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Optimization of the enzymatic hydrolysis conditions of waste from shortfin scad *(Decapterus Macrosoma)* for the production of angiotensin I-converting enzyme (ACE) inhibitory peptide using response surface methodology

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Abstract

This study aims to optimize enzymatic hydrolysis process for producing angiotensin I-converting enzyme (ACE) inhibitory peptides from protein hydrolysate of shortfin scad (Decapterus Macrosoma) waste (SWH). The enzymatic hydrolysis conditions, namely the temperature (40, 50, 60°C), time (B: 60, 120, 180 min), pH (C: 7, 8, 9) and enzyme substrate concentrations (D: 1, 2, 3%) on yield, degree of hydrolysis (DH) and ACE-inhibitory activity were analysed. Responses were optimized using the response surface methodology (RSM) by employing four factors, 3-levels and the Central Composite Design (CCD). The optimized conditions were further validated to indicate the validity of the prediction model. The optimal conditions obtained for the hydrolysis conditions were at temperature of 50°C, time of 60 min, pH of 9 and enzyme to substrate concentration of 2.92%. The experimental result for yield was lower than the predicted value, as generated by RSM. However, the degree of hydrolysis of SWH was higher than the predicted value. The ACE inhibitory activity of SWH was 79.34%, and showed lower than the predicted value. Therefore, the optimized conditions of SWH served as good conditions for the production of bioactive peptide with high ACE inhibitory activity.

Introduction

The fish processing industry produces more than 60% of by-products as waste, which includes the head, skin, trimmings, fins, frames, viscera and roe. Large amounts of fish waste were currently discarded or disposed as they were considered low value waste (FAO, 2014). Thus, the production of fish protein hydrolysates from fish waste was the most convenient method conducted by researchers for the utilization of fish waste (Olsen *et al*., 2014). There have been many studies on the production of fish protein hydrolysates such as the frame of the Alaska pollock (Hou *et al*., 2011), the backbone of the Ribbonfish (*Trichiurus haumela*) (Zou *et al*., 2014), and the mix by-product (i.e. the head, tail and viscera) of the Monterey sardine (*Sardinops sagax caerulia*) (Castro-Ceseña *et al*., 2012).

Enzymatic hydrolysis was frequently employed to produce fish protein hydrolysates. Enzymes were used to hydrolyse peptide bonds and were essential for the preparation of the functional attributes of fish protein hydrolysates from fish waste. Alcalase close to neutral with an optimal pH reaction of 7 to 9 have been reported to be the most efficient in the hydrolysis of fish proteins (Herpandi *et al*., 2011). During hydrolysis, the pH value, temperature, time and enzyme concentration played an important role, which affected the characteristics of the hydrolysates produced (Intarasirisawat *et al*., 2012).

The optimization of the hydrolysis process was conducted to gain a good product and better quality of the fish protein hydrolysates. The response surface methodology (RSM) was the method that was mostly selected by researchers for optimization in the study of fish hydrolysates (Halim *et al*., 2016). One of the reasons for using RSM in the determination of hydrolysis conditions was that it generated a mathematical model that accurately described the overall process with a significant ability for estimation (Wasswa *et al*., 2007). The optimization of enzymatic hydrolysis conditions for protein hydrolysate production was commonly controlled by four variables, such as time, temperature, pH and enzyme substrate concentration (E/S) on several responses, such as the degree of hydrolysis (DH), yield and ACE inhibitory activity.

In the cardiovascular system, ACE converted the inactive decapeptide (angiotensin I) to an octapeptide (angiotension II) that was a potent vasoconstrictor, and degraded the antihypertensive vasodilator bradykinin, a process that increased blood pressure

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(Brown and Vaughan, 1998). Research on ACE peptide inhibitors derived from fish waste, such as Skipjack roe (Intarasirisawat *et al*., 2012), Ribbonfish backbone (Zou *et al*., 2014) and Skate skin (Ngo *et al*., 2015) was conducted. However, research to investigate the effect of the process conditions on fish waste hydrolysates to identify the optimal hydrolysis conditions to improve the ACE inhibitory activity has been limited. Therefore, in this study, the optimization conditions of enzymatic hydrolysis with yield, degree of hydrolysis (DH) and the ACE inhibitory activity were investigated for shortfin scad waste using RSM. The production of fish protein hydrolysates from fish waste, including shortfin scad, is still presently limited.

Materials and Methods

Materials

The fish waste (bones and tails) of shortfin scad (*Decapterus Macrosoma*) was purchased from Maperow Sdn. Bhd. in Kuala Terengganu, Malaysia. The waste was washed with water and stored at -80°C until further use. The alcalase, Hippuryl-l-histidyl-l-leucine (HHL), hippuric acid (HA) and the angiotensin converting enzyme (ACE) from rabbit lung were purchased from Sigma–Aldrich, USA. All other reagents used in this study were of the analytical grade.

Preparation of the shortfin scad waste hydrolysate (SWH)

The frozen fish waste was thawed in a refrigerator (4°C) for overnight. The SWH was prepared following the method of Hamid *et al*. (2015) with a slight modification. About 44 g of homogenized fish waste were added with 44 g of distilled water. The mixture was then heated to 85°C and stirred for 20 min to inactivate the endogenous enzymes. Under varying conditions of temperature (40, 50, 60°C), time (60, 120, 180 min), pH (7, 8, 9) and enzyme substrate concentration (E/S) (1, 2, 3%) that were determined by the experimental design, the shortfin scad waste was hydrolysed with alcalase. The pH of the mixture was adjusted to the desired value using 1N NaOH. The reaction was stopped by heating the mixture to 85°C for 20 min and the mixture was centrifuged at 6000 rpm for 20 min. The supernatant (hydrolysate) was then filtered and freeze-dried.

Optimization of the enzymatic hydrolysis conditions of shortfin scad waste hydrolysate (SWH) using the response surface methodology (RSM)

The response surface methodology (RSM) was used to predict the optimal hydrolysis conditions of shortfin scad waste hydrolysate (SWH) using alcalase on three responses, which were yield, degree of hydrolysis (DH) and ACE inhibitory activity. The optimization process was conducted following Rafi *et al*. (2015) with a slight modification. Thirty hydrolysis trials were randomly run per Central Composite Design (CCD) with independent variables, including temperature (A: 40, 50, 60°C), time (B: 60, 120, 180 min), pH (C: 7, 8, 9) and enzyme substrate concentration (E/S) (D: 1, 2, 3%) employed at three equidistant levels (−1, 0, +1).

The degree of hydrolysis (DH) of shortfin scad waste hydrolysate (SWH)

The degree hydrolysis of SWH was determined using the trichloroacetic acid (TCA) method with a slight modification (Klompong *et al*., 2007). After the hydrolysis process, 0.5 g of SWH was mixed with 10 ml of distilled water. About 10 ml of 20% (w/v) TCA was added to the SWH mixture. It was left to stand for 30 min to allow for precipitation and then centrifuged at 4000 rpm for 15 min (Hitachi model CR22N, Japan). The supernatant was filtered and analysed for protein content using the Kjedahl method (AOAC, 2002). The degree of hydrolysis of the SWH was determined using the following formula:

$$\text{Degree hydrolysis (\%)} = \frac{\text{Soluble N in 10\% TCA (w/v)}}{\text{Total N in the sample}} \times 100$$

where N was the nitrogen and TCA the trichloroacetic acid.

ACE inhibitory activity assay

The ACE inhibitory activity assay was based on the liberation of hippuric acid (HA) from hippuryl-L-histidyl-L-leucine (HHL) catalysed by ACE. The ACE inhibitory activity assay was performed according to Baharuddin *et al*. (2016) with some modification. The reaction mixture was made up of 50 µl of 2.17 mM HHL, 10 µl of 2 mU of ACE and 10 µl of SWH solution (all prepared with 50 mM borate buffer, containing 300 mM NaCI, pH 8.3) giving a total volume of 70 µl . The SWH solution and HHL were combined and incubated at 37°C for 10 min in 2 ml polyethylene micro centrifuge tubes. The ACE was also treated in the same way and incubated at 37°C for 10 min before the two solutions (HHL and SWH solution) were mixed together and incubated at 37°C for 30 min with continuous agitation. After 30 min, 85 µl of 1 M HCL was added to terminate the reaction and the mixture was then vortexed. A positive control (HHL and enzyme) and blank (HHL

and buffer) were also prepared in the same manner. The ACE inhibitory activity was calculated as follows:

$$\text{ACE inhibitory activity (\%)} = 100-[(S-B)/(C-B) \times 100]$$

where C was the peak area of control (buffer added instead of test sample), B the peak area of the reaction blank (without ACE and sample), and S the peak area in the presence of the sample.

Statistical analysis

To optimize the enzymatic hydrolysis conditions, the RSM Design–Expert 6.0.10 software (Stat-Ease 2003) was used. The results were expressed as a mean (±SD) for each analysis. Comparative statistical analysis between means was calculated with ANOVA with the Minitab 14.0 to assess if there were significant differences between the treatments.

Results and Discussion

The optimization of enzymatic hydrolysis conditions on yield, degree of hydrolysis (DH) and ACE inhibitory activity using the response surface methodology (RSM)

The response surface methodology (RSM) was used to optimize enzymatic hydrolysis conditions of shortfin scad waste. The data of 30 experimental runs using central composite design (CCD) were obtained with four independent factors, namely temperature (°C, A), time (min, B), pH (C) and enzyme substrate concentration (%, D) on three responses; yield (%), degree of hydrolysis (DH, %) and ACE inhibitory activity.

The yield of freeze-dried SWH obtained from 30 experimental runs ranged from 7.42%-15.85%, which was similar with that reported from the yield of leed tree seed (7.97–10.29%) (Rafi *et al*., 2015). Meanwhile, the degree of hydrolysis (DH) of SWH ranged from 67.7%-99.52%, which was similar to the DH of fish hydrolysates of patin (60.33%-83.60%) (Najafian and Babji, 2014). The ACE inhibitory activity values ranged from 10.16% to 94.21%, which was similar with that of the lizard fish (84.45%) (Wu *et al*., 2012), and collagen (88.25%) (Kong *et al*., 2011). The difference in yield, DH and ACE inhibitory activity of fish hydrolysates could be due to the difference in fish species, fish parts, applied enzymes used and hydrolysis conditions applied.

Table 1. Analysis of variance (ANOVA) after choosing significant model for SWH yield

Source	Sum of Squares	DF	Mean Square	F Value	Prob>F
Model	135.17	9	15.02	10.65	<0.0001 significant
A	5.45	1	5.45	3.87	0.0633
B	12.10	1	12.10	8.58	0.0083
C	84.37	1	84.37	59.84	<0.0001
D	12.18	1	12.18	8.64	0.0081
AC	1.84	1	1.84	1.30	0.2673
AD	10.11	1	10.11	7.17	0.0145
BC	0.67	1	0.67	0.48	0.4978
BD	8.09	1	8.09	3.74	0.0265
CD	0.34	1	0.34	0.24	0.6276
Residual	28.20	20	1.41		
Lack of fit	55.22	15	1.68	2.83	0.1282 not significant
Pure Error	2.98	5	0.60		
Cor Total	163.37	29			
R-squared	0.8274				
Pred R-squared	0.5067				
Adj R-squared	0.7497				
Adeq precicion	13.753				

A = temperature (°C), B = time (min), C = pH, D = enzyme concentration (%)

Analysis of the yield of shortfin scad waste hydrolysate (SWH)

Summary of the model of statistics for the yield of shortfin scad waste hydrolysate (SWH)

A multiple regressions analysis technique included in the RSM was performed to determine the coefficients of the terms of the linear (A, B, C, D), quadratic (A^2, B^2, C^2, D^2) and two-factor interaction (AB, AC, AD, BC, BD, CD) to fit a full response surface model for the responses. In this experiment, the two-factor interaction (2FI) model was the model summary suggested for SWH yield. The 2FI model suggested for the SWH yield was not in agreement with previous studies that reported on the optimization of skate cartilage (Murado *et al*., 2010), which found that the predicted model for SWH yield was a quadratic model. The difference in the prediction model for SWH yield could be due to the differences in the raw materials, types of enzymes used and hydrolysis conditions applied.

Analysis of variance (ANOVA) for the yield of shortfin scad waste hydrolysate (SWH)

The ANOVA of the Response Surface Two-factor interaction model for SWH yield after model reduction is shown in Table 1. The model reduction was done to reduce the insignificant terms of the model (Ruangmee and Sangwichien, 2013). Compared to the unreduced model, the F-value for the reduced model was higher after the elimination of non-significant terms. The p-value for the lack of fit of the reduced model was also greater, confirming that the reduced model was more accurate to be

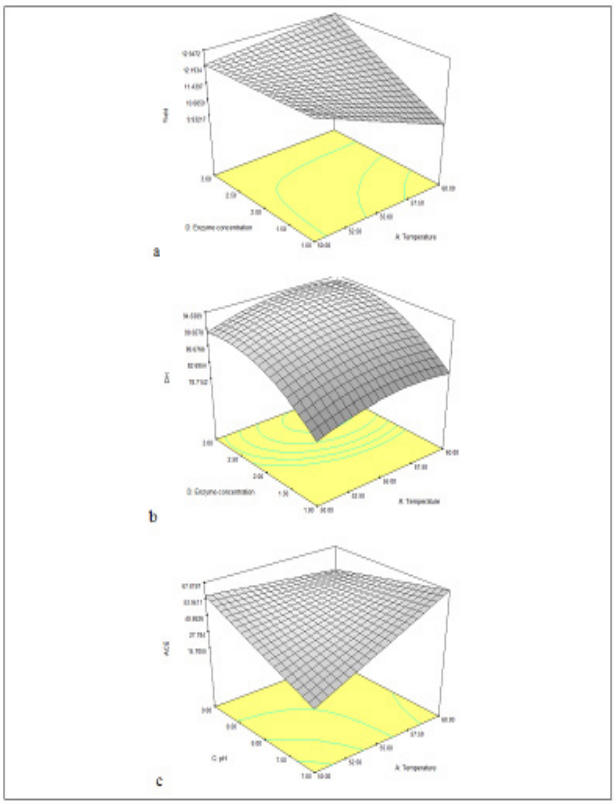

Figure 1. Response surface plot for a) yield (%) as function of temperature and enzyme concentration, b) DH (%) as function of temperature and enzyme concentration, c) ACE inhibitory activity (%) as function of temperature (°C) and pH

used for predictions. The results demonstrated that the model was significant at a 95% confidence level ($p<0.05$). This indicated that the 2FI model could explain a high percentage of the variability in the observed data. The Fisher's test (F-test) carried out on the experimental data made it possible to estimate the statistical significance of the proposed model (Maache-Rezzoug *et al*., 2011). Table 1 shows that the F-value (10.65) and the P-value of <0.00011 were less than 0.05 which indicated a significant model. There was only 12.82% chance that a "Model F-value" this large could occur due to noise. The lack of the fit test was used to predict the fitness of the model. In the model of SWH yield, it was found that the p-value for the lack of fit value was not significant ($p>0.05$) (0.1282). Thus, the model was fitted to determine the optimum hydrolysis conditions of SWH.

Guan and Yao (2008), reported that R^2 should be at least 0.80 for the good fit of a model. Based on the result presented in Table 1, the coefficient of determination (R^2) was 0.8274, indicating that 82.74% of the experimental results could be explained by the fitted model over the range of factors tested for the SWH hydrolysate. The value of "Pred R-Squared" (0.5067) was in reasonable agreement with "Adj R-Squared" (0.7497). "Adeq Precision" measured

Table 2. Analysis of variance (ANOVA) after choosing significant model for SWH degree of hydrolysis

Source	Sum of Squares	DF	Mean Square	F Value	Prob>F
Model	2098.23	11	190.75	2.50	0.0409 significant
A	70.60	1	70.80	0.93	0.3483
C	11.76	1	11.76	0.15	0.6993
D	753.54	1	753.54	9.87	0.0056
A^2	130.10	1	130.10	1.70	0.2081
B^2	96.39	1	96.39	1.26	0.2759
C^2	180.05	1	180.05	2.36	0.1420
D^2	494.36	1	494.36	6.48	0.0203
AB	13.73	1	13.73	0.18	0.6765
AC	17.10	1	17.10	0.22	0.6417
BC	93.90	1	93.90	1.23	0.2820
CD	99.00	1	99.00	1.30	0.2697
Residual	1373.85	18	76.33		
Lack of Fit	1256.12	13	96.62	4.10	0.0643 not significant
Pure Error	117.73	5	23.55		
Cor Total	3472.08	29			
R-squared	0.6043				
Pred R-squared	-0.6595				
Adj R-squared	0.3652				
Adeq precicion	7.210				

A = temperature (°C), B = time (min), C = pH, D = enzyme concentration (%)

the signal-to-noise ratio and a ratio greater than 4 was desirable (Canettieri *et al*., 2007). For this model, the "Adeq Precision" ratio was 13.753, which was an adequate signal-to-noise ratio. Therefore, this model proved to be powerful for navigating the design space.

The ANOVA results demonstrated that the linear model in terms of time (B), pH (C) and enzyme concentration (D) had a significant effect ($p<0.05$) on the yield of SWH. The interaction terms (AD, BD) also had significant effects ($p<0.05$) on SWH yield. Thus, a significant model ($p<0.05$) and a non-significant lack of fit test validated the SWH yield model.

Response surface plots and the effects of factors on the yield of shortfin scad waste hydrolysate (SWH)

The model equation for yield and the response variable (Y) of SWH obtained was derived using the regression coefficient of linear and interaction terms to fit a full response surface model. According to the model's regression analysis, the best explanatory model equation of SWH yield was given as follows:

$$Y = +11.92 - 0.48\,A + 0.71\,B + 1.87\,C + 0.71\,D - 0.34\,AC + 0.80\,AD + 0.21\,BC - 0.71\,BD - 0.15\,CD$$

A three-dimensional (3D) response surface

plot was developed to understand the effects of the interaction of the factors by evaluating two variables at a time on the yield of SWH. Figure 1a shows the effects of the interaction between temperature and enzyme concentration (AD) on SWH yield. When examining the surface plot of Figure 1a, the centre point was a saddle point where a combination of low temperature (A) and high enzyme substrate concentration (D) resulted in high SWH yields. A similar result was reported by Sindhu *et al*. (2014) that when temperature increased from 45°C to 61oC, the yield of sample decreased from 60% to 20%. The decreased SWH yield on increasing temperature could partially be explained by the loss of enzyme activity due to thermal inactivation (Sindhu *et al*., 2014). To preserve the SWH, hydrolysis temperatures were maximally set at 60°C as protein began to unfold at this condition (Bhaskar *et al*., 2008). Meanwhile, enzyme concentration was reported to be one of the most important factors and generally high enzyme concentration loading resulted in better hydrolysis probably by increasing the rate and yield of enzymatic hydrolysis.

Analysis for the degree of hydrolysis (DH) of shortfin scad waste hydrolysate (SWH)

Summary of the model of statistics for the DH of shortfin scad waste hydrolysate (SWH)

The summary of the model for the degree of hydrolysis (DH) of SWH was a quadratic model. The same model was reported in the study by Wu *et al*. (2012) from the enzymatic hydrolysis of different fish species, such as the lizard fish.

Analysis of variance (ANOVA) on the DH of SWH

The ANOVA of the Response Surface Quadratic model for DH of SWH after model reduction is shown in Table 2. The result demonstrated that the model was significant at a 95% confidence level ($p<0.05$). This indicated that the quadratic model could explain a high percentage of the variability in the observed data. The "Model F-value" of 2.50 and the P-value of 0.0409 were less than 0.05, implying that the model was significant. Meanwhile, the lack of fit value of 4.10 meant that there was 6.43% chance that a "Model F-value" this large could occur due to noise. The lack of fit test was designed to determine whether the selected model was adequate to describe the observed data, or whether a more complicated model should be used (Fang *et al*., 2012). The lack of fit for SWH model was not significant ($p>0.05$) (0.0643), hence, the model was fit to determine the optimum hydrolysis conditions of SWH.

Based on the result presented in Table 2, the model for DH of SWH had a significant ($p<0.05$) coefficient variation (R^2) value (0.6043). The value of 0.6043 indicated that 60.43% of the variation could be explained by the fitted model over the range of factor values tested for SWH. Since the R^2 value was high, this indicated that the models were well adapted to the responses. Meanwhile, the "Adeq Precision" ratio of the model was 7.210, indicating adequate signals. Therefore, this model could be used to navigate the design space for the determination of the hydrolysis conditions of SWH. Besides that, the ANOVA results after the model reduction demonstrated that only the linear model term of enzyme concentration (D) had a significant effect ($p<0.05$) on the DH of SWH (0.0056). In terms of the quadratic coefficients, enzyme concentration (D^2) had significantly affected ($p<0.05$) the DH of SWH (0.0203). This result showed that the more important parameter in the hydrolysis reaction was the enzyme concentration. In summary, the model presented was fit and was chosen to predict the enzymatic hydrolysis conditions of SWH.

Response surface plots and the effects of factors of DH on shortfin scad waste hydrolysate (SWH)

The model equation for DH and the response variable (Y) of SWH obtained was derived using the regression coefficient of the linear and quadratic models to fit a full response surface model. According to the model's regression analysis, the best explanatory model for DH of SWH equation was given as follows:

$$Y = + 92.46 + 1.72\,A - 0.70\,C + 5.60D - 2.18\,A2 + 1.87\,B2 + 2.56\,C2 - 4.25\,D2 - 0.93\,AB + 1.03\,AC + 2.42\,BC - 2.49\,CD$$

Figure 1b shows the response surface plot for the interaction between enzyme concentration and temperature during the hydrolysis of shortfin scad waste for DH. From Figure 1b, DH increased when temperature and enzyme substrate concentration (E/S) increased. Similar results were reported by Aziz *et al*. (2015) where the DH of sunflower oil increased from 64.1% at 30°C to a maximum of 84.1% at 45°C. This was consistent with the optimum temperature of alcalase which was from 50°C to 60°C. Heat treatment and higher enzyme concentrations caused the exposure and rapid cleavage of peptide bonds during enzymatic hydrolysis which led to the increase of DH (Haslaniza *et al*., 2013). At higher E/S, the enzyme initially attacked the most susceptible peptide bonds, continuously hydrolysing the bonds during the entire

Table 3. Analysis of variance (ANOVA) after choosing significant model for SWH ACE inhibitory activity

Source	Sum of Squares	DF	Mean Square	F Value	Prob>F
Model	7956.96	7	1136.71	12.61	< 0.0001 significant
A	1134.48	1	1134.48	12.58	0.0032
C	504.71	1	504.71	5.60	0.0330
D	97.40	1	97.40	1.08	0.3163
AB	3577.18	1	3577.18	39.67	< 0.0001
AC	2005.64	1	2005.64	22.24	0.0003
BD	94.04	1	94.04	1.04	0.3245
CD	451.16	1	451.16	5.00	0.0421
Residual	1262.39	14	90.17		
Lack of Fit	557.57	9	61.95	0.44	0.8659 not significant
Cor Total	704.82	5			
R-squared	0.8631				
Pred R-squared	0.6342				
Adj R-squared	0.7946				
Adeq precicion	13.805				

A = temperature (°C), B = time (min), C = pH, D = enzyme concentration (%)

hydrolysis period because of the availability of the substrate (Aziz *et al*., 2015). As E/S increased, hydrolysis became more rapid because of a relative increase in enzyme concentrations. This observation was attributed to the increased number of enzymes acting upon the surface of the substrate. Hence, more enzymes and optimum temperature were able to digest the proteins found in the different areas of the substrate surface and greater hydrolysis of protein occurred.

Analysis for ACE inhibitory peptide of shortfin scad waste hydrolysate (SWH)

Summary of the model of statistics for ACE inhibitory peptide of shortfin scad waste hydrolysate (SWH)

The summary of the suggested model for ACE inhibitory peptide of SWH was the two-factor interaction model. The two-factor interaction model suggested for the SWH ACE inhibitory activity was not in agreement with previous studies that reported on the optimization of collagen (Kong *et al*., 2011) which found that the predicted model for SWH ACE inhibitory activity was a quadratic model. The difference in the prediction model for SWH yield could be due to the differences in the raw materials, types of enzymes used and hydrolysis conditions applied.

Analysis of variance (ANOVA) on the ACE inhibitory activity of SWH

The ANOVA of the Response Surface two-factor interaction model for ACE inhibitory activity of SWH after model reduction is shown in Table 3. The "Model F-value" of 12.61 and the P-value of <0.0001 were less than 0.05, implying that the model was significant. The lack of fit test was designed to determine if the selected model was adequate to describe the observed data, or whether a more complicated model should be used (Fang *et al*., 2012). The P-value for the lack of fit test was not significant (0.8659) ($p>0.05$), indicating that the model was fit for predicting the enzymatic hydrolysis of SWH. Hence, the model was fit to determine the optimum hydrolysis conditions of SWH.

Based on the results presented, the model for ACE inhibitory activity of SWH had the coefficient of determination (R^2) of 0.8631, indicating that 86.31% of the experimental results could be explained by the fitted model over the range of factors tested for the SWH. The "Adj R-Squared" (0.7946) was within reasonable agreement with "Pred R-Squared" (0.6342), indicating that the model was well-adapted to the response. Meanwhile, the "Adeq Precision" ratio of the model was 13.805, indicating adequate signals. Therefore, this model could be used to navigate the design space for the determination of hydrolysis conditions of SWH for predicting the ACE inhibition for any combination of independent variables within the range studied.

The ANOVA results after model reduction in Table 3 also demonstrated that the linear model terms of temperature (A) and pH (C) had a significant effect ($p<0.05$) on the ACE of SWH. The interaction model term of (AC, CD) also had a significant effect ($p<0.05$) on the ACE inhibitory activity of SWH. Thus, this model was fit and was chosen to predict the enzymatic hydrolysis conditions of SWH.

Response surface plots and the effects of factors on the ACE inhibitory activity of shortfin scad waste hydrolysate (SWH)

The model equation for the ACE inhibitory activity and the response variable (Y) of SWH obtained were derived using the regression coefficient of the two-interaction factor to fit a full response surface model. According to the model's regression analysis, the best explanatory model for ACE inhibitory activity of the SWH equation was given as follows:

$$Y = +46.41 + 10.71\,A + 5.55\,C - 3.14\,D + 26.49\,AB - 15.45\,AC - 4.29\,BD - 7.33\,CD$$

Table 4. Recommended solutions for optimal shortfin scad waste hydrolysate (SWH)

No.	Temperature	Time	pH	Enzyme	Yield	Degree of hydrolysis	ACE-I activity	Desirability
1	50.00	60.00	9.00	2.92	14.1351	87.1957	85.2243	0.8257 Selected
2	50.17	60.88	9.0	3.00	9.77125	87.0595	84.7546	0.825
3	50.00	69.18	9.0	3.00	9.77104	86.7705	83.443	0.818
4	50.66	60.00	9.0	2.98	9.77107	87.9206	82.2593	0.816
5	50.28	60.00	8.91	3.00	9.77129	87.4039	82.7694	0.811

Figure 1c shows the response surface plot for the interaction between temperature and pH during the hydrolysis of shortfin scad waste for ACE inhibitory activity. Figure 1c showed that the ACE inhibitory activity increased when temperature and pH increased. An increase in the hydrolysate bioactivity suggested that the compact primary structure of the shortfin scad waste partially unfolded with the increase in temperature and pH. This assisted the enzyme to access the bioactive peptides in the primary sequence of the protein chains (Costa *et al*., 2007). Similar results were reported by Pan and Guo (2010) from sour milk. When temperatures rose close to the optimum temperature (i.e. 60°C), the enzyme activity accessed to the primary sequence of proteins, resulting in the increased release of α-amino groups and bioactive peptide (Goudarzi *et al*., 2012). Moreover, according to the results obtained by Costa *et al*. (2007), the best ACE inhibition activity was presented by hydrolysates obtained from isolated treatment at 65°C, whereas hydrolysates obtained from untreated isolates or isolates previously treated at 95°C had significantly lower activity.

The breakdown in the hydrolysate bioactivity as a result of the degradation of bioactive peptides to smaller inactive counterparts occurred with an increase in pH (Goudarzi *et al*., 2012). This could be due to the disruption of the compact three-dimensional structure of SWH which allowed for the further digestion of polypeptide chains (De la Fuente *et al*., 2002). Thus, the hindered parent sequence of bioactive peptides would be exposed and was available for the proteolytic reaction of the enzyme. Aziz *et al*. (2015) had also suggested that the pH could modify the ionization state of the enzyme and, as a result, the activity and selectivity of the enzyme could be altered. Since the value of pH 9 was suggested to be the optimum condition for activity for the alcalase, a major breakdown of the ACE inhibitory peptides to smaller inactive counterparts could have occurred.

The optimization of the shortfin scad waste hydrolysate (SWH) yield, DH and ACE inhibitory activity

Optimal response conditions

The desirability profiles for the optimum condition suggested by RSM are shown in Table 4. The desirability value close to 1 showed that the suggested conditions were the most suitable in obtaining optimum SWH responses (yield, DH and ACE inhibitory activity). Therefore, the suggested hydrolysis conditions for SWH were a temperature of 50°C, time of 60 min, pH of 9 and enzyme concentration of 2.92%. At these conditions, the predicted yield was 14.13%, the degree of hydrolysis was 87.20% while the predicted ACE inhibitory activity was 85.22%. The optimized process conditions gave an overall desirability value of 0.8257.

Validation test

To confirm the validity of the model, an experiment was conducted using the optimal conditions with three replicates for each response. The yield obtained was 11.16% which was lower than the predicted value generated by RSM (i.e. 14.13%). However, the DH of SWH was 88.81%, which was higher than the predicted value (87.20%). Meanwhile, the ACE inhibitory activity of SWH was 79.34%, and was also lower than predicted value (85.22%).

Conclusion

In conclusion, as it was expected the yield, the degree of hydrolysis (DH) and ACE inhibitory activity of SWH were significantly affected by the hydrolysis conditions including the temperature, time, pH and enzyme concentration. Based on the model, the optimum conditions were a temperature of 50°C, time of 60 min, pH of 9 and enzyme concentration of 2.92%. The corresponding responses were 87.19% of DH, 14.13% of hydrolysate yield and 85.22% of ACE inhibitory activity. Therefore, RSM was successfully used to investigate the effects of four processing conditions (i.e. pH, substrate concentration, temperature, and time) and to optimize the hydrolysis conditions for the production of ACE inhibitory peptides from SWH.

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