypsin and chymotrypsin hydrolysis of frozen and fresh shrimp both produced hydrolysates with high levels of the amino acids, alanine, proline, glycine, and arginine, considered to be important in crustacean flavours. While both enzymes were capable of producing reasonably high levels of essential amino acids, trypsin produced about 40% more arginine and lysine than chymotrypsin. The high levels of essential amino acids furnished by the two enzymes would enhance the value and potential of shrimp protein hydrolysates as food ingredients in the same way as fish protein hydrolysates.

The close agreement between predicted and observed results would also ensure the application of such mathematical models to obtain products of specific qualities without having to perform any further experiments.

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