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Identification of angiotensin I converting enzyme inhibitory and radical scavenging bioactive peptides from sea cucumber *(Stichopus vastus)* collagen hydrolysates through optimization

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Abstract

The sea cucumber *(Stichopus vastus)* is an underutilized species, as most of its parts, including the integument (high collagen content) are thrown away during processing. The aim of this study was to investigate the effects of different hydrolysis conditions (substrate to enzyme ratio (S/E), reaction temperature, and hydrolysis time) on the angiotensin I converting enzyme (ACE) inhibitory and radical scavenging (RSc) activities of the hydrolysates produced from trypsin hydrolysis of *S. vastus* collagen. Optimal conditions predicted by Box-Behnken Design modelling for producing ACE inhibitory and RSc hydrolysates were found to be S/E ratio (15), reaction temperature (55°C), and hydrolysis time (1 h). Under optimal conditions, ACE inhibitory and RSc activities were estimated to be as high as 67.8% and 77.9%, respectively. Besides, some novel bioactive peptides were identified through mass spectrometry analysis. These results indicate that *S. vastus* hydrolysates might be used as a functional ingredient in food and nutraceutical products.

Introduction

A multifunctional zinc-containing enzyme, angiotensin I converting enzyme (ACE; EC 3.4.15.1), converts inactive angiotensin I to the effective vasopressor angiotensin II, which regulates blood pressure through the rennin angiotensin system (Fujita *et al*., 2000). Thus, inhibiting ACE has becomes a popular therapeutic approach for the management of high blood pressure. Kamath *et al.* (2007) reported that some artificial ACE inhibitors (e.g., captopril, enalapril, and listinopril) are extremely useful as antihypertensive drugs, but they have unpleasant side effects such as allergic reactions, coughing, skin rashes, and taste disturbances. ACE inhibitory peptides that originate from food proteins are considered to be a safe substitute for mercantile ACE inhibitory drugs (Ariyoshi, 1993). More than 200 ACE inhibitory peptides have been identified to date, and they are mostly produced through digestion of diverse plant and animal proteins by different proteases (He *et al.*, 2004). These protein sources include Alaska pollack, tuna, algae, oysters, casein, mushrooms, peas, whey protein, soybeans, milk and soymilk, and sea cucumbers (Wu and Ding, 2002; Lee *et al*., 2004; Vermeirssen, 2004; Silva and Malcata, 2005; Apostolidis *et al.*, 2007; Wang *et al.*, 2008; Sheih *et al*., 2009; Lee *et al*., 2010; Liu *et al.*, 2011).

In recent years, researchers have reported that enzymatic hydrolysates (peptides) derived from food proteins such as patin, jellyfish, yellow stripe trevally, wheat protein, yellow fin sole, mackerel, herring, sea cucumber, unicorn leatherjacket, and milk casein possess antioxidant activities (Suetsuna *et al*., 2000; Sathivel *et al*., 2003; Wu *et al.*, 2003; Jun *et al*., 2004; Zhu *et al.*, 2006; Klompong *et al*., 2007; Zhuang *et al*., 2009; Najafian *et al*., 2013; Abedin *et al*., 2014; Sai-ut *et al*., 2014). The hydrolysis conditions for generating such antioxidant peptides

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from the original raw materials should be optimized. Hydrophobic amino acids are dominant in collagen, and this property results in high affinity for oil and good emulsifying ability (Rajapakse *et al*., 2005; Lin and Li, 2006). Consequently, collagen is likely to supply natural ACE inhibitory and antioxidant peptides with superior effects as compared to other proteins.

Processing conditions greatly affect the physicochemical properties of functional peptides. Thus, it is crucial to identify the best possible conditions for producing ACE inhibitory and radical scavenging (RSc) peptides through enzymatic hydrolysis of protein. Box-Behnken Design (BBD) is a type of response surface technique that can be used to optimize the biotechnological processes. Aslan (2008) reported that response surface methodology (RSM) is an inexpensive method that requires the minimum number of experiments and a short period of time for utmost information.

The sea cucumber *(S. vastus)*, which is rich in collagen, has never been explored as a latent source of bioactive peptides under optimized conditions. In this study, RSM was used to investigate the optimum hydrolysis parameters (S/E ratio, temperature, and time) for producing ACE inhibitory and RSc peptides through trypsin hydrolysis of collagen extracted from the integument of *S. vastus*. Peptide sequencing of the collagen hydrolysates produced under optimized hydrolysis conditions was also conducted using mass spectrometry analysis.

Materials and Methods

Materials and chemicals

Sea cucumbers *(S. vastus)* (Voucher specimen number: PPSK/USM/6139058-01-2009-SVT) were supplied by the Fishery Department of Malaysia, Terengganu, Malaysia. Trypsin powder (Porcine 1:250), ACE from rabbit lung, and hippuryl-L-histidyl-L-leucine were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). All other reagents used were of analytical grade.

Extraction of collagen

Collagen from the integument of *S. vastus* was extracted according to the method of Liu *et al.* (2010) with a slight modification as described in our preceding publication (Abedin *et al*., 2013).

Experimental design and optimization of enzymatic hydrolysis conditions by RSM

RSM (Box *et al.*, 1978) was used to identify the best possible conditions of three variables (S/E ratio, reaction temperature, and hydrolysis time) for optimal ACE inhibitory and RSc activities of the hydrolysates produced through trypsin hydrolysis of *S. vastus* collagen. BBD was drawn and a set (17 experiments) was plotted out (Table 1). Different values of the following three independent variables were tested: S/E ratio (15, 20, and 25, w/w), reaction temperature (45, 50, and 55°C), and hydrolysis time (1, 3, and 5 h). The following equation obtained from regression coefficient was used to fit the experimental results:

$$Y = \alpha_0 + \alpha_1 A + \alpha_2 B + \alpha_3 C + \alpha_{11} A^2 + \alpha_{22} B^2 + \alpha_{33} C^2 + \alpha_{12} AB + \alpha_{13} AC + \alpha_{23} BC \quad (1)$$

The enzymatic reaction was carried out under the hydrolysis conditions designated by BBD to produce collagen hydrolysates through trypsin hydrolysis of *S. vastus* integument collagen. The pH (8.0) of the reaction mixture was kept constant using a pH-stat. The reaction mixture was heated at 100°C (5 min) to stop enzyme digestion and then cooled down with running tap water. Following centrifugation at 5000 × g at 4°C for 30 min, the supernatants were collected and freeze dried (FreeZone12, Labconco Corporation, Kansas City, MO, USA). The dried collagen hydrolysates powder was preserved in an airtight container at 4°C until analysis.

ACE inhibitory activity assay

ACE inhibitory activity of the collagen hydrolysates was measured using the modified method described by Mugumura *et al*. (2009). The release of hippuric acid from hippuryl-L-histidyl-L-leucine is the key to this assay, as the reaction is catalyzed by ACE. An equal volume (50 µL each) of hydrolysate solution (1 mg/mL) and ACE solution (50 mU/mL) was mixed and left to incubate at 37°C for 10 min. Next, 150 µL of Hip–His–Leu (4.15 mM in 100 mM borate buffer maintaining 0.3 M NaCl, pH 8.3) as substrate was added to the reaction mixture and incubated at 37°C for 30 min. A solution of 1 M HCl (500 µL) was added to the reaction mixture to stop the reaction, except for the blank, to which 1 M HCl (50 µL) was added before incubation. Ethyl acetate (1.5 mL) was added to the mixture, which then was mixed vigorously with a vortex (1 min) for the extraction of hippuric acid liberated through ACE. After sitting for 5 min, the separated ethyl acetate layer containing hippuric acid (800 µL) was transferred to an Eppendorf tube followed by vacuum drying using a concentrator (Concentrator 5301, Eppendorf AG, Hamburg, Germany) for 30 min. Distilled water (1 mL) was added to the dried concentrated sample in the Eppendorf tube and mixed

thoroughly with a vortex. Deionized distilled water was used as the blank. The sample was placed in a quartz cuvette and absorbance was measured using a spectrophotometer (Molecular Devices SpectraMax M5, Sunnyvale, CA, USA) at 228 nm. The following equation was used to calculate the percentage of ACE inhibition:

$$\% \text{ ACE inhibition} = 100 \times \frac{Abs_{ctrl} - Abs_{smpl}}{Abs_{ctrl} - Abs_{blnk}} \quad (2)$$

where Abs_{ctrl} = absorbance of control (buffer), Abs_{smpl} = absorbance of sample, and Abs_{blnk} = absorbance of blank (HCl was added before ACE).

RSc activity assay

RSc activity of the collagen hydrolysates was assessed using 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) following the modified version of the method of Re *et al.* (1999). Freshly prepared ABTS solution (3.9 mL containing potassium persulphate, 80% ethanol, and distilled water) and 0.1 mL collagen hydrolysate (1 mg/mL) were transferred into test tubes and incubated at 23°C for 6 min in the dark. Methanol and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), an antioxidant-like vitamin E, were used as the control and the reference, respectively. The SpectraMax M5 spectrophotometer was used to measure the absorbance of the control and samples at 734 nm. The following equation was used to estimate the percentage of RSc activity:

$$\% \text{ RSc} = 100 \times \frac{Abs_{ctrl} - Abs_{smpl}}{Abs_{ctrl}} \quad (3)$$

where %RSc = percentage radical scavenging of collagen hydrolysates, Abs_{ctrl}= absorbance of methanol with ABTS, and Abs_{smpl}= absorbance of sample.

Identification of bioactive peptides using MS

For this analysis, 500 µL collagen hydrolysate samples (1 mg/mL) obtained under the optimized hydrolysis conditions were filtered through Whatman number 1 filter paper. Each sample was applied as an injection volume of 10 µL. Liquid chromatography-mass spectrometry (LC-MS) and mass spectrometry/mass spectrometry (MS/MS) analyses were performed using a Thermo LTQ/Orbitrap Velos (Thermo Scientific, San Jose, CA, USA) equipped with an Easy-nLC II system according to the method of Siow and Gan (2013). Chromatographic separation of hydrolysates was investigated using an Easy-Column C18-A2 (100 × 0.75 mm) in conjunction with a pre-column Easy-Column (20 × 0.1 mm) at a flow rate of 0.3 µL/min. The pre-column and analytical columns were equilibrated for 15 µL and 4 µL at flow rates of 3 µL/min and 0.3 µL/min, respectively. Formic acid (0.1%) in deionized distilled water (A) and formic acid (0.1%) in acetonitrile (B) were used as running buffers. The pump gradient elution of nano-LC occurred as follows: 0–70 min, 5–45% B; 70–85 min, 45–100% B, and 85–100 min, 100% B.

The source voltage of 2.3 kV and capillary temperature of 200˚C were kept constant to spray the eluant into the MS. Peptides were detected through full scan mass analysis from a range of m/z ratios of 200 to 2,000 at resolution power of 60,000 (at m/z 400, FWHM; 1-s acquisition) with data-dependent MS/MS analyses (ITMS) generated by the eight major abundant ions from the parent mass list of expected peptides with refusal of singly or unassigned charge state. The ITMS analysis was carried out with resolution power of 60,000, and collision induced dissociation (CID) was performed with a separation width of 2 Da, normalized collision energy of 35, activation q of 0.25, activation time of 50 ms, and charge state of 2 or higher. Data acquisition was executed using Xcalibur version 2.1 (Thermo Scientific, San Jose, CA, USA) with a mass tolerance threshold of 5 ppm. Data analysis was conducted using PEAKS studio version 6.0.

Statistical analysis

All experiments were conducted in triplicate. All data were subjected to analysis of variance (ANOVA) using Design Expert version 6.0.

Results and Discussion

Model development and outcome of optimization

Design Expert 6.0 statistical program was used to demonstrate the effect of various hydrolysis factors (S/E ratio, reaction temperature, and hydrolysis time) on ACE inhibitory and RSc activity of the hydrolysates produced from *S. vastus* collagen using trypsin. Table 1 shows the experimental design and their corresponding results. ANOVA revealed that the Prob > *F* value for the ACE inhibitory activity was 0.0356 (Table 2), which illustrates that the model itself was significant (96% confidence level). The extent of fitness of the quadratic model was tested by calculating the coefficient (R^2) of determination. The R^2 value of ACE inhibitory activity was 84.4%, which suggests that the statistical model might represent the genuine relationships among the parameters selected. For RSc activity, the Prob > *F* value was 0.0081, which indicates that the model itself was significant (99% confidence level). The R^2 value of RSc activity

Table 1. Box-Behnken experimental design, and predicted and response values of the two dependent variables under different hydrolysis conditions

Run	S/E ratio	Temperature	Time	%ACE inhibition		%RSc activity	
				Predicted values	Experimental values	Predicted values	Experimental values
1	15	50	1	64.51	64.05	77.81	78.25
2	20	45	1	62.92	63.87	72.59	73.79
3	20	45	5	67.88	68.66	70.51	71.75
4	20	50	3	64.01	65.34	73.26	71.38
5	15	50	5	67.84	67.55	77.25	77.65
6	25	50	1	63.54	63.83	76.20	75.80
7	15	55	3	71.29	72.53	83.20	84.00
8	25	45	3	69.56	68.32	75.15	74.35
9	20	55	5	65.78	64.83	81.68	80.48
10	20	55	1	65.30	64.52	77.52	76.28
11	25	55	3	65.87	66.36	84.18	85.82
12	20	50	3	64.01	64.34	73.26	74.57
13	20	50	3	64.01	62.46	73.26	74.57
14	20	50	3	64.01	65.46	73.26	71.20
15	25	50	5	65.63	66.09	78.84	78.40
16	15	45	3	67.32	66.83	76.14	74.50
17	20	50	3	64.01	62.46	73.26	74.57

Table 2. Analysis of variance of response surface quadratic model for ACE inhibitory activity (Y_1) and radical scavenging activity (Y_2)

	Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	R^2	Adjusted R^2
Y_1	Model	86.34	9	9.59	4.21	0.0356		
	Linear	19.84	3	6.61	1.04	0.4065		
	2FI	20.07	3	6.69	1.07	0.4041		
	Quadratic	46.43	3	15.48	6.79	0.0176	0.844	0.644
	Cubic	7.16	3	2.39	1.09	0.4502		
	Lack of Fit	7.16	3	2.39	1.09	0.4502		
Y_2	Model	244.56	9	27.17	7.23	0.0081		
	Linear	131.63	3	43.87	4.09	0.0299		
	2FI	13.24	3	4.41	0.35	0.7900		
	Quadratic	99.69	3	33.23	8.84	0.0089	0.903	0.778
	Cubic	13.34	3	4.45	1.37	0.3719		
	Lack of Fit	13.34	3	4.45	1.37	0.3719		

was 90.3%, indicating that the statistical model might correspond to the real relationships among the parameters selected.

Among the three independent variables tested, hydrolysis time had the greatest effect on ACE inhibitory activity ($P < 0.05$) (Table 3). There were no significant effects of S/E ratio and reaction temperature ($P > 0.05$). However, the following terms were significant: the quadratic terms of (S/E ratio)^2 and T^2 and the interaction terms of S/E ratio × T ($P < 0.05$). In contrast, the effects of (hydrolysis time)^2, S/E ratio × hydrolysis time, and reaction temperature × hydrolysis time were not significant ($P > 0.05$).

Reaction temperature had the greatest effect on RSc activity ($P < 0.01$) among the three independent variables tested (Table 3). There were no significant effects of S/E ratio and hydrolysis time ($P > 0.05$). The quadratic terms of (S/E ratio)^2 and (reaction temperature)^2 were also significant ($P < 0.05$), but the effects of (hydrolysis time)2, S/E ratio × temperature, S/E ratio × hydrolysis time and reaction temperature × hydrolysis time were not significant ($P > 0.05$).

The most descriptive model equation was determined based on the analysis of regression as follows:

$$Y_1 = 231.98 + 0.24A - 7.29B + 8.15C + 0.09A^2 + 0.09B^2 - 0.21C^2 - 0.08AB - 0.03AC - 0.11BC \quad (4)$$

$$Y_2 = 370.17 - 7.91A - 8.98B - 9.26C + 0.17A^2 + 0.09B^2 + 0.02C^2 + 0.02AB + 0.08AC + 0.15BC \quad (5)$$

Figures 1 and 2 show the response surface plots of collagen hydrolysates for ACE inhibitory activity and RSc activity, respectively. Liu *et al*. (2011) used such plots to show the responses towards two independent variables to identify the optimal activity

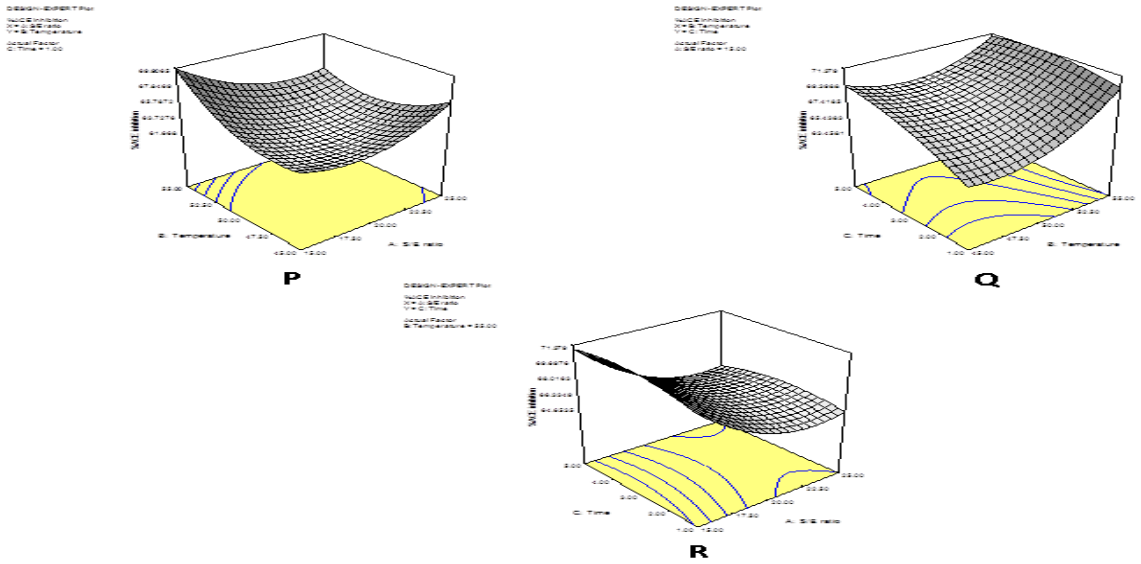

Figure 1. Response surface plots of sea cucumber *(Stichopus vastus)* collagen hydrolysates for ACE inhibitory activity. P: Consequence of different temperature and S/E ratio on ACE inhibitory activity at constant time (1 H); Q: Consequence of different hydrolysis time and temperature on ACE inhibitory activity at constant S/E ratio (15); R: Consequence of different hydrolysis time and S/E ratio on ACE inhibitory activity at constant temperature (55°C)

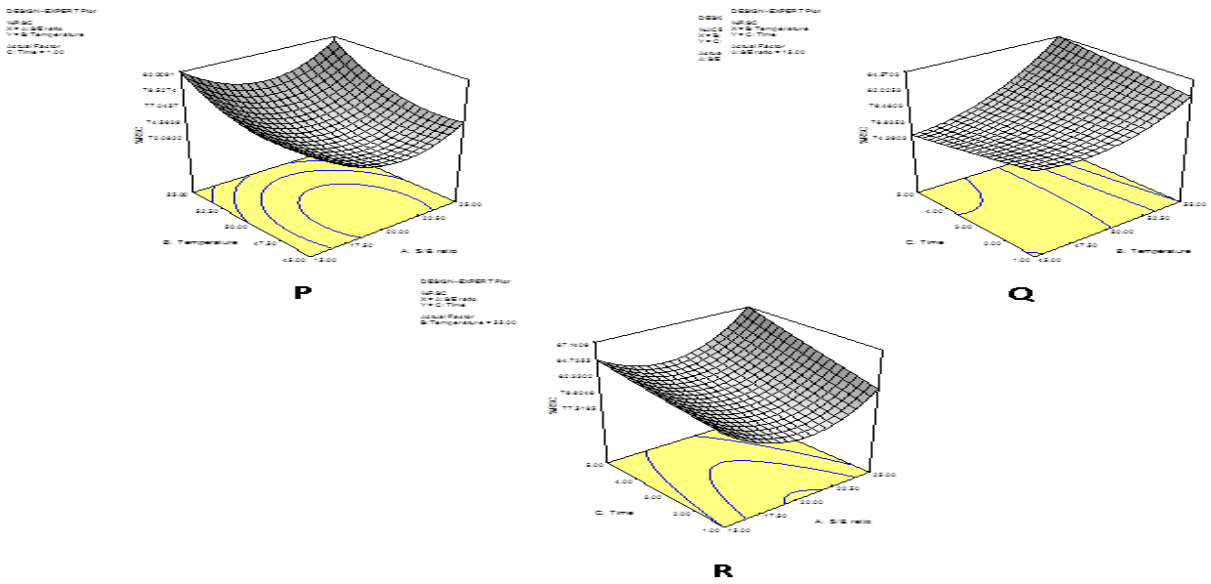

Figure 2. Response surface plots of sea cucumber *(Stichopus vastus)* collagen hydrolysates for radical scavenging (RSC) activity. P: Consequence of different temperature and S/E ratio on RSC activity at constant time (1 H); Q: Consequence of different hydrolysis time and temperature on RSC activity at constant S/E ratio (15); R: Consequence of different hydrolysis time and S/E ratio on RSC activity at constant temperature (55°C)

point. Every response surface plot had a specific point (the optimal point) at which the optimum value was within the experimental range. Numerical optimization using Design-Expert 6.0 software was used to analyze different independent variables to determine the optimum reaction conditions. The results indicated that the optimal conditions were S/E ratio of 15, reaction temperature 55°C, and hydrolysis time 1 h. The optimal ACE inhibitory and RSc activities were predicted to be 69.9% and 82%, respectively. To confirm the precision of the model, an experiment was conducted under the optimized hydrolysis conditions, and the ACE inhibitory and RSc activities were found to be 67.8% and 77.9%, respectively. Thus, the predicted ACE inhibitory and RSc activities exhibited error rates of 3–5%.

The ACE inhibitory and RSc activities measured in this study were higher than those of other marine species, possibly due to variations in chemical composition among species. Liu *et al.* (2011) reported that the maximal ACE inhibitory activity for collagen hydrolysates from the sea cucumber *Parastichopus californicus* was predicted to be 59.8% under optimized conditions. The highest

Table 3. Analysis of variance for the responses of ACE inhibitory and radical scavenging activities of the hydrolysates

Source	ACE-inhibitory activity		Radical scavenging activity	
	F Value	P Value	F Value	P Value
S/E ratio	2.219	0.1799	4.58E-05	0.9948
T	0.017	0.8993	34.446	0.0006**
Time	6.469	0.0385*	0.575	0.4731
$(S/E\ ratio)^2$	8.976	0.0201*	19.562	0.0031**
$(T)^2$	9.724	0.0169*	5.571	0.0503*
$(Time)^2$	1.291	0.2932	0.008	0.9298
S/E ratio×T	6.437	0.0388*	0.257	0.6275
S/E ratio×Time	0.169	0.6936	0.678	0.4375
T×Time	2.202	0.1814	2.587	0.1518

*Significant at 5% level; **Significant at 1% level

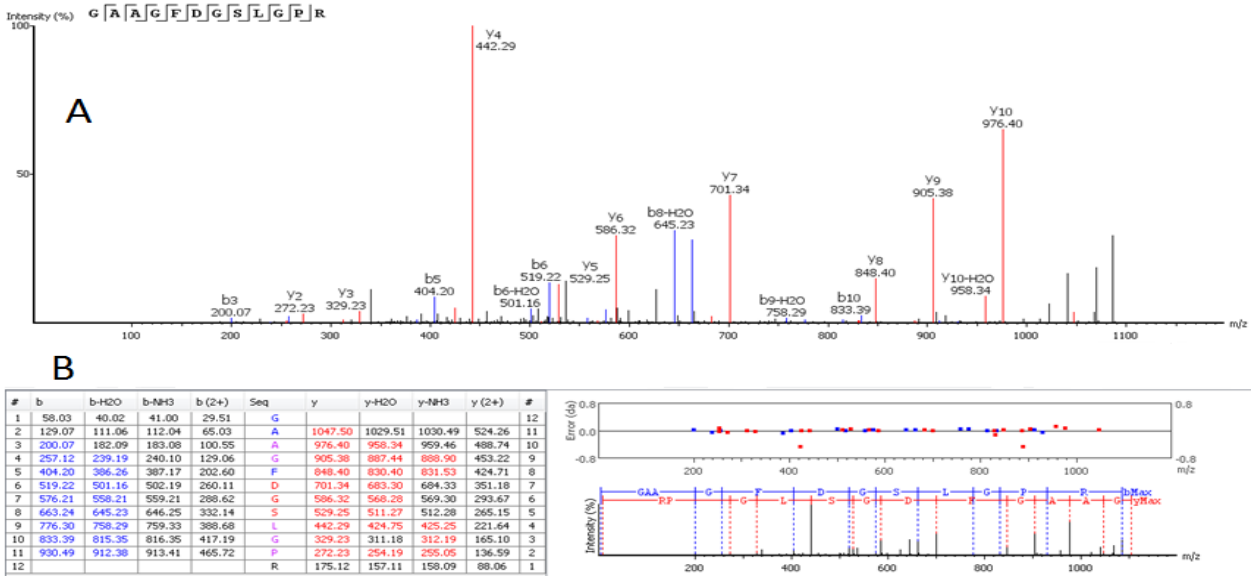

Figure 3. MS/MS spectra for one of the peptide of sea cucumber *(Stichopus vastus)* collagen hydrolysates. Panel (A) and panel (B) show the MS/MS spectra, and the ion tables, standard errors and MS/MS fragments for the peptide sequence, respectively

value of hydroxyl RSc activity for hydrolysates of the jellyfish *Rhopilema esculentum* was estimated to be 72.15% under optimized conditions (Zhuang *et al.*, 2009).

Identification of bioactive peptides

Peptides from the collagen hydrolysates that exhibited the highest biological activities were identified using a typical MS approach. Figure 3A shows the MS/MS spectra for peptides from the hydrolysates with a high resolution of 60,000. This resolution is crucial for identification and confirmation of the peptide sequences based on the molecular formula and subsequently the mass accuracy of the molecular ions. Mass spectral peaks with double or higher charges were subsequently selected for fragmentation. Figure 3B shows the MS/MS fragments along with the m/z tables. The GAAGFDGSLGPR sequence was found within the fragment spectra. It had high signal-to-noise ratio with complete or near complete backbone fragmentation and a mass accuracy of < 0.8 Da. The other MS/MS spectra were similarly of excellent quality and thus were used to identify peptide sequences.

Table 4 lists the 33 peptide sequences identified from the collagen hydrolysates that exhibit antioxidant and antihypertensive properties. These sequences consisted of 7 to 19 amino acid residues per peptide. Korhonen and Pihlanto (2006) previously reported that bioactive peptides are generally small peptides containing 2 to 20 amino acid residues per peptide, which supports our findings.

The majority of the peptide sequences from the hydrolysates had a high content of hydrophobic amino

Table 4. Identified peptide sequences from the sea cucumber *(Stichopus vastus)* collagen hydrolysates obtained under the optimized hydrolysis conditions

Peptide sequence	Molecular mass (Da)
Ala-Glu-Asp-Ser-Leu-Leu-Val-Pro-Arg-Lys	1126.6350
Ala-Thr-Thr-Gly-Ala-Ala-Gly-Arg	703.3613
Asp-Ala-Ala-Asn-Thr-Pro-Gly-Thr-Gly-Pro-Arg	1055.5000
Asp-Leu-Leu-Met-Pro-Gly-Thr-Gly-Pro-Arg	1055.5440
Glu-Leu-Asp-Ala-Gly-Ala-Ala-Gly-Pro-Lys	927.4661
Glu-Leu-Leu-Gly-Glu-Gly-Met-Met-Met-Glu-Gly-Pro-Leu-Lys	1533.7240
Phe-Gly-Thr-Leu-Gly-Leu-Gly-Glu-Leu-Gly-Leu-Leu-Arg	1344.7760
Gly-Ala-Ala-Gly-Phe-Asp-Gly-Ser-Leu-Gly-Pro-Arg	1103.5360
Gly-Pro-Ala-Gly-Gly-Lys-Val-Val-Arg-Arg	995.5988
Gly-Pro-Ala-Gly-Gly-Ser-Leu-Asp-Ala-Ala-Gly-Lys	999.4985
Gly-Leu-Ser-Ser-Ala-Ala-Gly-Arg	703.3613
His-Tyr-Gly-Val-Gly-Ala-Thr-Gly-Ala-Ala-Pro-Ala-Ala-Gly-Thr-Gln-Glu-Tyr-Lys	1847.8800
Lys-Ala-Ala-Gly-Glu-Pro-Ala-Asp-Leu-Gly-Glu-Arg	1212.6100
Lys-Gly-Thr-Ala-Ala-Gly-Ala-Ala-Gly-Met-Ser-Gly-Ala-Ala-Gly-Val-His-Arg	1568.7840
Lys-Asn-Asp-Ala-Gly-Leu-Tyr-Ala-Ala-Gly-Leu-Tyr-Pro-Lys	927.4774
Leu-Asp-Glu-Val-Gln-Thr-Gly-Ala-Ala-Trp-Thr-Gly-Ala-Pro-Gly-Leu-Leu-Lys	1825.9570
Leu-Thr-Gly-Asn-Gln-Gly-Pro-Ala-Ala-Leu-Thr-Asn-Leu-Asp-Arg	1539.8010
Met-Ala-Gly-Val-Val-Thr-Lys-Arg	860.4902
Met-Gly-Gly-Thr-Gly-Asp-Pro-Ser-Thr-Leu-Leu-His-Arg	1340.6510
Asn-Asp-Gly-Ala-Ala-Gly-Ala-Ala-Gly-Phe-Ala-Gly-Ala-Ala-Gly-His-Val-Arg	1568.7440
Asn-Asp-Gln-Val-Gly-Ala-Thr-Gly-Ala-Gln-Glu-Thr-Gly-Pro-Ala-Gly-Leu-Leu-Lys	1825.9170
Gln-Ala-Ala-Gly-Pro-Lys-Ala-Asp-Gly-Glu-Arg	1211.6260
Gln-Leu-Gly-Leu-Leu-Gly-Ala-Leu-Gly-Leu-Ala-Gly-Pro-Arg	1334.8040
Gln-Thr-Gly-Ala-Val-Gly-Ala-Thr-Gly-Ala-Ala-Asp-Val-Lys	1244.6360
Arg-Ser-Ser-Ala-Ala-Gly-Arg	703.3725
Ser-Glu-Met-Leu-Leu-Leu-Thr-Leu-Ala-Ala-Pro-Glu-Gly-Thr-His-Lys	1709.9020
Ser-Gly-Gly-Ala-Asp-Gly-Asn-Val-Ala-Ala-Arg	973.4577
Ser-Ser-Trp-Ala-Ala-Thr-Val-Asn-Pro-Gly-Lys	1116.5560
Thr-Gly-Ala-Leu-Gly-Gly-Ala-Ala-Asp-Gly-Arg	944.4675
Thr-His-Leu-Gly-Thr-Leu-Leu-Ala-Arg	980.5767
Val-Ala-Ala-Pro-Gly-Thr-Lys	642.3701
Val-Gly-Ala-Ala-Ser-Gly-Pro-Gly-Pro-Ser-Arg	954.4882
Val-Thr-Asp-Ser-Leu-Asn-Pro-Leu-Leu-Arg	1126.6350

acids (e.g., Gly, Ala, Val, Leu, and Pro). Their presence likely improves the solubility of the peptides in lipid, which could create a bridge between the peptides and radical species to enhance their interaction and thus help to increase lipid inhibitory activity. Cheung *et al.* (1980) reported that hydrophobic amino acids could confer better antihypertensive potential to the hydrolysates. The peptide sequences in our study also had a high content of repeating amino acids, such as Ala-Ala, Val-Val, and Leu-Leu. Kawashima *et al.* (1979) reported that some di- and tri-peptides have exhibited superior biological activity as compared to single constituent amino acids in the peptide sequences. Silk *et al*. (1980) also found that amino acid residues in di- and tri-peptides could be absorbed more quickly than free amino acid. It is therefore feasible that the antioxidant and antihypertensive activities of the protein hydrolysates might be related to the abundance of such repeating peptides.

The location of the N- or C-terminal amino acid residue of the peptide sequences is also a crucial factor in determining their biological activity. Cheung *et al*. (1980) reported that the presence of Pro, Phe, and Tyr at the C-terminal peptide sequence is mostly preferred for ACE inhibition. In addition, Val and Ile were found to be the most effective for increasing the binding capacity between peptides and ACE. Therefore, peptides with such structural features likely contributed to the bioactivity of the *S. vastus* collagen hydrolysates.

Conclusions

ACE inhibitory and RSc peptides can be prepared successfully from the sea cucumber *S. vastus* integument collagen through trypsin hydrolysis. BBD and RSM predicted optimal conditions (S/E ratio of 15, 55°C, and 1 h) for producing collagen hydrolysates with good ACE inhibitory and RSc activities. Some bioactive peptides were identified through MS analysis. These findings indicate that the hydrolysates of *S. vastus* integument collagen can be used as a bioactive ingredient in food and nutraceautical products.

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