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Optimization of enzymatic hydrolysis of shortfin scad *(Decapterus macrosoma)* myofibrillar protein with antioxidant effect using alcalase

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Abstract

By employing response surface methodology (RSM), this study aimed to describe conditions that optimized both yield and antioxidant activity for the hydrolysate of shortfin scad *(Decapterus macrosoma)* myofibrillar protein (SSMH). The study used a three level, factor-face centered, central composite design (CCD) to assess effects from four independent variables; namely, time (60 mins, 120 mins, 180 mins); temperature (45, 55 and 65°C); pH (8, 9, 10); and enzyme/substrate ratio (1, 2 and 3%). The hydrolysate products were then evaluated for (i) the yield; and (ii) the level of DPPH radical scavenging activity. Optimized hydrolysis conditions were established at 180 mins / temperature 59.49°C / pH 9.93 / and 1% enzyme concentration. Predicted response values (yield: 13.56% and DPPH antioxidant activity: 59.20%) were close to the experimentally validated values of 13.18 and 56.10%, respectively. Optimized SSMH demonstrated a DH of 40.56% along with higher chelating effects on ferrous ion (90.91%) (IC_{50} = 0.38 mg/ml) as well as greater hydroxyl radical scavenging activity (96.12%) (IC_{50} = 1.37 mg/ml). However, lower reducing activity (54.00%) (IC_{50} = 4.59 mg/ml) and DPPH radical scavenging activity (69.98%) (IC_{50} = 1.89 mg/ml) were observed. The higher chelating and radical scavenging activity values suggest that SSMH has potential characteristics as a natural antioxidant.

Introduction

Shortfin scad *(Decapterus macrosoma)* is an aquatic life form that is widely distributed throughout the Indo-western Pacific, ranging from East Africa to Malaysia (where it abounds) and Indonesia to the Arafura Sea; and from the Eastern Pacific to the Gulf of California and Peru (Paxton *et al.,* 1989). It is a source of protein, vitamins and minerals commonly marketed as 'keropok lekor' in Malaysia. Although not widely commercialized, it is considered a low value tropical fish normally used in salted fish production. Recently, Cheow *et al.* (2007) also demonstrated that shortfin scad skin is a viable gelatine source.

According to Hamid *et al*. (2015), protein hydrolysates are products derived from the enzymatic breakdown of protein compounds into smaller peptides containing 2–20 amino acids. Protein hydrolysates can be produced by the enzymatic hydrolysis of native protein and are considered a good source of amino acids that are thereby made readily available for human physiological functions when eaten (Hamid *et al.*, 2015). These include hydolysates that are derived from myofibrillar (muscle) fish proteins. Enzymatic hydrolysis involves enzymes that facilitate the cleavage of molecular bonds in molecules with watery elements. A variety of enzymes are employed in the preparation of protein hydrolysates. These include alcalase, bromelain, flavourzyme, neutrase, pepsin, trypsin and papain (Razali *et al*., 2013). Alcalase is used to hydrolyze myofibrillar fish proteins because it is a highly efficient bacterial protease, which is primarily due to its thermostability (Hamid *et al*., 2015). Alcalases can solubilize fish protein at optimized temperatures ranging from 50 to 70°C, and within an optimal pH range of 8–10 (See *et al.*, 2011).

In the present work the authors sought to optimize the enzymatic hydrolysis process to obtain a better fish protein hydrolysate yield and end-product quality. Such optimization involves a statistically derived experimental design approach that is commonly used in analytic chemistry to determine 'best alternatives' from a set of specified options (See *et al*., 2011). Several studies have reported optimized hydrolysis processes for salmon *(Salmo salar)* (See *et al.*, 2011); skipjack tuna *(katsuwonus pelamis)* (Herpendi *et al.*, 2013); and cockle *(Anadara Granosa)* (Haslaniza *et al*., 2013). Response Surface Methodology (RSM) is a relevant multivariate technique that is often used by researchers to obtain desireable outcomes from variables like pH, temperature and enzyme/substrate

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concentration (See *et al*., 2011).

Antioxidants are molecules or compounds with the ability to inhibit oxidation (Taghvaei *et al*., 2014). Sources of naturally occurring antioxidants include whole grains, fruits and vegatables. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are presently used to preserve food products from lipid oxidation (Hamid *et al.*, 2015). Although synthentic antioxidants have been widely used in food indutries, there are some arguments about their safety towards human and a search is on for 'natural' antioxidants, which has been a focus of considerable interest for several years (Taghvaei *et al*., 2014). This same quest has revealed that the protease breakdown of myofibrillar fish tissue to hydrolysates yields bioactive compounds with potential antioxidant peptides and furthermore, that these peptides can be isolated (Ahn *et al*., 2012). Reports of this nature include several fish protein sources including Salmon (Ahn *et al.*, 2012), *Exocoetus volitans* (Naqash and Nazeer, 2011), eel (Halim and Sarbon, 2017) and shortfin scad (Ishak and Sarbon, 2017), that can be used for the production of antioxidant protein hydrolysates. Hence, the current and growing demand for natural and safe antioxidants prompted the authors to explore the potential of shortfin scad *(Decapterus macrosoma)*.

This study's objectives were to determine the chemical composition of shortfin scad myofibrillar protein and optimize the condition for its enzymatic protein hydrolysis in terms of temperature, enzyme/substrate ratio, pH and time. Response Surface Methodology (RSM) was employed to optimize the cited paramaters for best outcomes in terms of antioxidant activity and hydolysate yield. The degree of hydrolysis (DH) output and the mechanism of the hydrolsates's antioxidant activity were also determined.

Materials and Methods

Materials

Shortfin scad *(Decapterus macrosoma)* was purchased from a local supplier in Kuala Terengganu, Malaysia. Chemicals used such as Alcalase® 2.4 L in liquid form (2.4AU/g) and other chemicals used were of analytical grade.

Sample preparation

Shortfin scad flesh was manually separated, washed and then minced in a Waring blender (model HGBTWTS3, Torrington, CT. USA). The minced flesh was then packaged and stored at -40°C pending further processing.

Chemical composition of shortfin scad flesh

The chemical compositions (moisture, crude protein, crude fat and ash content) of the raw material (shortfin scad flesh) were determined according to method described by AOAC (2002).

Preparation of shortfin scad myofibrillar protein hydrolysate (SSMH)

Shortfin scad myofibrillar protein hydrolysate (SSMH) was prepared according to the method described by Hamid *et al*. (2015). About 16.5g of minced flesh were mixed with 16.5 ml of distilled water and homogenized. The mixture was then heated to 85ºC for 15 mins to inactivate endogenous enzymes, after which it was cooled to 45, 55 and 65°C. Approximately 20 g of diluted alcalase (prepared by diluting the required enyzme mass to a final weight of 20 g with distilled water) at enzyme/substrate ratio of 1, 2 and 3% were added per mix once temperatures stabilized and pH was adjusted to 8, 9 and 10 with 1 N NaOH. Hydrolysis process times were then set at 60, 120 and 180 minutes. During hydrolysis, 1N NaOH was added to maintain a constant pH. After each specified hydrolysis time was reached, each slurry was heated to 90°C for 15 mins to inactivate the alcalase. The resultant slurry was then centrifuged at 5000 rpm at 4°C for 20 mins using a refrigerated centrifuge (Zentrifuge D-78532, Tuttlinyen, Germany). Each supernatant was filtered using filter paper, then collected and freeze dried (Scanvac Coolsafe 110-4, Lynge, Denmark). The freeze dried hydrolysates were then stored at -80°C pending further analysis.

Yield and DPPH radical scavenging activity for optimizing shortfin scad myofibrillar hydrolysate (SSMH)

Hydrosalate yield was calculated as follows:

Yield (%) = (Mass of freeze dried hydrolysate/Mass of Shortfin scad flesh) x 100

To assess DPPH radical scavenging activity, a sample solution (500 µl at 1 mg/ml) was mixed with 500 µl of distilled water and 125 µl (0.02% w/v) of DPPH in 500 µl of 99.5% ethanol. The mixture was vortexed and incubated in the dark for 60 minutes. Reaction absorbance of the resulting solution measured at 517 nm using the UV-Vis Spectrophotometer (Spectroquant® Pharo 300, Darmstadt, European Union). DPPH radical-

scavenging activity was then calculated as follows:

$$\text{DPPH radical-scavenging activity (\%)} = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

Where $A_{control}$ = absorbance of the control mixture
A_{sample} = absorbance of the hydrolysate.

Optimization of enzymatic hydrolysis conditions for shortfin scad myofibrillar protein hydrolysate (SSMH) using Response Surface Methodology (RSM)

Response Surface Methodology (RSM) and Central Composite Design (CCD) was employed to predict optimal hydrolysis conditions for SSMH as per Hamid *et al.* (2015). Four different independent variables for the enzymatic hydrolysis process included temperature (45, 55 and 65°C); time (60, 120 and 180 mins); pH (8, 9 and 10); and enzyme/ substrate ratio (1, 2 and 3%). These parameters were subjected to 30 trials at three equidistant levels (-1, 0, +1) with 6 central points. Projected yields and DPPH radical scavenging activity were then experimentally validated.

Degree of SSMH hydrolysis (DH)

The degree of SSMH hydrolysis (DH) was determined by using the percent trichloroacetic acid (TCA) ratio (Klompong *et al*., 2007). Twenty (20) ml of protein hydrolysate (supernatant) was added to 20 \ ml of 20% (w/v) TCA to produce a 10% TCA solution. The protein hydrolysate with added TCA was then left to stand for 30 mins to allow for precipitation. This was followed by centrifugation (7800 x g for 15 mins) (Gyrozen 1580R, Daejon, Korea). The supernatant was decanted and analyzed for nitrogen content, as per the Kjeldahl method (AOAC, 2002). The degree of hydrolysis was calculated as follows:

DH (%) = (Soluble N in 10% TCA/Total N in sample) x 100

Where N is nitrogen and TCA is trichloroacetic acid.

Determination of SSMH antioxidant activity (reducing power)

The ability of SSMH to reduce iron (III) was determined according to the method described by Hamid *et al*. (2015). Aliquots of 1.25 ml of hydrolysate at different concentrations (0.5, 1.0, 5.0, 10.0 and 20.0 mg/mL) were mixed with 1.25 ml of 0.2M phosphate buffer (pH 6.6) and 1.25 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 30 mins, followed by the addition of 1.25 ml (10%, w/v) trichloroacetic acid. This mixture was then centrifuged at 1650 x g for 10 minutes. Finally, 1.25 ml of the supernatant were mixed with 1.25 ml of distilled water and 2.5 ml of 0.1% (w/v) ferric chloride. After 10 mins, the resulting solution's reaction absorbance was measured at 700 nm using the UV-Vis Spectrophotometer (Spectroquant® Pharo 300, Darmstadt, European Union). Synthetic antioxidant BHT was used as a positive control. The reaction mixture's increased absorbance indicated an increase in reducing power. Outcome values are presented as the means of three assays.

Chelating effects on ferrous ions

The chelating effects on ferrous ion was measured using the method of Hamid *et al.* (2015). Briefly, about 0.025 ml of 2 mM $FeCl_2$ were added to hydrolysate aliquots of 0.5, 1.0, 5.0, 10.0 and 20.0 mg/mL in 0.5 ml distilled water. The addition of 5 mM ferrozine (0.1 ml) initiated the reaction as the mixture was vigorously shaken and left to stand at room temperature for 10 minutes. The control tube contained distilled water. Absorbance was measured at 562 nm with the UV-Vis Spectrophotometer (Spectroquant® Pharo 300, Darmstadt, European Union). The hydrolysate's metal chelating activity on ferrous ion was calculated as follows:

$$\text{Metal chelating effect (\%)} = [(Abs_1 - Abs_2)/ Abs_1] \times 100$$

Where Abs_1 is the absorbance of the negative control, and Abs_2 is the absorbance of the protein hydrolysate with BHT as the positive control. The negative control was prepared in the same manner, except that distilled water was used instead of the hydrolysate sample. All trials were conducted in triplicate. IC_{50} values (amount of antioxidant necessary to inhibit metal chelation by 50%) were also calculated.

DPPH radical-scavenging activity

The ability of SSMH's to scavenge free radicals with the synthetic free-radical compound, 1,1-diphenyl-2-picrylhydrazyl (DPPH) were measured according to the method described by Hamid *et al.* (2015). Approximately 500 µl of SSMH sample concentrations (0.5, 1.0, 5.0, 10.0 and 20.0 mg/mL) were mixed with 500 µl of ethanol and 125 µl (0.02%, w/v) of DPPH in 99.7% ethanol. These mixtures were vigorously vortexed and incubated in the dark for 60 minutes. Light absorbance measured at 517 nm by UV-Vis Spectrophotometer (Spectroquant® Pharo 300, Darmstadt, European Union) and DPPH radical-scavenging activity was calculated as follows:

$$\text{DPPH radical-scavenging activity (\%)} = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

Where $A_{control}$ is the absorbance of the control (DPPH solution without sample), and A_{sample} is the absorbance of the sample (DPPH solution plus sample of hydrolysate). Again, BHT was used as the positive control. A lower absorbance by the reaction mixture would indicate higher DPPH radical-scavenging activity. All trials were conducted in triplicate and IC_{50} values were also calculated.

Hydroxyl radical scavenging activity (HRSA)

Scavenging activity for hydroxyl radicals were measured by the Fenton Reaction as described by Hamid *et al.* (2015). Aliquots of 1.5 ml protein hydrolysate at 0.5, 1.0, 5.0, 10.0 and 20.0 mg/mL were mixed with 60 µl of 1.0 mM $FeCl_3$, plus 90 µl of 1 mM 1,10-phenanthroline, plus 2.4 ml of 0.2 M phosphate buffer (pH 7.8), and 150 µl of 0.17 M H_2O_2. Reactions were initiated with the addition of H_2O_2. After incubation at room temperature for 5 mins, absorbance at 560 nm was measured with the UV-Vis spectrophotometer (Spectroquant® Pharo 300, Darmstadt, European Union). Hydroxyl radical scavenging activity was calculated with the following equation:

$$\text{Inhibition (\%)} = (A_0\text{-}A_1/ A_0) \times 100$$

Where A_0 is absorbance of the control (without hydrolysate), and A_1 is absorbance in the presence of a hydrolysate sample. Again, the synthetic antioxidant, BHT, was used as the positive control and IC_{50} values were calculated. All results are presented as the means of three analytical trials.

Statistical analysis

The scad's myofibrillar tissue chemical composition analysis is presented as a mean (±SD) of three separate assays. RSM was employed to help optimize SSMH production. All reported results were statistically analyzed with Design-Expert 6.0.10 software, including the analysis of variance (ANOVA). All treatments and analyses of SSMH antioxidant activity were undertaken in triplicate, including the calculation of IC_{50} values.

Results and Discussion

Chemical composition of shortfin scad myofibrillar (SSM)

The composition analysis of shortfin scad myofibrillar tissue yielded moisture, protein, fat and ash contents of 75.38, 24.36, 5.14 and 2.13%, respectively. Chemical composition analysis of the scad's skin yielded moisture, protein, fat and ash contents of 60.43, 24.10, 9.63 and 5.9%, respectively (Cheow *et al*., 2007). Protein contents were nearly similar for both skin and flesh, a finding that disagreed with a study reporting higher concentrations of protein in the skin vs. flesh for mackerel, croaker and catfish (Makanjuola, 2012). Moreover, shortfin scad myofibrillar tissue had higher moisture but lower fat and ash contents than did its raw skin. The lower lipid content in the raw material appears to have affected the protein content in the hydrolysate end product, as higher lipid content has been related to lower volumes of solubilized protein (Khantaphant *et al*., 2011). Meanwhile, moisture content in the flesh was higher because water is the main constituent of fish flesh (Chalamaiah *et al*., 2012). The difference between fat and ash content is likely due to different concentrations of fatty acids and minerals found in shortfin scad myofibrillar tissue and skin.

RSM optimization of enzymatic hydrolysis conditions: yield and antioxidant activity

The used of RSM to integrate and project optimized parameters for the four cited variables that affect alcalase hydrolysis of shortfin scad myofibrillar protein. A total of thirty (30) hydrolysis trials were run based on a Central Composite Design with six (6) face-centred points and four independent variables: (A) for time; (B) for temperature: (C) for pH; and (D) for enzyme/substrate ratio — all based on two outcome responses, namely, percent yield and antioxidant activity as shown in Table 1.

Freeze dried SSMH yields from thirty experimental runs ranged from 7.25–14.52%, very similar to results reported by Quaglia and Orban (1990) for typical fish hydrolysate, and by Rafi *et al*. (2015) for lead tree sead (7.97–10.29%). Percent yield is affected by percent content of solubilised protein, which depends on the raw material's lipid content. Raw materials with the lowest lipid content yield the highest percent content of solubilised protein, thus increasing hydrolysate yield (Slizyte *et al*., 2005). Higher yields obtained from optimized enzymatic hydrolysis conditions also promise good reproducibility of hydrolysate and cost reduction (Maache-Rezzoug *et al*., 2011).

The present study's hydrolysate for DPPH antioxidant activity values ranged from 2.11–73.65%, lower than the range reported for golden apple snails (11.18–92.60%) by Hamid *et al*. (2015). This difference in SSMH antioxidant activity is likely due to the hydrolysate's source and enzyme catalyst, as well as dissimilar enzymatic hydrolysis conditions (Rafi *et al*., 2015). According to Wang *et al*. (2013), under optimized enzymatic hydrolysis conditions,

Table 1. Central composite experimental design and observed responses for DPPH (%) and Yield (%)

Standard	Run	A Time (min)	B Temp (°C)	C pH	D Enzyme (%)	Responses DPPH (%)	Yield (%)
7	1	60	65	10	1	67.92	8.35
3	2	60	65	8	1	16.32	9.20
22	3	120	55	10	2	33.50	11.07
21	4	120	55	8	2	2.11	9.87
9	5	60	45	8	3	25.35	8.51
16	6	180	65	10	3	56.51	13.85
17	7	60	55	9	2	4.49	10.18
24	8	120	55	9	3	12.56	14.16
23	9	120	55	9	1	20.35	12.67
14	10	180	45	10	3	8.31	12.93
4	11	180	65	8	1	73.65	11.06
25	12	120	55	9	2	36.28	9.80
2	13	180	45	8	1	9.95	8.12
10	14	180	45	8	3	13.89	8.49
20	15	120	65	9	2	66.59	9.75
15	16	60	65	10	3	18.24	11.84
19	17	120	45	9	2	8.52	7.25
12	18	180	65	8	3	3.62	10.17
11	19	60	65	8	3	28.55	9.23
28	20	120	55	9	2	49.13	14.52
18	21	180	55	9	2	35.93	14.30
13	22	60	45	10	3	12.42	10.59
26	23	120	55	9	2	54.59	11.17
1	24	60	45	8	1	20.62	8.02
5	25	60	45	10	1	21.45	10.00
8	26	180	65	10	1	63.08	10.88
29	27	120	55	9	2	53.20	13.49
27	28	120	55	9	2	34.36	13.69
30	29	120	55	9	2	54.83	14.22
6	30	180	45	10	1	49.94	10.23

hydrophobic interactions can cause functional and short amino acid sequence releases from parent proteins. It is also believed that hydrophobic amino acids with bulky and aromatic side chains can act as hydrogen donors and antioxidants (Chalamaiah *et al.*, 2012). Thus, the higher antioxidant activity observed in this study showed potential bioactive peptides with antioxidative properties can be obtained from SSMH.

Analysis of SSMH yield: summary statistics and analysis of variance (ANOVA)

The quadratic model was employed for the summary statistical analysis of SSMH yield. This model's results showed a low standard deviation (1.42), high 'R-Squared' index (0.78), and a low 'PRESS' value (80.42) for SSMH yields. Previous studies reported similar quadratic model analyses of enzymatic hydrolysate yields from fish protein (Chalamaiah *et al*., 2012; Halim and Sarbon, 2017).

Table 2 shows ANOVA results for the RSM quadratic model of SSMH yields after model reduction. The model's F-value for yield was 5.60, thus implying significance since only a 0.07% chance existed that the model's F-value could have occured due to noise. The Lack-of-Fit result was 0.28, which implied no significant error, further indicating that the model fit well with experimental data. Hence, the model fit experimental data with a determination coefficient of (R^2 = 0.78) ($p < 0.05$) (Table 2). R^2 should be >0.80 to obtain a good fit for the model

Table 2. Analysis of Variance (ANOVA): quadratic model for SSMH yield after model reduction

Source	**Sum of Squares**	**DF**	**Mean square**	**F value**	**p-value Prob > F**	
Model	105.89	11	9.63	5.60	0.0007	significant
A -time	11.10	1	11.10	6.45	0.0205	
B -temperature	5.77	1	5.77	3.36	0.0836	
C -pH	16.20	1	16.20	9.42	0.0066	
D -enzyme	7.03	1	7.03	4.09	0.0584	
B^2	26.62	1	26.62	15.48	0.0010	
C^2	3.39	1	3.39	1.97	0.1771	
D^2	9.77	1	9.77	5.68	0.0284	
AB	1.38	1	1.38	0.80	0.3831	
AC	1.11	1	1.11	0.65	0.4315	
BC	1.79	1	1.79	1.04	0.3208	
CD	5.95	1	5.95	3.46	0.0794	
Residual	30.96	18	1.72			
Lack of Fit	13.08	13	1.01	0.28	0.9697	not significant
Pure Error	17.88	5	3.58			
Cor Total	136.85	29				
Std.Dev.	1.42					
R-squared	0.78					
PRESS	80.42					

and thus explain data variation (Halim and Sarbon, 2017). Although the value of R^2 is low, however, due to the insignificant of lack of fit ($p<0.05$), therefore, the suggested model can be used to predict the hydrolysis condition of SSMH.

Moreover, the p-value (<0.05) indicated that the model's terms were significant while ANOVA results demonstrated that linear models for 'time' (A) and 'pH' (C) had significant affects on hydrolysate yield ($p < 0.05$). At the same time, quadratic models for temperature (B^2) and enzyme/substrate concentration (D^2) also showed significant effects on yield ($p < 0.05$). In terms of interaction coefficients, all interactions showed no significant effects on SSMH yield ($p > 0.05$).

Response surface plot and effect on SSMH yield

Using regression coefficients for intercepts as well as linear, quadratic and interaction terms to fit a full response surface model, the model's final yield equation, in terms of coded factors, is as follows:

$$\text{Yield} = 12.30 + 0.79^{*}A + 0.57^{*}B + 0.95^{*}C + 0.62^{*}D - 3.06^{*}B2 - 1.09^{*}C^2 + 1.86^{*}$$

$$D^2 + 0.29^{*}A^{*}B + 0.26^{*}A^{*}C - 0.33^{*}B^{*}C + 0.61^{*}C^{*}D$$

This equation can be used to predict and control SSMH yield for the alcalase hydrolysis process. A three-dimensional (3D) response surface plot was mapped to help us understand the effects on SSMH yield from two independent combined factors (time and temperature) and (pH and enzyme/substrate ratio).

Figure 1(a) shows the response surface plot of

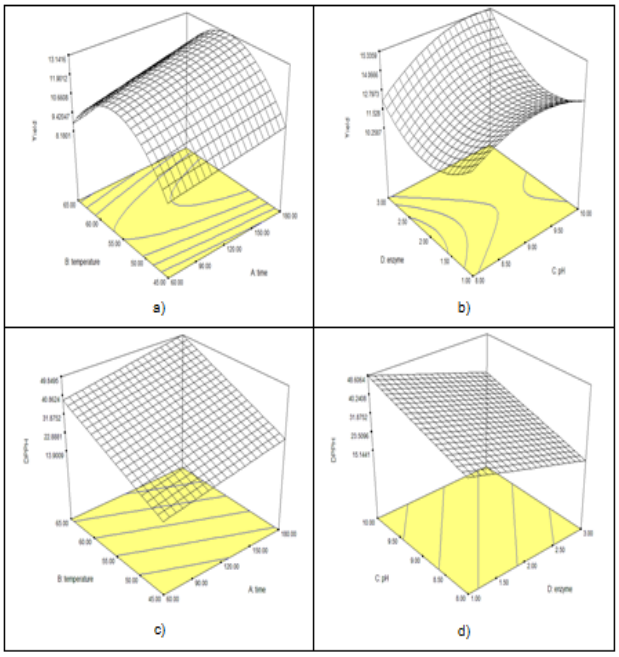

Figure 1. Response surface plot for (a) percent yield as a function of time (min) and temperature (ºC); (b) percent yield as a function of enzyme concentration (%) and pH; (c) percent antioxidant activity as a function of time (min) and temperature (ºC); (d) percent antioxidant activity as a function of enzyme concentration (%) and pH.

temperature and time vs. SSMH yield. The increased hydrolysis time presented was resulted in higher yield, with the highest hydrolysate yield obtained at 180 minutes. This result was in the same agreement with a study conducted by Hamid *et al.* (2015). Hence, it can be concluded that a longer enzymatic hydrolysis period yielded higher amounts of end product. In terms of temperature, hydrolysate yield increased with increased temperature. However, at 60 to 65ºC, the yield slowly decreased, a finding suggesting greater yield at temperatures less than 60ºC. According to Halim and Sarbon (2017), temperatures required to breakdown fish protein will vary according to fish type. Studies conducted by See *et al.* (2011) and Hamid *et al.* (2015) also showed that optimal hydrolysis periods and temperatures varied in range depending on the enzymatic substrate utilized.

Figure 1(b) shows the response surface plot for SSMH yield as a function of pH and enzyme concentration at 120 mins and 55ºC, indicating that increased pH produced higher SSMH yields. According to Hamid *et al.* (2015), alcalase solubilize fish protein at a pH of 10. Figure 1b demonstrates that enzymatic proteolysis of shortfin scad myofibrillar tissue in the present work concurred with Hamid's findings (pH 10) and that the highest yields obtained were at pH of 9.93. Moreover, the highest yields were also obtained with an alcalase enzyme/substrate ratio of 3% and decreased at 2 and 1%, respectively. According to Liu *et al.* (2013), higher enzyme/substrate ratio allowed greater proteolysis during enzymatic hydrolysis.

Table 3. Analysis of Variance (ANOVA): linear model for SSMH DPPH radical scavenging activity after model reduction

Source	Sum of Squares	DF	Mean square	F value	p-value Prob > F	
Model	5877.15	4	1469.29	4.56	0.0067	significant
A -time	550.19	1	550.19	1.71	0.2033	
B -temperature	2788.09	1	2788.09	8.65	0.0070	
C -pH	1047.84	1	1047.84	3.25	0.0835	
D -enzyme	1491.04	1	1491.04	4.62	0.0414	
Residual	8059.72	25	322.39			
Lack of Fit	7623.22	20	381.16	4.37	0.0546	not significant
Pure Error	436.50	5	87.30			
Cor Total	13936.87	29				
Std.Dev	17.96					
R-squared	0.4217					
PRESS	11916.29					

Analysis of SSMH antioxidant activity: summary statistics and analysis of variance (ANOVA)

Table 3 presents ANOVA results for the linear RSM of SSMH antioxidant activity after model reduction. The model's F-value for antioxidant activity was 4.56, implying significance with a 0.67% chance that results were due to noise. The "Lack of Fit" index was 4.37 for this F-value, which implied no significance due to pure error. Hence, this non-significant "Lack of Fit" result indicated that the model fit well with experimetal findings. The determination coefficient (R^2) was 0.4217 ($p < 0.05$), a value indicating that 42.17% of varience could be explained by the fitted SSMH model for a range of factor values. The low R^2 obtained was in similar observation with Rafi *et al.* (2015) was due to experimental error in controlling the parameter such as temperature and pH during the preparation of SSMH. Moreover, the ANOVA for the regression model demonstrated significance at a CI of 95% ($p < 0.05$), confirming that temperature (B) and enzyme concentration (D) were significantly relevant model terms.

Response surface plot and effects on SSMH antioxidant activity

The model's equation for SSMH antioxidant activity were derived by using regression coefficients for linear terms. According to the model's regression analysis, the best explanatory model equation is:

$$DPPH = 31.88 + 5.53^{*}A + 12.45^{*}B + 7.63^{*}C - 9.10^{*}D$$

This equation was used to predict and control the antioxidant activity of SSMH produced by alcalase. Influences from independent variables (time, temperature, pH and enzyme/substrate ratio) on antioxidant activity were prensented with three-dimensional (3D) response surface and respective contour plots [Figures 1(c) and (d)]. The 3-dimensional (3D) response plot was developed to study effects from two independent factors (time and temperature) on the DPPH antioxidant activity of SSMH when pH and enzyme/substrate ratio were fixed at 9 and 2%, respectively.

Figure 1(c) shows the response surface plot for percent SSMH antioxidant activity as a function of time and temperature. Moreover, it demonstrates that as hydrolysis time increased as well as SSMH's DPPH scavenging activity, most likely due to increased peptide content in the supernatant. This finding concurred with Halim and Sarbon (2017) who reported decreased DPPH scavenging activity from eel protein hydrolysate as hydrolysis time increased. According to Chalamaiah *et al*. (2012), hydrolysis time affects proteolysis and can result in various peptide products with different DPPH scavenging capabilities. The present work obtained the highest percent DPPH radical scavenging activity for SSMH at 180 min of hydrolysis, higher than that reported for golden apple snail hydrolysate at 120 minutes (Hamid *et al*., 2015). During hydrolysis, proteolysis releases hydrophobic peptides (Cheow *et al.*, 2007) with the ability to inhibit free radicals and lipid peroxidation (Song *et al.*, 2011). Thus, higher temperature allows for greater peptide breakdown whereby more hydrophobic peptides are made available to inhibit free radicals in DPPH.

Figure 1(d) shows the response surface plot for percent DPPH antioxidant activity by SSMH as a function of pH and enzyme concentration. The increased of pH during enzymatic hydrolysis resulted in increased DPPH antioxidant activity. Alcalase solubilizes fish protein in an alkaline environment (pH 8–10) (See *et al*., 2011). Enzymatic proteolysis of the myofibrillar tissue in a more alkaline state allowed for the release of more and smaller peptides to the hydrolysate end product. Hence, SSMH produced in a more alkaline condition yields hydrolysate with higher DPPH antioxidant activity.

In terms of enzyme/substrate ratio, DPPH antioxidant activity decreased as enzyme/substrate ratio increased. The enzyme's ability to lyse proteins into peptides is delimited by enzyme saturation at which point the hydrolysis process remains constant (Thuy *et al*., 2014). This might account for the decreased DPPH antioxidant activity observed as alcalase enzyme/substrate ratio increased.

Optimizing hydrolysate yield and antioxidant activity for shortfin scad myofibrillar protein

After setting limitations, optimization trials and evaluated results were conducted with Design-Expert 6.0.10 software (Stat-Ease 2003); hence, the proposed solutions for optimized hydrolysis conditions were assessed for validation. Selected optimum conditions were as follows: 180 mins / 59.49ºC / enzyme concentration (1%) / pH 9.93. These conditions aligned with those set for golden apple snail hydrolysate: 180 mins / 45°C / alcalase enzyme/substrate ratio (2%) / pH 10 (Hamid *et al*., 2015).

Validation trials

To confirm the proposed model's validity, experiments were undertaken to determine actual yield and antioxidant activity under the suggested optimized conditions. There was no significant difference ($p>0.05$) between the experimental value (13.18%) and predicted value (13.56%) of yield. However, SSMH antioxidant activity was 56.20% vs. predicted value of 59.20% exhibited a significant difference ($p<0.05$). Nevertheless, the respectable agreement suggests that RSM was successfully employed to project the effects of all four variables. Hence, the present study provides beneficial information on the production of peptides from SSMH for the potential functional foods.

Degree of hydrolysis (DH) of optimized SSMH

The degree of hydrolysis (DH) of optimized SSMH was determined as an indicator of peptide bond cleavage due to hydrolysis. To obtain maximum DH, the optimization model suggested ideal enyzmatic hydrolysis parameters for temperature, enzyme/substrate ratio (E/S) and pH that yielded a DH of 40.56%. During enzymatic hydrolysis, heat treatment and enzyme/substrate ratio expose proteins to the rapid cleavage of peptide bonds, thus leading to increased DH (Haslaniza *et al*., 2013). At higher enzyme/substrate ratio, the enzyme initially attacks the more susceptible peptide bonds and continuously hydrolyzes bonds, thus forming amino acids and smaller peptides (Aziz *et al.,* 2015).

SSMH antioxidant activity: reducing power

Potassium ferricyanide ($K_3[Fe(CN)_6]$) is widely used to measure the reducing power of a hydrolysate's antioxidant activity. Higher UV absorbance indicates higher reducing power. Hence, antioxidant activity

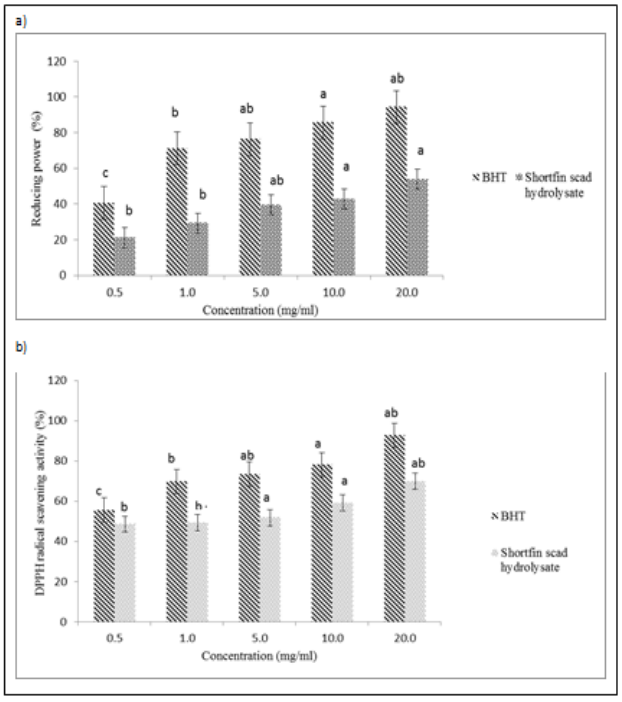

Figure 2. (a) Reducing Power; (b) DPPH radical scavenging activity for different concentrations of SSMH and BHT (positive control). All results expressed as the mean (± SD) of three trials.
($^{a-c}$) indicate significant differences between means ($p < 0.05$).

reduces Fe^{3+} ions to the ferrous form. This causes a colour change of the yellow solution to various shades of green and blue, depending on the reducing power of the tested compound. Figure 2(a) show the reducing power of SSMH (with BHT as the positive control) at different concentrations. Results showed significant differences ($p<0.05$) between each concentration of BHT and hydrolysate. Overall, percent reducing power increased with increased concentrations for both protein and BHT, with BHT showing higher reducing ability compared to SSMH. This result concurred with a report by Hamid *et al.* (2015) where the reducing power of BHT was also higher than golden apple snail hydrolysate. Hamid also reported that the hydrolysate's reducing power depended on protein substrate and enzyme type, as well as on amino acid composition, DH, and peptide sequence. IC_{50} for the SSMH reducing power in the present study was 4.59 mg/ml.

DPPH radical scavenging activities

The DPPH radical scavenging activity also were analyzed for different SSMH concentrations with BHT as a positive control. Figure 2(b) demonstrates that the inhibition of DPPH radicals increased with increasing concentrations of both BHT and SSMH. Significant differences were observed ($p<0.05$) between SSMH and the positive control for each concentration level. At 20 mg/ml, SSMH exhibited a DPPH radical-scavenging activity of 69.98%, much lower than BHT results. Results demonstrated SSMH's ability to donate hydrogen ions that react with free radicals to form stable compounds and thus terminate radical chain reactions (Razali *et al*., 2014). This finding mirrored a study by Rafi *et al*. (2015) who reported DPPH free radical scavenging by lead tree hydrolysate due to the presence of hydrogen donors.

The DPPH radical scavenging activity's IC_{50} for SSMH was 1.89 mg/ml, which is higher than the 4.88 mg/ml reported by Nakajima *et al*. (2009) for Alaska pollack myofibrillar hydrolysate, but lower than the IC_{50} (0.91 mg/mL) reported for sardine myofibrillar hydrolysate by García-Moreno *et al.* (2014). Differences in radical scavenging values can be attributed to differences in protein composition and surface hydrophobicity for the respective hydrolysates. The present study demonstrated that SSMH terminates a free radical chain reaction by converting free radicals into more stable products.

SSMH chelating effects on ferrous ions

For some compounds, Fe^{2+} chelating ability is related to antioxidant activity. Chelation begins when ferrozine quantitatively forms a complex with Fe^{2+} (Razali *et al.*, 2014). The increased of SSMH chelating effects on ferrous ion activity were observed with increased hydolysate concentrations (Figure 3(a)). Results also demonstrated significant differences in activity between concentration levels ($p< 0.05$); showing stronger chelating activity (90.91%) compared to the positive control (BHT) at 10 mg/mL. Similarly, a study by Hamid *et al.* (2015) also reported that golden apple snail hydrolysate demonstrated stronger chelating effects (46.80%) than BHT (36.09%), further suggesting that the metal chelating effects of the hydrolysate hindered oxidation. The IC_{50} for SSMH's chelating effect on ferrous ions for the present study was 0.38mg/ml. Thus, SMMH can indeed prevent metallic ion-dependent oxidative damage to food lipids and thus act as a food preservative.

Hydroxyl radical scavenging activity

Figure 3(b) shows a comparison of SSMH vs. BHT hydroxyl radical scavenging activity at different concentrations (0.5, 1.0, 5.0, 10.0 and 20.0 mg/ml). The authors observed higher SSMH hydroxyl scavenging activity compared to the positive control, BHT for each concentration level ($p < 0.05$). The SSMH IC_{50} index for radical scavenging activity was 1.37 mg/ml, higher than the 2.45 mg/ml (IC_{50})

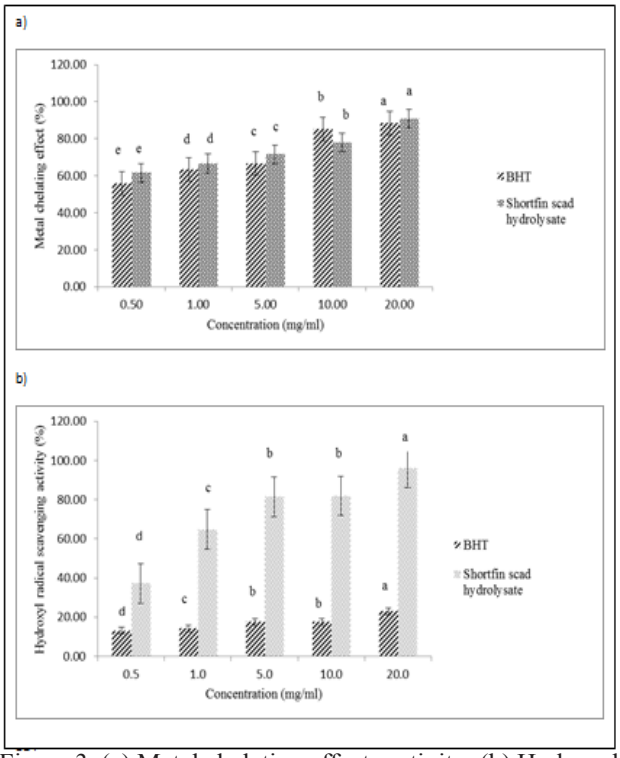

Figure 3. (a) Metal chelating effects activity; (b) Hydroxyl radical scavenging activity for different concentrations of SSMH and BHT (positive control). All results expressed as the mean (± SD) of three trials. ($^{a-e}$) indicate significant differences between means ($p < 0.05$).

reported for lead tree seed hydrolysate by Rafi *et al.* (2015). This higher scavenging ability is most likely due to the antioxidant activity of intrinsic SSMH amino acids and peptide sequences, especially hydrophobic amino acids that react with peptides and fatty acids to inhibit oxidation (Hamid *et al*., 2015). These findings therefore indicate that the potential of SSMH to be employed as a hydroxyl radical scavenger in food processing.

Conclusion

This study's analysis of shortfin scad myofibrillar tissue reported its ash, fat and relatively high protein and moisture content. It also established that the yield and antioxidant activity of shortfin scad protein hydrolysate (SSMH) is significantly influenced by hydrolysis conditions, including temperature, time, pH and enzyme/substrate ratio. Final recommendation of hydrolysis conditions for the optimization of both yield and DPPH antioxidant activity are time 180 min, temperature 59.49°C, pH 9.93 and 1% enzyme concentration. The validation trials also confirmed the estimation of SSMH yield and antioxidant activity predicted by RSM. More importantly, these results show that optimized SSMH shows excellent potential as a free radical scavenger due to its high hydroxyl radical scavenging activity and chelating effect on ferrous ions. Hence, this hydrolysate has potential for exploitation as a natural antioxidant.

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