International Food Research Journal 22(4): 1615-1623 (2015)
Journal homepage: *http://www.ifrj.upm.edu.my*

Optimization of enzymatic hydrolysis conditions of Golden Apple snail *(Pomacea canaliculata)* protein by Alcalase

Hamid, S.A., Halim, N.R.A. and *Sarbon, N.M.

School of Food Science and Technology, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia

Article history

Received: 27 October 2014
Received in revised form: 12 January 2015
Accepted: 22 January 2015

Keywords

Golden apple snails
Hydrolysate
Optimization
RSM
Yield
Antioxidant activity

Abstract

The objective of this study is to establish conditions that allow optimal yield and antioxidant activity for Golden Apple Snail (GAS) *(Pomacea canaliculata)* protein hydrolysate by employing response surface methodology (RSM). A three level, face-centered, central composite design (CCD) was adapted to assess the effects of temperature (45–65˚C); pH (8–10); the ratio of enzyme to substrate (2–4%); and hydrolysis time (60–180 min). The antioxidative activity of the hydrolysate obtained under optimized conditions was then evaluated via the following metrics: hydroxyl radical scavenging, reducing power, and chelating effects on ferrous ion. Established optimal conditions for the enzymatic protein hydrolysis of GAS were a temperature of 45˚C, a pH of 10, an enzyme concentration of 2%, and hydrolysis time of 159 minutes. The optimized GAS protein hydrolysate produced an experimental yield of 9.72% and antioxidant activity of 73.54%—slightly less than the predicted yield of 11.36% and antioxidant activity of 78.88%. The optimized GAS protein hydrolysate formed demonstrated both higher chelating effects and hydroxyl scavenging activity but had lower reducing power. These results suggest that GAS protein hydrolysate holds potential as a natural antioxidant for use in food processing.

© All Rights Reserved

Introduction

Generally, protein hydrolysates produced by enzymatic hydrolysis of native proteins are small peptide fragments that contain from two to twenty amino acids (Pihlanto *et al*., 2000). Enzymatic hydrolysis is affected by several factors including temperature, pH, enzyme/substrate concentration, and time; each of which cooperatively influence enzymatic activity and thereby make the process more controllable (Viera *et al*., 1995; Liaset *et al*., 2002). Protein hydrolysis decreases peptide size making hydrolysates the most available amino acid source for various physiological functions and are readily available sources of protein for humans and animals due to their excellent functional properties (Benjakul and Morrissey, 1997). Several studies have reported promising results for fish protein hydrolysates such as threadfin bream (Normah *et al*., 2005), and grass carp skin (Wasswa *et al*., 2007).

The term optimization has been commonly used in analytical chemistry to describe applicable conditions that produce the best possible result (Myers and Montgomery, 2008). Prior studies have reported on optimized hydrolysates from chicken meat (Kurozawa *et al*., 2008); the visceral waste of catla (Bhaskar *et al*., 2008); and mud snail (Xia *et al*., 2007). The Response Surface Method (RSM) is widely employed for the simultaneous optimization of variables to attain ‘best system performance’ (Madamba, 2002). RSM comprises commonly used mathematical and statistical techniques that optimize the hydrolysis process and is readily applied when a desired response or a set of responses is/are influenced by several variables including temperature, pH, enzyme/substrate concentration, and time.

Golden apple snails (GAS) are aquatic organism and easily found in and around rice paddy fields. Although they are not originally from Malaysia, the snails have adapted to the environment after being imported some time ago for food and decorative purposes. It is now considered a paddy crop pest across the nation, particularly in northern regions of Vietnam (UAF, 2004). In Laos, crop damage from GAS has been prevented in the past by collecting them in the fields, which has since become inefficient in several regions. Furthermore, due to labor constraints farmers have turned to nonspecific chemicals to alleviate the problem (e.g. Niclosamide or Baylucide, and Copper Sulfate). Paddy farmers in Laos use GAS for food (85%), animal feed (14%), and liquid bio-fertilizer or compost (1%). According to assays from other snail species such as channeled apple snail *(Pomacea canaliculata)*,

*Corresponding author.
Email: *norizah@umt.edu.my*
Tel: +609 668 4968; Fax: +609 668 4949

their chemical content includes 62.0% crude protein (flesh, excluding shell) and 14.9% dry matter (Rice Technology Bulletin, 2001) with high mineral and vitamin content (Yahya *et al*., 2006). GAS appears to be a good mineral source as indicated by their calcium (35% in the shell) and phosphorus content (1.2%), and they are also a good source of energy (13.94 MJ kg^{-1}) (Kaensombath, 2005). Hence, studies on potential uses for GAS are needed.

Antioxidants widely used in food industries are defined as any substance with the ability to delay or inhibit oxidation (Chalamaiah *et al*., 2012). Some synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate are commonly added to prevent lipid oxidation (Umayaparvathi *et al*., 2014). Recently, the search for natural antioxidants is growing due to health hazards from synthetic antioxidants. Fish protein hydrolysates exhibiting antioxidant activity such as yellow stripe travelly (Klompong *et al.*, 2007) and round scad (Thiansilakul *et al*., 2007) have been reported. However, no study has been conducted on the potential of GAS (Pomacea canaliculata) protein hydrolysate as an antioxidant.

Therefore, our objectives for the current effort are to (i) determine the chemical composition of Golden apple snails (GAS); (ii) establish the optimal conditions for the production of GAS protein hydrolysate produced from different combinations of independent parameters (temperature, ratio of enzyme substrate level, pH and hydrolysis time) on yield and antioxidant activity and iii) to compare the antioxidant activity of the optimized GAS hydrolysate with the commercial antioxidant.

Material and Methods

Materials

Golden apple snails (GAS) (Pomacea canaliculata) were purchased from a local supplier in Pekan, Pahang, Malaysia and brought alive to the laboratory. The flesh was separated manually, washed, and minced by using a Waring blender (model HGB2WTS3, Connecticut, USA) and stored at -21˚C before further analysis. Alcalase 2.4L FG, a bacterial endoprotease produced by Bacillus licheniformis was obtained from Novozymes. All chemical reagents used were of analytical grade.

Chemical composition analysis of GAS

The chemical composition (protein, fat, moisture and ash) of the GAS were estimated by the AOAC (2002) method. Protein measurements were conducted by the Kjeldahl method using the Kjeltec protein analyzer (Foss–Tecator AB, Sweden). Carbohydrate content was calculated by 'difference' method. Where 100% - (protein, fat, moisture and ash) %.

Preparation of GAS protein hydrolysate

GAS protein hydrolysate was prepared using the method described by Adler-Nissen (1986) with slight modification. About 22 g of minced GAS flesh was mixed in 121 g of distilled water and minced in a blender. The mixture was then placed in a water bath. Temperatures were set to 45, 55 and 65˚C while the pH was adjusted 8, 9 and 10 (Table 1). Once the temperatures and the pH stabilized, alcalase at enzyme-to-substrate ratios (E/S) of 2, 3 and 4% were added. Hydrolysis processing times were set for 60, 120 and 180 min. pH were kept constant throughout the hydrolysis period by adding 4N NaOH. After each mixture completed its specified hydrolysis period they were inactivated at 90˚C for 15 minutes. The mixture was then centrifuged at 5000 rpm at 4˚C for 20 minutes. Respective supernatants were collected then freeze dried (Labconco, USA) and stored in freezer at -80˚C for further analysis.

Optimizing GAS hydrolysis conditions

Response Surface Methodology (RSM) was employed to predict optimal conditions for GAS protein hydrolysis with alcalase while observing four independent variables: pH (A); time in min (B); temperature in °C (C); enzyme to substrate concentration in % (v/w) (D). A total of thirty (30) runs at three equidistant levels (−1, 0 and +1) were accomplished. The optimized parameter design was then validated by measuring yield percentages and antioxidant scavenging activity [2, 2-diphenyl-1-picrylhydrazyl (DPPH)] for each GAS protein hydrolysate end product.

Determination of antioxidant activity

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical-scavenging activity was measured following Yen and Wu's method (1999) with some modification. An amount of 4 ml hydrolysate solution (20 mg/ml) were mixed with 1 ml of 0.2 mM DPPH and vigorously mixed. The mixture was incubated for 30 min in the dark at room temperature before the absorbance was determined at 517 nm using a spectrophotometer (Spectroquant Pharo 300, Europe). A control sample was similarly treated using distilled water without the addition of sample. DPPH radical scavenging activity was calculated as:

(1- A_{517} of sample) / (A_{517} of control) x 100.

GAS protein hydrolysate chelating effect on ferrous ion

The chelation of ferrous ions by GAS protein hydrolysate was determined by the method described by Dinis *et al*. (1994) with slight modification. Briefly, 0.025 ml of 2 mM $FeCl_2$ was added to 1 mg/ml hydrolysate extracts. The reaction was initiated by the addition of 5 mM ferrozine (0.1 ml), vigorously shaken and then left to stand at room temperature for 10 min with BHT as the control. Spectrophotometric (Spectroquant Pharo 300, Europe) absorbance was measured at 562 nm and the metal chelating activity of each sample was calculated as follows:

Metal chelating effect (%) = [(Abs_1-Abs_2)/Abs_1] x 100

Where Abs_1 was the absorbance of the control and Abs_2 was the observed absorbance for the sampled GAS protein hydrolysate concentration.

GAS protein hydrolysate hydroxyl radical scavenging activity (HRSA)

Fenton Reaction was used in order to measure the scavenging of hydroxyl radicals according to the method described by Yu *et al.* (2004). Sampled reaction mixtures contained 60 µl of 1.0 mM $FeCl_3$, 90 ul of 1mM 1,10-phenanthroline, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 150 µl of 0.17 M H_2O_2 and 1.5 ml of GAS protein hydrolysate for 1 mg/ml. The reaction began with the addition of H_2O_2. After incubation at room temperature for 5 min, a spectrophotometer (Spectroquant Pharo 300, Europe) was used to measure the absorbance at 560 nm. The following equation was employed to calculate hydroxyl radical scavenging activity:

Inhibition (%) = (A_0-A_1) / A_0 x 100)

Where A_0 represents control absorbance (a blank without peptides), and A_1 represents the sample's absorbance. Final assay values are presented as means taken from separate assays of three samples for each concentration.

Reducing power

GAS protein hydrolysate's reduction of iron (III) was determined by the method of Yildirim *et al.* (2001). Aliquots of 1.25 ml of hydrolysate for 1 mg/ml was mixed with 1.25 ml of 0.2 M phosphate buffer (pH 6.6) and 1.25 ml of 1% potassium ferricyanide. Each mixture was incubated at 50ºC for 30 min after which 1.25 ml of 10% (w/v) trichloroacetic acid was added and then centrifuged at 1650 x g for 10 minutes. Finally, 1.25 ml of the supernatant was mixed with 1.25 ml of distilled water and 2.5 ml of 0.1% (w/v) ferric chloride. After allowing a 10 min reaction time, absorbance for the resulting solution was measured at 700 nm by using spectrophotometer (Spectroquant Pharo 300, Europe). The synthetic antioxidant, BHT, was used as reference. Increased absorbance of the reaction mixture indicated an increased in reducing power. Final assay values were presented as means averaged from separate assays of three samples for each of the five concentrations.

Statistical analysis

Data was analyzed using Design Expert 6.0.10 software (Stat-Ease 2003) to provide model summary statistics; model sum of squares; ANOVA table; final equations; diagnostic case statistics; response surface plots; and suggested solutions. Randomized experiments were run for thirty sets of protein hydrolysis trials conducted according to the face-centered Central Composite Design (CCD). Parameters studied were temperature (˚C), enzyme concentration (%), time (min), and pH in addition to hydrolysate yield (%) and antioxidant activity (%) (DPPH assay). For chemical composition analysis, all treatments and analysis were carried out in triplicate.

Results and Discussion

Chemical composition of GAS

The chemical composition analysis of GAS flesh content yielded the following results: moisture (83.85%); ash (1.54%); protein (10.79%); fat (1.40%); and carbohydrate (2.42%). A study by Kaensombath (2005) reported lower moisture content for GAS (Pomacea canaliculata) at 18.1%. Higher moisture content was reported by Xia *et al.* (2007) for snail foot protein (78.82%). Seasonal factors and GAS size likely account for higher moisture content.

The observed protein content (10.79%) was lower than that reported by Kaensombath (2005) for GAS *(Pomacea canaliculata)* (62.1%), however, Ogungbenle and Omowole (2012) reported that periwinkle *(Tympanotonus fuscatus var radula)*, which has same class with Nigerian snail, had protein content of 74.74%. Meanwhile, Xia *et al.* (2007) found that different species of gastropods yield higher protein content (up to 59.58%). Lower protein content may due to different environmental factors and feeding habits. The predominant feeding habit of the Pomacea canaliculata is macrophytophagous, which, from an agricultural and environmental perspective, is highly significant. Nevertheless, the

Table 1. Variance analysis (ANOVA) of model and variables chosen on yield of GAS protein hydrolysate

Source	Sum of squares	Df	Mean square	F-value	p-value Prob>F	
Model	113.84	4	28.46	3.20	0.0299	significant
B	19.61	1	19.61	2.20	0.1501	
C	10.34	1	10.34	1.16	0.2914	
B^2	42.70	1	42.70	4.80	0.0380	
BC	41.18	1	41.18	4.63	0.0413	
Residual	222.47	25	8.90			
Lack of Fit	204.43	20	10.22	**2.83**	0.1257	
Pure Error	18.04	5	3.61			
Cor Total	336.30	29				
			28.46	3.20	0.0299	Not significant

higher protein content reported by Kaensombath (2005) was likely due to an environment where temperature (Philippines) was about 20˚C and in which climate GAS requires more food for survival (Cowie and Thiengo, 2003). Although protein content observed in this study was lower than results obtained for Golden apple snails from other locales, it should be noted that species type, age, season, nutritional status and environmental conditions all affect snail composition (Anthony *et al.*, 1995).

The ash content of a food sample indicates the amount of minerals present. This study's GAS samples contained about 1.54% ash. Ogungbenle and Omowole (2012) found periwinkle ash was 9.56% and Xia *et al*. (2007) reported an ash content of 16.48%. The lower ash content obtained in this study could possibly be due to soil type and other environmental factors.

The GAS carbohydrate content measured in this study was 2.42%. However, carbohydrate content of snail flesh *(Achatina achatina)* reported by Xia *et al*. (2007) was 22.86% while 3.26% was reported by Engmann *et al.* (2013) which was relatively low and as expected because snail metabolic activity and locomotion is slow requiring only small stores of glycogen. Furthermore, these animals have limited glycogen storage capacity and any excess is metabolized to fat (Berg *et al*., 2012). Consequently, low percentages of fat were also observed in this study (1.4%), which similar to findings reported by Ogungbenle and Omowole (2012) (1.32%) and by Xia *et al*. (2007) of 0.65%.

Optimization of enzymatic protein hydrolysis

Optimization of hydrolysis condition on GAS showed the protein hydrolysate yield ranged from 5.69 to 19.52% with the greatest yield (19.52%) resulting under the following conditions: 45˚C, 4% enzyme concentration, pH 8, and hydrolysis time of 180 minutes. While the antioxidant scavenging activity on DPPH ranged from 11.8% to 92.60% with the highest activity (92.60%) was recorded at 65˚C with a 2% enzyme concentration, a pH of 10, and hydrolysis time of 60 minutes.

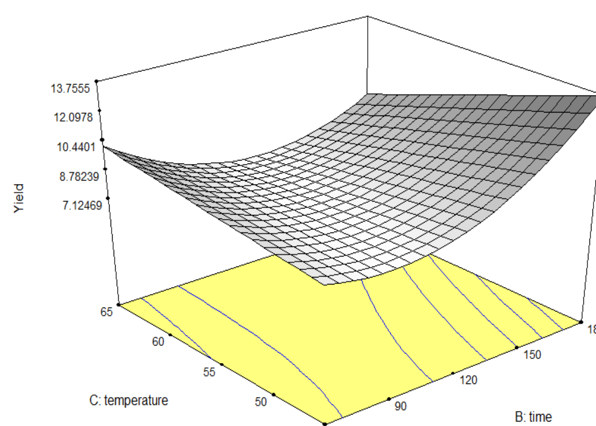

Figure 1: Response Surface Graph of GAS Protein Hydrolysate Yield (%) as a function of time and temperature (˚C).

Analysis on yield of GAS protein hydrolysate

Model summary statistics for yield of GAS protein hydrolysate

A quadratic model summary was suggested for GAS protein hydrolysate yield. Prior studies have also reported a quadratic model for enzymatic hydrolysis of fish waste (Nilsang *et al*., 2005; Bhaskar *et al.*, 2008).

Variance analysis (ANOVA) on yield of GAS protein hydrolysate

Variance analysis (ANOVA) of yields from the Response Surface Reduced Quadratic Model after the model's reduction are shown in Table 1. Terms excluded from the model were A, B, C, D, A^2,C^2, D^2, AB, AC, AD, BD,CD, A^2B, A^2C, A^2D, and AB^2. Terms 'B' and 'C' remain and indicate effects from time and temperature, respectively. The Lack-of-Fit 'F-value' was 2.83 which implies significance with

Table 2. Variance analysis (ANOVA) of model and variables chosen on antioxidant activity of GAS protein hydrolysate

Source	Sum of squares	Df	Mean square	F-value	p-value Prob>F	
Model	13083.48	12	1090.29	3.38	0.0111	significant
A	2303.17	1	2303.17	7.14	0.0161	
B	711.65	1	711.65	2.21	0.1557	
C	624.34	1	624.34	1.94	0.1821	
D	446.11	1	446.11	1.38	0.2558	
B^2	892.67	1	892.67	2.77	0.1145	
AB	34.93	1	34.93	0.11	0.7461	
AD	3102.49	1	3102.49	9.62	0.0065	
BC	343.73	1	343.73	1.07	0.3164	
BD	1130.30	1	1130.30	3.50	0.0785	
CD	1322.05	1	1322.05	4.10	0.0589	
AB^2	2492.67	1	2492.67	7.73	0.0128	
BCD	1974.91	1	1974.91	6.12	0.0242	
Residual	5483.06	17	322.53			
Lack of Fit	3796.07	12	316.34	0.94	0.5747	Not significant
Pure Error	1686.99	5	337.40			
Cor Total	18566.54	29				

respect to error. The "F-value" for our quadratic model was 3.20 with R^2 value of 0.8070 (data not shown), indicating the model's significance with only a 2.99% chance of its efficacy due to noise. Meanwhile, the p-value (<0.05) also implied that the model's terms were significant—in this case, B^2 and BC for protein hydrolysate yield—while the 'lack of fit' p-value was also insignificant. The final equation, in terms of coded factors given by the software, was as follows:

$$Yield = 2.09 + 2.11^{*}B+0.25^{*}C+ 6.76^{*}B^2 - 2.67^{*}B^{*}C$$

The result cited demonstrated that time was the most influential factor, followed by temperature. Hence, the equation can be used to predict and control GAS protein hydrolysate production with alcalase.

Response surface plot and effect of factors on yield of GAS protein hydrolysate

This study found that time and temperature was the most significant factors affecting GAS protein hydrolysate yield. Observed influences from independent variables on protein hydrolysate yield are best illustrated by a three dimensional views of 'response surface' and 'contour' plots as shown in Figure 1 which demonstrate the functions of time and temperature. Based on the response surface 3-D plot, yields increased as temperature rose to 65°C and higher yields were also observed as hydrolysis time increased to 180 min. The maximum yields were observed at 65°C and 180 minutes.

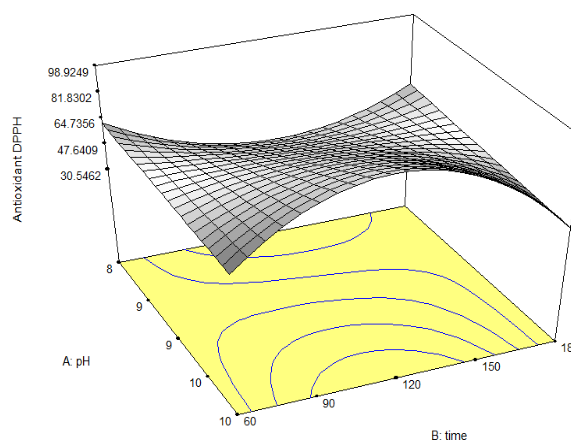

Figure 2. Response surface graph of the antioxidant activity of GAS protein hydrolysate (%) as a function of time (min) and pH

Analysis on antioxidant activity of GAS protein hydrolysate

Model summary statistics for antioxidant activity on GAS protein hydrolysate

The model summary suggested by the statistical analysis of the antioxidant activity of GAS protein hydrolysate was the 2FI model.

Variance analysis (ANOVA) on antioxidant activity of GAS protein hydrolysate

The variance analysis (ANOVA) of the Response Surface Reduced 2FI Model for antioxidant activity of GAS protein hydrolysate is shown in Table 2. Terms excluded from the model were B, C, D, A^2, B^2, C^2, D^2, AB, AC, BC, BD,CD, A^2B, A^2C,A^2D, and ABC. Terms B, C and D remained, thus, indicating effects from three factors: time (B), temperature (C) and enzyme concentration (D). The Lack of Fit 'F-value' was insignificant (0.26), implying that results were not due to error with a 77.74% chance

Table 3. Recommended solutions to optimize conditions for the production of GAS protein hydrolysate

No	pH	Time	Temperature	Enzyme concentration	Yield	Antioxidant activity	Desirability	
1	10	159	45	2	11.3575	78.8771	0.584	Selected
2	10	159	46	2	11.292	79.5643	0.583	
3	10	163	49	2	10.9476	82.5038	0.577	
4	9	180	45	4	13.7109	57.2634	0.573	
5	10	158	45	2	11.142	78.0022	0.589	

the lack-of-fit 'F-value' (3.38) was due to noise, thus, demonstrating that the model fit the data well with only a 1.11% chance for random occurrence.

The p-value (<0.05) implied significant relevance for the model's selected terms. In this case, A, AD, AB^2 and BCD for antioxidant activity on GAS protein hydrolysate while, at the same time, the p-value for lack-of-fit was insignificant. In an optimization study, insignificant results are preferable as they show the model fits well with observed experimental data. The final equation, in terms of coded factors given by the software, was as follows:

$$\text{Antioxidant activity of DPPH} = 770.54-71.12^{*}A-23.82^{*}B - 2.42^{*}C+64.94^{*}D+0.09^{*}B^{2} + 2.47^{*}A^{*}B13.93^{*}A^{*}D + 0.05^{*}B^{*}C + 0.88^{*}B^{*}D + 1.31^{*}C^{*}D-0.01^{*}A^{*}B2-0.02^{*}B^{*}C^{*}D.$$

These results demonstrated that the most influential factors affecting the antioxidant activity of GAS protein hydrolysate were time followed by enzyme concentration, pH and temperature, respectively. This equation can be used to confidently predict and control the production of GAS protein hydrolysate with alcalase.

Response surface plot and effect of factors on antioxidant activity of GAS protein hydrolysate

Figure 2 illustrates factors that significantly affected antioxidant activity on GAS protein hydrolysate, specifically, time and pH. These independent variable influences on antioxidant activity are best illustrated by 3-D views of the response surface and contour plots (Figure 2). Hence, we may conclude that as hydrolysis time increased and as temperature approached a maximum of 153˚C, the percentage of antioxidant activity also increased. However, after reaching 153˚C, a slight decrease in antioxidant activity was noted, most likely due to the thermal denaturation of the protease structure, thus, also reducing its enzymatic activity (Liaset *et al.*, 2002).

Optimization of percentage yield and antioxidant activity of GAS protein hydrolysate

After limitations cited above were determined, optimization protocols were established by using the Design Expert 6.0.10 software which produced the five most favorable sets of conditions for further evaluation (Table 3). A 'desirability value' approaching 1.0 is considered the best solution. Table 4 shows that optimum conditions included a temperature of 45˚C; an enzyme to substrate level of 2%; a pH of 10; and hydrolysis time of 159 min.

Validation tests

Validation trials were run to determine the actual yield and antioxidant activity under the stated optimized conditions. Experimental results for optimized hydrolysis produced a 9.72% yield with an averaged antioxidant activity of 73.54%. Both values were less than predicted values which anticipated a yield of 11.36% and antioxidant activity of 78.88%. These lower results were likely due to losses occurring during the process of freeze drying as a consequence of small batch drying (Abdul Hamid and Bee, 2002).

Antioxidant activity of optimized GAS protein hydrolysate

Antioxidants play an important role in food industries for purposes of nutritional preservation and prevention of color and flavor deterioration. Analysis of antioxidant activity on GAS protein hydrolysate showed higher ferrous chelating (46.80%) and hydroxyl radical scavenging activities (80.58%) compared to BHT (36.09% and 51.93%), respectively (data not shown). However, BHT showed higher reducing power (2.52%) compared to GAS protein hydrolysate (0.62%). The ferrous ion (Fe^{2+}) is a pro-oxidant that interacts with hydrogen peroxide (Fenton reaction) to produce reactive oxygen species (ROS) and the hydroxyl (OH) free radical, which may initiate and/or accelerate lipid oxidation (Stohs, 1995). The complex formation of the ferrous ion is

disrupted when chelating agents are present, resulting in decreased of color (Thiansilakul *et al*., 2007). As for reducing power, the presence of antioxidants in GAS protein hydrolysate causes the reduction of the ferricyanide complex to its ferrous form. These results suggested that GAS protein hydrolysate possibly contained amino acid or peptides which act as electron donors that could react with free radicals to form a more stable compound.

Conclusion

This study shows that GAS flesh has high moisture, relatively high protein, as well as low fat and ash content and that the GAS protein hydrolysate is significantly influenced by hydrolysis time and temperature while antioxidant activity was considerably affected by hydrolysis, pH and temperature. Results also showed that the antioxidant activity of GAS protein hydrolysate has potential as a radical scavenger due to its high chelation of the ferrous ion as well as hydroxyl radical scavenging activity. In addition, although lower than that of BHT, GAS protein hydrolysate has reducing power activity.

References

Abdul-Hamid, J. B. and Bee, G. H. 2002. Nutritional quality of spray-dried protein hydrolysate from Black Tilapia. Food Chemistry 78: 69–74.

Adler-Nissen, J. 1986. Enzymic hydrolysis of food proteins. Elsevier Applied Science, N. Y.

Anthony, P. U., Edet, O. A. and Ironge, E. I. 1995. Nutrients and anti-nutrients in small snails *(Limicolaria aurora).* Food Chemistry 53: 239–241.

AOAC. 2002. Official methods of analysis (16th ed.). Association of Official Analytical Chemists, Washington, DC.

Benjakul, B. and Morrissey, M. T. 1997. Protein hydrolysates from Pacific whiting solid wastes. Journal of Agricultural and Food Chemistry 45: 3423-3430.

Berg, J. M., Tymoczko, J. and Stryer, L. 2012. Biochemistry (7th ed.). pp. 338. W. H. Freeman, New York.

Bhaskar, N., Benila, T., Radha, C. and Lalitha, R. G. 2008. Optimization of enzymatic hydrolysis of visceral waste proteins of catla *(Catla catla)* for preparing protein hydrolysates using a commercial protease. Bioresource Technology 99: 335–343.

Chalamaiah, M., Dinesh Kumar, B., Hemalatha, R. and Jyothirmayi, T. 2012. Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A review. Food Chemistry 135: 3020–2038.

Cowie, R. H. and Thiengo, S. C. 2003. The apple snails of the Americas (Mollusca: Gastropoda: Ampullariidae: Asolene, Felipponea, Marisa, Pomacea, Pomella): a nomenclatural and type catalog. Malacologia 45(1): 41–100.

Dinis, T. C. P., Madeira, V. M. C. and Almeida, L. M. 1994. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitor of membrane lipids peroxidation and as peroxyl radical scavengers. Archives of Biochemistry and Biophysics 315: 161–169.

Engmann, F. N., Afoakwah, N. A., Darko, P. O. and Sefah, W. 2013. Proximate and mineral composition of snail *(Achatina achatina)* meat; Any nutritional justification for acclaimed health benefits? Journal of Basic and Applied Scientific Research 3(4): 8–15.

Kaensombath, L. 2005. Evaluation of the nutritive value of ensiled and fresh Golden Apple snails (*pomacea* spp) for growing pigs. Research Papers, National University of Laos, Vienetiane, Laos.

Klompong, V., Benjakul, S., Kantachote, D. and Shahidi, F. 2007. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally *(Selaroides leptolepis)* as influenced by the degree of hydrolysis and enzyme type. Food Chemistry 102: 1317-27.

Kurozawa, L. E., Park, K. J. and Hubinger, M. D. 2008. Optimization of the enzymatic hydrolysis of chicken meat using Response Surface Methodology. Journal of Food Science 73: 405–412.

Liaset, B., Nortvedt, R. and Lied, E. 2002. Studies on the nitrogen recovery in enzymatic hydrolysis of Atlantic salmon (*Salmosalar L.*) frames by Protamex protease. Process Biochemistry 37: 1263–1269.

Madamba, P. S. 2002. The response surface methodology: An application to optimize operations of selected agricultural crops. Food Science and Technology 35: 584–592.

Myers, R. H. and Montgomery, D. C. 2008. Response Surface Methodology: Process and product optimization using Design Expert. (3rd ed.). pp. 704. Washington: Wiley Press.

Nilsang, S., Lertsiri, S., Suphantharika, M. and Assavanig, A. 2005. Optimization of enzymatic hydrolysis of fish soluble concentrate by commercial proteases. Journal of Food Engineering 71: 571–578.

Normah, I., Jamilah, B., Saari, N. and Yaakob, B. C. 2005. Optimization of hydrolysis conditions for the production of threadfin bream *(Nemipterus japonicus)* hydrolysate by Alcalase. Journal of Muscle Foods 16: 87–102.

Ogungbenle, H. N. and Omowole, B. M. 2012. Chemical, functional and amino acid composition of periwinkle *(Tympanotonus fuscatus var radula)* meat. International Journal of Pharmaceutical Sciences Review and Research 13(2): 128-132.

Pihlanto, L. A., Koskinen, P., Piilola, K., Tupasela, T. and Korhonen, H. 2000. Angiotensin I-converting enzyme inhibitory properties of whey protein digests: concentration and characterization of active peptides. Journal of Dairy Research 67: 53–64.

Rice Technology Bulletin. 2001. Management option for

the Golden Apple Snail. Department of Agriculture, Philippine Rice Institute (Phil Rice). No 33. Retrieved from *http://www.applesnail.net/pestalert/management_guide/pest_management.php.*

Stohs, S. J. 1995. Oxidative stress induced by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). Free Radical Biological Medicine 9: 79–90.

Thiansilakul, Y., Benjakul, S. and Shahidi, F. 2007. Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad *(Decapterus maruadsi).* Food Chemistry 103(4): 1385–1394.

UAF. 2004. Chemical composition of flesh Golden apple snails (GAS). Animal Nutrition Laboratory of University of Agriculture and Forestry UAF (Nom Lam University). Ho Chi Minh City, Vietnam.

Umayaparvathi, S., Meenakshi, S., Vimalraj, V., Arumugam, M., Sivagami, G. and Balasubramanian, T. 2014. Antioxidant activity and anticancer effect of bioactive peptide from enzymatic hydrolysate of oyster *(Saccostrea cucullata)*. Biomedicine and Preventive Nutrition 233: 1–11.

Viera, G. H. F., Martin, A. M., Saker-Sampaiao, S., Omar, S. and Goncalves, C. F. 1995. Studies on the enzymatic hydrolysis of Brazilian lobster (*Panulirus* spp.) Processing wastes. Journal Science Food Agricultural 69: 61–65.

Wasswa, J., Tang, J., Gu, X. and Yuan, X. 2007. Influence of the extent of enzymatic hydrolysis on the functional properties of protein hydrolysate from grass carp *(Ctenopharyngodonidella)* skin. Food Chemistry 104: 1698–1704.

Xia, S. H., Wang, Z. and Xu, S. Y. 2007. Characteristics of *Bellamya purificata* snail foot protein and enzymatic hydrolysates. Food Chemistry 101: 1188–1196.

Yahya, H., Wan Z. M. and Mohd, I. H. S. 2006. The golden apple snail (*pomacea sp.*) as a potential animal and aquaculture feed. Malaysian Agricultural Research and Development Institute, MARDI, Serdang, Malaysia.

Yen, G. and Wu, J. 1999. Antioxidant and radical scavenging properties of extract from *Ganoderma tsugae.* Food Chemistry 65: 375-379.

Yildirim, A., Mavi, A. and Kara, A. A. 2001. Determination of antioxidant and antimicrobial activities of *Rumexcrispus L*. extracts. Journal of Agricultural and Food Chemistry 49: 4083–4089.

Yu, W., Zhao, Y. and Shu, B. 2004. The radical scavenging activities of radix *(Puerariaeisoflavonoids: Chemiluminescence)* study. Food Chemistry 86: 525–529.