A THESIS

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Purification and Characterization of Angiotensin I Converting Enzyme Inhibitory Peptide from Enzymatic Digests of *Styela plicata*

Seok-Chun Ko

Department of Aquatic Life Medicine

GRADUATE SCHOOL

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Purification and Characterization of Angiotensin I Converting Enzyme Inhibitory Peptide from Enzymatic Digests of *Styela plicata*

Seek-Chun Ko

(Supervised by professor You-Jin Jeon)

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This thesis has been examined and approved by

Thesis director, In-Kyu Yeo, Professor of Aquatic Life Medicine

---

Kwang-Sik Choi, Professor of Marine Life Science

You-Jin Jeon, Professor of Aquatic Life Medicine

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Date

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CHEJU NATIONAL UNIVERSITY

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CONTENTS

국문초록 v
LIST OF FIGURES viii
LIST OF TABLES xi
ABSTRACT 1
1. INTRODUCTION 2
2. MATERIALS AND METHODS 9
2. 1. Materials 9
2. 2. Approximate chemical composition of dried *S. plicata* 9
2. 3. Preparation of enzymatic digests from *S. plicata* 10
2. 4. Measurement of enzymatic digestion yield 10
2. 5. Determination of protein content 10
2. 6. Optimum-conditions assay for the active digestion 10
2. 7. ACE inhibitory activity 13
2. 8. Purification of ACE inhibitory peptide 13
2. 8. 1. Molecular weight fractionation of active enzymatic digest 13
2. 8. 2. Gel filtration chromatography 14
2. 8. 3. Isolation of peptide by reversed-phase HPLC (RP-HPLC) 14
3. RESULTS AND DISCUSSION 16
3. 1. Approximate chemical composition 16
3. 2. Enzymatic digestion yield 16
3. 3. Protein content 16
3. 4. ACE inhibitory activity 17
3. 5. Optimum-conditions for the production of ACE inhibitory activity digest by Protamex 22
3. 5. 1. Yield 22
3. 5. 2. Protein contents 22
3. 5. 3. ACE inhibitory activity 22

3. 6. Purification and characterization of ACE inhibition peptides......................31
3. 6. 1. ACE inhibitory activity of Molecular weight fractions..........................31
3. 6. 2. Size exclusion chromatography..34
3. 6. 3. Purification of peptide by reverse-phase HPLC...................................38
4. CONCLUSION...48
REFERENCE...49

국문초록

최근 들어 현대인의 성인병으로 크게 대두되는 고혈압은 동맥경화, 뇌졸중, 심근경색 등의 질병을 초래할 뿐만 아니라 뇌출혈과 심장병 및 신장병 등과 합병증으로 나타날 경우 치사율이 매우 높은 질병이다. 이러한 고혈압은 그 자체가 직접적인 증상을 보이지는 않지만 혈압을 정상적으로 유지시키는 일은 위험한 성인병의 발병을 방지하는데 중요하다.

고혈압의 발병과 정상혈압 유지에는 많은 인자가 관여하고 있으며, 인체에서 혈압을 조절하는 기구인 renin-angiotensin system (RAS)의 항상성이 유지되지 않을 때 혈압조절에 문제가 생기는 것으로 알려져 있다. renin-angiotensin system (RAS)에서 renin에 의해 angiotensinogen으로부터 활성화된 angiotensin I을 angiotensin II로 전환시키는데 angiotensin I converting enzyme (ACE, EC 3. 4. 15. 1)이 직접 관여하고 있다고 알려져 있다. 고혈압 억제는 angiotensin I converting enzyme (안지오텐신 전환 효소)의 활성을 억제할 수 있는 물질에 초점이 맞추어져 있다. 이러한 결과로 생성된 angiotensin II는 계속하여 부신과 혈관평활근세포 및 신장 그리고 심장 등에 존재하는 4종의 AT receptor에 작용하며, AT receptor는 혈관수축과 aldosterone 및 vasopressin 방출 그리고 세뇨관의 나트륨 흡수와 신장으로의 혈류량 감소 등을 유발함으로써 심혈관과 신장 및 중추에서 여러 가지 병변을 가져온다. 그리하여 angiotensin I converting enzyme (ACE, EC 3. 4. 15. 1)은 혈관과 심장 및 폐 그리고 뇌조직 등에서 발견되는 dicarboxy peptidase로서 혈압조절 기구인 renin-angiotensin system (RAS)에서 중요한 역할을 한다고 하겠다. 이러한 RAS에서 ACE의 작용으로 심혈관계에 여러 문제가 생기므로 ACE 작용에 대한 저해물질인 ACE inhibitor는 고혈압뿐만 아니라 만성신장병과 동맥경화 및 심장발작 등을 효과 적으로 감소시킬 수 있다.

이러한 ACE 저해제는 captopril, alapril, fosinopril, moexipril 및 tandolapril 등의 화학합성 치료제로 많은 연구가 이루어 졌다. 그러나 이러한 합성 치료제들은 헛기침과 미각장애 및 피부 홍진 등의 부작용이 나타나기 때문에 천연물 유래의

ACE 저해제를 찾는데 많은 관심을 기울이고 있다. 최근에는 다양한 단백질 가수분해물로부터 ACE 저해 peptide를 찾기 위해 많은 연구가 이루어지고 있으며 다양한 해양생물자원을 이용하여 ACE 저해 peptide에 대한 연구가 많이 이루어지고 있다.

이 연구의 재료로 사용되어진 주름미더덕(*Styela plicata*)은 척색동물문, 해초강, 측성해초목, 미더덕과에 속하며 우리나라와 극동 아시아 지역에서 서식하고 있으며, 우리나라 바다 동해와 남해 연안에 주로 분포하며 수심 5-15m의 조하대 바닥이나 각종 고형물에 부착하여 서식하는 독립형 멍게류 이다. 이들은 우리나라에서 양식이 이루어지고 있으며 식용으로 이용하고 있다.

이 연구는 주름미더덕을 이용한 단백질의 효소적 가수분해물을 재료로 하여 ACE 저해 peptide를 찾기 위해 분리와 정제 과정을 통하여 ACE 저해에 직접 관여하는 peptide를 검색하였다. 이 주름미더덕의 단백질 가수분해물에 의한 ACE 저해 peptide를 검색하기 위한 첫 단계는 단백질 가수분해효소 10종을 이용하여 단백질 가수분해물을 제조하고 ACE 저해활성을 측정하여 가장 적합한 효소 Protamex를 선정하고, 선정한 Protamex 효소를 이용하여 가수분해물 제조에 최적 조건을 검색하기 위해 가수분해의 시간과 효소 농도를 다양하게 하여 가수분해물을 제조한 후 ACE 저해 활성을 측정하여 최적의 조건을 선정하였다. 이러한 단계를 거쳐 제조된 단백질 가수분해물을 한외여과와 겔 여과 크로마토그래피 및 RP-HPLC를 이용하여 분리와 정제를 실시하였고 확인된 단일 peptide의 ACE 저해활성을 측정 하였고, 활성을 나타내는 순수 peptide를 분리하기 위한 단계에서는 우수한 ACE 저해활성을 확인 하였고, 보고된 여러 가수분해물 보다 뒤지지 않는 ACE 저해활성이 나타났다.

이러한 결과를 종합하면, 주름미더덕의 단백질 가수분해물은 ACE 저해 효과가 우수하였으며, 이 연구에서 행한 분리와 정제 방법이 효과적이라 판단된다. 천연물 유래의 ACE 저해제를 찾기 위해 이 연구에서 재료로 사용되어진 주름미더덕은 앞에서 언급한 바와 같이 우리나라에서만 양식이 되고 있으며, 또한 식용으로 이용된다. 이들은 항고혈압 기능성 식품으로 개발 된다면 세계적으로 원천기술 확보는 물론 현재 우리나라만이 전적으로 자원을 확보하고 있으므로 세계 시장에서 크게 활용가치를 증진시킬 것으로 판단된다.

이 외에도 주름미더덕은 해양생물 유래 신 혈압개선용 식품소재의 개발과 기존 혈압치료제라는 의약품 개념에서 기능성 식품으로 안전하게 복용이 가능하고 식품용으로 사용 가능한 효소적 추출공정이 적용 되므로 부작용이 없을 것으로 기대된다. 또한 미이용 수산물의 다양한 용도 개발과 해양 생물 산업의 활성화는 물론 최근 세계적으로 해양생물 선호에 따라 개발제품의 수출 증대도 기대 된다.

LIST OF FIGURE

Fig. 1. Proposed reaction mechanism catalyzed by angiotensin I converting enzyme (Kininase II).

Fig. 2. The photograph of *Styela plicata*.

Fig. 3. Flow chart for the separation of ACE inhibitory peptide from *Styela plicata*.

Fig. 4. Preparation of enzymatic digests.

Fig. 5. Ultra-filtration membrane system for fraction of *S. plicata* enzymatic digest according to the molecular weight of constituents.

Fig. 6. ACE inhibitory activity of enzymatic digests from *S. plicata*. A, Neutrase; B, Alcalase; C, Flavourzyme; D, Protamex; E, Kojizyme; F, Protase; G, α-chimotrypsin; H, Trypsin; I, Papain; J, Pepsin; K, Aqueous.
Mean±SE of determinations was made in triplicate experiments.

Fig. 7. The 0.2% ratio of enzyme to substrate on ACE inhibitory activity of Protamex digest from *S. plicata*. The Digestion times for ACE inhibitory assay were 6, 12, 24 and 48 h.
Mean±SE of determinations was made in triplicate experiments.

Fig. 8. The 1% ratio of enzyme to substrate on ACE inhibitory activity of Protamex digest from *S. plicata*. The Digestion times for ACE inhibitory assay were 6, 12, 24 and 48 h.
Mean±SE of determinations was made in triplicate experiments.

Fig. 9. The 5% ratio of enzyme to substrate on ACE inhibitory activity of Protamex digest from *S. plicata*. The digestion times for ACE inhibitory assay were 6, 12, 24 and 48 h.
Mean±SE of determinations was made in triplicate experiments.

Fig. 10. The 10% ratio of enzyme to substrate on ACE inhibitory activity of

Protamex digest from *S. plicata*. The Digestion times for ACE inhibitory assay were 6, 12, 24 and 48 h.
Mean±SE of determinations was made in triplicate experiments.
Fig. 11. ACE inhibitory activity of molecular weight fractions of Protamex digest.
Mean±SE of determinations was made in triplicate experiments.
Fig. 12. Gel filtration chromatography of below 5 kDa molecular weight fraction from Protamex digest on Sephadex G-25 column. Separation was performed with 1.5 ml/min and collected at a fraction volume (7.5 ml). The peaks isolated on Sephadex G-25 gel column were designated F1-F4. Elution was monitored at 215 nm.
Fig. 13. ACE inhibitory activity of peaks on gel filtration chromatography from below 5 kDa molecular weight fraction.
Mean±SE of determinations was made in triplicate experiments.
Fig. 14. RP-HPLC chromatogram of potent ACE inhibitory peak (F2) isolated from Sephadex G-25. Separation was performed with linear gradient of acetonitrile from 0% to 25% in 30 min at a flow rate of 1.0 ml/min. The fractions isolated on RP-HPLC were designated A-C. Elution was monitored at 215 nm.
Fig. 15. ACE inhibitory activity of peaks on RP-HPLC from active peak (F2) by gel filtration chromatography.
Mean±SE of determinations was made in triplicate experiments.
Fig. 16. RP-HPLC re-chromatogram of potent ACE inhibitory fraction (F2-B) isolated from RP-HPLC. Separation was performed with linear gradient of acetonitrile from 0% to 100% in 50 min at a flow rate of 1.0 ml/min. The peaks isolated by RP-HPLC were designated I-V. Elution was monitored at 215 nm.
Fig. 17. ACE inhibitory activity of peaks on re-chromatography from active

peak (F2-B) by RP-HPLC.

Mean±SE of determinations was made in triplicate experiments.

Fig. 18. RP-HPLC re-chromatogram of potent ACE inhibitory peak (F2-B-I) isolated from RP-HPLC. Separation was performed with linear isocratic of 20% acetonitrile for 50 min at flow rate 1.0 ml/min. Elution was monitored at 215 nm.

LIST OF TABLE

Table 1. Optimal conditions of proteases.

Table 2. Chemical compositions of *Styela plicata*.

Table 3. Yield of enzymatic digests from *S. plicata*.

Table 4. Protein contents of enzymatic digests from *S. plicata*

Table 5. Yield of various conditions of Protamex digest from *S. plicata*.

Table 6. Protein contents of various conditions of Protamex digest from *S. plicata*.

Table 7. IC_{50} value of Protamex digests from *S. plicata*.

Table 8. IC_{50} value of molecular weight fractions of Protamex digest.

Table 9. IC_{50} value of peaks of gel filtration chromatography from below 5 kDa fraction.

Table 10. IC_{50} value of peaks of RP-HPLC from F2 peak.

Table 11. IC_{50} value of peaks of RP-HPLC from F2-B peak.

Table 12. Purification of ACE inhibitory peptides from *S. plicata* enzymatic digest.

Abstract

Enzymatic digests prepared from *Styela plicata* by treatment with various proteases including Protamex, Kojizyme, Neutrase, Flavourzyme, Alcalase, Trypsin, α-Chymotrypsin, Protease, Pepsin and Papain, and their the angiotensin I-converting enzyme (ACE) inhibitory activities were investigated. Under the same concentration, the Protamex digest had the highest ACE inhibitory activity (72.21% at 2.5 mg/ml) compared to the other digests. The Protamex digest was performed optimum conditions assay for production of ACE inhibition peptides from *S. plicata*. The Protamex digest had the highest ACE inhibitory activity by conditions of enzyme concentrations of 10% for 48 h compared to the other conditions. We attempted to isolate ACE inhibitory peptides from Protamex digest of *S. plcata* using ultra-filtration and ACE inhibitory activity was measured. Below 5 kDa molecular weight fraction showed the highest ACE inhibitory activity (IC_{50} value 0.828 mg/ml). Next, below 5 kDa molecular weight fraction was separated on Sephadex G-25 gel filtration chromatography and ACE inhibitory activity was measured. The F2 peak showed the highest ACE inhibitory activity (IC_{50} value 0.319 mg/ml). The F2 peak was separated on reverse-phase column by high performance liquid chromatography (HPLC) and ACE inhibitory activity was measured. F2-B peak showed the highest ACE inhibitory activity (IC_{50} value 0.076 mg/ml). The F2-B-I from F2-B was divided into single peaks on RP-HPLC. The IC_{50} value of purified ACE inhibitory peptide was 0.014 mg/ml. The results of this study suggested that peptides derived from enzymatic digests of *S. plicata* would be useful new antihypertension compounds in functional food. However, further work is being carried out to confirm its *in vivo* physiological effects in blood pressure regulation.

1. Introduction

Recently, hypertension is major problem threatening human health in the world. It was estimated that there is about 20% of the world's adult population (Alper *et al,* 2001). Hypertension kind of a chronic disease causes problems of the circulatory organ as well as acts as the dangerous factor such as arteriosclerosis, stroke and myocardial infarction. In case hypertension develops the complication with cerebral hemorrhage, heart disease and nephropathy, the lethality can be very high (Unger *et al*, 2002; Je *et al.*, 2005). Hypertension has been mainly explained as the physiological and chemical mechanism by renin-angiotensin system (RAS) and substance that can inhibit the activation of angiotensin I converting enzyme is focused to directly inhibit hypertension.

Angiotensin I converting enzyme (ACE, EC 3. 4. 15. 1) discovered in vascular, heart, lung and brain tissue plays the important role as dicarboxy peptidase (Lapointe and Rouleau, 2002) in renin-angiotensin system (RAS) and kallikrein-kinin system (KKS) (Fig. 1). ACE converts angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) activated from angiotensinogen by renin in RAS into angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) that continuously acts in various kinds of AT receptor existing in adrenal, kidney and heart. AT receptor causes the contraction of vascular, the release of both aldosterone and vasopressin, the reduction of blood flow in the kidney and the renal tubule to absorb sodium and these lead to various diseases in the heart vascular (Unger, 2002). The ACE is a zinc-containing exopeptidase enzyme located in the endothelial lining of the vasculature of the lung and cleaves dipeptides at the C-terminus of oligopeptides (Jung *et al.*, 2006). In particular, ACE involves cleaving histidyl-leucine of angiotensin I forming angiotensin II, and degrades bradykinin (vasodepressor) to inactive peptides

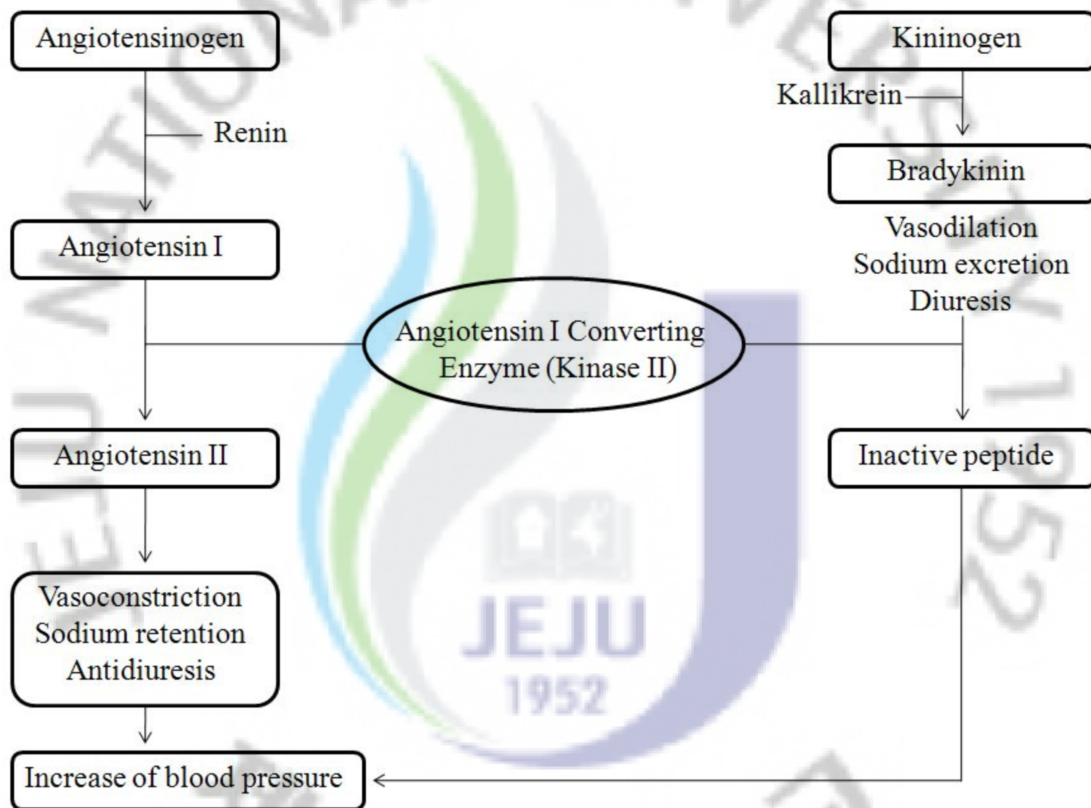

Fig. 1. Proposed reaction mechanism catalyzed by angiotensin I converting enzyme (Kininase II).

angiotensin II, and degrades bradykinin (vasodepressor) to inactive peptides (Dzau, 2001). These activities of ACE in RAS and KKS cause various problems in relation of the heart's blood, therefore, ACE inhibitor can effectively reduce hypertension as well as nephropathy, arteriosclerosis and heart attack (Thurman *et al.*, 2003; Bakris, 2001).

To help prevent and treat hypertension, inhibition of ACE has been a widely using therapeutic strategy and the literature on ACE inhibitory compound is quite extensive.

Since the discovery of ACE inhibitor, snake venom (Ondetti *et al.*, 1971), many studied have been directed the attempted synthesis of ACE inhibitors have been used extensively in the treatment of essential hypertension and heart failure in humans, such as alacepril, captopril, benazepril, enalapril, fosinopril, ramipril and zofenopril. However, these synthesis compounds are believed to have certain side effects, such as cough, taste disturbances and skin rashes (Kato and Suzuki, 1971; Sawayama *et al.*, 1990; Ondetti MA., 1977). Therefore a search for ACE inhibitors from natural products has become a major area of research.

Many ACE inhibitory peptide have been isolated from various protein digests such as canola (Jianping *et al.*, 2008), peanut (Wang *et al.*, 2008), bovine casein (Contreras *et al.*, 2009), porcine muscle (Miguel and Fidel, 2007), egg white (Miguel *et al.*, 2007), yak milk casein (Maoa *et al.*, 2007), sheep milk yoghurt (Papadimitriou *et al.*, 2007) and soybean (Chen *et al.*, 2002). Also various ACE inhibitory peptides have been isolated from marine resources protein such as oyster (Wang *et al.*, 2007), sardinelle (Ali *et al.*, 2008), hard clam (Tsai *et al.*, 2008), Alaska pollak (Byun and Kim, 2001).

S. plicata used for this study, a marine resource, inhabits in Korea and the Far East Asia. Aquaculture of *S. plicata* also has been made only in Korea and used for food. Studies on bio active from *S. plicata* such as anticoagulant

Fig. 2. The photograph of *Styela plicata*.

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activity (Joana *et al*., 2008), antioxidant and anticancer activity (Kim *et al*., 2005) have been performed before.

The purpose of the work reported here was to isolate ACE inhibitory peptides derived from enzymatic digests of *S. plicata* and the characterization of the isolated peptides with respect to their inhibitory effects on ACE (Fig. 3).

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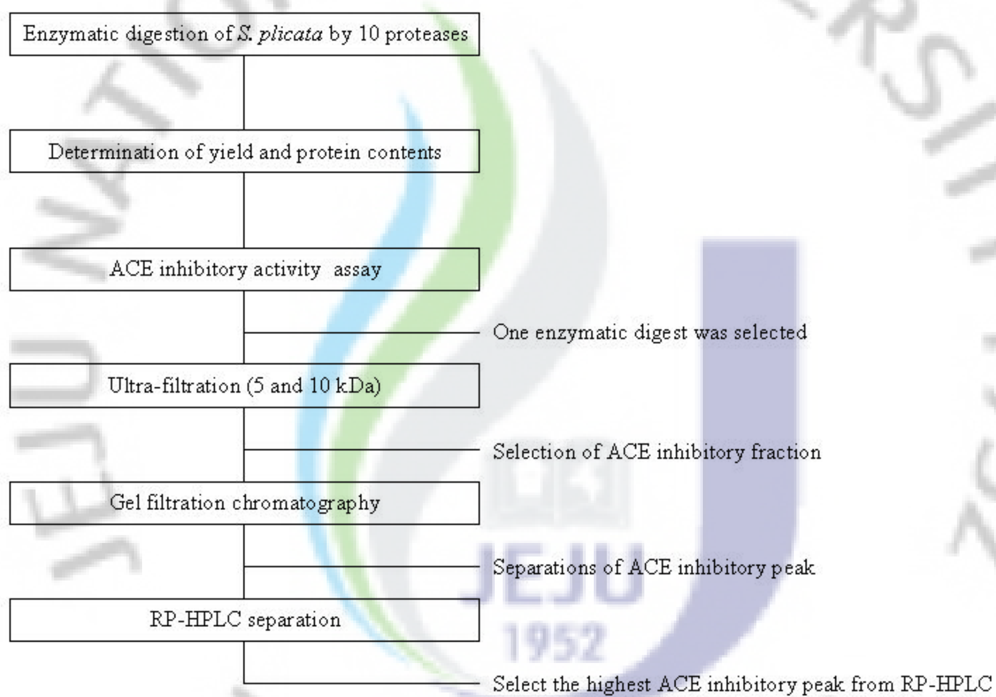

Fig. 3. Flow chart for the separation of ACE inhibitory peptide from *Styela plicata*.

2. Materials and Methods

2. 1. Materials

Styela plicata was collected along the coast of Masan in Korea, during the period from August to October 2008. Salt, sand and epiphytes were removed using tap water. Finally, the *S. plicata* were rinsed carefully in freshwater and stored at -20℃ for further experiments. The frozen sample was lyophilized and homogenized with a grinder before extraction. Ten proteases including Protamex, Kojizyme 500 MG, Neutrase 0.8L, Flavourzyme 500MG, Alcalase 2.4L FG were obtained from Novo Co. (Novozyme Nordisk, Bagsvaerd) and Trypsin, α-Chymotrypsin, Protase, Pepsin, Papain were obtained from Sigma chemical Co. (St. Louis, Mo, USA). The optimum pH, optimal temperature and characters of those enzymes were summarized in Table 1-1. N-Hippuryl-His-Leu tetrahydrate (HHL) and Angiotensin I Converting Enzyme (from rabbit lung) were obtained from Sigma chemical Co. (St. Louis, Mo, USA). The other chemicals and reagents used were of analytical grade.

2. 2. Approximate chemical composition of dried *S. plicata*

Approximate chemical composition of dried *S. plicata* was determined according to AOAC method (1990). Crude carbohydrate was determined by phenol-sulfuric acid reaction (absorbance at 480 nm, using glucose as the calibration standard), crude lipid was performed by Soxhlet method and crude ash was prepared at 550℃ in the dry-type furnace. The moisture was determined keeping in a dry oven at 105℃ for 24 h and the crude protein was determined by Kjeldahl method.

2. 3. Preparation of enzymatic digests from *S. plicata*

The enzymatic digests from *S. plicata* were obtained according to the method used by Jung *et al* (2006) and Heo *et al* (2002). Ten enzymes (Protamex, Kojizyme, Neutrase, Flavourzyme, Alcalase, Trypsin, α-Chymotrypsin, Protease, Pepsin and Papain) were used to treated to the powder of *S. plicata* under optimal conditions (Table 1) for 24 h. One gram of the ground dried *S. plicata* powder was homogenized with water (100 ml), and then 10 mg or 100 ㎕ enzyme was added. The pHs of the homogenate were adjusted to its optimal ph value before the digestion. The enzymatic reactions were performed for 24 h to achieve optimum degree of the digest. As soon as the enzymatic reaction is complete, the digests were boiled for 10 min at 100℃ to inactive the enzyme. Each enzymatic digest was clarified by centrifugation (3500 rpm, for 20 min at 4℃) to remove the residue (Fig. 4). All the enzymatic digests were kept -20℃ for further experiments.

2. 4. Measurement of enzymatic digestion yield

Yield of the enzymatic digests of *S. plicata* were calculated by dividing the dry weight of extracts filtrate over dry weight of the *S. plicata* enzymatic digest used.

2. 5. Determination of protein content

The amount of crude protein was determined by the Lowry method, absorbance at 540 nm using bovine serum albumin as the calibration standard (Lowry *et al.*, 1951).

2. 6. Optimum-conditions assay for the active digestion

Active digestion was performed optimum conditions assay for ACE inhibition digest from *S. plicata*. One gram of the ground dried *S. plicata* powder was homogenized with water (100 ml), and then various enzyme rate (0.2, 1, 5

Table 1. Optimal conditions of proteases.

Enzyme	Sources	Optimal	
		pH	Temperature(℃)
Protamex	*Bacilus* sp.	6.0	40
Kojizyme	A. *oryzae*	6.0	40
Netrase	*Bacillus amyloliquefaciens*	6.0	50
Flavourzyme	A. *oryzae*	7.0	50
Alcalase	*Bacillus* sp.	8.0	50
Pepsin	Porcine gastric mucosa	2.0	37
Protease	A. *oryzae*	2.8	37
Trypsin	Bovine pancreases	7.6	25
α-Chymotrypsin	Bovine pancreases	7.8	25
Papain	Papaya latex	6.2	25

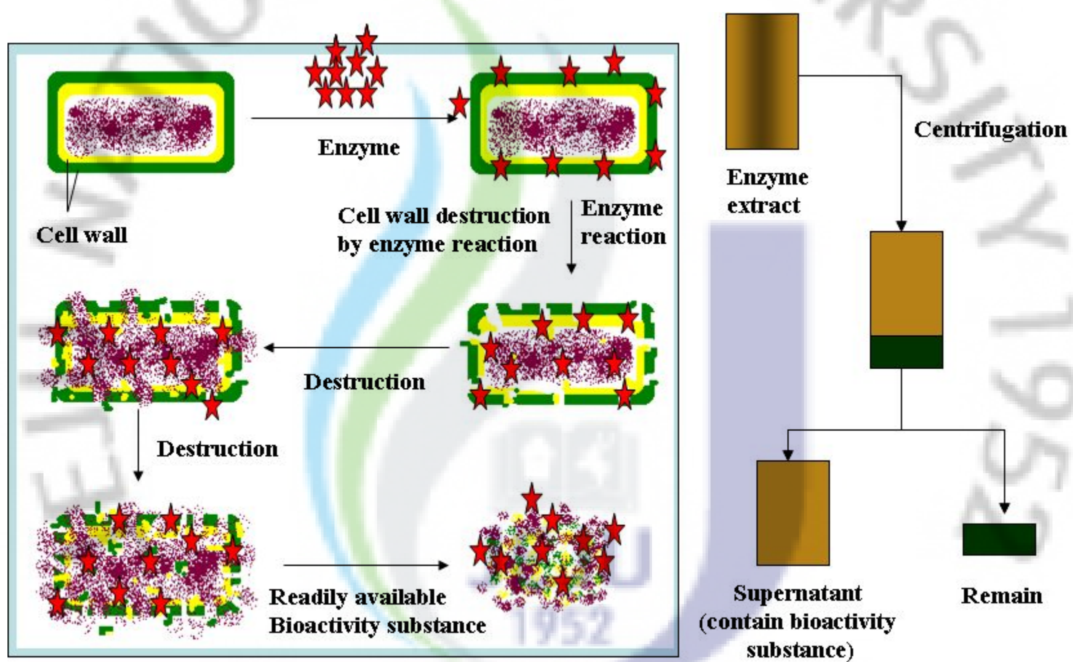

Fig. 4. Preparation of enzymatic digests.

and 10%) was added. The pHs of the homogenate were adjusted to its optimal ph value before the digestion of *S. plicata.* The enzymatic reactions were performed for various times from 6, 12, 24 and 48 h. Yield, protein contents and ACE inhibitory activities of the active digest formed at different enzyme rate and incubation times upon digestion were measured.

2. 7. ACE inhibitory activity

For each assay, 50 ㎕ of the sample solution with 50 ㎕ of ACE solution (25 mU/ml) was pre-incubated at 37℃ for 10 min, and then incubated with 100 ㎕ of substrate (25 mM hippuryl-His-Leu in 50 mM sodium borate buffer containing 500 mM NaCl at pH 8.3) at 37℃ for 60 min. The reaction stopped by adding 250 ㎕ of 1 N HCl. Hippuric acid was extracted with 500 ㎕ of ethyl acetate. Then a 200 ㎕ aliquot of the extract was removed by evaporation in an dry-oven at 80℃. The residue was dissolved in 1 ml distilled water and its UV spectra density was measured at 228 nm. The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of ACE inhibitory activity.

The extent of inhibition was calculated as follows:

Inhibition %=(Ac-As)/(Ac-Ab)

Ac=Absorbance of control sample

As=Absorbance of sample solution

Ab=Absorbance of blank solution

2. 8. Purification of ACE inhibitory peptide

2. 8. 1. Molecular weight fractionation of active enzymatic digest

In all the tested enzymatic digests for ACE inhibitory activity of enzymatic digests, one enzymatic digest from *S. plicata* subjected to molecular weight fractionation to obtain the peptide with molecular weight of above 10 kDa,

5-10 kDa and below 5 kDa. Enzymatic digest was passed through ultra-filtration membranes (5 and 10 kDa) using Millipore's Labscale TFF system (Millipore Corporation, Bedford, Massachusetts, USA) at 4℃ (Fig. 5). The resultant, fractions were then collected according to molecular weights (above 10 kDa, 5-10 kDa and below 5 kDa) were lyophilized and stored at -20℃ for use in further experiments.

2. 8. 2. Gel filtration chromatography

The most active fraction by Ultra-filtrated digest was again filtered and applied to a column (∅ 2.5 × 75 cm) saturated in Sephadex G-25 resin which was previously equilibrated with distilled water. The flow rate was 1.5 ml/min, and elution peaks were monitored at 215 nm. The peaks were collected at 5 min intervals with a peak collector, and peaks showing ACE inhibitory activity were pooled and lyophilized. The active ACE inhibition peak was applied onto the RP-HPLC for further purification.

2. 8. 3. Isolation of peptide by reversed-phase HPLC (RP-HPLC)

The fraction with the highest ACE inhibitory activity was dissolved in distilled water, and separated by reversed-phase high performance liquid chromatography (HPLC) on a symmetry Grom-sil 120 ODS-5 ST column (Alltech, C_{18}, ∅10 × 250 mm). For RP-HPLC analysis, mobile phases used in the gradient elution consisted of eluant A consisting of 0.1% trifluoroacetic acid (TFA) in distilled water (v/v); eluent B of 0.1% trifluoroacetic acid (TFA) in acetonitrile. The separation was performed with a linear gradient from 0 to 25% eluent B at a flow rate of 2.0 ml/min. The UV absorbance of the eluent was monitored at 215 nm. The fractions showing ACE inhibitory activity were pooled and lyophilized.

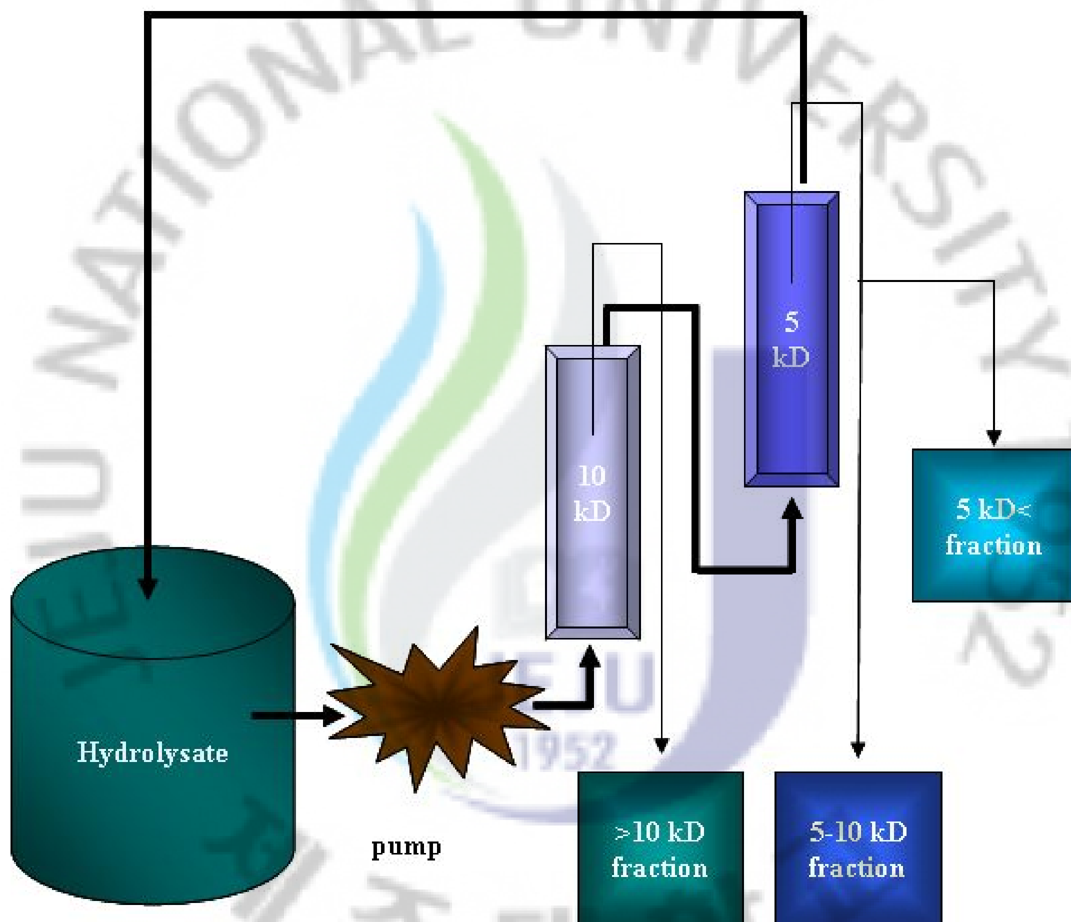

Fig. 5. Ultra-filtration membrane system for fraction of *S. plicata* enzymatic digest according to the molecular weight of constituents.

3. Results and Discussion

3. 1. Approximate chemical composition

Approximate chemical compositions *S. plicata* was shown in Table 2. The major chemical component of *S. plicata* tested was found to be carbohydrate whose content occupied over 40% of the total dry weight. Protein contents determined from *S. plicata* was 33.12%. Ash content determined from *S. plicata* was 10.77%. Moisture and lipid content determined from *S. plicata* was 9.34 and 4.25%.

3. 2. Enzymatic digestion yield

The yield of enzymatic digests were determined by subtracting the dried weight of residue from one gram of *S. plicata* sample dried and was expressed as a percentage (Heo *et al*., 2005). The Enzymatic digestion yield from *S. plicata* was shown in Table 3. The each enzymatic digests were showed different yield and almost enzymatic digests showed relatively higher yield compared with the aqueous extract. But, Pepsin digest was showed lower yield compared with the aqueous extract. The highest yield (36.8%) was recorded in the Neutrase digest from *S. plicata,* whereas the lowest yield (18.7%) was shown the by Pepsin digest from *S. plicata.*

3. 3. Protein content

All enzymatic digests were subjected to Lowry method (1951) to determine their protein contents. The protein contents of each of the enzymatic digests are shown in the Table 4. The each enzymatic digests were showed different yield and almost enzymatic digests showed relatively higher yield compared with the aqueous extract. But, Kojizyme digest was showed lower protein content compared with the aqueous extract. The proteases can work primarily

by macerating the tissues and breaking down the cell walls and complex interior storage materials (Siriwardhana *et al.*, 2008). Therefore, enzymatic digests have higher yield and protein content than aqueous extract. The highest protein content (435.8 mg/g) was recorded in the Protamex digest from *S. plicata,* whereas the lowest protein content (171.61 mg/g) was shown the by Kojizyme digest from *S. plicata.*

3. 4. ACE inhibitory activity

The ACE inhibitory activity was assayed by measuring the concentration of hippuric acid liberated from hippuryl-His-Leu by the method of Cushman and Cheung (1970). The result of the ACE inhibitory activities of different enzyme digests of ten proteases (Protamex, Kojizyme, Neutrase, Flavourzyme, Alcalase, Trypsin, α-Chymotrypsin, Protease, Pepsin and Papain) and aqueous extract are shown in Fig. 6. All enzymatic digests of *S. plicata* showed more than 50% ACE inhibitory activities and dose-dependant activity over ACE inhibition. Specially, Protamex digest showed higher activities (65.25%) than that other digests. ACE inhibitory activity of Protamex digest from *S. plicata* increased gradually with the increased concentrations and were 37.24, 45.38, 58.94 and 65.25%. Compared to enzymatic digests, aqueous extract showed the lowest ACE inhibition (17.24%) for ACE inhibitory assay. Also, ACE inhibitory activity was dose-dependently increased with protein content.

Previous reports have shown that ACE inhibitory activity is affected by hydrolysis of protein. However, the reduction in the ACE inhibitory activity is assumed to be mainly due to aggregation of the protein caused by the high temperature treatment (Youshie-Stark *et al.*, 2006). Therefore, the Protamex digest was selected for use in further experiments.

Table 2. Chemical compositions of *S. plicata*.

Chemical composition	Content (%)
Moisture	9.34±0.2[a]
Ash	10.77±0.3
Protein	33.12±0.2
Carbohydrate	42.52±0.4
Lipid	4.25±0.4

[a]The values are averages of triplicate determinations.

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Table 3. Yield of enzymatic digests from *S. plicata*.

Enzymatic digests	Yield (%)
Neutrase	36.8±0.2[a]
Alcalse	29.3±0.3
Flavourzyme	25.3±0.1
Protamex	29.7±0.2
Kojizyme	20.8±0.2
Protase	25.2±0.2
α-chymotrypsin	34.3±0.2
Trypsin	25.7±0.3
Papain	23.3±0.1
Pepsin	18.7±0.2
Aqueous	19.8±0.1

[a]The values are averages of triplicate determinations.

Table 4. Protein contents of enzymatic digests from *S. plicata*

Enzymatic digests	Protein contents (mg/g)
Neutrase	421.61±4.46[a]
Alcalse	349.84±3.35
Flavourzyme	361.67±2.23
Protamex	435.80±2.23
Kojizyme	171.61±1.12
Protase	343.71±3.11
α-chimotrypsin	369.41±2.11
Trypsin	312.89±4.33
Papain	299.33±1.99
Pepsin	201.49±1.93
Aqueous	183.22±3.39

[a]The values are averages of triplicate determinations.

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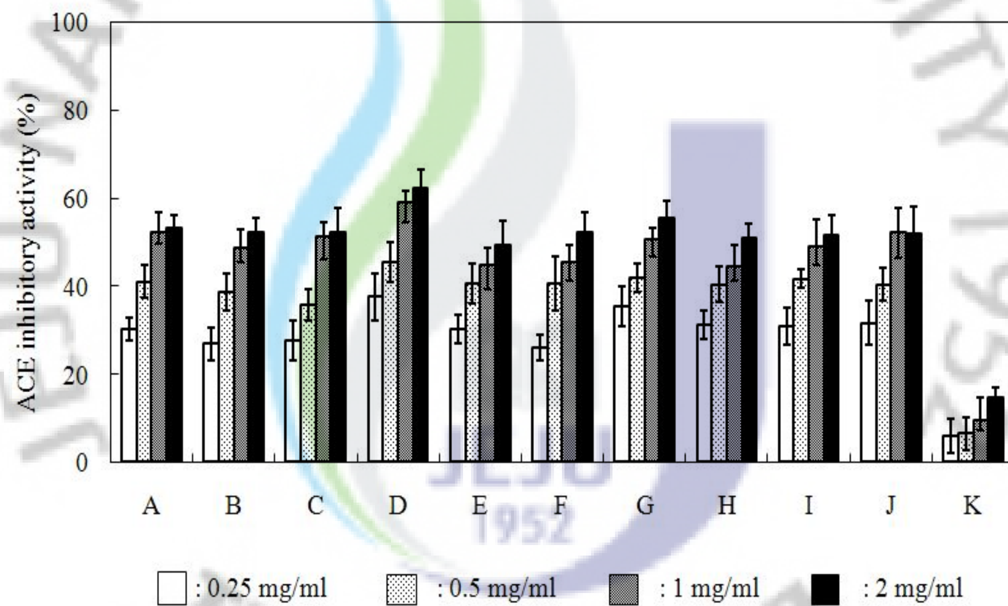

Fig. 6. ACE inhibitory activity of enzymatic digests from *S. plicata*. A, Neutrase; B, Alcalase; C, Flavourzyme; D, Protamex; E, Kojizyme; F, Protase; G, α-chimotrypsin; H, Trypsin; I, Papain; J, Pepsin; K, Aqueous.

Mean±SE of determinations was made in triplicate experiments.

3. 5. Optimum-conditions for the production of ACE inhibitory activity digest by Protamex

3. 5. 1. Yield

The yield of Protamex digests were determined by subtracting the dried weight of residue from one gram of *S. plicata* sample dried and was expressed as a percentage (Heo *et al*., 2005). The optimal digestion conditions for production of ACE inhibitory activity digest from *S. plicata* were investigated in the various enzyme rate (0.2, 1, 5 and 10%) and digestion times from 6, 12, 24 and 48 h. As the enzyme rate and digestion time were increased, the yield of Protamex digests was increased (Table 5). The highest yield of the Protamex digest (40.5%) of *S. plicata* was obtained when the digestion was performed at 10% for 48 h, whereas the lowest yield (11.5%) was shown the by Protamex digest at 0.2% for 6 h.

3. 5. 2. Protein contents

All Protamex digests were subjected to Lowry method (1951) to determine their protein contents. The optimal digestion conditions for production of ACE inhibitory activity digest from *S. plicata* were investigated in the various enzyme rate (0.2, 1, 5 and 10%) and digestion times from 6, 12, 24 and 48 h. As the enzyme rate of 0.2% and digestion time was increased, the of Protamex digests was increased (Table 6). The highest protein content of Protamex digest (435.59 mg/g) of *S. plicata* was obtained when the digestion was performed at 10% for 48 h, whereas the lowest protein content (182.99 mg/g) was shown the by Protamex digest at 0.2% for 6 h.

3. 5. 3. ACE inhibitory activity

The ACE inhibitory activity was assayed by measuring the concentration of hippuric acid liberated from hippuryl-His-Leu by the method of Cushman

and Cheung (1970). The ACE inhibitory activity of all Protamex digests were measured. The optimal digestion conditions for production of ACE inhibitory activity digest from *S. plicata* were investigated in the various enzyme rate (0.2, 1, 5 and 10%) and digestion times from 6, 12, 24 and 48 h. The enzyme rate of 0.2% and as the digestion time were increased, the ACE inhibitory activity of Protamex digests was increased (Fig. 7). The highest ACE inhibitory activity of Protamex digest (54.26%) of *S. plicata* was obtained when the digestion was performed for 48 h, whereas the lowest yield (30.25) was shown the by Protamex digest for 6 h. And, the enzyme rate of 1% and as the digestion time were increased, the ACE inhibitory activity of Protamex digests was increased (Fig. 8). The highest ACE inhibitory activity of Protamex digest (58.77%) of *S. plicata* was obtained when the digestion was performed for 48 h, whereas the lowest yield (39.44%) was shown the by Protamex digest for 6 h. Also, the enzyme rate of 5% and as the digestion time were increased, the ACE inhibitory activity of Protamex digests was increased (Fig. 9). The highest ACE inhibitory activity of Protamex digest (68.08%) of *S. plicata* was obtained when the digestion was performed for 48 h, whereas the lowest yield (45.24%) was shown the by Protamex digest for 6 h. Finaly, the enzyme rate of 10% and as the digestion time were increased, the ACE inhibitory activity of Protamex digests was increased (Fig. 10). The highest ACE inhibitory activity of Protamex digest (72.21%) of *S. plicata* was obtained when the digestion was performed for 48 h, whereas the lowest yield (46.43%) was shown the by Protamex digest for 6 h. The IC_{50} value of All Protamex digests shown in Table 5. The result, As the digestion time was increased, the yield, protein content and ACE inhibitory activity of the Protamex digest was increased. Previous reports have shown that the ACE inhibitory activity of digests was increased when the digestion time was increased (Lee *et al.*, 2004).

Table 5. Yield of various conditions of Protamex digest from *S. plicata*

Enzyme concentrations[a] (%)	Incubation time (h)			
	6	12	24	48
0.2	11.2±0.1[b]	20.3±0.1[c]	22.1±0.2	27.5±0.1
1	13.2±0.1	24.2±0.1	26.5±0.3	37.5±0.1
5	19.4±0.2	26.5±0.1	29.7±0.3	38.3±0.2
10	20.6±0.3	33.2±0.2	39.5±0.1	40.5±0.1

[a]The extent of enzyme concentration was calculated as follows:

Enzyme concentration %=(Enzyme/Substrate)×100

[b]The values are averages of triplicate determinations.

[c]Unit: %

Table 6. Protein contents of various conditions of protamex digest from *S. plicata*.

Enzyme concentrations[a] (%)	Incubation time (h)			
	6	12	24	48
0.2	182.99±1.88[b]	220.33±2.11[c]	267.66±4.22	301.32±1.55
1	196.36±0.95	242.67±1.08	295.12±1.23	331.34±2.23
5	200.18±3.31	277.97±0.9	331.11±3.45	383.32±2.91
10	200.14±3.33	312.39±1.89	415.22±1.39	435.59±3.23

[a]The extent of enzyme concentration was calculated as follows:

Enzyme concentration %=(Enzyme/Substrate)×100

[b]The values are averages of triplicate determinations.

[c]Unit: mg/g

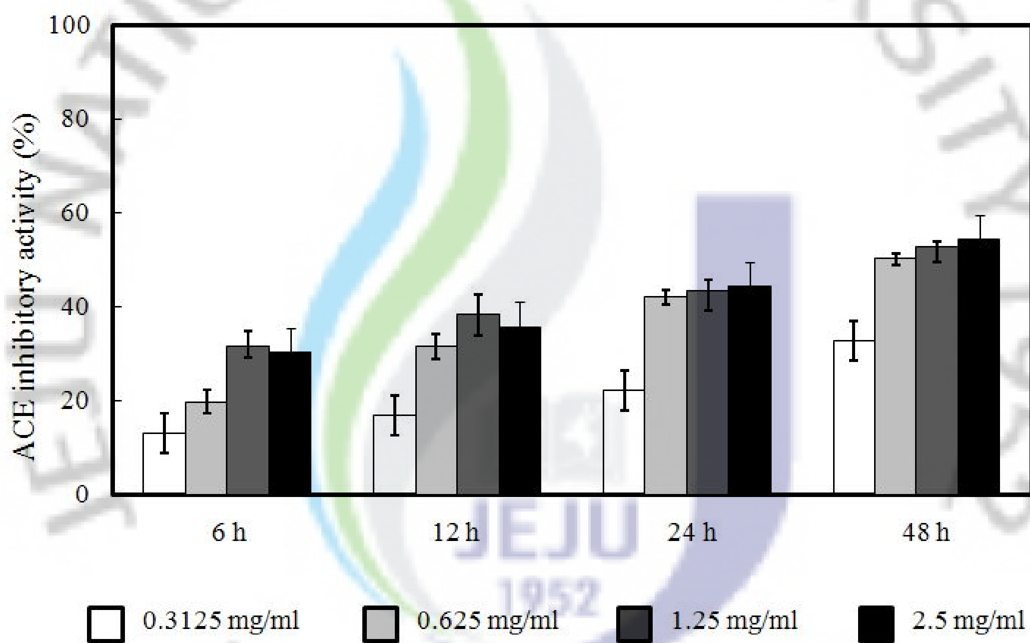

Fig. 7. The 0.2% ratio of enzyme to substrate on ACE inhibitory activity of Protamex digest from *S. plicata*. The Digestion times for ACE inhibitory assay were 6, 12, 24 and 48 h.

Mean±SE of determinations was made in triplicate experiments.

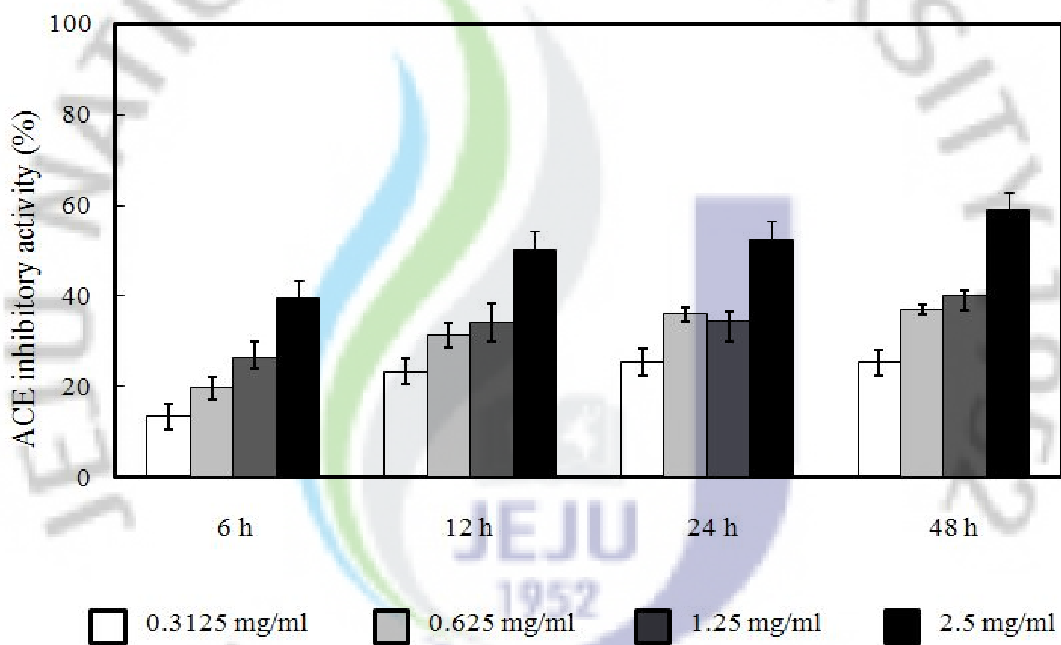

Fig. 8. The 1% ratio of enzyme to substrate on ACE inhibitory activity of Protamex digest from *S. plicata*. The Digestion times for ACE inhibitory assay were 6, 12, 24 and 48 h.

Mean±SE of determinations was made in triplicate experiments.

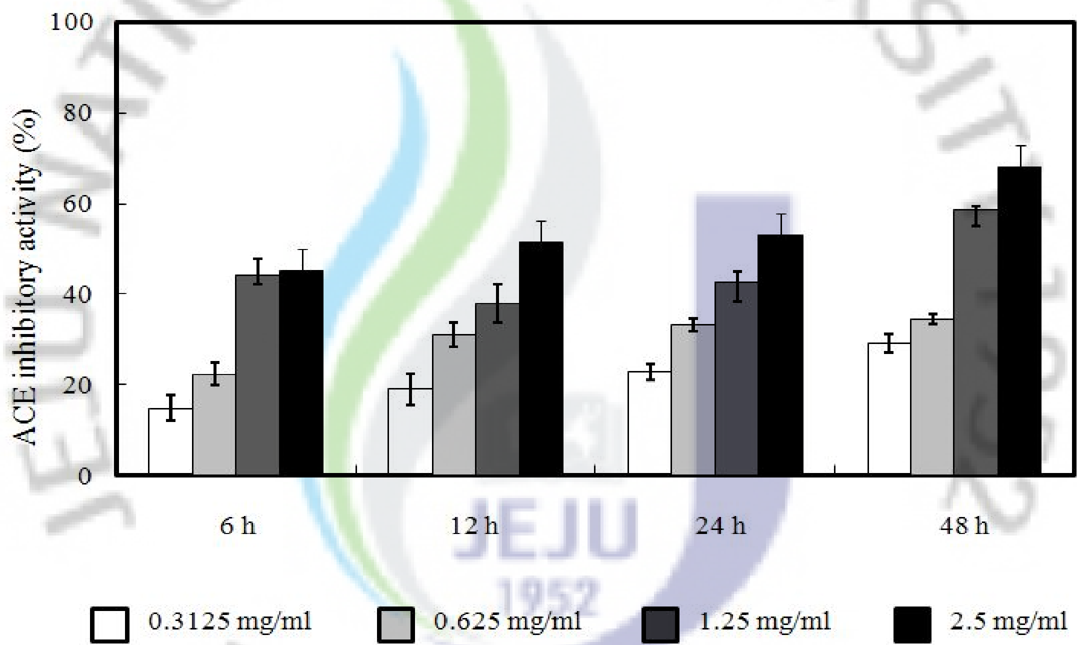

Fig. 9. The 5% ratio of enzyme to substrate on ACE inhibitory activity of Protamex digest from *S. plicata*. The digestion times for ACE inhibitory assay were 6, 12, 24 and 48 h.

Mean±SE of determinations was made in triplicate experiments.

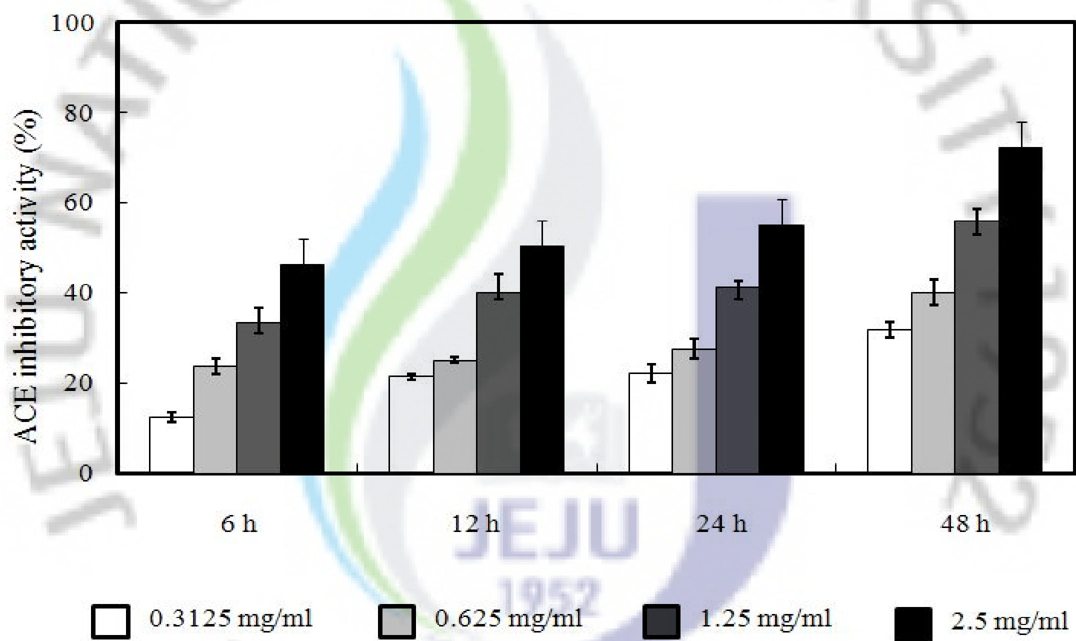

Fig. 10. The 10% ratio of enzyme to substrate on ACE inhibitory activity of Protamex digest from *S. plicata*. The Digestion times for ACE inhibitory assay were 6, 12, 24 and 48 h.

Mean±SE of determinations was made in triplicate experiments.

Table 7. IC_{50} value of Protamex digests from *S. plicata*

Enzyme concentration[a] (%)	IC_{50} values (mg/ml) Incubation time (h)			
	6	12	24	48
0.2	2<	2.499	2.233	2.333
1	2<	2.498	2.367	1.916
5	2<	2.313	2.126	1.245
10	2<	2.399	2.065	1.171

[a]The extent of enzyme concentration was calculated as follows:

Enzyme concentration %=(Enzyme/Substrate)×100

3. 6. Purification and characterization of ACE inhibition peptides

3. 6. 1. ACE inhibitory activity of Molecular weight fractions

The fractionation different molecular weights of the digests is the use of a membrane reactor using ultra-filtration (UF) membranes with different pore size (Byun and Kim, 2001). The molecular weight of the hydrolyzed protein is important factors in producing protein hydrolysates with desired functional materials. The UF system has the major advantage that the molecular weight distribution of the desired digests can be controlled by adoption of an appropriate UF membrane (Jeon *et al.*, 2000). In this study, the fractionation of enzymatic digest of *S. plicata* using UF membranes is described. The characterization of the separated fractions having functional peptides such as ACE inhibitory activity was carried out.

Protamex digest was separated by using tow kinds of UF membranes (10 kDa and 5 kDa MWCO membranes) according to molecular size and three kinds of permeates (above 10 kDa, 5-10 kDa and below 5 kDa digests) were obtained. Thus, individual fractions were assayed for ACE inhibitory activity. The result of this study is shown in Fig. 11. Below 5 kDa molecular weight fraction showed highest ACE inhibitory activity (IC_{50} value 0.828 mg/ml) (Table 6). And at the same concentration, below 5 kDa molecular weight fraction showed higher ACE inhibitory activity than the above 10 and 5-10 kDa molecular weight fractions. In addition, the ACE inhibitory activity of those molecular weight fractions increased with increasing concentrations from 0.3125 mg/ml to 2.5 mg/ml. This observation suggests that continuous enzymatic digest with different fractionation of resulting digests using different UF membranes produced small molecular weight digests having ACE inhibitory activity.

Previous reports have shown that yellowfin frame protein hydrolysate was fractionated of high and low molecular weight by UF membranes. The low

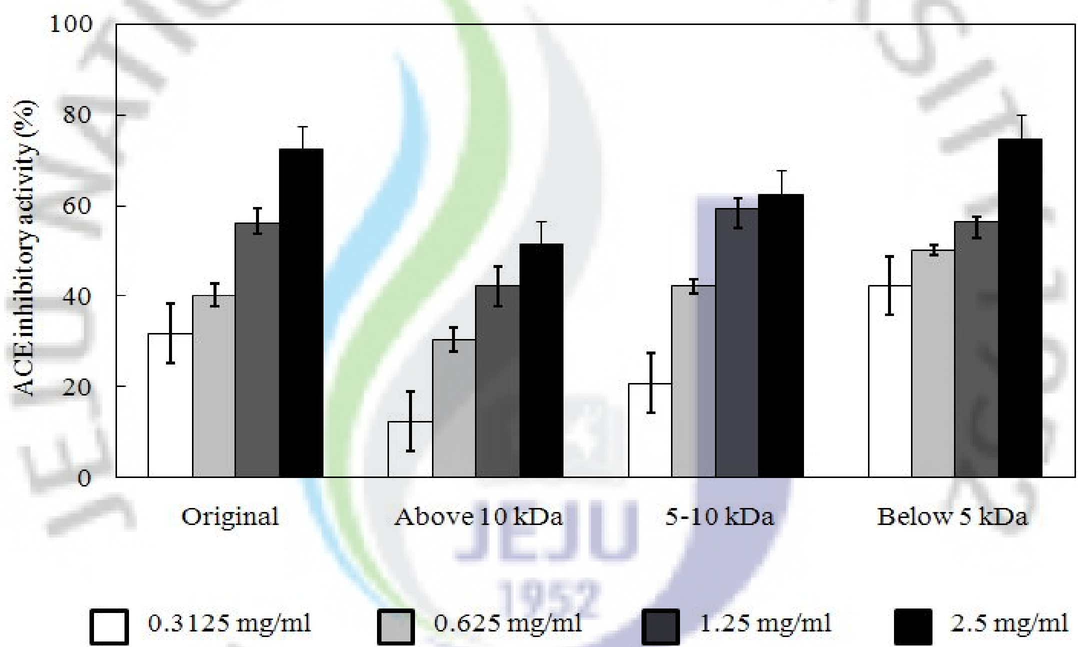

Fig. 11. ACE inhibitory activity of molecular weight fractions of Protamex digest.

Mean±SE of determinations was made in triplicate experiments.

Table 8. IC_{50} value of molecular weight fractions of Protamex digest.

Fractions	IC_{50} value (mg/ml)
Original	1.171
Above 10 kDa	2.209
5-10 kDa	1.023
Below 5 kDa	0.828

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molecular weight fraction had more potent ACE inhibitory activity than that fractionated of high and low molecular weight by UF membranes. The low molecular weight fraction had more potent ACE inhibitory activity than that of the high molecular weight (Jung and Kim, 2006). Zhao *et al* (2007) reported that, with respect to sea cucumber gelatin hydrolysate, ACE inhibitory activity markedly increased with increasing proteolysis. In this study, Protamex contributed to a high degree of digest due to their different enzymatic actions, and the UF was an successful method to enhance its ACE inhibitory activity of enzymatic digest from *S. plicata*. Therefore, the was selected Bellow 5 kDa molecular weight fractionfor use in further experiments.

3. 6. 2. Size exclusion chromatography

For the purification of ACE inhibitory activity peptide, the most activity fraction was filtered through a UF membrane (molecular weight cut-off; 5 kDa) by UF system. The below 5 kDa molecular weight fraction was lyophilized and concentrated in distilled water. It was load on a Sephadex G-25 column, and then selected four peaks (Fig. 12) that were measured to have ACE inhibitory activity. The ACE inhibitory activity of fractions from F2 were investigated in the various concentrations (0.3125, 0.625, 1.25 and 2.5 mg/ml). All peaks as the concentration were increased, the ACE inhibitory activity of fractions were increased. The ACE inhibitory activity is shown in Fig. 13. But, not all peaks showed ACE inhibitory activity. Specially, 2nd-fraction (F2) showed higher activities (IC_{50} value 0.319 mg/ml) (Table 6) than that other fractions. ACE inhibitory activity of F2 from below 5 kDa molecular fraction increased gradually with the increased concentrations and were 43.75, 65.25, 80.69 and 88.65%. Compared to fractions, 4th-peak (F4) showed the lowest ACE inhibition (20.28, 27.82, 28.57 and 35.33%) for ACE inhibitory assay.

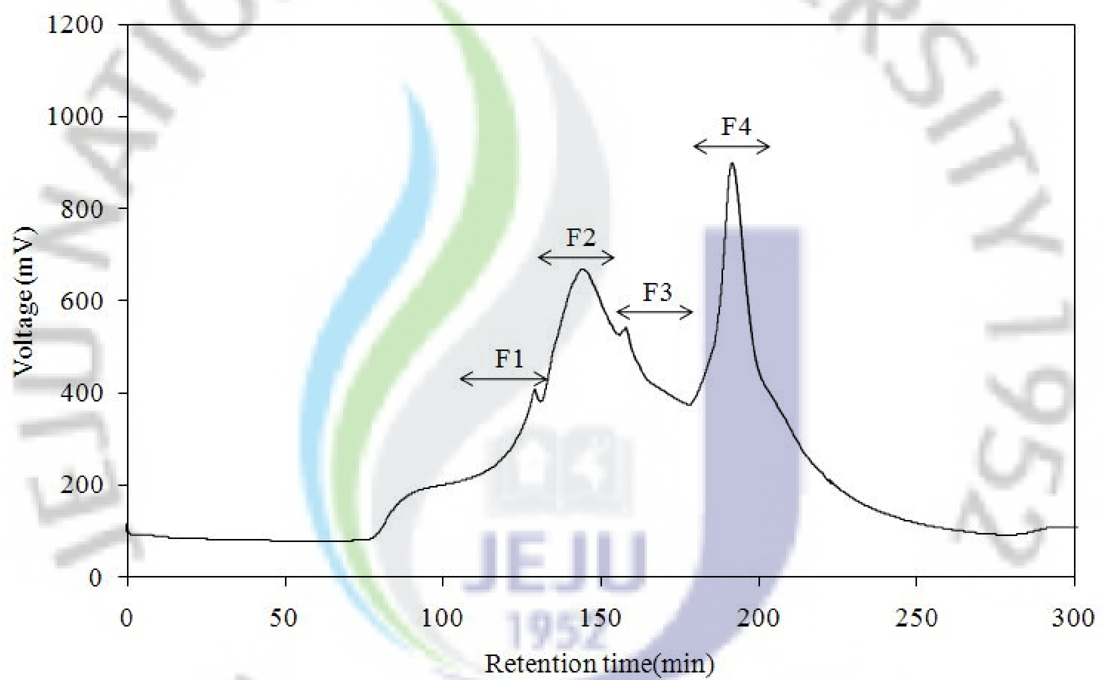

Fig. 12. Gel filtration chromatography of below 5 kDa molecular weight fraction from Protamex digest on Sephadex G-25 column. Separation was performed with 1.5 ml/min and collected at a peak volume (7.5ml). The peaks isolated by Sephadex G-25 gel column were designated F1-F4. Elution was monitored at 215 nm.

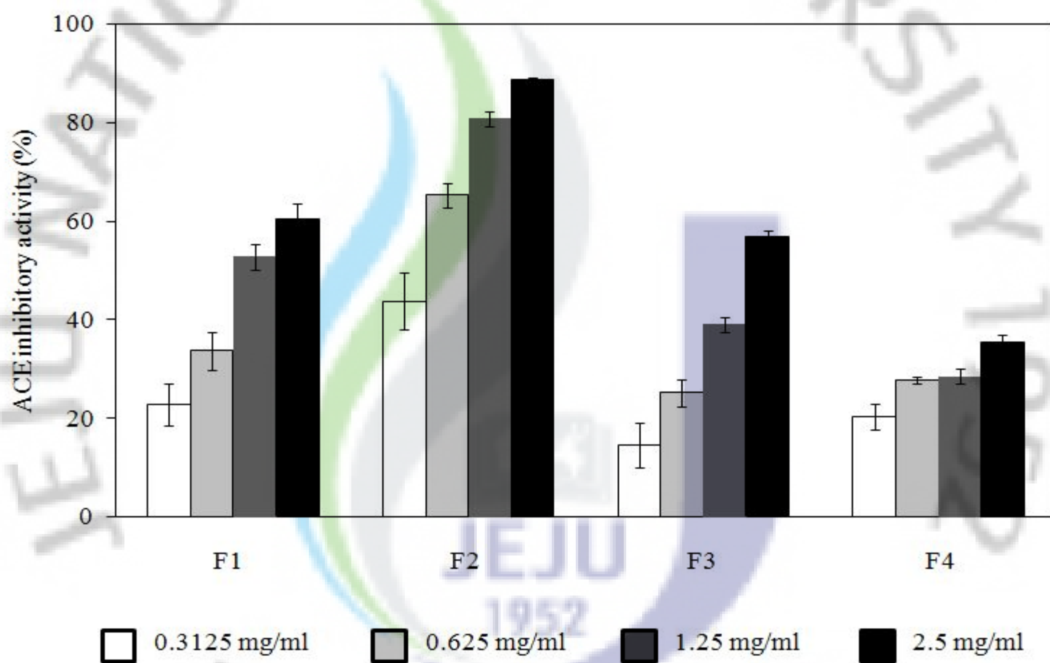

Fig. 13. ACE inhibitory activity of peaks on gel filtration chromatography from below 5 kDa molecular weight fraction.

Mean±SE of determinations was made in triplicate experiments.

Table 9. IC_{50} value of peaks of gel filtration chromatography from below 5 kDa fraction.

Peaks	IC_{50} value (mg/ml)
F1	1.15
F2	0.39
F3	2.035
F4	2.5<

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3. 6. 3. Purification of peptide by reverse-phase HPLC

The F2 (Fig.12), identified as the most active peak by gel filtration chromatography, was performed reverse-phase column by high performance liquid chromatography (HPLC) on Grom-sil 120 ODS-5 ST column, and then eluted with acetonitrile under linear gradient elution condition of 0-25%, in order to improve the separation of the peptide. The F2 was divided into three major peaks on RP-HPLC (Fig. 14), all of which were collected, concentrated and the ACE inhibitory activity was measured. The ACE inhibitory activity of peaks from F2 were investigated in the various concentrations (0.0781, 0.1563, 0.3125, 0.625, 1.25 and 2.5 mg/ml). All peaks as the concentration were increased, the ACE inhibitory activity of peaks was increased. The ACE inhibitory activity is shown in Fig. 15. But, not all fractions showed ACE inhibitory activity. Specially, 2nd-peak (F2-B) showed higher activities (IC_{50} value 0.076 mg/ml) (Table 7) than that other peaks. ACE inhibitory activity of F2-B from F2 increased gradually with the increased concentrations and were 46.62, 53.12, 60.78, 71.67 and 79.02%. Compared to peaks, 1st-fraction (F2-A) showed the lowest ACE inhibition (5.68, 12.57, 19.43, 31.78 and 37.38 mg/ml) for ACE inhibitory assay. The active peak F2-B was subjected to re-chromatography on the same column using eluted with acetonitrile under linear gradient elution condition of 0-100%, in order to improve the separation of the peptide. The F2-B was divided into four major peaks on RP-HPLC (Fig. 16), all of which were collected, concentrated and the ACE inhibitory activity was measured. Specially, 1st-peak (F2-B-I) showed higher activities (IC_{50} value 0.015 mg/ml) (Table 8). ACE inhibitory activity of F2-B-I from F2-B increased gradually with the increased concentrations (0.0125, 0.025, 0.05 and 0.1 mg/ml) and were 43.25, 63.28, 77.47 and 89.25%. Compared to peaks, 3th-peak (F2-A-III) showed the lowest ACE inhibition (4.35, 17.45, 36.22 and 51.34%l) for ACE inhibitory assay (Fig. 17). The active peak F2-B-I was

subjected to re-chromatography on the same column using a linear isocratic of 20% acetonitrile for 50 min, at flow rate 1 ml/min. The F2-B-I was divided into single peaks on RP-HPLC (Fig. 18). Based on this result, the IC_{50} value of the purified inhibitor was 0.014 mg/ml. Table 12 summarizes the results of purification of the ACE inhibitor from *S. plicata.* The ACE inhibitor was purified 83.64-fold from *S. plicata* using a four-step purification procedure.

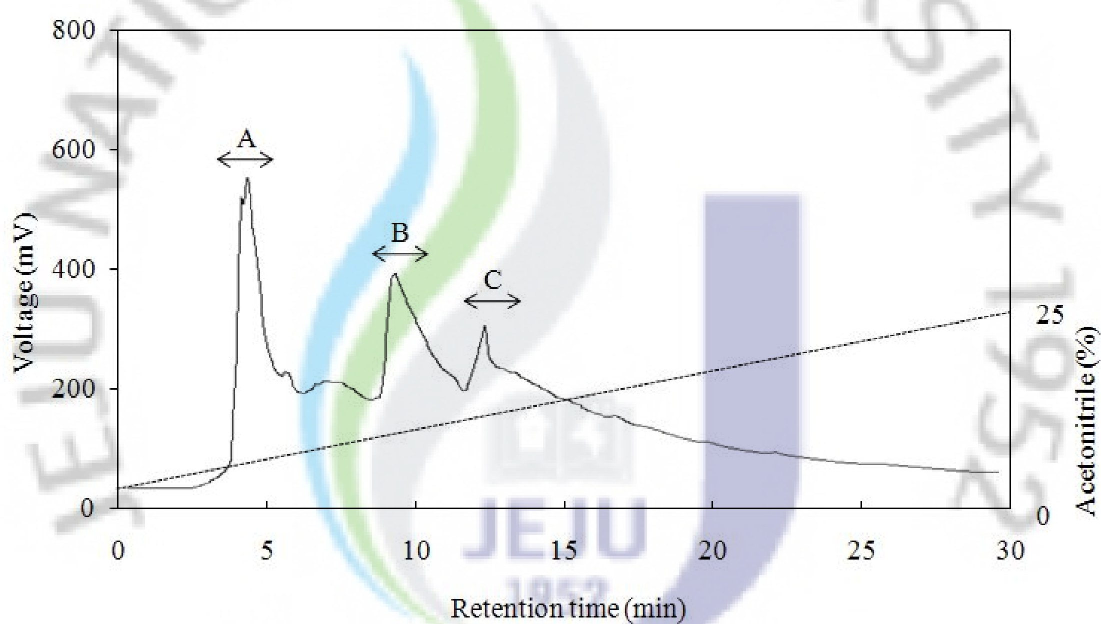

Fig. 14. RP-HPLC chromatogram of potent ACE inhibitory peak (F2) isolated from Sephadex G-25. Separation was performed with linear gradient of acetonitrile from 0% to 25% in 30 min at a flow rate of 1.0 ml/min. The fractions isolated by RP-HPLC were designated A-C. Elution was monitored at 215 nm.

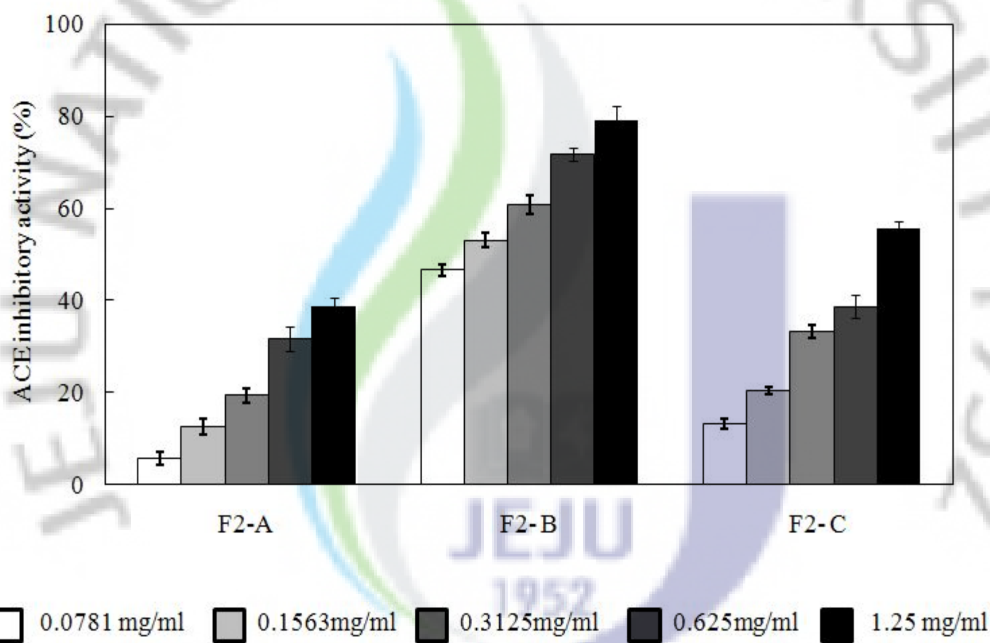

Fig. 15. ACE inhibitory activity of peaks on RP-HPLC from active peak (F2) by gel filtration chromatography.

Mean±SE of determinations was made in triplicate experiments.

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Table 10. IC_{50} value of peaks of RP-HPLC from F2 peak.

Peaks	IC_{50} value (mg/ml)
F2-A	1.25<
F2-B	0.076
F2-C	1.043

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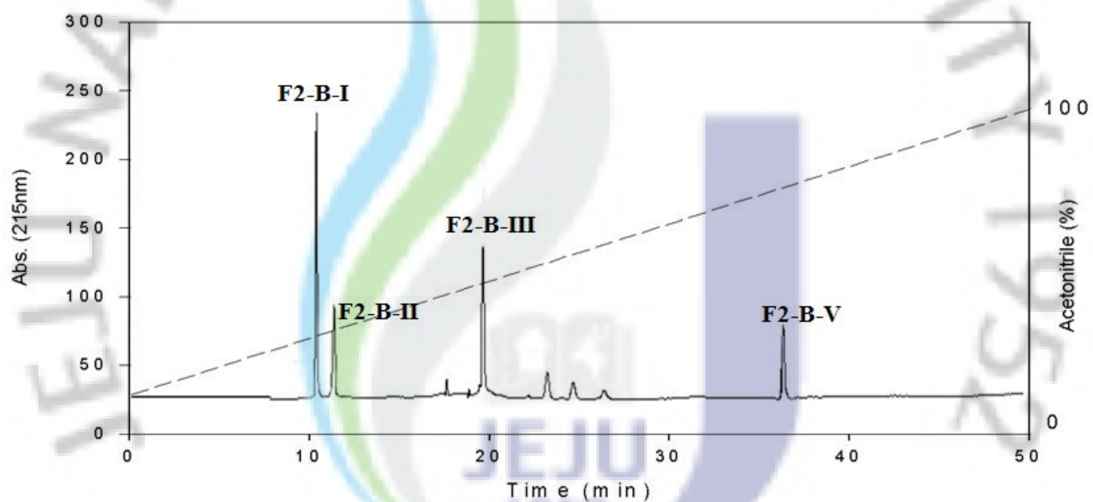

Fig. 16. RP-HPLC re-chromatogram of potent ACE inhibitory peak (F2-B) isolated from RP-HPLC. Separation was performed with linear gradient of acetonitrile from 0% to 100% in 50 min at a flow rate of 1.0 ml/min. The peaks isolated by RP-HPLC were designated I-V. Elution was monitored at 215 nm.

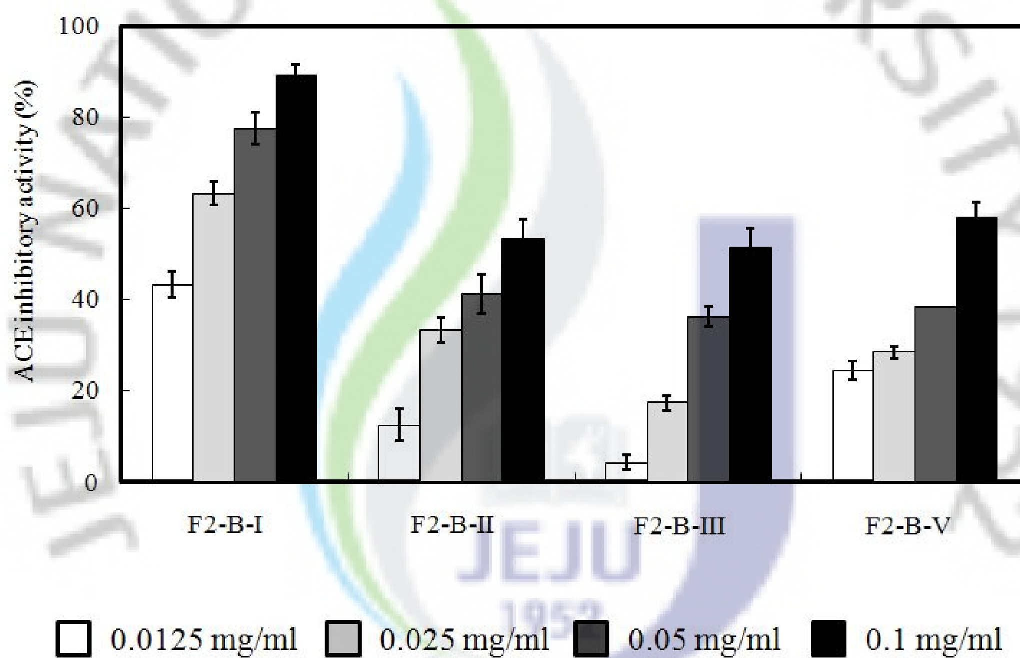

Fig. 17. ACE inhibitory activity of peaks on re-chromatography from active peak (F2-B) by RP-HPLC.

Mean±SE of determinations was made in triplicate experiments.

Table 11. IC_{50} value of peaks of RP-HPLC from F2-B peak.

Peaks	IC_{50} value (mg/ml)
F2-B-I	0.015
F2-B-II	0.086
F2-B-III	0.091
F2-B-V	0.08

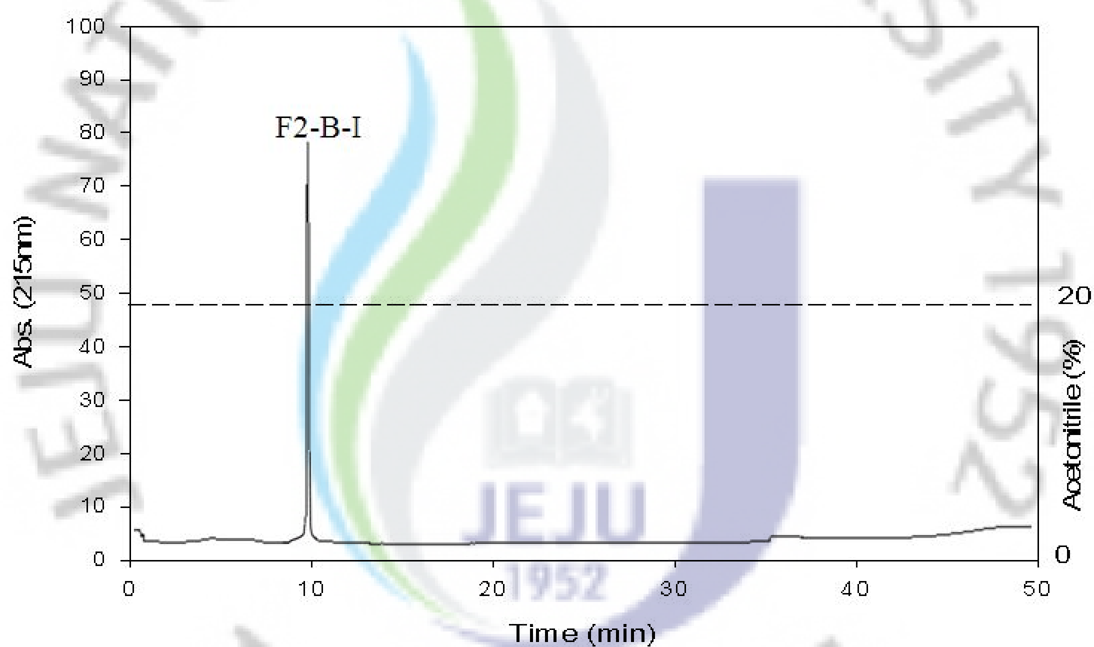

Fig. 18. RP-HPLC re-chromatogram of potent ACE inhibitory fraction (F2-B-I) isolated from RP-HPLC. Separation was performed with linear isocratic of 20% acetonitrile for 50 min at flow rate 1.0 ml/min. Elution was monitored at 215 nm.

Table 12. Purification of ACE inhibitory peptides from *S. plicata* enzymatic digest.

Purification step	IC_{50} value (mg/ml)	Purification (Fold)
Enzymatic digest (*S. plicata*)	1.171	1.00
Ultra-filtration (below 5 kDa)	0.823	1.42
Gel filtration chromatography (Sephadex G-25)	0.390	3.00
1st RP-HPLC (ODS reverse phase)	0.076	15.41
2nd RP-HPLC (ODS reverse phase)	0.014	83.64

4. Conclusion

S. plicata, a solitary ascidian, is a popular seafood in Korea. In this study, we looked at ACE inhibitory activity peptide purified from *S. plicata* protein digest obtained by enzymatic digest. The Protamex digest had the highest ACE inhibitory activity compared to the other digests. We attempted to isolate ACE inhibitory peptides from Protamex prepared *S. plcata* digest using ultra filtration, gel permeation chromatography on a Shepadex G-25 column and RP-HPLC on an ODS column. The F2-B-I was divided into single peaks on RP-HPLC. The IC_{50} value of purified ACE inhibitory peptide was 0.014 mg/ml. Based on the results of this study, it appears that this peptide may be beneficial to the nutraceutical and pharmaceutical industries. However, further work is being carried out to confirm its *in vivo* physiological effects in blood pressure regulation.

REFERENCE

Ali B. a, Naima N-A., Rozenn R-P., Yves L., Didier G., Ahmed B., & Moncef N. (2008). Angiotensin I-converting enzyme (ACE) inhibitory activities of sardinelle (*Sardinella aurita*) by-products protein hydrolysates obtained by treatment with microbial and visceral fish serine proteases. *Food Chemistry*, 111, 350-356.

Alper, A. B., Calhoun, D. A., Oparil, S. (2001). Hypertension. In: Encyclopedia of life sciences. *Nature Publishing Group*. http://www.els.net, pp. 1 - 8.

Bakris, G. L. (2001). Angiotensin-converting enzyme inhibition to enhance vas cular health-clinical and research models. *Am. J. Hypertension*, 14, 264S-269S.

Byun H. G. & Kim S. K. (2001). Purification and chracterization of angiotensin I converting enzyme (ACE) inhibitory peptides from Alaska pollack (*Theragra chalogramma*) skin. *Process Biochemistry.* 36. 1155-1162.

Chen JR, Okada T, Muramoto K. (2002). Identification of angiotensin I-converting enzyme inhibitory peptides derived from the peptic digest of soybean protein. *J Food Biochem*, 6, 543-544.

Cherry Lin, MD, and William H. Frishman, MD Bronx, N.Y.(1996). Renin inhibition: A novel therapy for cardiovascular disease. *American Heart Journal*. 131. 1024-1034.

Cheung, H. S., F. L. Wang, M. A. Ondetti, E. F. Sabo, and D. W. Cushman (1980). Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Importance of the COOH-terminal dipeptide sequence. *J. Biol. Chem.*, 255, 401-407.

Cushman, D. W. and Cheung, H. S. (1970). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem.*

Pharmaco. 20. 1637-1648.

Dzau, V. J. (2001). Tissue angiotensin and pathobiology of vascular disease a unifying hypothesis. *Hypertension*. 37. 1047-1052.

Fahmi A, Morimura S, Guo HC, Shigematsu T, Kida K, Uemura Y. (2004). Production of angiotensin I converting enzyme inhibitory peptides from sea bream scales. *Process Biochemistry,* 39, 1195-1200.

Heo, S. J., Park, E. J., Lee, K.. W., Jeon, Y. J. (2005). Antioxidant activities of enzymatic extracts from brown seaweeds. *Bioresource Technology*. 96. 1613-1623.

Je, J. Y., Park, J. Y., Jung, W. K., Park, P. J., & Kim, S. K. (2005). Isolation of angiotensin I converting enzyme (ACE) inhibitor from fermented oyster sauce, C*rassostrea gigas*. *Food Chemistry*, 90, 809-814.

Jeon, Y. J., Byun, H. G., Kim, S. K. (2000). Improvement of functional properties of cod frame protein hydrolysates using ultrafiltration membranes. *Process Biochemistry*. 35. 471-478.

Jianping, Wu., Rotimi, E., Aluko, 1., Alister, D., Muir. (2008). Purification of angiotensin I-converting enzyme-inhibitory peptides from the enzymatic hydrolysate of defatted canola meal. *Food Chemistr*y, 111, 942-950.

Joana C. S., Juliana M. F. Mesquita, Celso L. R. Belmiro, Carolina B. M. da Silveira, Christian V., Pierre A. Mourier, Mauro S. G. Pavão. (2008). Isolation and characterization of a heparin with low antithrombin activity from the body of *Styela plicata* (Chordata-Tunicata). Distinct effects on venous and arterial models of thrombosis. *Thrombosis Research*, 121, 213-223.

Jung, W. K., Mendis, E., Je, J. Y., Park, P. J., Son, B. W., Hyoung Chin Kim, H. C., Choi, Y. K., Kim, S. K. (2006). Angiotensin I-converting enzyme inhibitory peptide from yellowfin sole (*Limanda aspera*) frame protein and its antihypertensive effect in spontaneously hypertensive rats. *Food*

Chemistry. 94. 26-32.

Kato, H., Suzuki, T., (1971). Bradykinin-potentiating peptides from the venom of Agkistrodon halys blomhoffii: Isolation of five bradykinin potentiators B and C. *Biochem*, 10, 972-980.

Kim, J. J., Kim S. J., Kim, S. H., Park, H. R. & Lee, S. C. (2005). Antioxidant and Anticancer Activities of Extracts from *Styela plicata*. *J Korean Soc Food Sci Nutr*. 34. 937-941.

Lapointe, N., and J. L. Rouleau. (2002). Activation of vascular tissue angiotensin-converting enzyme (ACE) in heart failure. *J. Am. College of Cardiol*, 39, 776-779.

Lee, D. H., Kim, J. H., Park, J. S., Choi, Y. J. and Lee, J. S. (2004). Isolation and characterization of a novel angiotensin I-converting enzyme inhibitory peptide derived from the edible mushroom *Tricholoma giganteum*. *Peptides*. 25. 621-647.

Lowry, O. H., Rosebrough, N. J., Farr, L. and Rindall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem*. 193. 256-259.

Ma, M. S., Bae, I. Y., Lee, H. K., & Yang, C. B. (2006). Purification and identification of angiotensin I-converting enzyme inhibitory peptide from buckwheat (*Fagopyrum esculentum Moench*). *Food Chemistry*. 96. 36-42.

Maoa, X., Ni, J., Sun, W., Hao, P., & Fan, L. (2007). Value-added utilization of yak milk casein for the production of angiotensin-I-converting enzyme inhibitory peptides. *Food Chemistry*, 103, 1282 - 1287.

Matsui T., Yukiyoshi A., Doi S. Sugimoto H., Yamada H., Matsumoto K. (2002). Gastrointestinal enzyme production of bioactive peptide from royal jelly protein an their antihypertensive ability in SHR. *Journal of Nutritional Biochemistry*. 13. 80-86.

Miguel, M., Alonso, J. M., Salaices, M., Aleixandre, A., & Lopez-Fandin, R. (2007). Antihypertensive, ACE-inhibitory and vasodilator properties of an egg white hydrolysate: Effect of a simulated intestinal digestion. *Food*

Chemistry. 104. 163-168.

Miguel, M., Contreras, M. M., Recio, I., & Aleixandre, A. (2009). ACE-inhibitory and antihypertensive properties of a bovine casein hydrolysates. *Food Chemistry.* 112. 211 - 214.

Ondetti MA. 1977. Design of specific inhibitors of angiotensin converting enzyme: New class of orally active antihypertensive agents. *Science*, 196, 441-444.

Papadimitriou, G. C., Vafopoulou-Mastrojiannaki, A., Silva, V. S., Gomes, A., Malcata, X. F., & Alichanidis, E. (2007). Identification of peptides in traditional and probiotic sheep milk yoghurt with angiotensin I-converting enzyme (ACE)-inhibitory activity. *Food Chemistry.* 105. 647 - 656.

Sawayama, T., Itokawa, A., Shimada, K., Doi, Y., Kimura, K., Nishimura, H., (1990). Synthesis of 1-[(s)-3-acetylthio-2-methyl propanoyl]-L-propyl-L-phenylalanine (Alacepril) and one of its active metabolites, the desacetyl derivative (DU-1227). *Chem Pharm Bull*, 38, 529-531.

Siriwardhana, N., Kim, K. N., Lee, K. W., Kim, S. H., Ha, J. H., Song, C. B., Lee, J. B. and Jeon, Y. J. (2008). Optimisation of hydrophilic antioxidant extraction from *Hizikia fusiformis* by intergrating treatments of enzymes, heat and pH control. *International Journal Food Science and Technology*. 43. 587-596.

Thurman J. M., and R. W. Schrier. (2003). Comparative effects of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers on blood pressure and the kidney. *Am. J. med*, 114, 588-598.

Tsai J. S., Chen J. L., Bonnie S. P. (2008). ACE-inhibitory peptides identified from the muscle protein hydrolysate of hard clam (*Meretrix lusoria*). *Process Biochemistry*, 43, 743-747.

Unger, T. (2002). The role of rennin-angiotensin system in the development

of cardiovascular disease. *Am J Cardiol*, 89, 3 - 9.

Unger, T. (2002). The role of the renin-angiotensin system in the development of cardiovascular disease. *Am. J. Cardiol,* 89(suppl), 3A-10A.

Wang, NF., Li,C. Yang., & Yan, Z. (2008). Purification and characterization of angiotensin I-converting enzyme inhibitory peptide from lactic acid fermented peanut flour. *Journal of Biotechnology*, 136, S723.

Wang, J., Hu, J., Cui, J., Bai, X., Du, Y., Miyaguchi, Y., et al. (2008). Purification and identification of a ACE inhibitory peptide from oyster proteins hydrolysate and the antihypertensive effect of hydrolysates in spontaneously hypertensive rats. *Food Chemistry*, 111, 302 - 308.

Yoshie-Stark, Y., Bez, J., Wasche, A. (2006). Effect of different pasteurization conditions on bioactivities of Lupinus albus protein isolates. *LWT-Food Science and Technology*. 39. 118-123

Zhao, Y., Liu, Z., Dong, S., Zhao, X., Zeng, M. (2007). Antihypertensive effect and purification of an ACE inhibitory peptide from sea cucumber gelatin hydrolysate. *Process Biochemistry*. 42 . 1586 - 1591.

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제주대학교

JEJU NATIONAL UNIVERSITY 1952
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