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Characteristics of *Bellamya purificata* snail foot protein and enzymatic hydrolysates

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Abstract

The foot muscle protein of *Bellamya purificata* (mud snail, named Luosi in Chinese) was investigated. Its conformation change and increase in solubility were researched during enzymatic hydrolysis. The protein conformation was looser following an increase in pH (from 10 to 12), while the β-sheet was the main conformation at pH 12. Blending, ultrasonic extraction, ultradispersing and alkaline treatment increased the solubility of the foot muscle protein. The effects of several proteases on its hydrolysis were compared and Proleather FG-F was chosen. The relative molecular mass distribution, the free amino acids (FAA) content and the angiotensin-I converting enzyme (ACE) inhibitory activity of the hydrolysates were quantitatively analyzed and compared. In the Proleather FG-F hydrolysates, the percentage of the peptides with molecular weight between 150 and 2000 Da were 84.65%, much more than that in the Alcalase 2.4L hydrolysates (68.44%). Proleather FG-F released much less FAA (5.80%), than Alcalase 2.4L (17.01%). The IC_{50} of the Proleather FG-F hydrolysate was 0.69 mg/ml, whereas for the Alcalase 2.4L hydrolysate the value was 3.30 mg/ml. Finally, response surface methodology (RSM) was used to optimize the factors (pH, enzyme: substrate ratio- E/S- and temperature) affecting Proleather FG-F hydrolysis.

Keywords: *Bellamya purificata*; Foot muscle protein; Proleather FG-F; Enzymatic hydrolysis; Angiotensin-I converting enzyme inhibition

1. Introduction

Due to increasing cost, animal protein supply is limited, while the potential for improving the situation through increased animal production is limited and the need for efficient utilization of cheap and available food resources is inevitable. *Bellamya purificata* is a species of mud snail found in fresh water in China. It belongs to phylum Mollusca, class Gastropoda, subclass Prosobranchia and family Viviparidae. It has a flattened muscular foot for locomotion. Its total annual yield in China is over 10 million tonnes. It is abundant in fresh water in the Yangtze River drainage area and most of the provinces in east-China.

It is 2–3 cm in shell length and much smaller than *Cipangopaludina chinensis* and *Cipangopaludina cathayensis* (5–6 cm in shell length), which are favourite mud snails to the Chinese. Its muscle is tough and small quantities have traditionally been cooked or salted for food purposes. In addition, it is an important animal protein feedstuff for poultry, crab, turtle and predatory fish, such as herring. The shell is hard to break and can be used to increase the mineral content of animal feeds when powdered. However, it is still under-utilized. Its consumption is limited owing to lack of adequate information about its nutritional and biological potential. There is a desire to better utilize it and the interest in the use of the snail protein for human consumption has been increasing.

One of the approaches for improving and upgrading the functional and nutritional properties of proteins is enzymatic hydrolysis. Proteins can be hydrolyzed to produce biologically active peptides. Protein hydrolysates are

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diverse in their functional and bioactive properties. Several bioactive properties of interest include inhibition of the angiotensin-I converting enzyme (ACE), which results in blood pressure decrease. Enzymatic hydrolysis of the snail produces protein hydrolysates rich in soluble low molecular weight peptides.

The fact that protein is absorbed in the form of both peptides and amino acids, and that the transport mechanism of peptides in the intestinal mucous membrane is better than that of free amino acids, suggests that protein hydrolysates may offer physiological advantages of speed and efficiency over free amino acids (Matthews, 1975; Silk et al., 1980). Furthermore, free amino acids make the diets hyperosmotic, causing intestinal secretion and hence diarrhoea (Mahmoud, 1994).

The molecular weight or, even more importantly, the number of peptide residues remaining in the peptide chains after hydrolysis is essential in producing protein hydrolysates with desired functional properties to use as functional components. Since di- and tri-peptides are more rapidly absorbed than tetra- and penta-peptides (Silk, Grimble, & Rees, 1985; Webb, 1990). The physiological function of protein hydrolysates is also related to various other factors, including the protein source and the enzyme used (Fairclough, Hearty, Silk, & Clark, 1980; Keohane, Grimble, Brown, Spiller, & Silk, 1985). Amano Enzyme Inc. (Japan) claims Proleather FG-F (*Bacillus subtilis*, EC 3.4.21.62) can produce hydrolysates consisting mainly of di- to penta-peptides. Few reports exist on its prior use (Aluko & McIntosh, 2005; Lino, Maria, & Jonathan, 2002) while its hydrolysates have not been characterized, nor is there a report on its comparison with other enzymes. The enzyme has not been utilized in hydrolyzing *Bellamya purificata* muscle protein. In this study, the effect of Proleather FG-F was compared with five other proteases (namely Neutrase, Alcalase 2.4L, Protamex, Papain and A.S.1398) and the relative molecular mass distribution of the hydrolysates was studied. To the best of our knowledge, neither the protein source in our research (the muscle protein of *Bellamya purificata*) nor its hydrolysates have been described in the literature before.

The peptide composition of the hydrolysates depends on the specificity of the proteolytic enzyme as well as on the hydrolysis conditions. The five often studied parameters comprise source, enzyme to substrate ratio (E/S), hydrolysis time, pH and temperature of hydrolysis. Response surface modelling has been proven to be a valuable tool for simultaneous optimization of several process parameters for hydrolysis processes. For optimization of the hydrolysis process, the effects of enzyme to substrate ratio, pH and temperature of hydrolysis on degree of hydrolysis (DH) and protein recovery were studied, using a central composite design.

2. Materials and methods

2.1. Materials and reagents

The mud snail (*Bellamya purificata*) was obtained live from a local supplier near the campus. Neutrase 0.5L, Alcalase 2.4L and Protamex were obtained from Novo Enzymes (Bagsvaerd, Denmark). Papain and a crude protease preparation A.S.1398 were purchased from Genencor International Inc. (Wuxi, China). Proleather FG-F was obtained from Amano Enzyme Inc. (Nagoya, Japan). The enzymes' compositions and optimum hydrolysis conditions are presented in Table 1. Hippuryl-L-histidyl-L-leucine (Hip-His-Leu) and ACE (EC 3.4.15.1, from rabbit lung) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals and reagents were obtained from local manufacturers and made available at the university chemical store.

2.2. Preparation of the Bellamya purificata flesh powder

The snails were deshelled and the foot muscle was manually separated. Next, the flesh was washed 2–3 times under tap water to remove sand and other dirt. Excess water was then drained and the flesh was packed in PVC bags and stored at −18 °C until required for further use. Before use, the muscle was thawed and homogenized using a home blender for 3 min; and the slurry was lyophilized in a freeze dryer. The powder was obtained after pulverization.

Table 1
Composition and optimum hydrolysis conditions of individual enzymes

Enzyme	EC Number	Enzyme composition	Optimum conditions		Source
			pH	T (°C)	
Protamex	3.4.24.28	Endoprotease	6.5–7.0	40–50	*Bacillus protease*
Neutrase	3.4.24.28	Endoprotease	6.5–7.5	45–55	*Bacillus* sp.
Alcalase 2.4L	3.4.21.62	Subtilisin Carlsberg A serine endopeptidases	8.0	50–60	*Bacillus licheniformis*
Papain	3.4.22.2	Cysteine endopeptidases (including amidase, esterase, transamidase and transesterase)	6.5–7.5	55	*Carica papaya*
A.S.1398[a]	Crude mixture	Endoprotease	7.0	45	*Bacillus* sp.
Proleather FG-F	3.4.21.62	Endopeptidases	9.5–10.5	60	*Bacillus* sp.

[a] Given by Shen et al. (2002).

2.3. Circular dichroism (CD) spectroscopy

The flesh powder was extracted by stirring for 1 h in alkaline solutions (1:40, w/v) at either pH 10, 11, or 12. After centrifugation at 10,000*g* for 15 min, an additional extraction was carried out for another hour with half the volume of alkaline solutions. The supernatants were pooled. The pH of the protein extracts was adjusted to 4.7 using 0.5 M HCl and centrifuged as above. The precipitates were freeze-dried.

Protein samples were solubilized in 5 mM Na_2HPO_4-NaOH buffer at extraction pH and the CD measured at 20 °C on a Jasco J-500 spectropolarimeter (JASCO International Co., Tokyo, Japan). In the 192–250 nm wavelength region five scans were accumulated with a scan rate of 100 nm/min. Fractions of secondary structures were estimated using the secondary structure estimation program (SSEAX) of Jasco Corp. Each observed spectrum was expressed as a linear combination of reference spectra of four types of secondary structures: α-helix (α), β-sheet (β), β-turn (t) and coil (c, random).

2.4. Hydrolysis of the muscle with proteases

The powder in Section 2.2 was homogenized in distilled water using an Ultra-Turrax T25 (IKA Labortechnik, Stauffen, Germany) at 24,000 r/min for 0.5 min for three cycles to get a 5% (w/v) protein blend. The pH was adjusted using 1 N of either NaOH or HCl, according to the hydrolysis conditions of each enzyme. The blended protein, at 60 °C, was treated in an ultrasonicator at 20 kHz for 20 min.

Six proteases (Neutrase, Alcalase 2.4L, Protamex, Papain, A.S.1398 and Proleather FG-F) were tried (E/S was 3:100, w/w) at the optimum conditions recommended by the manufacturers (Table 2). The muscle protein was suspended in 250 ml distilled water (5% protein concentration) and hydrolyzed with proteases in a batch reactor and the degree of hydrolysis (DH) monitored using the pH-stat technique, according to Adler-Nissen (1986). After 4 h hydrolysis, the proteases were inactivated by dipping in boiling water (90 °C) for 15 min. After cooling, the mixture was centrifuged at 3000*g* for 20 min (4 °C). The supernatants were lyophilized and stored in a desiccator until use without further treatment. The hydrolysates obtained were analyzed for protein recovery (with Kjeldahl N*6.25).

Table 2
Protein recovery of the muscle hydrolysates using various proteases

Proteases	Condition	Protein recovery (%, w/w)
Neutrase	55 °C, pH 7.0	66.42
Alcalase 2.4L	55 °C, pH 8.0	80.02
A.S.1398	45 °C, pH 7.0	76.53
Protamex	50 °C, pH 7.0	51.74
Papain	55 °C, pH 7.0	34.66
Proleather FG-F	60 °C, pH 10.0	79.23

2.5. Molecular weight determination

Hydrolysates were analyzed for molecular weight distribution using a Waters™ 600E Advanced Protein Purification System (Waters Corporation, Milford, MA, USA). A TSK gel, 20005 μ × L, (6.5 × 300 mm) column was used with 10% acetonitrile + 0.1% TFA in HPLC Grade water as the mobile phase. The calibration curve was obtained by running bovine carbonic anhydrase (29,000 Da), horse heart cytochrome C (12,400 Da), bovine insulin (5800 kDa), bacitracin (1450 Da), gly-gly-tyr-arg (451 kDa) and gly-gly-gly (189 Da). The results were obtained and processed with the aid of Millennium^{32} Version 3.05 software (Waters Corporation, Milford, MA 01757, USA).

2.6. Free amino acid analysis

The hydrolysates were dissolved in 3.5% 5-sulfosalicylic acid (SSA). After filtration and centrifugation, the supernatants were submitted to online derivatization by *o*-phthaldialdehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC-Cl, for proline analysis). Reversed phase high performance liquid chromatography (RP-HPLC) analysis in an Agilent 1100 (Agilent Technologies, Palo Alto, CA 94306, USA) assembly system was done using the conditions prescribed by the equipment manufacturer, using a Zorbax 80A C_{18} column (4.6 id × 180 mm).

2.7. Assay of ACE inhibitory activity

The ACE inhibitory activity was measured according to the method of Cushman and Cheung (1971) as modified by Wu, Aluko, and Muir (2002), using RP-HPLC in a Waters™ 600E Advanced Protein Purification System (Waters Corporation, Milford, MA 01757, USA). A peptides sample solution (approximately 2.5 mg/ml) was dissolved in deionized water and diluted to make five different concentrations. Then, from each dilution, 10 μl was mixed with 40 μl of 50 mM sodium borate buffer (pH 8.3), containing 6.5 mM Hip-His-Leu and 300 mM NaCl, and then preincubated for 5 min at 37 °C. The reaction was initiated by the addition of 20 μl of ACE dissolved in distilled water (100 m units/ml), while the reaction mixture was incubated for 40 min at 37 °C. The reaction was stopped by adding 80 μl of 1 N HCl. The hippuric acid liberated by ACE was determined directly at 228 nm. Plots of the peptide concentrations against the % inhibition were plotted and from it the IC_{50} value was estimated. The concentration of ACE inhibitors needed to inhibit 50% of ACE activity was defined as the IC_{50} value. The respective peptide concentration in each dilution was further determined, according to the method of Lowry, Rosebrough, Farr, and Randall (1951).

Table 3
Independent variables and their levels in optimization of hydrolysis for the snail muscle

Independent variables	Symbol	Coded variable levels		
		−1	0	1
pH	X_1	9	10	11
E/S (%, w/w)	X_2	3	5	7
Temperature (°C)	X_3	50	60	70

2.8. Optimization of proleather FG-F hydrolysis

After Proleather FG-F was chosen, its hydrolysis of the muscle protein was optimized with three hydrolysis parameters being varied: enzyme to substrate ratio (E/S), the pH and temperature. The process parameters were optimized using response surface methodology (RSM), varied at three levels for each process parameter at coded levels −1, 0 and +1 (Table 3).

The responses (DH and protein recovery) were analyzed using the SAS (Statistical Analysis System Institute Inc., 1989, Cary, NC, USA) program. A quadratic polynomial regression model was assumed for predicting the responses. The model proposed for the response was

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + e \quad (1)$$

where X_1, X_2 and X_3 are the independent variables. The model goodness-of-fit was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA). The response surface and contour plots were developed using the fitted full quadratic polynomial equations, obtained by holding one of the independent variables at a constant value and changing the levels of the other two variables using Matlab 6.0 software (MathWorks Co., New Mexico, USA).

2.9. Proximate analysis

Crude protein and fat contents as well as moisture and ash were determined according to the AOAC (1990) method. For protein calculation a normal protein concentration Kjeldahl factor of 6.25 was used.

3. Results and discussion

Table 4 shows the main chemical components of the snail foot muscle. The crude protein and ash levels were higher while the other indices were lower than those of another kind of mud snail (*Limicolaria aurora*) reported by Anthony, Edet, and Ironge (1995). It should be noted, however, that factors such as species type, age, season, nutritional status and environmental conditions all affect snail composition.

3.1. CD spectroscopy

Circular dichroism (CD) spectroscopy plays an important role in the study of protein folding as it allows the characterization of secondary and tertiary structures of proteins in native, unfolded and partially folded states (Arnoldus & Willem, 2000). In this study, CD was used to detect the effect of pH on the structures of the snail muscle protein, which was isolated by alkaline extraction-acid precipitation method.

The far UV-CD spectra of the protein extracted at different pH (pH 10, 11 and 12) are shown in Fig. 1. They exhibit clearly different spectroscopic properties. Higher pH leads to an increase of the negative ellipticity around 208 nm. The bands peak here (typical α-helical peak) has significantly higher magnitude and is slightly shifted towards lower wavelengths. Only the spectrum at pH 12 exhibits a large positive band peak at approximately 195 nm, which is a typical β-sheet character. These results indicate drastic changes in the structural composition of the protein as extraction pH increased.

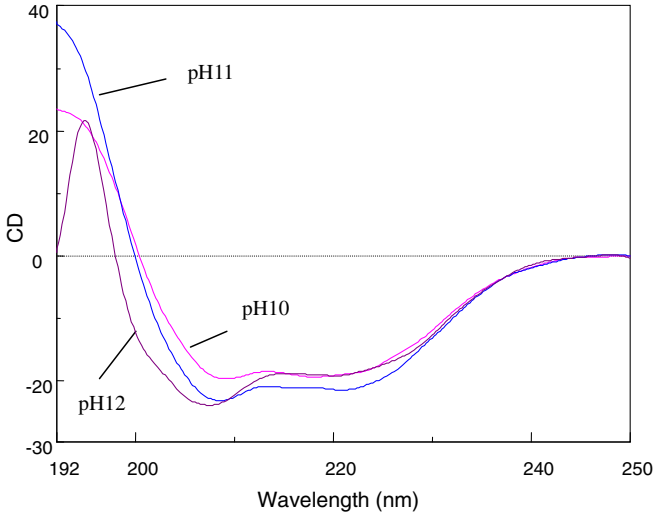

Fig. 1. The far UV-CD spectra of the protein (0.2 mg/ml) extracted at different pH (pH 10, 11 and 12) solubilized in 5 mM phosphate buffer (corresponding pH) at 20 °C. Instrumental conditions: path length 0.01 cm, scanning speed 100 nm/min, sensitivity 20 mdeg.

Table 4
The main compositions of the snail foot muscle[a]

Composition (%)	Protein[b]	Carbohydrate[b]	Fat[b]	Ash[b]	Moisture
Snail foot muscle	59.58 ± 0.33	22.86 ± 0.12	0.65 ± 0.02	16.48 ± 1.02	78.82 ± 0.44

[a] Data are mean values of triplicate determination ± standard deviation.
[b] Calculated based on dry weight basis.

Table 5
Circular dichroism spectroscopy data analysis to determine the secondary structure composition of the protein extracted at different pH

Extract condition	Secondary structure content (%)			
	α	β	t	c
pH 10	28.3	27.9	11.7	32.2
pH 11	28.8	37.6	0.7	33.0
pH 12	13.2	48.7	6.1	32.0

The data in Table 5 were obtained from the spectra shown in Fig. 1, which are the fractions of residues in α-helix (α), β-sheet (β), β-turn (t) and random coil (c) conformation in per cent for the protein extracted at three different pH values. The analysis indicates a higher percentage of β-sheet conformation (27.9% in the pH 10 sample vs. 48.7% in the pH 12 sample) with a lower contribution of α-helix (28.3% vs. 13.2%, respectively) with an increasing extraction pH, while the contributions of the random coils are similar. That is to say, the conformation of the protein loosened due to an increase in extraction pH from 10 to 12. The reason maybe the electrostatic interaction among the molecules is stronger at high pH because of net negative charge, which produces short-range repulsive forces and, consequently, contributes to protein solubility.

3.2. Solubilization of muscle protein

The snail muscle is tough and the native protein (pH 8.8) solubility is low, being only 19.9%. In this research, several methods were used to enhance protein solubilization. Besides alkaline treatment (pH 10) and heating (60 °C, alkali treatment shifted the maximum solubility to temperature value of 60 °C), blending, ultrasonic extraction and ultradispersing also greatly increased the solubility of the muscle protein (to 55.6% before hydrolysis). Generally, protein solubility is affected largely by environmental factors (i.e., pH and temperature). Alkaline treatment can induce excess net negative charges in the peptide chains, producing repulsion among the molecules, leading to more hydration of the protein molecules. Heating partially denatures the protein and the peptide chains may unfold, which also allows them to hydrate more easily. Therefore, a combination of both the alkaline and heat treatments contributed to greater solubility of the muscle protein and improved its extraction. Enzymatic hydrolysis of protein needs to be in a bulk liquid phase, meaning solubility is of primary importance.

3.3. Molecular weight characteristics of the hydrolysates

Fig. 2 shows the molecular weight distribution of Proleather FG-F hydrolysates. The major components in the hydrolysate had a molecular weight (Mw) of 150–2000 Da within which lie the fractions grouped in the range 150–550 Da, that are assumed to be di- to penta-peptides, which was consistent with the manufacturer's claims. In our research, the contents of short oligopeptides and free amino acids (FAA) were determined, respectively, which can be used to justify the effect of enzymatic hydrolysis. Compared with Alcalase 2.4L, Proleather FG-F was much more effective for producing smaller peptides with those in the range of 150–2000 Da being 84.65%, much more than for Alcalase 2.4L hydrolysates (68.44%).

The fraction of less than 150 Da was assumed to correspond to the FAA. With respect to FAA, Proleather FG-F released much less (5.80%) compared with Alcalase 2.4L (17.01%), although the DH of Proleather FG-F and Alcalase 2.4L hydrolysates were 19.65% and 16.70%, respectively, following hydrolysis at their optimum conditions for 4 h. Although release of FAA is theoretically inevitable at high DH, it is also affected by the quality of the enzyme. Therefore, it can be concluded that the characters of the two proteases are markedly different. Indeed both Alcalase 2.4L and Proleather FG-F are mainly composed of an endopeptidase, as shown in Table 1. However, the high FAA content in Alcalase 2.4L in this study as contrasted with when it is used in other substrates (Aspmo, Horn, & Eijsink, 2005; Guerard, Dufosse, Broise, & Binet, 2001; Park, Jin, & Hyun, 2002; Synowiecki & Al-Khateeb,

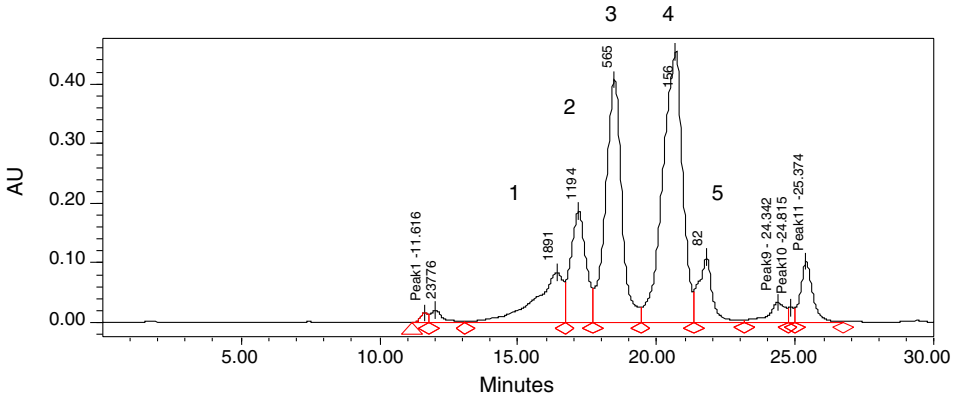

Fig. 2. The molecular weight distribution of Proleather FG-F hydrolysates. The molecular weight range for each peak: (1) 12,850–1,620 Da; (2) 1,620–880 Da; (3) 880–330 Da; (4) 330–110 Da; and (5) 110–40 Da.

2000) may be explained by the influence of pH on ease of protein solubilization, although the substrate in this study probably presented unique hydrolysis challenges to the different enzymes. Hence the Proleather FG-F hydrolysis process was done at pH 10, as compared to pH 8.0 for the Alcalase 2.4L hydrolysis.

3.4. ACE inhibitory activity of hydrolysates

Fig. 3 shows ACE inhibition by Alcalase 2.4L and Proleather FG-F hydrolysates at different concentrations. The ACE inhibitory activity of Proleather FG-F hydrolysate was much higher than that of Alcalase 2.4L hydrolysate with a lower IC_{50} value of 0.69 mg/ml (IC_{50} value of Alcalase 2.4L hydrolysate was 3.30 mg/ml). The content of N-compounds in the hydrolysate was 62%, as determined using the method of Lowry et al. (1951), and the remainder was salt and carbohydrate. Since the hydrolysate was determined without any purification, ACE inhibition by the hydrolysate was the result of the inhibitory action of various peptides present with some groups probably having no ACE inhibitory activity.

The fact that ACE inhibition is found in hydrolysates produced from different proteins, with different proteases and hydrolysis conditions, indicate that a variety of peptides, with various amino acid sequences, are able to inhibit ACE. Despite the traditional means of identifying already existing ACE inhibitors from milk (van der Ven, Gruppen, Bont, & Voragen, 2002), soy bean (Kuba, Tana, Tawata, & Yasuda, 2005), fish (Byun & Kim, 2001), porcine sources (Arihara, Nakashima, Mukai, Ishikawa, & Itoh, 2001) and beef sources (Jang & Lee, 2005), few efforts have been made to develop novel ACE inhibitors from other natural bioresources, such as mud snail. Comparison of ACE inhibition measured in the present study with results obtained in other studies (Arihara et al., 2001; Byun & Kim, 2001; Jang & Lee, 2005; Kuba et al., 2005; van der Ven et al., 2002) is not completely without error, since they were obtained from different proteins, while the IC_{50} values calculated depend on the assay conditions, which vary between studies (Wu et al., 2002).

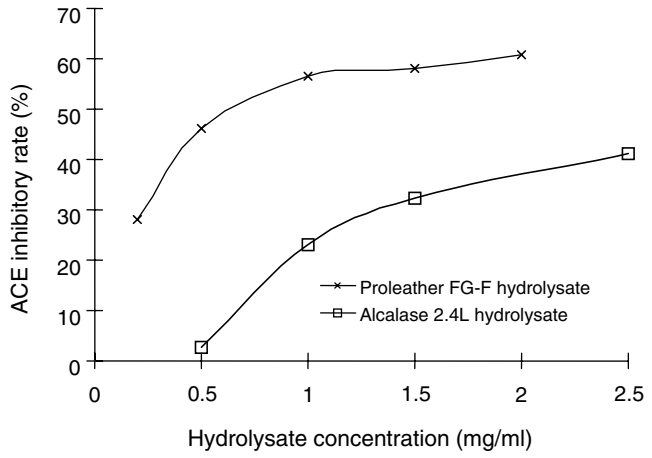

Fig. 3. The effect of hydrolysate concentrations on ACE inhibition.

There is indeed an intimate relationship between chain length and bioactive properties of peptides. Additionally, evidence suggests fairly higher parenteral absorption rate for the peptide-bound amino acids than for FAA and in our study we desire a minimal release of FAA. Furthermore, the antihypertensive peptides are those with amino acid units of 2–5 (Hong, Feng, & Miguel, 1980). Probably, Proleather FG-F hydrolysates had much higher ACE inhibitory activity because of it contained more short peptides.

3.5. Optimization of enzymatic hydrolysis

The protein hydrolysate yields obtained from use of different proteases are given in Table 2. Although many factors affect the yields of the hydrolysates, the type of enzyme used had a marked effect on the yield and properties of the final product. Among the six proteases, the protein recoveries of Alcalase 2.4L and Proleather FG-F were highest. Combined with comparison of the molecular weight characteristics and ACE inhibitory activity of the hydrolysates, Proleather FG-F was found to be superior and hence chosen for use in hydrolysis. It was the most suitable for induction of high ACE inhibition activity.

Thus response surface methodology was used to optimize protein hydrolysis using Proleather FG-F. Regression coefficients and the response surfaces were used to study the effects of various parameters on Proleather FG-F hydrolysis. Table 6 shows the responses, i.e., the DH (%) and protein recovery (%, w/w) of the hydrolysis process. The responses and variables were fitted to each other by multiple regressions. After elimination of model factors

Table 6
RSM test design and experiment data for Proleather FG-F enzymatic hydrolysis

Run	Independent variables[a]			Responses[b]	
	X_1	X_2	X_3	Y_1	Y_2
1	0	−1	−1	16.59	72.90
2	0	−1	1	14.65	64.28
3	0	1	−1	18.78	80.23
4	0	1	1	17.14	75.02
5	−1	0	−1	16.05	74.06
6	−1	0	1	15.78	71.94
7	1	0	−1	14.71	63.97
8	1	0	1	13.16	60.44
9	−1	−1	0	16.95	76.82
10	−1	1	0	19.03	80.95
11	1	−1	0	15.08	69.07
12	1	1	0	17.77	78.51
13	0	0	0	19.47	84.02
14	0	0	0	19.56	84.96
15	0	0	0	19.35	83.48

[a] Independent variables X_1, X_2 and X_3 represent pH, enzyme to substrate ratio (E/S) and temperature, respectively.

[b] Responses Y_1 and Y_2 represent DH (%) and protein recovery (%), respectively.

Table 7
The analysis of variance (ANOVA)

Parameter	DF	Sum of squares		R^2		F value		Pr > F	
		Y_1	Y_2	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2
Linear	3	25.7026	355.7961	0.4020	0.4073	29.06	13.23	0.0014	0.0082
Quadratic	3	35.7625	462.6969	0.5593	0.5297	40.43	17.20	0.0006	0.0046
Cross product	3	0.9976	10.2111	0.0156	0.0117	1.13	0.38	0.4215	0.7725
Total model	9	62.4627	828.7040	0.9769	0.9487	23.54	10.27	0.0014	0.0098
X_1	4	27.9487	316.9857	6.9872	79.2464	23.70	8.84	0.0019	0.0108
X_2	4	13.2075	168.5701	3.3019	42.1425	11.20	4.70	0.0104	0.0602
X_3	4	25.0284	394.9393	6.2571	98.7348	21.22	11.01	0.0025	0.0172

with $p > 0.05$, the coefficients of quadratic polynomial regression model fitted into Eq. (1) are shown in Eq. (2) corresponding to DH (%) and Eq. (3), corresponding to protein recovery (%)

$$Y_1 = -276.61 + 44.4900X_1^{***} + 2.6290^{**}X_3 - 2.2463X_1^{2\,***} - 0.0231X_3^{2\,***} + e \quad (2)$$

$$Y_2 = -866.72 + 132.0571X_1^{**} + 10.5419^{**}X_3 - 6.8604X_1^{2\,**} - 0.0934X_3^{2\,**} + e \quad (3)$$

***Significant at 0.001 level. **Significant at 0.05 level.

The pH (X_1) and temperature (X_3) had both linear and quadratic effects to Y_1 (DH) and Y_2 (Protein Recovery). The effect of pH was the most important, as is reflected by the highest absolute regression coefficient. The net effect of the linear and quadratic terms of pH and temperature suggested that the amount of DH and protein recovery increased until either pH or temperature reached an optimum point and then decreased following further pH or temperature increase. The Y_2 response was more significantly influenced by pH and temperature than was Y_1, as shown by the coefficients 44.4900 and 132.0571, respectively, which were much more than those of Y_1, DH, (2.6290 and 10.5419). That is probably because pH and temperature affected the solubility of substrate significantly, besides favouring enzyme hydrolysis (Proleather FG-F has a high optimum pH, 9.5–10.5) as discussed in Section 3.2. More of the protein may stay in the solution with the increased pH and temperature before 60 °C, thus increasing its recovery.

Table 7 illustrates the analysis of variance (ANOVA) results for the polynomial model. For pH and temperature the effects were highly significant; this means that their optimal values existed in the experimental range, while for enzyme concentration the maximum was apparently not within the experimental area because we used economical enzyme amounts. The R^2 values of Y_1 and Y_2 are very high (0.9769 and 0.9487, respectively, which indicated that 2% and 5% of total variation was not explained by the model, respectively). The models were considered adequate with satisfactory R^2 values (>0.85). The probability (p) values of regression models were 0.0014 and 0.0098 respectively, with no significant lack-of-fit, except for interactions regression ($p > 0.05$). A good fit was obtained,

Table 8
The optimized hydrolysis conditions fitted from the regression equation and predicted and experimental maximum DH and protein recovery

Responses	Conditions		Maximum (%)	
	pH	Temperature (°C)	Predicted	Experimental
DH	9.8	58.5	19.78	19.65 ± 0.05
Protein recovery	9.6	58.5	86.28	86.02 ± 0.19

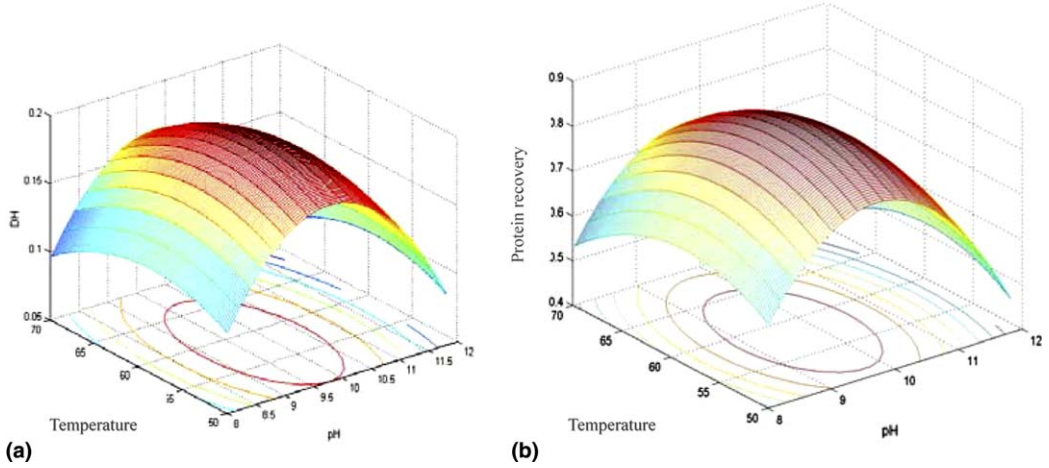

Fig. 4. Response surface and contour plot of DH (a) and protein recovery (b) in Proleather FG-F hydrolysis as a function of the interactions of pH and temperature. The other parameters: E/S 5% (w/w); hydrolysis time 4 h.

which means that the generated models adequately explained the data variation and significantly represented the actual relationships between the reaction parameters. The relationship between the two responses gave a positive covariance of 13.48 indicating that a higher DH was favourable for a higher protein recovery.

Since enzyme to substrate ratio was the most important factor in reducing the production cost, it is suggested that the hydrolysis should be carried out using an intermediate level (5%, w/w). Even though other combinations might slightly increase the DH, much more enzyme and/or longer reaction times are required. Table 8 shows the optimum conditions of the hydrolysis reaction to yield maximum DH and protein recovery, respectively, by holding E/S at 5%. It was noted that the optimum conditions for DH and protein recovery were slightly different, but the maximum DH and protein recovery were both obtained with pH and temperature located within the medium levels. According to the models the predicted results were obtained at the optimum condition for DH and protein recovery (19.78% and 86.28%, respectively) was close to the observed experimental responses (19.65% and 86.02%, respectively).

The contour and three-dimensional response surface plots are presented in Fig. 4. They illustrate the effects of pH and temperature, whereas the E/S was taken at its middle level while the hydrolysis time was set at 4 h.

4. Conclusion

The character and bioactivity of *Bellamya purificata* (mud snail) muscle protein was investigated both in intact and in enzymatic hydrolysate forms. Before hydrolysis, the snail protein needed to be solubilized by alkaline treatment. Compared with Alcalase 2.4L hydrolysates, the Proleather FG-F hydrolysates provided a higher proportion of short peptides from 2000 Da to dipeptides with fewer FAA and higher ACE inhibitory activity. The Proleather FG-F hydrolysates from the mud snail muscle protein have promising bioactivity. The optimum conditions obtained with RSM with regard to protein recovery were: pH 9.6, temperature 58.5 °C, E/S 5% and hydrolysis time 4 h. Further work will focus on the further purification of antihypertensive peptide from hydrolysate. The hydrolysates provide a versatile supply of the benefits of mud snail proteins and may be included in food and or pharmaceutical formulations as well as in feeds for livestock.

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