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Antioxidant and angiotensin I-converting enzyme inhibitory activities of northern shrimp (*Pandalus borealis*) by-products hydrolysate by enzymatic hydrolysis

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Abstract

In the present study, we investigated to the antioxidant and angiotensin I-converting enzyme (ACE) inhibitory activities of the northern shrimp (*Pandalus borealis*) by-products (PBB) hydrolysates prepared by enzymatic hydrolysis. The antioxidant and ACE inhibitory activities of five enzymatic hydrolysates (alcalase, protamex, flavourzyme, papain, and trypsin) of PBB were evaluated by the 2, 2′-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] ($ABTS^+$) radical scavenging and superoxide dismutase (SOD)-like activities, reducing power and Li's method for ACE inhibitory activity. Of these PBB hydrolysates, the protamex hydrolysate exhibited the most potent ACE inhibitory activity with IC_{50} value of 0.08 ± 0.00 mg/mL. The PBB protamex hydrolysate was fractionated by two ultrafiltration membranes with 3 and 10 kDa (below 3 kDa, between 3 and 10 kDa, and above 10 kDa). These three fractions were evaluated for the total amino acids composition, antioxidant, and ACE inhibitory activities. Among these fractions, the < 3 kDa and 3–10 kDa fractions showed more potent $ABTS^+$ radical scavenging activity than that of > 10 kDa fraction, while the > 10 kDa fraction exhibited the significant reducing power than others. In addition, 3–10 kDa and > 10 kDa fractions showed the significant ACE inhibitory activity. These results suggested that the high molecular weight enzymatic hydrolysate derived from PBB could be used for control oxidative stress and prevent hypertension.

Keywords: Northern shrimp, Enzymatic hydrolysate, Antioxidant, Angiotensin I-converting enzyme

Abbreviations: ACE, Angiotensin I converting enzyme; PBB, Pandalus borealis by-products; $ABTS^+$, 2,2'-Azino-bis[3-ethylbenzothiazoline-6-sulfonic acid]; SOD, Superoxide dismutase; RAS, Renin angiotensin system; OPA, *o*-phthaldialdehyde; DMSO, Dimethyl sulfoxide; EDTA, Ethylenediaminetetraacetic acid; $K_3Fe(CN)_6$, Potassium ferricyanide; TCA, Trichloroacetic acid; HHL, Hippuryl-L-histidyl-L-leucine; BSC, Benzene sulfonyl chloride; $FeCl_3$, Iron (III) chloride; MWCO, Molecular weight cut-offs; ROS, Reactive oxygen species

Background

Hypertension is one of the primary causes of cardiovascular disease which leads to stroke, coronary artery disease, and sudden cardiac death (Bhuyan and Mugesh 2011). The renin-angiotensin system (RAS) plays a key role in regulating blood volume and hypertension (Hall 1991; Hall et al. 1989). Angiotensin I-converting enzyme (ACE) is important in the functioning of the RAS. Renin converts angiotensinogen to angiotensin I, and ACE catalyzes cleavage of angiotensin I into angiotensin II which is the main active component of hypertension (Paul et al. 2006; Takahashi et al. 2011). The increased angiotensin II causes vasoconstriction along with increased blood volume and water retention (Parish and Miller 1992). Several ACE inhibitors have been tested and developed in order to inhibit angiotensin II-mediated hypertension. Most synthetic ACE inhibitors such as captopril, enalapril, lisinopril, ramipril, and benzapril on the market have been reported with their side effects including skin rash, loss of taste, and dry cough (Dr and Lisa 2012). Moreover, a recent research has found that oxidative stress is a principal factor for hypertension (Bagatini et al. 2011). Excess reactive oxygen species affects cellular functions and reduces the

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bioavailability of endothelial nitric oxide and enhances low-density lipoprotein oxidation in the vascular system (Ray et al. 2012; Toeroek 2008; Mattson 2009). Therefore, there is a necessity for the development of new ACE inhibitors with potent oxidative stress inhibition and fewer side effects from natural resources.

Northern shrimp (*Pandalus borealis*), one of the most popular shrimp species in Korea, belongs to family Pandalidae and distributes widely in the deep sea at depths of 20–1330 m with a temperature of 2–14 °C found in the water around the eastern coast of Korea (Bauer 2004). This is rich in nutrients such as proteins, minerals, and vitamins. However, the inedible parts of shrimp by-products including head, shell, and tail portions account for approximately 50 % of the catch and constitute valuable and useful bioactive materials, such as carotenoprotein, pigments, chitin, and chitosan (Chakrabarti 2002; Babu et al. 2008; Younes et al. 2015). Recently, much research has been carried out on the utilization of protein-rich fisheries by-products as nutraceuticals and nutritional supplements with high nutrient (Chae et al. 1998; Guerard et al. 2001; Arvanitoyannis and Kassaveti 2008).

The aim of the present study was to determine antioxidant and ACE inhibitory activities of enzymatic hydrolysates and its molecular weight cut-off fractions of the *P. borealis* by-products for prevention of hypertension.

Methods

Materials

P. borealis was purchased from the market of Yang yang-gun, Gangwon-do, Korea, in May, 2012. Alcalase® 2.4 L, papain, trypsin, serine, *o*-phthaldialdehyde (OPA), pyrogallol, 2, 2′-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] ($ABTS^+$), dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), potassium ferricyanide ($K_3Fe(CN)_6$), trichloroacetic acid (TCA), hippuryl-L-histidyl-L-leucine (HHL), angiotensin I-converting enzyme (ACE), sodium borate, sodium chloride, hydrochloric acid, pyridine, benzene sulfonyl chloride (BSC), captopril, and L-ascorbic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and iron(III) chloride ($FeCl_3$) was obtained from Junsei Chemical Co. (Tokyo, Japan). Protamex and flavourzyme were purchased from Novo Co. (Novozyme Laboratories, Copenhagen, Denmark).

Enzymatic hydrolysis and fractionation of shrimp by-product

The by-products of *P. borealis* (PBB) were lyophilized and stored at −20 °C until use. The crude protein content of PBB was 44.50 ± 0.35 % by AOAC method (AOAC, 2000) and enzymatic hydrolysis of PBB was performed using five enzymes, alcalase, papain, trypsin, protamex, and flavourzyme, under their optimal conditions (Table 1). A 100-g sample (on the basis of protein weight) and 1 % enzyme were mixed and then the mixture was incubated for 8 h at each optimal temperature with stirring. After incubation, the mixture was heated at 100 °C to inactivate the enzyme. The inactivated mixture was centrifuged at 2000×*g* for 20 min. After centrifugation, the supernatant was lyophilized and stored at −20 °C until use.

Table 1 The proximate compositions including moisture, crude fat, ash, and crude protein of PBB

	Moisture (%)	Crude fat (%)	Ash (%)	Crude protein (%)
PBB	21.07 ± 0.22	12.19 ± 0.12	17.18 ± 0.64	44.50 ± 0.35
Protamex	7.05 ± 0.21	1.12 ± 0.15	18.35 ± 0.21	73.92 ± 0.95
< 3 K	14.04 ± 0.13	4.66 ± 0.21	15.85 ± 0.18	62.07 ± 3.23
3–10 K	7.26 ± 0.17	2.27 ± 0.25	11.75 ± 0.26	69.31 ± 2.11
> 10 K	5.48 ± 0.22	2.30 ± 0.17	12.69 ± 0.31	74.02 ± 2.63

The proximate composition of PBB was measured by AOAC methods

The protamex hydrolysate (38 g) was dissolved in 50 mL deionized water and filtered by two ultrafiltration membranes (Amicon Ultra-filter devices; Millipore, Billerica, MA, USA) with 3 and 10 kDa molecular weight cut-offs (< 3 kDa, 3–10 kDa, and > 10 kDa). The soluble fractions were prepared by centrifuging at 3000×*g* for 20 min and passed through the membrane sequentially, beginning with the largest molecular weight cut-off membrane cartridge (10 kDa). The retentate and permeate were collected separately, and the retentate was recirculated into the feed until the maximum permeate yield was reached. Permeate from the 10 kDa membrane was then filtered through the 3 kDa membrane with recirculation until the maximum permeate yield was reached.

Degree of hydrolysis (DH)

The DH of enzymatic hydrolysates of PBB was calculated by determining free amino groups with OPA (Nielsen 2001).

$$\mathrm{DH} = h / h_{\mathrm{tot}} \times 100$$

where h_{tot} is the total number of peptide bonds per protein equivalent, and h is the number of hydrolyzed bonds. The factor h_{tot} is dependent on the amino acids composition of the raw material (Adler-Nissen 1986).

Total amino acids contents

The total amino acids composition was determined using an amino acid analyzer (S43000; Sykam, Eresing, Germany). Samples were hydrolyzed in hydrochloric acid (6 N) in vacuum-sealed tubes at 110 °C for 24 h.

Tryptophan is measured after alkaline hydrolysis (Sato et al. 1984). Samples (9–10 mg, 0.1 mg precision) were dissolved in 10 mL of 4.2 N sodium hydroxide and hydrolysed in an oven at 110 °C for 20 h. The hydrolysates were filtered using filter paper (ADVANTEC No. 5B) and mass up 50 mL by 0.2 N sodium citrate buffer (pH

4.2). The 1–5 mL of solution was dried in a waterbath at 70 °C. The pH of the hydrolysates was adjusted to 4.2 with 6 N HCl and 0.2 N sodium citrate buffer and mass up 25 mL. The solutions were filtered through 0.2 μm membrane filter and analyzed by amino acid analyzer (Sykam 4300, Sykam, Germany).

$ABTS^+$ radical scavenging activity

The $ABTS^+$ radical scavenging activity was determined using the method of Roberta et al. (1999). The ABTS solution was diluted with water to achieve an absorbance of 0.75 ± 0.03 at 734 nm. Then, 180 μL of ABTS solution was added to 20 μL of different concentrations of samples. The mixture was incubated in the dark for 10 min and measured the absorbance by spectrophotometer (BIO-TEK US/MQX 200, USA) at 734 nm. L-ascorbic acid was used as a positive control and the $ABTS^+$ radical scavenging activity of each sample was expressed as IC_{50} value.

Superoxide dismutase (SOD)-like activity

The SOD-like activity of the sample was evaluated according to the procedure of Marklund and Marklund (1974) with a slight modification. One hundred microliters of sample solutions were mixed with 100 μL of pyrogallol (7.2 mM) and 100 μL of 50 mM Tris-HCl buffer at pH 8.5 containing 0.2 mM EDTA. After 10 min, 50 μL of 1 N HCl was added to the mixture to stop the reaction and measured the absorbance at 420 nm. L-ascorbic acid was used as a positive control, and the SOD-like activity of the PBB was expressed as IC_{50} value.

Reducing power

The reducing power of shrimp shell extracts were measured by the method of Oyaizu (1986). Different concentrations of samples in 10 % DMSO were mixed with 50 μL of 0.2 M sodium phosphate buffer (pH 6.6) and 50 μL of potassium ferricyanide (10 mg/mL). The mixtures were incubated at 50 °C for 20 min. Then, 50 μL of TCA (100 mg/mL) was added and centrifuged at 2000×*g* for 10 min. After centrifugation, 100 μL of the supernatant was mixed with 20 μL of iron(III) chloride (1 mg/mL) and the mixture was measured at 700 nm. Reducing power was expressed as the 0.5 of absorbance (EC_{50}) and L-ascorbic acid was used as a positive control.

ACE inhibitory activity

The inhibitory activity of ACE was monitored according to the method of Li et al. (2005). A 20 μL of sample, 50 μL of 5 mM HHL, and 100 mM of sodium borate buffer (pH 8.3) containing 300 mM NaCl were pre-incubated at 37 °C for 5 min. The reaction was initiated by the addition of 10 μL of ACE solution (100 mU/mL), and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 100 μL of 1 M HCl, and then sodium borate buffer (320 μL), pyridine (600 μL), and BSC (200 μL) were added to the reaction mixture. After incubation at room temperature for 30 min, the absorbance of reaction mixture was measured at 492 nm and the captopril was used as a positive control.

Statistical analysis

The data were analyzed using analysis of variance through the general linear model procedure (SAS Institute, Cary, NC, USA). Duncan's multiple range test was applied to determine the significance of the differences between means ($P < 0.05$).

Results and discussion

Enzymatic hydrolysis and fractionation

Recently, the enzymatic hydrolysates have been studied and utilized as nutraceutical resources. In particular, the interest of many researchers on the enzymatic hydrolysates derived from fish and shrimp processing by-products and the various biological activities such as antioxidant, antibacterial, antiobesity, and antihypertensive activities is growing (Sila et al. 2015; Benoit et al. 2008; Cancre et al. 1999).

In the present study, PBB was hydrolyzed by five specific enzymes including alcalase, papain, trypsin, protamex, and flavourzyme for 8 h, respectively, and DH values of their hydrolysates were given in Table 2. The cleavage of peptide bonds by protease leads to decomposition of protein tertiary structure and reduction of the molecular weight of proteins (Adler-Nissen 1986). This reaction also increases in the concentration of free amino and carboxyl groups and its functional properties of proteins (Kristinsson and Rasco 2000). The DH values of PBB hydrolysates were as follows: protamex (59.85 ± 0.09 %) > papain (58.86 ± 0.08 %) > trypsin (58.31 ± 0.08 %) > alcalase (55.96 ± 0.04 %) > flavourzyme (55.68 ± 0.08 %). Among the enzymatic hydrolysates, protamex hydrolysate of PBB showed the highest DH value.

Table 2 The conditions of enzymatic hydrolysis and degree of hydrolysis of the enzymatic hydrolysates of PBB

Enzyme	Crude protein (%)	pH	Temperature (°C)	DH (%)
Alcalase	70.57 ± 2.05	8.0	50	55.96 ± 0.04[1,d]
Protamex	73.92 ± 0.95	8.0	45	59.85 ± 0.09[a]
Flavourzyme	67.40 ± 0.16	7.0	50	55.68 ± 0.08[e]
Papain	69.45 ± 1.33	6.0	37	58.86 ± 0.08[b]
Trypsin	66.62 ± 0.66	8.0	37	58.31 ± 0.08[c]

Enzymatic hydrolysates were obtained from 8 h under the optical conditions.
[1]Means within the same row with different superscripts are significantly different by Duncan's multiple range test ($P < 0.05$)

Table 3 Total amino acids composition for the molecular weight cut-off fractions of protamex hydrolysate (g/100 g)

Amino acids	< 3 kDa	3–10 kDa	> 10 kDa
Asp	3.66	4.77	3.65
Thr	1.96	2.21	1.94
Ser	1.91	2.18	1.10
Glu	6.14	7.16	6.11
Pro	2.95	0.34	3.22
Gly	5.06	4.72	5.15
Ala	4.44	4.10	4.43
Cys	0.08	0.18	0.17
Val	2.53	2.62	2.11
Met	1.44	1.24	1.74
Ile	2.21	2.54	1.88
Leu	3.80	3.68	3.73
Tyr	1.51	1.43	1.50
Phe	2.24	2.27	2.25
His	1.29	1.73	1.24
Lys	3.06	3.49	3.89
Arg	2.94	3.36	3.07
Trp	0.22	0.15	0.30
Total	47.44	48.17	47.48

Total amino acids contents

Total amino acid composition of the three MWCO fractions of protamex hydrolysate was presented in Table 3. As shown in Table 3, the total amino acid contents of three MWCO fractions were 47.44 g/100 g (< 3 kDa), 48.17 g/100 g (3–10 kDa), and 47.48 g/100 g (>10 kDa), respectively. The total amino acid compositions of three MWCO fractions were similar to each other. All MWCO fractions were rich in Glu, Gly, Asp, Ala, Leu, and Lys, while these fractions contained low levels of Cys and Trp.

Antioxidant activity

Reactive oxygen species (ROS), containing superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and singlet oxygen (O_2^-), can cause the oxidative damage to the important components such as protein, lipid, nucleic acids and have been associated with the occurrence of various diseases including hypertension (Ngo et al. 2011a, b).

The antioxidant activities of the five PBB enzymatic hydrolysates were measured by the scavenging activity on $ABTS^+$ radicals, SOD-like activity, and reducing power (Table 4). The alcalase and protamex hydrolysates of PBB exhibited noticeable $ABTS^+$ radical scavenging activity with IC_{50} value of 0.16 ± 0.02 and 0.17 ± 0.00 mg/mL, respectively. However, all hydrolysates showed lower $ABTS^+$ radical scavenging activity than that of L-ascorbic acid.

SOD, an important antioxidant defense enzyme, catalyzes the dismutation of the superoxide anion (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2). The SOD-like activity is widely used for assay to measure the inhibition of pyrogallol autoxidation. The results of SOD-like activity of the five enzymatic hydrolysates were shown in Table 4. The protamex hydrolysate exerted the greatest SOD-like activity (2.04 ± 0.15 mg/mL) among hydrolysates, while the alcalase hydrolysate exhibited moderate activity.

The reducing power is the ability to donate an electron or hydrogen (Dorman et al. 2003). The electron donation capacity of the five PBB enzymatic hydrolysates were evaluated and showed in Table 4. All the PBB hydrolysates exhibited moderate reducing power.

The antioxidant activities of the three MWCO fractions of protamex hydrolysate were evaluated and showed in Table 5. The < 3 kDa and 3–10 kDa fractions showed potent $ABTS^+$ radical scavenging activity ($IC_{50} = 0.22 \pm 0.01$ mg/mL and 0.22 ± 0.00 mg/mL),

Table 4 Antioxidant and ACE inhibitory activities for the enzymatic hydrolysates of PBB

Sample	$ABTS^+$scavenging activity IC_{50} (mg/mL)[1]	SOD-like activity IC_{50} (mg/mL)	Reducing power EC_{50} (mg/mL)[2]	ACE inhibitory activity IC_{50} (mg/mL)
Alcalase	$0.16 \pm 0.02^{a, c}$[3]	2.82 ± 0.72^{a}	9.42 ± 0.82^{a}	0.11 ± 0.01^{b}
Protamex	$0.17 \pm 0.00^{b, c}$	2.04 ± 0.15^{a}	6.75 ± 0.94^{b}	0.08 ± 0.00^{c}
Flavourzyme	$0.20 \pm 0.00^{a, b}$	3.13 ± 0.51^{a}	4.63 ± 0.15^{c}	0.11 ± 0.00^{b}
Papain	0.21 ± 0.03^{a}	2.59 ± 0.48^{a}	4.46 ± 0.31^{c}	0.11 ± 0.01^{b}
Trypsin	0.22 ± 0.02^{a}	2.44 ± 0.13^{a}	6.62 ± 0.06^{b}	0.13 ± 0.00^{a}
L-Ascorbic acid[A]	0.004 ± 0.000^{d}	0.02 ± 0.00^{b}	0.04 ± 0.00^{d}	
Captopril[B]				0.00002 ± 0.00000^{d}

[1] IC_{50} (50 % inhibitory concentration) values of $ABTS^+$ scavenging, SOD-like, and ACE inhibitory activities were expressed as a mean ± SD
[2] The reducing power was expressed as an EC_{50} (concentration of the 0.5 absorbance) value
[3] Means within the same row with different superscripts are significantly different by Duncan's multiple range test ($P < 0.05$)
[A] L-ascorbic acid was used as a positive control of $ABTS^+$ radical scavenging and SOD-like activities and reducing power
[B] Captopril was used as a positive control of ACE inhibitory activity

Table 5 Antioxidant and ACE inhibitory activities for the molecular weight cut-off fractions of the protamex hydrolysate

Sample	$ABTS^+$ scavenging activity IC_{50} (mg/mL)[1]	SOD-like activity IC_{50} (mg/mL)	Reducing power EC_{50} (mg/mL)[2]	ACE inhibitory activity IC_{50} (mg/mL)
< 3 K	0.22 ± 0.01[3, b]	>10	20.14 ± 0.39[a]	0.06 ± 0.00[a]
3–10 K	0.22 ± 0.00[b]	>10	13.84 ± 0.16[b]	0.03 ± 0.00[b]
> 10 K	0.24 ± 0.01[a]	>10	7.04 ± 0.83[c]	0.03 ± 0.00[b]
L-Ascorbic acid[A]	0.005 ± 0.000[c]	0.07 ± 0.00[d]	0.04 ± 0.00[d]	
Captopril[B]				0.00001 ± 0.0000[c]

Antioxidant and ACE inhibitory activities were measured using the molecular weight cut-off fractions of the protamex hydrolysate
[1]IC_{50} values of $ABTS^+$ scavenging, SOD-like, and ACE inhibitory activities were expressed as a mean ± SD
[2]The reducing power was expressed as an EC_{50} value
[3]Means within the same row with different superscripts are significantly different by Duncan's multiple range test ($P < 0.05$)
[A]L-ascorbic acid was used as a positive control of $ABTS^+$ radical scavenging and SOD-like activities, and reducing power
[B]Captopril was used as a positive control of ACE inhibitory activity

while the > 10 kDa fraction was exhibiting stronger reducing power with EC_{50} value of 7.04 ± 0.83 mg/mL than those of the < 3 kDa and 3–10 kDa fractions. However, the three MWCO fractions showed not high antioxidant activities as much as protamex enzymatic hydrolysate.

ACE inhibitory activity

The inhibition of ACE, a key enzyme regulating the blood pressure, has been recognized as the most effective therapy for the treatment of hypertension. However, many synthetic ACE inhibitors including captopril, enalapril, alacepril, fosinopril, and lisinopril cause side effects such as cough, taste alterations, skin rashes, and angioneurotic edema (Alderman 1996; Cicoira et al. 2001; Vyssoulis et al. 2001). Therefore, it is necessary to develop safe and effective ACE inhibitors from natural products.

The ACE inhibitory activity of the enzymatic hydrolysates of PBB was shown in Table 4. Among the five PBB hydrolysates, the protamex hydrolysate exhibited the most potent ACE inhibitory activity with IC_{50} value of 0.08 ± 0.00 mg/mL, followed by flavourzyme (IC_{50} = 0.11 ± 0.00 mg/mL) > alcalase (IC_{50} = 0.11 ± 0.01 mg/mL) > papain (IC_{50} = 0.11 ± 0.00 mg/mL) > trypsin (IC_{50} = 0.13 ± 0.00 mg/mL).

The ACE inhibitory activity of the three MWCO fractions of protamex hydrolysate was measured and shown in Table 5. Among the MWCO fractions, the > 3 kDa fraction including 3–10 kDa and > 10 kDa fractions showed significant ACE inhibitory activity with IC_{50} value of 0.03 ± 0.00 mg/mL. This result also indicated that high molecular weight fraction, 3–10 kDa and > 10 kDa, exhibited potent ACE inhibitory activity compared with protamex enzymatic hydrolysate.

Recently, many researchers have reported that various bioactive peptides derived from fisheries processing by-products including crab shell (Yoon et al. 2013), Pacific cod skin (Ngo et al. 2011a, b), squid skin and muscle (Mendis et al. 2005; Rajapakse et al. 2005), and tuna (Qian et al. 2007; Je et al. 2005; Lee et al. 2010). In the present study, PBB protamex hydrolysate showed notable $ABTS^+$ radical scavenging and ACE inhibitory activities. In addition, its MWCO fractions exerted potent ACE inhibitory activities. Therefore, more detailed investigations are necessary to isolate and identify the peptides from active enzymatic hydrolysate and to clarify the mechanism of active peptides.

Conclusions

In this study, the five enzymatic hydrolysates of PBB derived from fisheries processing by-products were investigated on the antioxidant and ACE inhibitory activities. The PBB Protamex hydrolysate which showed the most potent ACE inhibitory activity than other fractions was fractionated as the below 3 kDa, between 3 and 10 kDa, and above 10 kDa fractions respectively to isolate the active materials. But these fractions showed lower antioxidant activities than those of enzymatic hydrolysate, while these fractions showed the significant ACE inhibitory activity. In addition, 3-10 kDa and >10 kDa fractions showed the better ACE inhibitory activity than <3 kDa fraction. These results suggested that the high molecular weight enzymatic hydrolysate derived from PBB could be used for prevent hypertension.

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Authors' contributions

SB carried out the anti-oxidant and ACE inhibitory activities assay. NY performed the enzymatic hydrolysis and fractionation. KB analyzed the total amino acids composition. CW participated in the design of the study and performed the statistical analysis. All authors read and approved the final manuscript.

Competing interests

The authors have no pecuniary or other personal interest, direct or indirect, in any matter that raises or may raise a conflict with our duties.

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