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Angiotensin I Converting Enzyme Inhibitory Activity of Krill (*Euphausia superba*) Hydrolysate

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Angiotensin I converting enzyme inhibitory activities of shelled krill (*Euphausia superba*) hydrolysates by autolysis and by hydrolysis with commercial proteases were analyzed. Among the proteases, Alcalase was the most effective protease for the hydrolysis of krill considering the degree of hydrolysis (87.5%) and the ACE inhibitory activity (60%). Four hour hydrolysis suggested as the most suitable and economic. In order to establish the optimum hydrolysis condition of krill, degree of hydrolysis and ACE inhibitory activity as affected by Alcalase concentration and water amount added were statistically analyzed by response surface methodology (RSM). The optimum hydrolysis condition was 2.0% Alcalase hydrolysis in 2 volumes (v/w) of water at 55℃ for 4 hr. The hydrolysate prepared from the optimum hydrolysis condition was fractionated by molecular weight. The lower molecular weight fraction showed the higher ACE inhibitory activity. IC_{50} of the fraction under 500 Da was 0.57 mg protein/mL.

Key words: Krill, Angiotensin I converting enzyme (ACE), ACE inhibition, RSM

Introduction

Angiotensin I converting enzyme (ACE) in renin -angiotensin system is a cause of essential hypertension, which covers most hypertension, one of the major adult diseases (Ondetti and Cushman, 1982; Garbers and Dubois, 1999). Thus, the inhibition of ACE would be indispensable for the prevention and cure of hypertension. Therefore, a lot of studies on the ACE inhibitor have been conducted. Peptides from the protein hydrolysate have been reported as an remarkable inhibitor. Especially, various ACE inhibitory peptides were isolated and identified from marine products for their utilization as value added products. Kohama et al. (1988) isolated ACE inhibitor from tuna muscle, and Matsui et al. (1993) and Lee et al. (1998) prepared ACE inhibitory hydrolysate from sardine muscle and anchovy muscle, respectively. Suetsuna (1998) purified ACE inhibitory peptide from *Hizikia fusiformis*. Also, Turban shell (Kim et al., 2000) and Mackerel (Do, 2000) were used for the purification of ACE inhibitory peptide.

Antarctic krill (*Euphausia superba*) could be a good food resource in view of high nutritive value (Watanabe et al., 1976) as well as abundant catchable amount, which were suggested over sixty million tons a year (Ross and Quetin, 1986).

Therefore, this study analyzed ACE inhibitory activity of shelled krill hydrolysates by autolysis and commercial proteases, and established the optimum hydrolysis condition statistically by response surface methodology (RSM).

Materials and methods

Materials

Antarctic krill immediately frozen after catch was donated by In Sung Co. (Korea) and the krill was shelled by meat separator and then stored below −40℃. The shelled krill was thawed and homogenized for hydrolysis. Nine kinds of enzymes, such as Alcalase 0.6 L (Novo Korea, Korea), Flavourzyme 500 MG (Novo Korea, Korea), Protamex 1.5 MG

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(Novo Korea, Korea), Neutrase 0.5 L (Novo Korea, Korea), Maxazyme NNP (Bision Biochem, Korea), Sumizyme LP (Bision Biochem, Korea), Collupulin (Bision Biochem, Korea), Delvolase (Bision Biochem, Korea) and Protease-NP (Pacific, Korea) were used for the hydrolysis of krill.

Autolysis and Hydrolysis

Autolysis was conducted in 4 volumes (v/w) of distilled water at 37℃ for 8 hr. Hydrolyses by commercial proteases were conducted with 2% (w/w, dry basis) enzyme in 4 volumes (v/w) of distilled water at 50℃ for 12 hr. Hydrolysates were separated from precipitates by centrifugal method (1,400×g). Degree of hydrolysis was defined as the percentage ratio of the weight of soluble fraction after hydrolysis to the weight of the whole sample on dry basis.

Assay for Angiotensin I converting enzyme inhibitory activity

Angiotensin I converting enzyme inhibitory activity was analyzed by the method of Yamamoto et al. (1980), an advanced one of Cushman and Cheung (1971). Aliquot (100 μL) of sample solution, crude ACE solution 100 μL and borate buffer solution (pH 8.3, containing 400 mM NaCl) 200 μL were pre-incubated at 37℃. After addition of hippuryl-histidyl-leucine (12.5 mM) solution 100 μL, the solution was incubated at 37℃ for 1 hr then stopped the reaction with 1 N HCl 300 μL (Borate buffer solution 100 μL was used instead of sample solution in blank, and 1 N HCl 300 μL then crude ACE solution 100 μL were added in control). Ethyl acetate 1.5 mL was added to this solution, mixed for 15 seconds followed by centrifugation at 1,400×g for 10 min. Aliquot (1 mL) of the supernatant was dried at 140℃ for 20 min, allowed to stand at an ambient temperature for 5 min. It was dissolved with 1 M NaCl 3 mL for 15 seconds then measured the absorbance at 228 nm. IC_{50} (mg protein/mL) represents the concentration of potent ACE inhibitor required to inhibit 50% of the ACE activity under the foregoing conditions.

Response surface analysis

Optimum hydrolysis condition of the shelled krill was established by response surface methodology (RSM). A central composite design was conducted on dependent variables, degree of hydrolysis and ACE inhibitory activity as affected by the independent variables, Alcalase concentration and water amount added. The Alcalase concentration was from 0 to 2% (w/w, dry basis) and the water amount added was from 0 to 4 volumes (v/w) on the homogenate of the shelled krill. Domain, spacing and coded values of the independent variables were shown in Table 1.

Table 1. Experimental domain and spacing of Alcalase concentration and the water amount added expressed in coded and natural units

Code units	Experimental factor	
	Alcalase concentration (%, w/w)[1)]	Water amount added (volumes, v/w)
−2	0.5	0
−1	1.0	1
0	1.5	2
1	2.0	3
2	2.5	4

[1)] Dry basis.

Ultrafiltration

The hydrolysate of shelled krill obtained from the optimum hydrolysis was fractionated as affected by molecular weight. Ultrafiltration was conducted with membranes (molecular weight cut off 100,000, 10,000, 3,000 and 500 Da; Amicon, USA) at 5℃ under nitrogen gas (<50 psi). Yield of each fraction was defined as the percentage ratio of the volume of each fraction after ultrafiltration to the whole volume of the hydrolysate.

Analysis of protein content

Protein content was analyzed by the method of Lowry et al. (1951) and the absorbance was detected at 280 nm.

Statistical analysis

Data were analyzed by analysis of variance using the SAS. Means were separated by the least significant difference (LSD) method at the 5% level.

Results and Discussion

Degree of hydrolysis and ACE inhibitory activity of krill autolysate

Shelled krill was autolyzed as affected by autolysis time then degree of hydrolysis and ACE inhibitory activity of the hydrolysate were analyzed (Fig. 1). The degree of hydrolysis increased from 77.3% of pre-autolysis to 81.2% of 2 hr autolysis and then kept almost constant level of 81~82% ($p<0.05$) until 8 hr autolysis. ACE inhibitory activity decreased from 28% of pre-autolysis to 11% of 3 hr autolysis then increased to 34% until 8 hr. The relatively high inhibitory activity in pre-autolysis was presumed owing to the existence of inhibitory peptides could be prepared from autolysis enzymes in krill. Once diminution of the inhibitory activity was supposed owing to dilution effect of the initial active inhibitors by autolysis of krill then reincrease of the activity would be caused from the increased potent ACE inhibitory peptides through 8 hr autolysis. Finally, autolysis of krill was statistically not effective on the ACE inhibitory activity during 8 hr autolysis.

Degree of hydrolysis and ACE inhibitory activity of krill hydrolysate by commercial proteases

Shelled krill was hydrolyzed by commercial proteases then degree of hydrolysis and ACE inhibitory activity of the hydrolysates were analyzed (Fig. 2). Degree of hydrolysis was high in order of Protamex

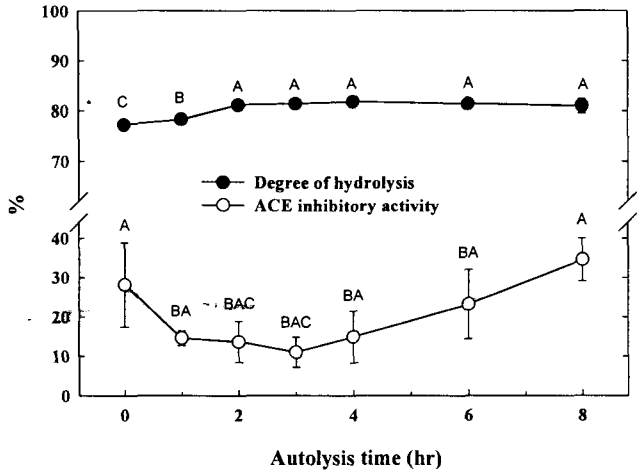

Fig. 1. Degree of hydrolysis and ACE inhibitory activity of hydrolysates by autolysis of krill, *Euphausia superba*.
Krill was autolyzed in 4 volumes (v/w) of distilled water at 37℃ for 8 hr.
Each point shows the mean ± S.D. (n=2~3).
Means with the same letter are not significantly different ($p<0.05$).

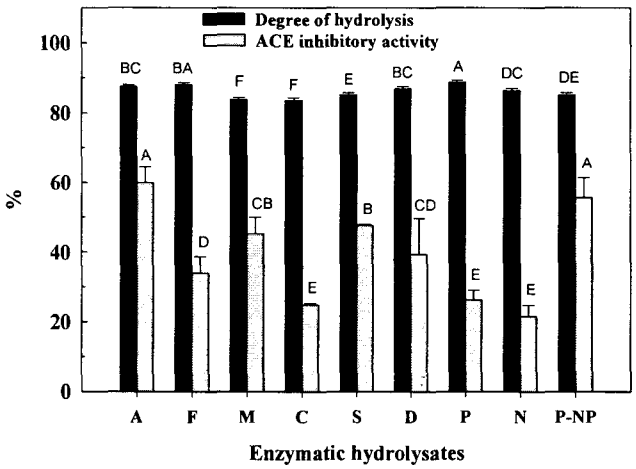

Fig. 2. Degree of hydrolysis and ACE inhibitory activity of enzymatic hydrolysates of shelled krill, *Euphausia superba*.
Krill was hydrolyzed by 2% (w/w, dry basis) enzyme in 4 volume (v/w) of distilled water at 50℃ for 12 hr.
A: Alcalase 0.6 L, F: Flavourzyme 500 MG, M: Maxazyme NNP, C: Collupulin, S: Sumizyme LP, D: Delvolase, P: Protamex 1.5 MG, N: Neutrase 0.5 L, P-NP: Protease NP.
Each bar shows the mean ± S.D. (n=2).
Means with the same letter are not significantly different ($p<0.05$).

(88.8%), Flavourzyme (88.0%), and Alcalase (87.5%) and Delvolase (86.9%). Others also showed high degree of hydrolysis at least 83.5%. In case of ACE inhibitory activities, Alcalase hydrolysate showed the highest inhibitory activity of 60% followed by Protease-NP (56%) and Sumizyme (48%), especially, the activities of both Alcalase and Protease-NP hydrolysate were not significantly different. Flavourzyme (34%), Protamex (26%) and Devolase (39.4%) were significantly low even though high degree of hydrolysis. From these results, Alcalase and protease-NP were superior in view of both ACE inhibitory activity and degree of hydrolysis. Moreover, Alcalase is the cheapest. Therefore, Alcalase was the most effective enzyme among the tested proteases for the hydrolysis of krill in view of not only degree of hydrolysis (87.5%) and ACE inhibitory activity (60%) but economic consideration. This result was similar to the report of Lee et al. (1998) that ACE inhibitory activity (>70%) of Alcalase hydrolysate of anchovy muscle was the highest.

Degree of hydrolysis and ACE inhibitory activity of krill hydrolysate by Alcalase

Shelled krill was hydrolyzed by Alcalase, the most effective protease, as affected by hydrolysis time of 1, 2, 4, 6, 8 and 10 hr. Degree of hydrolysis and ACE inhibitory activity of the hydrolysate were determined as shown in Fig. 3. Degree of hydrolysis was not significantly different from 1 to 10 hr hydrolysis and showed above 85%. ACE inhibitory activity was also not significantly different and showed 70% in 4 hr hydrolysis. Matsui et al. (1993) reported that ACE inhibitory activity of 1 hr hydrolysate of sardine muscle by alkaline protease reached the highest (70%) then kept a constant degree. Degree of hydrolysis and ACE inhibitory activity could be affected by the condition of raw krill and the degree of its shelling. Considering these factors, in spite of no significant difference in this result, 4 hr hydrolysis by Alcalase was suggested for the enough hydrolysis of krill considering degree of hydrolysis, ACE inhibitory activity and economics. In this conditon, the hydrolysate showed degree of hydrolysis of 87% and ACE inhibitory activity of 70%.

Optimum hydrolysis condition of krill

In order to establish the optimum hydrolysis condition of shelled krill, response surface methodology (RSM) was conducted. The optimum hydrolysis condition of krill was determined from the relation of the dependent variables, degree of hydrolysis and ACE inhibitory activity, to the independent variables, Alcalase concentration and water amount added, by the RSM. Degree of hydrolysis and ACE inhibitory activity as affected by Alcalase concentration and water amount added were shown in Table 2, and the results of RSM treatment of degree of hydrolysis and ACE inhibitory activity were shown in Fig. 4 and 5, respectively. In the ranges of the experimental conditions, degree of hydrolysis increased from the lowest 76% to the highest 86%. Degree of hydrolysis above 82% could be estimated in condition of 2.0% (w/w) Alcalase and above 2 volumes (v/w) of water. Regression equation of the degree of hydrolysis were shown in Table 3. It was significant ($p<0.01$) and R^2 was 0.8698.

ACE inhibitory activity also increased from the lowest 55% to the highest 85% as affected by the increase of Alcalase concentration and water amount added. The inhibitory activity above 80% could be estimated in condition of 2% (w/w) of Alcalase and above 2 volumes (w/w) of water. The regres-

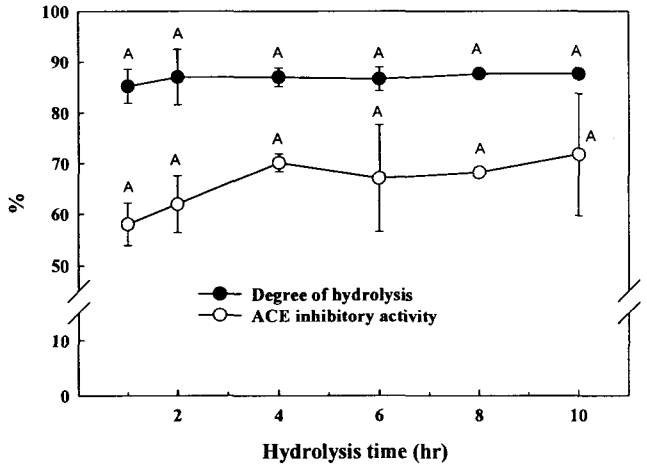

Fig. 3. Degree of hydrolysis and ACE inhibitory activity of hydrolysates by Alcalase of krill, *Euphausia superba*.
Krill was hydrolyzed by 2% (w/w, dry basis) Alcalase 0.6 L in 4 volumes (v/w) of distilled water at 55℃.
Each bar shows the mean ± S.D. (n=2~3).
Means with the same letter are not significantly different ($p<0.05$).

Table 2. Response of dependent variables to the reaction conditions for the hydrolysis of krill, *Euphausia superba*

Run No.	Independent variable		Dependent variable	
	X_1[1)]	X_2[2)]	Degree of hydrolysis (%)	ACE inhibitory activity (%)
1	−1	−1	79.17	70.29
2	1	−1	82.52	83.20
3	−1	1	82.42	78.47
4	1	1	86.33	82.57
5	−1	0	81.12	79.35
6	1	0	83.50	85.54
7	0	−1	77.91	72.09
8	0	1	81.28	79.97
9	−1	−2	74.81	57.74
10	1	−2	79.50	71.06
11	−1	2	84.30	75.05
12	1	2	85.73	85.69
13	−2	−1	75.96	50.17
14	2	−1	83.72	85.64
15	−2	1	80.01	56.47
16	2	1	85.70	84.02
17	0	0	79.17	72.90

[1,2)] X_1: Alcalase concentration (%, w/w; dry basis), X_2: Water amount added (volumes, v/w). Krill was hydrolyzed by Alcalase at 55℃ for 4 hr.

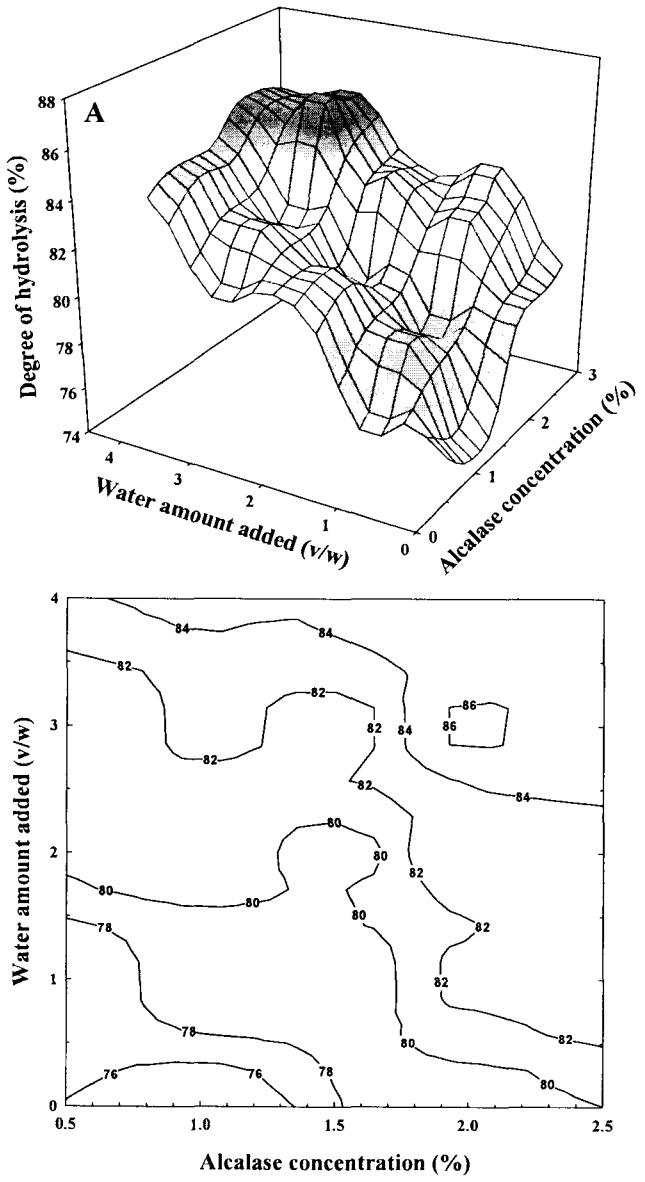

Fig. 4. Response surface (A) and contour plot (B) for degree of hydrolysis of krill (*Euphausia superba*) hydrolysate as affected by Alcalase concentration and water amount added. Krill was hydrolyzed in D.W. at 55℃ for 4 hr.

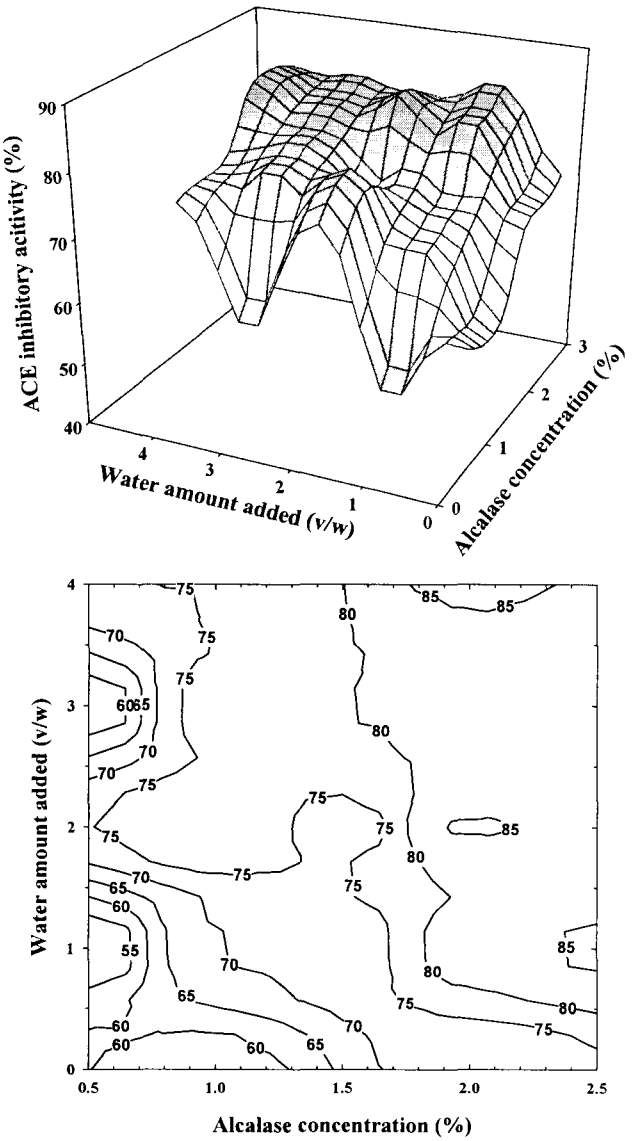

Fig. 5. Response surface (A) and contour plot (B) for ACE inhibitory activity of krill (*Euphausia superba*) hydrolysate as affected by Alcalase concentration and water amount added. Krill was hydrolyzed in D.W. at 55℃ for 4 hr.

Table 3. Regression equation calculated by RSM for the degree of hydrolysis and ACE inhibitory activity of krill, *Euphausia superba* hydrolysate by Alcalase 0.6 L

Response variables	Regression equation	R^2	Significance
Degree of hydrolysis (%)	$Y_1 = 80.248533 + 6.661538X_1 + 3.230385X_2 - 2.161605X_1^2 - 0.833611X_1X_2 - 1.453974X_2^2$	0.8698	0.01
ACE inhibitory activity (%)	$Y_2 = 81.234865 - 1.640769X_1 + 1.842308X_2 + 0.154381X_1^2 - 0.280556X_1X_2 - 0.071408X_2^2$	0.8870	0.01

X_1, Alcalase concentration; X_2, water amount added.

sion equation of ACE inhibitory activity was shown in Table 6. It was significant ($p<0.01$) and R^2 was 0.8870. From these results, the optimum condition of shelled krill hydrolysis was 2.0% (w/w; dry basis) Alcalase hydrolysis in 2 volumes (v/w) of water at 55℃ for 4 hr to prepare hydrolysate showing high degree of hydrolysis and ACE inhibitory activity conveniently and economically. Estimate values of the degree of hydrolysis and ACE inhibitory activity calculated from the regression equation were 82.2% and 80.8%, respectively, and experimental values obtained from mean values of 4 experiments conducted under the optimum condition were 81.9.% and 79.0%, respectively.

ACE inhibitory activity of fractions from krill hydrolysate

Shelled krill was hydrolyzed in the optimal condition, afterward, fractionated by membrane (molecular weight cut off 100,000, 10,000, 3,000 and 500 Da) as affected by molecular weight. Yield of the fraction and IC_{50} were shown in Table 4. Yield of fraction above 100,000 Da was 20.4%, that from 100,000 to 10,000 Da 1.1%, from 10,000 to 3,000 Da 13.1%, from 3,000 to 500 Da 3.3% and that below 500 Da was the highest 62.0%. The lower molecular weight fraction showed the higher ACE inhibitory activity and IC_{50} of the fraction below 500 Da was 0.57 mg protein/mL. Related this result, Kim et al. (2000) found that the lower molecular weight under 10,000 Da of Turban Shell (*Turbo cornutus*) hydrolysate exhibited the higher ACE inhibitory activity. Also, the lower molecular weight showed the lower IC_{50} in κ-casein hydrolysate (Oh et al., 1997). IC_{50} of alkaline protease hydrolysate of sardine muscle was 240 μg/mL (Matsui et al., 1993) and ACE inhibitory activity of fraction under 10,000 molecular weight was 2 times higher than that of fraction above the molecular weight from mackerel and IC_{50} was 138 μg (Do, 2000). Conclusively, the optimal hydrolysis condition of shelled krill suggested was 2.0% (w/w, dry basis) Alcalase hydrolysis in 2 volumes (v/w) of water at 55℃ for 4 hr and IC_{50} of fraction below 500 Da prepared from the optimal hydrolysis was 0.57 mg protein/mL.

Table 4. Fractionation of ACE inhibitors by ultrafiltration from krill, *Euphausia superba* hydrolysate[1)]

Fraction[2)]	Yield (%, v/v)	IC_{50} (mg protein/mL)
I	20.4	1.98
II	1.1	1.61
III	13.1	1.56
IV	3.3	0.79
V	62.0	0.57

[1)] It was hydrolyzed by 2% (w/w, dry basis) Alcalase at 55℃ for 4 hr.

[2)] I, M.W.>100,000; II, 10,000~100,000; III, 3,000~10,000; IV, 500~3,000; V, <500.

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