International Food Research Journal 25(4): 1541-1549 (August 2018)
Journal homepage: *http://www.ifrj.upm.edu.my*

Optimization of enzymatic hydrolysis conditions and characterization of Shortfin scad *(Decapterus Macrosoma)* skin gelatin hydrolysate using response surface methodology

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Article history

Received: 10 April 2017
Received in revised form: 24 May 2017
Accepted: 25 May 2017

Keywords

Shortfin scad
Fish gelatin hydrolysate
Enzymatic hydrolysis
Angiotensin converting enzyme
Response surface methodology

Abstract

This study employed response surface methodology (RSM) to optimize alcalase-enzymatic hydrolysis conditions for the production of an angiotensin I-converting enzyme (ACE) inhibitory peptide from Shortfin scad *(Decapterus Macrosoma)* skin gelatin (SSGH). Using Central Composite Design (CCD) with four factors and three levels, a statistical modeling equation was developed to predict effects from the following variables:(i) temperature (40, 50, 60°C); pH (7, 8, 9);(ii) enzyme/substrate (E/S) concentration (1, 2, 3%); and (iii) hydrolysis time (60, 120, 180 min) with respect to yield, degree of hydrolysis (DH) and ACE-inhibitory activity. Optimum hydrolysis conditions obtained were 60°C, pH 9, 2.92% and 114.56 minutes. Experimental yield for SSGH was higher (90.05%) than the predicted value of 54.38%. The degree of SSGH hydrolysis (DH = 90.48%) was also higher than the RSM predicted value of 72.04%. Experimental SSGH ACE inhibitory activity (79.61%) was lower than predicted (89.19%).This study demonstrates the viability of using RSM to optimize conditions for the enzymatic hydrolysis of SSGH to yield gelatin with high ACE inhibitory peptide activity.

Introduction

Shortfin scad *(Decapterus macrosoma)* from the Carangidae family are readily found in Malaysia and are locally known as selayang, basung and sardine (Mohsin and Ambak, 1999). Total tonnage captured in Malaysia increased from 102,644 in 2014 to 117,155 in 2015 (Department of Fisheries, 2015). Shortfin scad is a very important pelagic species and is used as the main ingredientto produce 'keropok lekor' and fish crackers in Terengganu, Perlis and East Johore. However, numerous environmental problems have arisen from fish processing to include nearly 60–70% of post-processing leftovers (flesh, head, skin, bones, scales and viscera) (Clement and Lovell, 1994). The fish's entire body of rounded flesh is considered the main product while head, backbone, trimmings (cut-offs), skin and guts constitute by-products or wastage (Gildberg *et al.*, 2002). A solution for fish waste is the production of various value-added products such as proteins (Arnesen and Gildberg, 2006), bioactive peptides (Wang *et al*., 2013), collagen (Chi *et al*., 2014) and gelatin (Ghaly *et al.,* 2013).

Contemporary fish gelatin hydrolysates deriving from skin, bone, head and scales yield gelatin alternatives via enzymatic hydrolysis and recover valuable components from fish by-products (Slizyte *et al.*, 2005). Researchers have studied several enzymes from plants, animals and microbes to optimize parameters for the production of the ACE inhibitory peptide. Alcalase is the most preferable enzyme employed due to its ability to yield shorter peptide sequences and grant the highest range of ACE inhibitory activity (60–80%) (Ngo *et al.*, 2014). Relevant work in this field includes studies on fish gelatin hydrolysates deriving from skin such as pacific cod *(Gadus macrocephalus)* (Himaya *et al*., 2012); Korean rockfish *(Sebastes schlegelii)* (Ngo *et al*., 2011); scale of Nile tilapia *(Oreochromis niloticus)* (Ngo *et al*., 2010); sea bream (Akagündüz *et al*., 2014); and the bones of sea bream (Akagündüz *et al.*, 2014).

Angiotensin Converting Enzyme (ACE) plays an important role in the regulation of blood pressure as well as fluid and salt balance in mammals. Regarding the cardiovascular system, ACE converts the inactive decapeptide, angiotensin I, to the active octapeptide, angiotensin II, which is potent vasoconstrictor that degrades the antihypertensive vasodilator, bradykinin. This process results in increased blood pressure (Brown and Vaughan, 1998). Hence, ACE inhibitors are believed to lower blood pressure and help prevent cardiovascular disease. Many researchers have successfully isolated ACE-inhibitory peptides from

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various fish protein hydrolysates such as brown stripe red snapper muscle (Khantaphanta *et al.*, 2012); lizard fish *(Saurida elongata)* and seaweed pipefish *(Syngnathus schlegeli)* (Wijeskera *et al.*, 2011); and salmon byproducts (Ahn *et al.,* 2012). ACE inhibitory peptides deriving from fish protein hydrolysates are considered natural alternative bioactive peptides that are safer than synthetically produced ACE inhibitors.

The authors examined hydrolysis process optimization to obtain gelatin hydrolysate with quality bioactive peptides. RSM was employed to specifically produce a peptide hydrolysate to inhibit ACE activity because RSM quickly and efficiently requires a minimum of experimental runs while maintaining high degrees of statistically significant outcomes (Qi *et al.*, 2009). Although RSM has several alternative designs, namely, full factorial design, central composite design (CCD), D-optimal designs, Taguchi's contribution to experimental design, and Latin's hypercube design, researchers studying fish hydrolyates mostly use CCD. RSM efficacy has also been demonstrated by the use of models such as quadratic, 2FI, linear and cubic. The quadratic model has been reported as the fit test for tilapia skin gelatin (Goudarzi *et al*., 2012). The 2FI model fits Golden apple snail (Hamid *et al*., 2015) and the linear model fits both Nemipterus japonicus and Exocoetus volitans (Naqash and Nazeer, 2012). The present study aimed to optimize alkalase enzymatic hydrolysis conditions with a view on outcomes including yield, degree of hydrolysis (DH) and ACE inhibitory activity. Comparing expected and experimental results for all tested parameters, projected outcomes were experimentally validated for optimized hydrolysis conditions.

Materials and Methods

Materials

Shortfin scad *(Decapterus Macrosoma)* were purchased fresh from Lembaga Kemajuan Ikan Malaysia (LKIM) in Kuala Terengganu, Malaysia and transported in ice to the laboratory for beheading and gutting. Fish skins were manually removed after filleting and stored at -40ºC. Alcalase, Hippuryl-l-histidyl-l-leucine (HHL), hippuric acid (HA) and angiotensin converting enzyme (ACE) from rabbit lung were purchased from Sigma–Aldrich, USA. All reagents used in this study were of analytical grade.

Preparation of the Shortfin scad skin gelatin (SSG)

Gelatin was extracted from Shortfin scad skin by following the method described by Cheow *et al.* (2007), with slight modification. Frozen fish skins were cleaned and copiously rinsed with water after overnight thawing at 4°C. The skins were then soaked in sodium hydroxide (0.15%, w/v) for 40 min followed by sequential soaks in sulphuric acid (0.15%, w/v) and citric acid (0.7%, w/v). Each soaking and washing was repeated three times; thus taking a total of 2 h for each treatment. The ratio of skin to solution was 1 kg / 6L of acid or alkali for each treatment. The skins were washed in distilled water after treatments to remove all residues. Final extraction was undertaken overnight in distilled water at 45°C. The clear extract was filtered through a Buchner funnel using Whatman filter paper (No. 4) and then evaporated under a vacuum and freeze-dried.

Preparation of the Shortfin scad skin gelatin hydrolysate (SSGH)

SSGH was prepared following the method described by Razali *et al*. (2015), with slight modification. One gram of gelatin was added to 100g of distilled water. Using alcalase, SSGH samples were prepared at different temperatures, time periods, pH levels and enzyme substrate concentrations (E/S). The pH of a mixture was adjusted to a desired value with 1N NaOH. Reactions were stopped by heating each mixture at 85°C for 20 min to inactive enzyme activity. Each hydrolysate was then cooled and centrifuged at 6000 rpm for 20 min, after which the supernatant of the hydrolysate was filtered and freeze dried.

RSM optimization of enzymatic hydrolysis process variables and conditions for SSGH

RSM was employed to optimize alcalase hydrolysis conditions to produce SSGH with specific views on three outcomes: yield, degree of hydrolysis (DH), and ACE inhibitory activity. The optimization process followed the protocol of Rafi *et al.* (2015) with slight modification. As per Central Composite Design (CCD), 30 hydrolysis trials were randomly undertaken at three equidistant levels (−1, 0, +1) for each independent variable: 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%)

Degree of hydrolysis (DH) for SSGH

The DH were determined for SSGH by following protocols outlined by Baharuddin *et al.* (2016), with slight modification. After hydrolysis, ~0.5 g of SSGH were mixed with 10 ml of distilled water and then added 10 ml of 20% (w/v) TCA. The solution was left standing for 30 min to allow precipitation and

then centrifuged for 15 min (Hitachi model CR22N, Japan) at 4000 rpm. The supernatant was filtered and analyzed for protein content following the Kjedahl method (AOAC, 2002). DH for SSGH was determined as follows:

$$DH\ (\%) = \frac{\text{Soluble N in 20\% TCA (w/v) x 100}}{\text{Total N in the sample}}$$

Where N is nitrogen and TCA is trichloroacetic acid.

ACE inhibitory activity assay

ACE inhibitory activity were measured according the method reported by Wu *et al.* (2002), with some modifications. Total reaction volume (70 µl) comprised 50 µl of 2.17 mM HHL, 10 µl of 2 mU of ACE and 10 µl of SSGH, all prepared with 50 mM of borate buffer and 300 mM of NaCI at pH 8.3. The SSGH solution was added to HHL and incubated at 37°C for 10 min in 2 ml polyethylene micro-centrifuge tubes after incubating ACE at 37°C for 10 min. Both solutions were mixed and incubated at 37°C for 30 min with continuous agitation and then vortexed after adding 85 µl of 1 M HCL to terminate enzyme activity. Positive control (HHL and enzyme) and blank (HHL and buffer) samples were prepared in the same manner. The final samples were analysed using HPLC.

High-performance liquid chromatography (HPLC)

The HPLC unit consists of a pump (Shimadzu 2LC-10AT), an auto-sampler (ShimadzuSIL-30AC) and a detector (Shimadzu SPD 20-A). Approximately 20µl of samples for each assay were injected into a C18 column (4.6 x 150 mm – 5 µm, Fisher). HA and HHL elution peaks were detected at 228 nm. The column was eluted (1 ml/min) by using a bi-solvent system: (a) 0.05% (v/v) TFA in water, and (b) 0.05% (v/v) TFA in acetonitrile with a 5–60% acetonitrile gradient for the first 10 min. This was maintained for 2 min at 60% acetonitrile and then returned to 5% acetonitrile for 1 min, followed by isocratic elution for 4 min at a constant flow-rate of 1 ml/min. ACE inhibitory activity was calculated as follows:

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

C= peak area of control (buffer added instead of test sample);

B= peak area of the reaction blank (without ACE and sample);

S= peak area in the presence of sample.

IC_{50}=concentration of inhibitor required to inhibit 50% of ACE activity under assay conditions as determined by regression analysis for ACE inhibition (%) vs. inhibitor concentration (µM or mg/ml).

Statistical analysis

The authors used RSM Design–Expert 6.0.10 software (Stat-Ease 2003) to analyze optimal enzymatic hydrolysis conditions. Results are expressed as a mean (±SD) for each analysis. A comparative statistical analysis between means (ANOVA) were conducted using Minitab 14.0 in order to determine significant differences ($p<0.05$).

Results and Discussion

RSM optimization of enzymatic hydrolysis results for yield, degree of hydrolysis (DH) and ACE inhibitory activity

Using RSM, the optimal hydrolysis conditions was projected for the specific production of SSGH containing peptides with enhanced ACE inhibitory activity. Table 1 presents data from 30 experimental runs undertaken according to CCD for four independent variables and three outcomes: A for temperature (°C); B for time (min); C for pH; and D for enzyme substrate concentration (%); yield (%), degree of hydrolysis (DH, %) and ACE inhibitory activity, respectively. SSGH yields ranged from 41.58 to 94.38% on a dry basis. The highest yield (94.38%) was similar to that obtained for duck skin gelatin hydrolysate (93.20%) (Lee *et al*., 2012). A higher hydrolysate yield is likely due to a gelatin's lower lipid content. Slizyte *et al.* (2005) submitted that raw materials with the lowest amount of lipids allow the highest percentages of solubilized protein, which increases yield.

DH depends on hydrolysis time, temperature and enzyme concentration (Baharuddin *et al.,* 2016). DH results for SSGH obtained in this study ranged from 42.72 to 98.15%, similar to reports on salmon skin (77.03%) (See *et al*., 2011); sole and squid gelatin (35-50%) (Gimenez *et al.*, 2009); and patin (60.33–83.60%) (Najafian and Babji, 2014).The higher DH presented in this study likely stems from decreased peptide size, which exposes hydrophilic groups of amino acids to the solvent; thereby increasing solubility (Kristinsson and Rasco, 2000a).

ACE inhibitory activity ranged from 19.67 to 95.06%, similar to reports on Pacific cod skin gelatin (60.40%) (Himaya *et al*., 2012); skate (Okamejei kenojei) skin gelatin (72.8%) (Ngo *et al.*,2014); and lizard fish (84.45%) (Wu *et al*., 2012).This finding indicated that the hydrolysis of Shortfin scad skin gelatin probably releases a significant number of ACE

Table 1.Experimental design results for CCD using RSM.

No.	A: Temperature (°C)	B: Time (min)	C:pH	D: E/S (%)	Yield (%)	DH (%)	ACE-I activity (%)
1	60	180	9	3	43.87	66.84	85.26
2	60	60	9	1	83.42	89.28	77.67
3	55	120	8	2	52.81	82.92	79.73
4	65	120	8	2	47.55	64.63	86.09
5	55	240	8	2	55.82	89.84	78.99
6	50	60	7	1	67.23	92.45	81.63
7	55	120	6	2	71.35	79.41	59.6
8	45	120	8	2	48.78	88.63	52.13
9	50	60	7	3	65.15	65.27	68.11
10	60	60	7	3	88.30	85.95	62.07
11	55	120	8	0	70.60	61.08	41.8
12	50	180	7	3	88.18	56.95	83.04
13	60	60	7	1	68.46	90.05	50.55
14	50	180	7	1	78.39	43.57	70.38
15	55	120	8	2	53.57	88.47	58.45
16	55	120	8	2	88.49	76.66	88.76
17	55	0	8	2	64.47	80.02	19.67
18	55	120	8	2	56.09	42.72	71.02
19	55	120	8	4	90.40	91.42	53.81
20	50	60	9	3	52.57	86.52	74.39
21	50	180	9	1	61.76	56.01	58.92
22	50	180	9	3	64.64	98.15	65.42
23	60	180	7	1	70.01	81.4	57.65
24	55	120	8	2	53.33	93.73	58.61
25	60	60	9	3	66.31	88.49	95.06
26	55	120	10	2	94.38	59.69	51.15
27	60	180	9	1	41.58	50.78	61.28
28	55	120	8	3	54.28	70.08	77.42
29	50	60	9	1	65.03	89.21	50.93
30	60	180	7	3	76.87	74.65	37.30

inhibitory peptides. Prior researchers have submitted that the highest degrees of enzyme induced ACE inhibition attended the highest DH levels and lowest peptide molecular weights (150–800Da) (Zhang, Wangand Xu, 2009; Baharuddin *et al*., 2016). Ngo *et al.* (2014) also reported that most researchers preferred alcalase because it produced shorter peptide sequences and yielded the highest ACE inhibitory activity range (60–80%). Hence, using RSM to project different conditions and outcomes for temperature, pH, time and E/S concentration, the authors prepared SSGH by enzymatic hydrolysis to optimize outcomes for DH and ACE inhibitory activity.

Analysis of SSGH yield: RSM summary statistics

Our multiple regression analyses included all coefficients for linear (A, B, C, D), quadratic (A^2, B^2, C^2, D^2) and two-factor interaction (AB, AC, AD, BC, BD, CD) terms to fit full RSM outcomes. The quadratic model's summary suggested optimized SSGH yield in agreement with hydrolysate models reported for golden apple snail by Hamid *et al.* (2015) and for lead tree seed as reported by Rafi *et al.* (2015).

Analysis of variance (ANOVA) for Shortfins cad skin gelatin hydrolysate (SSGH) yields

Table 2 summarizes ANOVA results for RSM SSGH yields after linear reduction of the model's proposed polynomial, by which the F-test determined statistical significance (Maache-Rezzoug *et al*.,2011). The F-value for yield equaled 2.91, implying significance since only a 4.00% chance existed that an F-value this large could occur due to noise. A 'lack of fit' test yielded a value of 0.11, implying no significance due to error. Hence, a 98.74% chance remained that a 'lack of fit' F-value this large could occur due to noise. Thus, the model fit experimental data with a significant determination coefficient of $R^2 = 0.7270$ ($p< 0.05$; See: Table 2). Ideally, R^2 should be >0.80 to obtain the best explanatory 'fit model' for data variables (Halim and Sarbon, 2015). Moreover, a 'Pred R-Squared'value of 0.2014 did not approximate the 'Adj R-Squared'value of 0.4768. Generally, an 'Adeq Precision' ratio >4 is desired to measure the signal-to-noise ratio. The present model's ratio equaled 6.119, indicating an adequate signal. Thus, our RSM derived model qualified for design space navigation. Based on ANOVA results, the linear model and interaction terms (A and BC) would have significant effects on SSGH yield ($p<0.05$).

Response surface plots for variables and their effects on SSGH yield

Using regression coefficients for linear and interaction terms to fit a full RSM, the model's polynomial equation for SSGH yield as a response variable (Y) were derived. According to regression analysis, the best explanatory equation for SSGH yield was:

$$Y = +62.67 - 8.00\,A - 4.20\,B + 0.69\,D + 1.66\,B^2 - 2.78\,C^2 + 4.86\,D^2 - 4.5\,AB - 1.33\,AC - 3.19\,AD - 7.81\,BC + 2.59\,BD. \quad (1)$$

A 3-dimensional (3D) RSM were developed to help analyze the effects from all independent variables on SSGH yield. Figure 1a summarizes interactive effects from temperature and time on SSGH yield. Based on this response surface plot, can be concluded that increasing hydrolysis time would increase yield,

Table 2. Analysis of variance (ANOVA) of chosen model for significant SSGH yield

Source	Sum of Squares	DF	Mean Square	F Value	Prob>F
Model	3799.031	11	345.3664	2.905631	0.0400**significant**
A	1063.305	1	1063.305	8.94578	0.0113
B	265.8995	1	265.8995	2.237061	0.1606
D	10.55181	1	10.55181	0.088774	0.7708
B2	33.10854	1	33.10854	0.278548	0.6073
C2	168.1603	1	168.1603	1.414764	0.2573
D2	488.4662	1	488.4662	4.109556	0.0654
AB	225.3193	1	225.3193	1.895652	0.1937
AC	19.83655	1	19.83655	0.166889	0.6901
AD	113.0765	1	113.0765	0.951333	0.3486
BC	679.9287	1	679.9287	5.720365	0.0340
BD	74.84509	1	74.84509	0.629685	0.4429
Residual	1426.333	12	118.8611		
Lack of Fit	789.8018	11	71.80016	0.112799	0.9874**not significant**
Pure Error	636.5312	1	636.5312		
Cor Total	5225.364	23			
Pred R-squared	0.2014				
Adj R-squared	0.4768				
Adeq precicion	6.119				

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

Table 3. Variance (ANOVA) and significance of chosen model for DH of SSGH.

Source	Sum of Squares	DF	Mean Square	F Value	Prob>F
Model	2916.074	8	364.5093	2.815391	0.0400 **significant**
B	1344.871	1	1344.871	10.3875	0.0057
D	151.4021	1	151.4021	1.169397	0.2966
B2	567.8058	1	567.8058	4.38561	0.0536
C2	23.46064	1	23.46064	0.181205	0.6764
AC	811.3088	1	811.3088	6.266375	0.0243
AD	490.3974	1	490.3974	3.787724	0.0706
BD	706.1557	1	706.1557	5.454195	0.0338
CD	88.5639	1	88.5639	0.684049	0.4212
Residual	1942.053	15	129.4702		
Lack of Fit	1922.459	14	137.3185	7.008263	0.2887 **not significant**
Pure Error	19.5938	1	19.5938		
Cor Total	4858.127	23			
Pred R-squared	0.6002				
Adj R-squared	0.3870				
Adeq precicion	7.5570				

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

which proved highest at 180 min in agreement with a report for golden apple snail hydrolysate by Hamid *et al*. (2015). Results from increasing the temperature to 60ºC also increased yield in agreement with Roslan *et al.* (2014), who reported optimized yield at 60ºC. Low hydrolysate yields have been reported for higher temperatures due to denatured proteins, and also for lower temperatures that do not allow alcalase to complete the hydrolysis reaction (Roslan *et al.*, 2014). Moreover, temperatures required for fish protein hydrolysis differ as to fish type and substrate (Halim and Sarbon, 2015; Hamid *et al.*, 2015).

Degree of SSGH hydrolysis (DH) analysis for the quadratic model with summary statistics

The 3D RSM approach suggested that the quadratic model would ideally optimize the DH for SSGH in concurrence with studies on protein hydrolysates for Catla (Catla catla) (Bhaskar & Mahendrakar, 2008); eel (*Monopterus* sp.) (Baharuddin *et al.*, 2016); and lizard-fish (Wu *et al.*, 2012).

Analysis of variance (ANOVA) for the DH of SSGH

Table 3 summarizes ANOVA results for the RS Quadratic Model's projections for SSGH DH yields after model reduction. A DH F-value of 2.82 implied significance with only a 4.00% risk that an F-value this large would occur from noise. The 'lack of fit' test value was 7.01, implying no significance due to error. Moreover, a 28.87% probability indicated that a 'lack of fit' F-value this large could result from noise. The determination coefficient (R^2) for the model's fit to experimental data was significant at 0.6002 ($p<0.05$). Nonetheless, an R^2 value >0.80 is preferred for a fit that ideally explains data variation (Halim and Sarbon, 2015). The 'Pred R-Squared' value at -0.2313 did not approximate 'Adj R-Squared'(0.3870),and an 'Adeq precision' value of 7.1557 indicated an adequate

Table 4. Variance (ANOVA) and significance of chosen model for SSGHACE inhibition activity

Source	Sum of Squares	DF	Mean Square	F Value	Prob>F
Model	4264.569	12	355.3808	3.190897	0.0321 **significant**
A	225.6066	1	225.6066	2.025679	0.1824
B	265.4865	1	265.4865	2.383754	0.1509
C	115.8929	1	115.8929	1.04058	0.3296
D	229.845	1	229.845	2.063735	0.1787
B2	109.8169	1	109.8169	0.986025	0.3421
D2	887.3624	1	887.3624	7.96746	0.0166
AB	119.1684	1	119.1684	1.06999	0.3232
AC	312.3505	1	312.3505	2.804536	0.1222
AD	819.8079	1	819.8079	7.360901	0.0202
BC	490.6897	1	490.6897	4.40581	0.0597
BD	107.6364	1	107.6364	0.966447	0.3467
CD	162.5164	1	162.5164	1.459204	0.2524
Residual	1225.11	11	111.37		
Lack of fit	1184.34	10	118.43	2.90	0.4296 **not significant**
Pure error	40.77	1	40.77		
Cor total	5489.68	23			

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

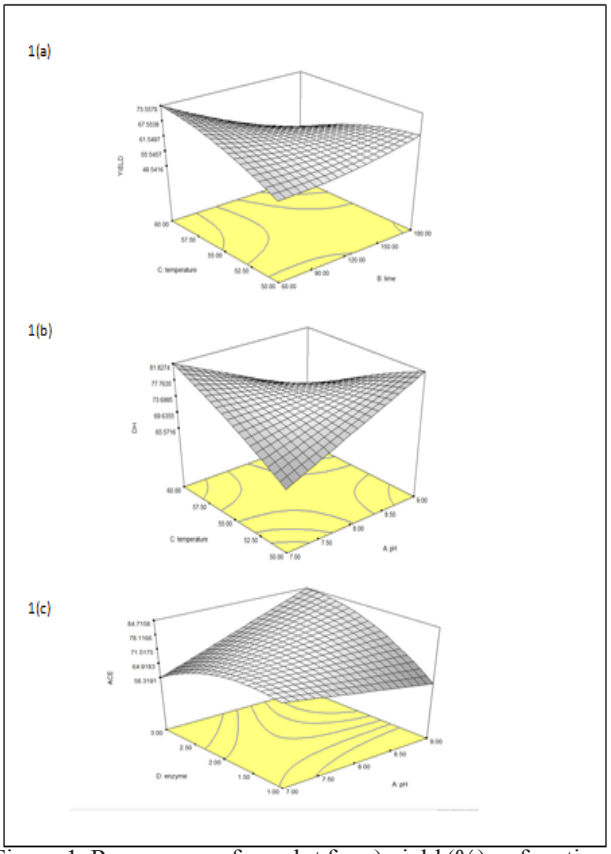

Figure 1. Response surface plot for a) yield (%) as function of temperature and enzyme concentration,b) DH (%) as function of temperature and enzyme concentration,c) ACE inhibitory activity (%) as function of temperature and pH.

signal. Therefore, we deemed the model qualified for design space navigation. ANOVA results also showed that linear terms for B (time) and variable interactions (AC and BD) would have significant effects on the DH for SSGH ($p<0.05$).

Response surface plots for variables and their effects on the DH of SSGH

The model's equation for DH and the response variable 'Y' for SSGH by using regression coefficients for linear and interaction terms to fit the full RSM were derived. Accordingly, and after regression analysis, the model's best explanatory equation for SSGH yield is as follows:

$$Y = +74.67 - 9.30\ B + 2.59\ D + 6.55\ B^2 - 0.97\ C^2 - 8.13\ AC + 6.32\ AD + 7.58\ BD - 2.69\ CD. \quad (2)$$

Figure 1b shows that the DH of SSGH increased as a result of interactive effects between pH and increased temperature. Such an outcome agreed with a report by Aziz *et al*. (2015) on the DH of sunflower oil hydrolysate (DH = 84.1% at 45 ºC). Enzymatic hydrolysis exposes peptide bonds and increases DH with heat treatment (Nielsen, 1995). Moreover, during hydrolysate production, the total number of cleaved peptide bonds in a protein determines the level of DH. Benjakul and Morrisey (1997), for example, reported that optimized hydrolysis conditions for pacific whiting solid waste proteins were a pH of 9.5 and a hydrolysis temperature of 60ºC. The observations also demonstrated that pH had a significant effect on SSGH resulting in increased DH when raising the pH from 7 to 9. Saidi *et al.* (2013) reported similar results.

Analysis of Angiotensin converting enzyme (ACE) inhibition by SSGH: RSM summary statistics

The authors used the quadratic model suggested by RSM to assay ACE inhibition by SSGH. The same model was similarly employed for hydrolysates from rice dregs (He *et al*., 2005) and collagen (Kong *et al.*, 2011).

Analysis of variance (ANOVA) for ACE inhibition by SSGH

Table 4 summarizes ANOVA results after model reduction for the RS Quadratic Model's projections for ACE inhibition by SSGH. A 'Model F-value' of 3.91 and p-value of 0.0321 (<0.05) implied significance. The p-value for 'lack of fit' test (0.4296) (p> 0.05) indicated the model's predictive fitness for the enzymatic hydrolysis of SSGH. The model's coefficient of determination (R^2) was 0.7768 for ACE inhibition by SSGH. Moreover, the 'Pred R-Squared'value of 0.1802 did not approximate the 'Adj R-Squared' value of 0.5334. Furthermore, the 'Adeq precision'value of 7.012 indicated an adequate signal. Hence, the model was deemed appropriate for design space navigation. These ANOVA results inferred significance for the quadratic model's terms, 'D^2' and 'AD interactions', for the inhibition of ACE by SSGH (p<0.05).

RSM plots for variables' effects on ACE inhibition by SSGH

The authors derived the model's equation for the determination of DH and the response variable 'Y'by using the regression coefficientsfor linear and interaction terms to fit a full RSM. After regression analysis, the best explanatory polynomial equation for SSGH yield was:

$$Y = +74.45+3.68\,A - 4.25\,B + 2.34\,C + 3.22\,D + 2.99\,B^2 - 6.15\,D^2 + 3.63\,AB + 5.87\,AC + 9.52\,AD - 7.63\,BC + 3.45\,BD - 4.24\,CD \quad (3)$$

Figure 1c summarizes interactive effects from enzyme concentration and pH for ACE inhibition by SSGH, which increased as enzyme concentration increased until reaching an optimum level at an enzyme concentration of 2%, after which ACE inhibition activity tended to decrease with E/S levels approaching 3%.This result disagreed with a study by Abedin *et al.* (2015) on sea cucumber *(Stichopus vastus)* collagen hydrolysate, which found that increased enzyme content increased ACE inhibition. The latter results were likely due to a greater degree of protein hydrolysis as proteases were added. Moreover, ACE inhibition activity also increased slightly as pH was increased; similar to a study by Guo *et al.* (2009) that reported higher pH levels allowed for increased ACE inhibitory activity. Aziz *et al*. (2015) suggested that the pH level modified the enzyme's ionization state resulting in altered enzyme activity and selectivity.

Optimal RSM response conditions

The obtained desirability value (~1.0) indicated that the model's suggested conditions were suitable for optimizing SSGH outcomes for yield, DH and ACE inhibition. These projected conditions were a temperature of 60°C; a hydrolysis time of 113.79 min; an enzyme concentration of 2.92%; and a pH of 9. Predicted responses for yield, DH and ACE inhibitory activity were 54.38%, 72.04% and 89.19%, respectively.

Validation

Validation trials were undertaken in triplicate for each response. SSGH yield averaged 51.01%, lower than predicted (54.38%); DH averaged 90.48%, higher than predicted (72.04%); and ACE inhibitory activity averaged 79.61%, lower than predicted (89.19%). These results demonstrated no significant difference in yield, but significant differences for DH and ACE inhibition.

Conclusion

SSGH yield, DH and ACE inhibitory activity were significantly affected by alcalase hydrolysis conditions in this study. These variables included temperature, hydrolysis time, pH level and enzyme concentration. The RSM approach undertaken suggested the quadratic model to optimize all outcomes. Predicted values for yield and ACE inhibition were higher while DH was lower on validation outcomes tests. These confirmed RSM results suggest that SSGH can be used as a natural ACE inhibitor.

Acknowledgments

With sincere appreciation, the authors gratefully acknowledge the Ministry of Higher Education for its financial support through the Fundamental Research Grant Scheme (FRGS/1/2014/59337); as well as the Universiti Malaysia Terengganu for the use of its laboratory facilities and technical support.

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