A THESIS
FOR THE DEGREE OF MASTER OF SCIENCE

Small molecular preparation of flounder fish(*Paralichthys olivaceus*) muscle and its biological activities.

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GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

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국문 초록

제주도는 사면이 바다라는 지리적인 조건과 무공해 청정해역, 어류의 회유로 및 월동장으로 좋은 어장을 형성하며, 지하해수 개발로 육상양식을 하기에는 최적의 조건을 갖추고 있다. 제주도의 중요한 산업요소 중 하나인 넙치는 국내 양식장의 98%를 차지하고 있는 주요 양식어종으로 매년 25,000 톤을 생산하고 있으며, 약 4,000 톤을 수출하고 있다. 또한 넙치는 감칠맛을 내는 IMP, glutamic acid 의 함량이 높아 횟감으로서 기호성이 높은 어종으로 그에 대한 연구가치가 크다. 현재 Capelin (Amarowicz and Shahidi, 1997), 참치 (Jao and Ko, 2002), 고등어 (Wu et al., 2003), 명태 (Je et al., 2005), 대구 (Thiansilakul et al., 2007), chum salmon (Ono et al., 2003) 및 hoki (Je et al., 2005)등으로부터 얻어진 가수분해물이 항산화, 항고혈압 활성에 탁월하다는 효능이 밝혀져 있지만, 아직까지 넙치에 대한 연구는 되어있지 않다. 따라서 이 연구에서는 넙치 효소적 가수분해물에 대한 생리활성의 평가와 넙치를 우리가 섭취하였을 때, 항산화, 항고혈압에 어떠한 영향을 미치는지 알아보기 위하여 복합효소 가수분해를 이용해 평가하였다.

첫 번째 파트에서는 일반적으로 이용되고 있는 단백질 가수분해효소 8 종을 이용하여 넙치 근육으로부터 얻은 가수분해물의 항산화 및 ACE 소거 활성을 측정하였다. 단백질 가수분해물 8 개 중에서 α-chymotrypsin 가수분해물의 활성이 가장 높았으며 이렇게 선정된 효소를 이용하여 가수분해물의 최적 조건을 설정하기 위해 가수분해 시간(6, 12, 18, 24 시간) 과 기질 대 효소비 (1000:1, 500:1, 100:1)를 다양하게 처리하여 가수분해물을 제조하였다. 제조한 가수분해물들의 활성을 측정하여 최적조건 (가수분해 시간 18 시간, 기질 대 효소비율 1000:1)을

선택한 후 분자량 크기에 따라 분리를 할 수 있는 한외여과와 겔 여과 크로마토그래피를 순차적으로 이용하여 분리를 하였다. 그 결과 5 kDa 이하의 저분자 물질에서 우수한 항산화 효과와 ACE 소거능 측정을 통해 항고혈압에 대한 가능성을 확인하였다.

두 번째는, 넙치를 섭취하였을 때, 우리 몸에 존재하는 장내효소 (Pepsin 및 α-chymotrypsin)에 의해 소화가 진행되면서 어떠한 기능을 갖는지 알아보고자 하였다. 먼저 위에 존재하고 있는 Pepsin 단백질 가수분해효소를 이용하여 기질 대 효소비 (1000:1, 500:1, 100:1) 및 가수분해 시간 (6, 12, 18, 24 시간)에 따라 활성을 측정하여 최적조건 (가수분해 시간 18 시간, 기질 대 효소 비율 100:1)을 선택한 후 한외여과를 통해 분자량 크기별로 분리를 하였다. 그 결과 5 kDa 이하의 저분자 물질에서 항산화 효과와 ACE 소거 효과가 나타난 것을 확인하였다. 두 번째로는 위에 존재하는 Pepsin 과 췌장에 존재하는 α-chymotrypsin 단백질 가수분해 효소에 의한 복합 가수분해 작용에 의한 생리활성을 확인하고자 하였다. Pepsin 과 α-chymotrypsin 의 복합 가수분해 시간 (0.5, 1, 3, 6, 12 시간)에 따른 최적조건을 선택 (Pepsin 가수분해 시간 1 시간, α-chymotrypsin 가수분해 시간 12 시간)한 후 한외여과를 통해 분자량 크기별로 분리를 하였다. 그 결과 5 kDa 이하의 저분자 물질에서 높은 항산화 효과와 항고혈압에 대한 가능성을 확인할 수 있었다.

위의 결과들을 종합하여 보면, α-chymotrypsin 에 의한 넙치 가수분해물로부터 얻어진 저분자 물질은 우수한 항산화 효과와 항고혈압에 대한 가능성을 보여주었고, 장내 효소인 pepsin 과 α-chymotrypsin 의 복합효소 가수분해물에서의 저분자 물질, 또한 항산화 효과와 항고혈압에 대한 가능성을 보여줌으로서 ,

우리는 넙치를 섭취하였을 때, 이와 같은 효능을 기대할 수 있을 것으로 사료되어진다.

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INTRODUCTION

Free radicals must be produced from oxygen by aerobic organisms during the normal course of respiration. Reactive oxygen species (ROS) have variety of free radicals, such as superoxide anion (O_2^-), hydroxyl radical (HO^-), nitric oxide radical (NO^-), alkyl radical (RO^-) and non free radical species like hydrogen peroxide (H_2O_2). In the normal cells can be effectively eliminated by an enzyme-mediated system such as superoxide dismutase, peroxidase, catalase and glutathione peroxidase when ROS occur. However, if these are excessively produced by many factors such as exposure to radiation, major operation, smoking, drinking, food additive, stress, pollution, infections and excessive exercise, they are induced many disease such as cancer (Leanderson et al., 1997), gastric ulcers (Debashis et al., 1997), Alzheimer's, arthritis and ischemic reperfusion (Vajragupta et al., 2000). Moreover, oxidation of fatty acids and lipids induced by free radicals also leads to the deterioration of food quality (Liceaga-Gesualdo and Li-Chan., 1999; Kristinsson and Rasco., 2000). An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Kind of synthetic antioxidants is butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG). Synthetic antioxidants show stronger antioxidant activity. But the use must be under strict regulation due to potential health hazards (Park et al., 2001).

Hypertension or high blood pressure is a chronic medical condition in which the systemic arterial blood pressure is elevated. Also high blood pressure is called a 'life-style related disease', which sometimes causes cerebrovascular and cardiac infarction (Hiroyuki Enari et al., 2008). Hypertension, which affects 15-20% of all adults, is worldwide problem of epidemic proportions (Jung et al., 2006). Major complications are hypertensive encephalopathy (confusion, headache and convulsion), elevated sugar levels, retinopathy, heart failure and chronic renal failure. Angiotensin I-converting enzyme (EC 3. 4. 15. 1;

ACE) plays an important physiological role in the regulation of blood pressure (Skeggs et al., 1957). This enzyme discovered in vascular, heart, lung and brain tissue plays the important role as dicarboxy peptidase (Lapointe and Rouleaul., 2002) in rennin-angiotensin system (RAS) and kallikrein-kinin system (KKS). Angiotensin II, a potent vasoconstrictor, is a major contributor to high blood pressure in the rennin angiotensin system. Vasoconstriction occurs an enzyme liberated by the kidneys, proteolytically acts on circulating angiotensinogen and converts it to angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu). In the presence of angiotensin converting enzyme (ACE), angiotensin I is cleaved to the octapeptide, angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) resulting in arterial constriction and blood pressure elevation. ACE also breaks down bradykinin, a vasodilator, further contributing to the elevation in blood pressure (Fig. 1-1). Inhibition of ACE is, therefore, important for the lowering of blood pressure as this results in a decrease in the concentration of angiotensin II and an increase in the levels of bradykinin (Erdos, 1975; Yang et al., 1970; Joyce et al., 2010). Well known synthetic ACE inhibitors are captopril, enalapril, alacepril, lisinopril and quinapril. These are used extensively in the treatment of essential hypertension and heart failure in human (Ondetti et al., 1977; Patchett et al., 1980). But they have side effects including cough, allergic reactions, taste disturbances and skin rashes. As mentioned above synthetic antioxidants and ACE inhibitors have many problem. Therefore, the alternatives of synthetic antioxidants and ACE inhibitors take notice that development of natural antioxidants.

Recently, researchers consider bioactive peptides from food proteins. Bioactive peptides can be released from proteins by enzymatic proteolysis of proteins, and may act as potential physiological modulators in metabolism process during the intestinal digestion of the diet. The possible regulatory effects of peptides relate to nutrient uptake, immune defense, opioid and antihypertensive activities and antioxidant activities (Pihlanto-Leppälä, 2001). Fish protein hydrolysates (FPH) have been reported to possess nutritional, antioxidative,

antihypertensive, antimicrobial and immunomodulatory properties (Fujita and Yoshikawa. 1999, Shahidi et al., 1995). FPH have been reported about antioxidant peptides hydrolyzed from various fish sources such as capelin (Amarowicz and Shahidi, 1997), tuna (Jao and Ko, 2002), mackerel (Wu et al., 2003), Alaska pollack (Je et al., 2005) and scad (Thiansilakul et al., 2007). Also there are reported about ACE inhibition peptides hydrolyzed from tuna (Kohama et al., 1988), bonito (Matsumura et al., 1993), channel catfish (Theodore and Kristinsson, 2007) and chum salmon (Ono et al., 2003).

In Jeju islands, Flounder fish (*Paralichthys olivaceus*) is one of the most important industrial factors and major cultivated fish species in Korea with 98% of the domestic aquaculture. Annual production is about 25,000 tones and export is about 4,000 tones. Like this, flounder fish has occupied aquaculture industry in Jeju island. However, recently, many consumers avoid flounder fish because price fluctuation and indiscreet antibiotic use about flounder fish. So, Many people try to contribute to solve these problems. But, research about bioactive peptides of flounder fish has not investigated until now. Therefore In this study, we investigated antioxidative activity and ACE inhibition activity of protein hydrolysates from flounder fish muscle by various protease and changes of physiological property of protein hydrolysates by gastro-intestinal proteinase after human's oral intake.

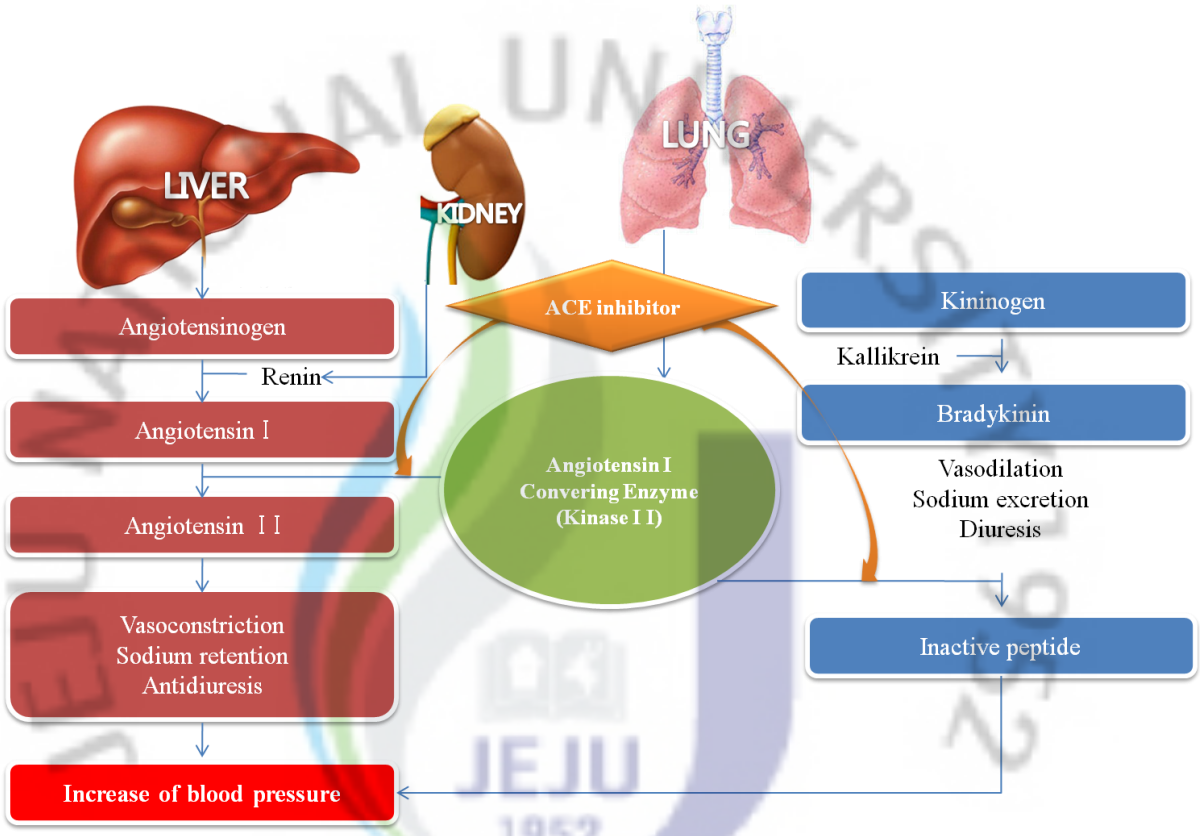

Fig. 1-1. Formation mechanism of hypertension by Angiotensin I converting enzyme.

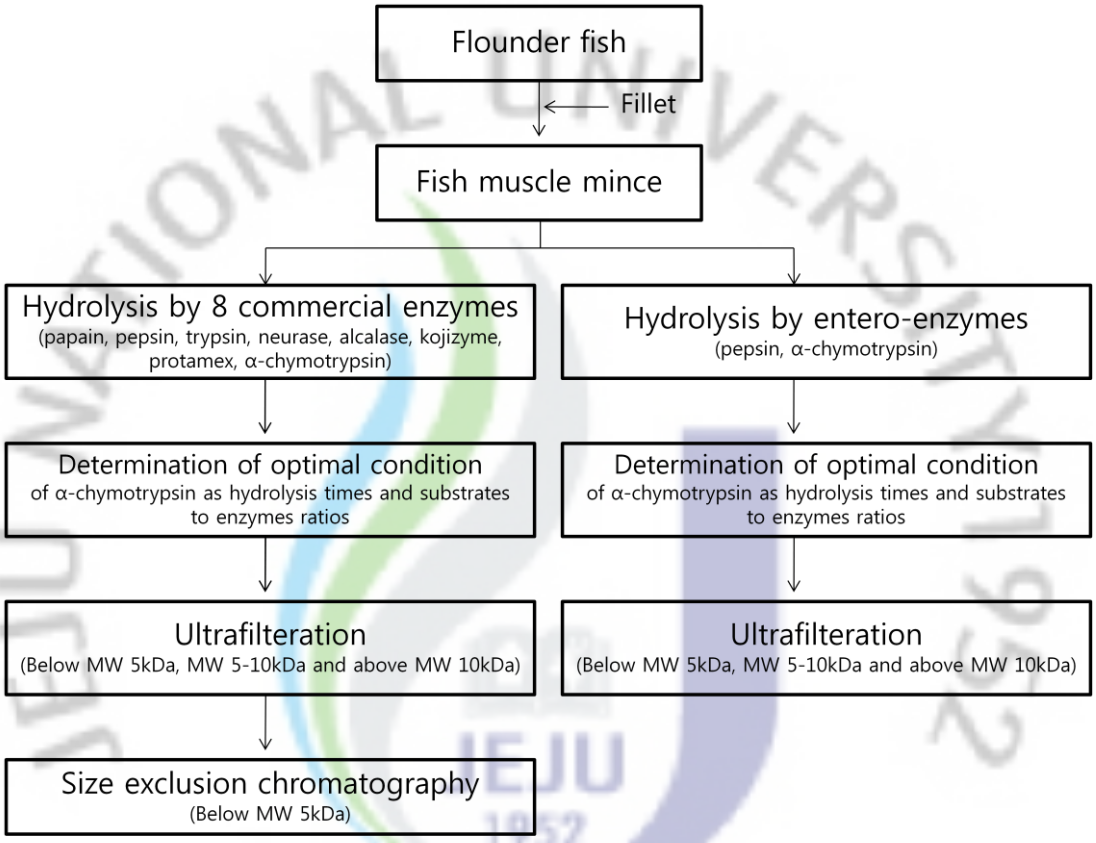

Fig.1-2. Schematic representation of the process for enzymatic hydrolysates from flounder fish muscle.

Part I

Antioxidant and ACE inhibition activities on enzymatic hydrolysate of flounder fish muscle

ABSTRACT

Flounder fish (*Paralichthys olivaceus*) is one of the most important industrial factors and major cultivated fish species in Korea. This study investigated the effects of bioactive-peptide from flounder fish muscle on antioxidant activity and angiotensin converting enzyme (ACE) inhibition activity. The hydrolysates were prepared by eight commercial enzymes such as Papain, Pepsin, Trypsin, Neutrase, Alcalase, Kojizyme, Protamex and α-chymotrypsin. The antioxidant activity was determined by measuring free radical such as 1, 1 diphenyl-2-picrylhdrazyl (DPPH), alkyl radical, hydroxyl radical using electron spin resonance (ESR). Hydrogen peroxide, ACE inhibition activity was determined by colorimetric assay. α-chymotrypsin hydrolysate was shown the strongest antioxidant activity and ACE inhibition activity among eight enzymatic hydrolyastes (DPPH radical IC_{50} value 2.775 mg/ml, Hydroxyl radical IC_{50} value 0.403 mg/ml, Alkyl radical IC_{50} value 0.156 mg/ml, Hydrogen peroxide IC_{50} value 0.711 mg/ml and ACE inhibition IC_{50} value 0.859 mg/ml). And then, in results of bio-activity of α-chymotrypsin hydrolysate preparing as hydrolysis times (6h, 12h, 18h and 24h) and substrate to enzyme ratio (1000:1, 500:1 and 100:1), α-chymotrypsin hydrolysate which was 1000:1 (substrate : enzyme) and 18hr (hydrolysis time), was exhibited higher level of antioxidant activity (DPPH radical IC_{50} value 1.968 mg/ml, Hydroxyl radical IC_{50} value 0.347 mg/ml, Alkyl radical IC_{50} value 0.148 mg/ml and Hydrogen peroxide IC_{50} value 0.678 mg/ml) and ACE inhibition activity (ACE inhibition IC_{50} value 0.780 mg/ml) compared with hydrolysates of other condition. Ultrafilteration was performed for separating of as α-chymotrypsin hydrolysate (1000:1, 18hr) as molecular weight (Above MW 10 kDa, MW 5-10 kDa and below MW 5 kDa). Although α-chymotrypsin hydrolysate of above MW 10 kDa exhibited the highest hydroxyl radical scavenging activity(IC_{50} value 0.165 mg/ml). Alkyl radical scavenging activity (IC_{50}

value 0.130 mg/ml) and ACE inhibition activity (IC_{50} value 0.732 mg/ml) were exhibited the strongest activity at α-chymotrypsin hydrolysate of below MW 5 kDa. In results performed Gel-chromatography, it was collected four fractions from α-chymotrypsin hydrolysate of below MW 5 kDa and fraction 4 (Fr. 4) exhibited the highest antioxidant activity against alkyl radical (90.54%). In results about Amino acid analysis of Fr. 4, we could confirm high levels of phenylalanine, histidine, lysine which are antioxidant amino acid. In conclusion, we have known that small molecular weight material from flounder fish has good antioxidant activity and possibility about anti-hypertension.

MATERIALS and METHODS

2. 1. Materials

Flounder fish (*Paralichthys olivaceus*) were cultivated in Jeju island. Fish after capture were filleted and muscle was collected. It was washed twice with freshwater, then immediately frozen and stored at -20℃ until used. The frozen sample was lyophilized and homogenized with a grinder before hydrolysis.

2. 2. Chemicals and reagent

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 5,5-Dimethyl-1-pyrrolin N-oxide (DMPO), α-(4-pyridyl-1-oxide)-N-t-butylnitrone (4-POBN), Folin-Ciocalteu reagent, peroxidase, 2,2-Azino-bis(3-ethylbenz-thiazolin)-6-sulfonic acid (ABTS), 2,2-azobis-(2-amidinopropane) hydrochloride (AAPH) and Albumin were purchased from Sigma Chemical Co. (USA). $FeSO_4 \cdot 7H_2O$, Hydrogen peroxide were purchased from Dae Jung Chemical Co. (Korea). 30% Acrylamide/Bis solution (29:1) were purchased from Bio-rad Laboratories, Inc. (USA). Ammonium persulfate (APS) and TEMED were purchased from Amresco Inc. (USA). N-Hippuryl-His-Leu tetrahydrate (HHL) and Angiotensin I converting enzyme (from rabbit lung) were purchased from Sigma chemical Co. (USA). Protein protease such as Papain, Pepsin, Trypsin, α-chymotrypsin were purchased from Sigma chemical Co. (USA) and Neutrase, Alcalase, Kojizyme, Protamex were purchased from Novozyme Co. (Denmark). Optimum hydrolysis conditions indicated Table 1-1. All the other chemicals used were analytical grade.

2. 3. Proximate composition

The proximate composition of dried flounder fish muscle powder was determined

according to the AOAC methods (AOAC, 1990). Moisture content was determined keeping in a dry oven at 105℃ for 24h. Crude ash content was determined by calcinations in furnace at 550℃ and crude protein content was determined by Kjeldahl method. Crude lipid was content was determined by Soxhlet method.

2. 4. Preparation of enzymatic hydrolysate

2. 4. 1. Preparation of enzymatic hydrolysates by eight protein protease

Freeze dried flounder fish muscle was homogenized with a grinder. Five gram of the dried flounder fish powder put into distilled water 100 ml and was adjusted pH of protease optimum condition (Table 1-1). And then protease of substrate to enzyme ratio 500:1 was added. Protein hydrolysis enzyme used eight proteinase such as Papain, Pepsin, Trypsin, Neutrase, Alcalase, Kojizyme, Protamex and α-chymotrypsin. Hydrolysis was carried out at protease optimum temperature (Table 1-1) for 24h. At the end of the reaction, the hydrolysates were adjusted to pH 7.0 and heating at 97℃ for 10 min in order to inactivate the enzyme. The hydrolysates were centrifuged at 3,500 rpm for 20 min to separate insoluble and soluble fractions. The soluble phase was freeze dried using freeze dryer and stored at -20℃ for further use.

2. 4. 2. Optimum conditions assay for the active enzymatic hydrolysate

It was carried out optimum conditions assay (Ko, 2009) for antioxidant and ACE inhibition hydrolysate from flounder fish. Five gram of the dried flounder fish powder put into distilled water 100 ml and was adjusted optimal pH value. And then various substrate to enzyme ratio (1000:1, 500:1 and 100:1) was added. Hydrolysis time was carried out various times (6h, 12h, 18h and 24h). At the end of the reaction, the hydrolysates were adjusted to pH 7.0 and heating at 97℃ for 10 min in order to inactivate the enzyme. The hydrolysates were centrifuged at 3,500 rpm for 20 min to separate insoluble and soluble fractions. The

soluble phase was freeze dried using freeze dryer and stored at -20℃ for further use.

2. 4. 3. Molecular weight fractionation of active enzymatic hydrolysate

In all the enzymatic hydrolysates tested antioxidant and ACE inhibition activities. The superior enzymatic hydrolysates were selected. And then selected hydrolysate separated molecular weight by Ultra filtration membranes using Millipore's Lab scale TFF system (Millipore Corporation, Bedford, Massachusetts, USA) at 4℃. The collected molecular weight fractions are above 10 kDa, 5-10 kDa and below 5 kDa. This was lyophilized and stored at -20℃ for use.

2. 4. 4. Size exclusion chromatography

The superior active fraction (100 mg) by Ultra filtration membranes was dissolved with 1 ml of distilled water. The fraction was loaded onto a Sephadex G-25 column (2.5 X 100 cm). Previously the column equilibrated with distilled water. And then elution was carried out with distilled water at a flow rate was 1.5 ml/min. The fractions absorbance was read at 220 nm and collected peaks. This was lyophilized and stored at -20℃ for use.

2. 5. Measurement of protein content

The soluble protein content was determined by the Lowry method. One milliliter of the digests, 1 ml of alkaline-copper working solution and 3 ml of 50% Folin-Ciocalteu's phenol reagent were mixed. The mixtures were allowed to react for 45 min and absorbance at 540 nm was measured with a spectrophotometer. Calibration standard is used bovine serum albumin (Lowry et al., 1951).

2. 6. SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was

performed on the protein hydrolysates using a 15% Tris/Hcl gel to characterize the hydrolysates based on their molecular weights (MW). The MW of the hydrolysates was calculated with reference to the migration of SDS-PAGE wide range molecular weight standard. Samples were heated at 100℃ for 5 min prior to the electrophoresis run. After electrophoresis, the gels were stained with Bio-Rad Coomassie Blue R-250. The bands in the samples were compared with known bands of protein standards.

2. 7. Free radical scavenging capacities using ESR spectrometer

2. 7. 1. DPPH radical scavenging activity

DPPH radical scavenging activity was measured using the method described by Nanjo et al. (1996). A distilled water solution of 60 ul of each sample (or distilled water solution itself as a control) was added to 60 ul DPPH (60 uM) in methanol solvent. The sample mixed roughly. And then after 2 minutes transferred into a capillary tube and recorded spectrum by ESR (electron spin resonance) spectrometer (JES-FA machine, JEOL, Tokyo, Japan). The experimental conditions were as followed; magnetic field 336.5±5 mT, power 1 mW, modulation frequency 100 kHz, amplitude 10 X 100, modulation width 0.8 mT, sweep width 10 mT, sweep time 30 sec, time constant 0.03 sec. The extent of scavenging activity was calculated as followed.

Scavenging activity % = (HC –HS) / HC X 100

HC : Relative peak heights of the radical signals without sample

HS : Relative peak heights of the radical signals with sample

2. 7. 2. Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by the iron-catalyzed Haber-Weiss reaction (Fenton-driven Haber-Weiss reaction; $Fe^{2+} + H_2O_2 \rightarrow {}'OH + OH^-$) and the hydroxyl radicals rapidly reacted with nitrone spin trap DMPO (Rosen and Rauckman, 1980). The resultant DMPO-

OH adducts was detectable with the ESR spectrometer. Sample 20 ul of various concentrations were mixed with 0.3 M DMPO 20ul in phosphate buffer solution (PBS; pH 7.4), 10 mM $FeSO_4$ 20 ul and 10 mM H_2O_2 20 ul. And then after 2.5 minutes transferred into a capillary tube and recorded spectrum by ESR. The experimental conditions were as followed; magnetic field 336.5±5 mT, power 1 mW, modulation frequency 100 kHz, amplitude 1 X 200, modulation width 0.1 mT, sweep width 10 mT, sweep time 30 sec, time constant 0.03 sec.

2. 7. 3. Alkyl radical scavenging activity

Alkyl radicals were generated by AAPH and their scavenging effects were investigated by the method described by Hiramoto et al. (1993) Sample 20 ul of various concentrations were mixed with distilled water 20 ul, 40 mM AAPH 20 ul and 40 mM 4-POBN 20ul in the PBS (pH 7.4). And then after 30 minutes incubated at 37℃ in a water bath. So transferred into a capillary tube and recorded spectrum by ESR. The experimental conditions were as followed; magnetic field 336.5±5 mT, power 1 mW, modulation frequency 100 kHz, amplitude 1 X 1000, modulation width 0.2 mT, sweep width 10 mT, sweep time 30 sec, time constant 0.03 sec.

2. 7. 4. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was carried out according to the method of Muller (1995). Sample 100 ul was mixed with 0.1 M phosphate buffer (pH 5.0) 100 ul and 10 mM hydrogen peroxide 20 ul in a 96 microwell plate and incubated at 37℃ for 5 minutes. Thereafter, 1.25 mM ABTS 30 ul and peroxidase (1 U/ml) 30 ul were mixed and incubated at 37℃ for 10 minutes. The absorbance was measured at 405 nm.

2. 8. ACE inhibition activity

The ACE inhibition activity assay was performed according to the method of Cushman and Cheung (1971) with slight modification. The amount of hippuric acid (HA) formed from HHL by is determined by a spectrophotometric assay. A sample solution 50ul with 50 ul of ACE solution (25 mU/ml) was pre-incubated at 37℃ for 10 minutes, and then incubated with 100 ul 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 minutes. The reaction stopped by added 250 ul of 1 N HCl. Hippuric acid was extracted with 350 ul of ethyl acetate. After centrifugation (4,000 rpm, 10 minutes), 200 ul of the upper phase was transferred into a ependorf tube and evaporated in dry-oven at 80℃. The hippuric acid was dissolved in 1 ml distilled water, and the absorbance was measured at 228 nm. The extent of inhibition was calculated as followed.

Inhibition activity % = (AC – AS) / (AC – AB) X 100

AC : Absorbance of control

AS : Absorbance of reaction sample solution

AB : Absorbance of blank solution

2. 9. Amino acid analysis

The amino acid profiles were determined according to the method of Bidlingmeyer et al. (1987) and Dong et al. (2008) with a slight modification. Amino acid composition was determined by high performance liquid chromatography equipped with a PICO.TAG column (Waters, Milford, MA, USA). Total amino acid residues were determined after hydrolysis at 110℃ for 24 h with 6 N hydrochloric acid prior to the derivatization with phenyl isothiocyanate. The amino acid standards included the following components; L-alanine, L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-

leucine, $_{L}$-lysine, $_{L}$-methionine, $_{L}$-phenylalanine, $_{L}$-proline, $_{L}$-serine, $_{L}$-threonine, $_{L}$-tyrosine, $_{L}$-valine and ammonium choride (Sigma Co., St. Louis, Mo, USA). These standards were at equal concentration except the ammonium chloride. All of these standards were run once a day before the experimental runs.

2. 10. Statistical analysis

All experiments were conducted in triplicate (n=3) and an ANOVA test (using SPSS 11.5 statistical software) was used to analyze the data. Significant differences between the means of parameters were determined by using the Duncan's test ($p < 0.05$).

Table 1-1. Optimal hydrolysation conditions.

Enzyme	Sources	Optimal	
		pH	Temperature (℃)
Papain	Papaya latex	6.2	37
Pepsin	Porcine gastric mucosa	2.0	37
Trypsin	Bovine pancreases	7.6	37
Neutras	Bacillus amyloliquefaciens	6.0	50
Alcalase	Bacillus sp.	8.0	50
Kojizyme	A. oryzae	6.0	40
Protamex	Bacillus sp.	6.0	40
α-chymotrypsin	Bovine pancreases	7.8	37

RESULTS and DISCUSSION

3. 1. Proximate composition

Proximate compositions of flounder fish muscles by freeze-dried were determined according to the AOAC methods (1990) and presented in Table 1-2. The values showed moisture 1.00 %, ash 1.63%, crude protein 91.82%, crude lipid 5.55 %.

3. 2. Preparation of enzymatic hydrolysate from flounder fish muscles

The Yield of enzymatic hydrolysates by commercial proteinase including papain, pepsin, trypsin, neutrase, alcalase, kojizyme, protamex and α-chymotrypsin, and distilled water extracts presented in Fig 1-3. The enzymatic hydrolysates generally showed highly yield than distilled water extracts (9.13%). Enzymatic hydrolysates had high yield among the eight enzymatic hydrolysates were neutrase, alcalase, protamex and α-chymotrypsin digests, and showed values 54.73%, 59.92%, 61.71% and 56.60%, respectively.

Protein contents showed values in the Fig 1-4. Like the preceding, protein contents also showed highly value than distilled water extracts (49.02 mg/g). α-chymotrypsin digest clearly had the highest protein contents (446.51 mg/g) and other digests generally indicated similar protein contents (papain 225.06 mg/g, pepsin 227.16 mg/g, trypsin 301.54 mg/g, neutrase 266.41 mg/g, alcalase 264.55 mg/g, kojizyme 225.01 mg/g, protamex 271.02 mg/g).

Table 1-2. Proximate composition of dried flounder fish muscles.

Composition	Sources (%)
Moisture	1.00±0.01
Ash	1.63±0.14
Crude protein	91.82±1.37
Crude lipid	5.55±1.29

Mean ± SD from triplicate determinations.

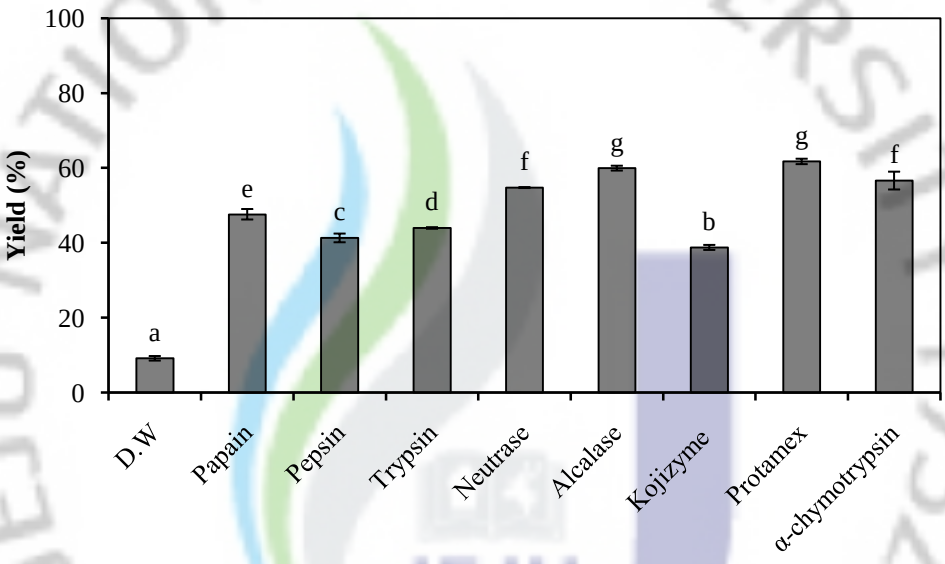

Fig. 1-3. Yield of enzymatic hydrolysates with eight proteinase enzyme. Mean ± SD from triplicate determinations. Significant differences at $p<0.05$ indicated with different letters.

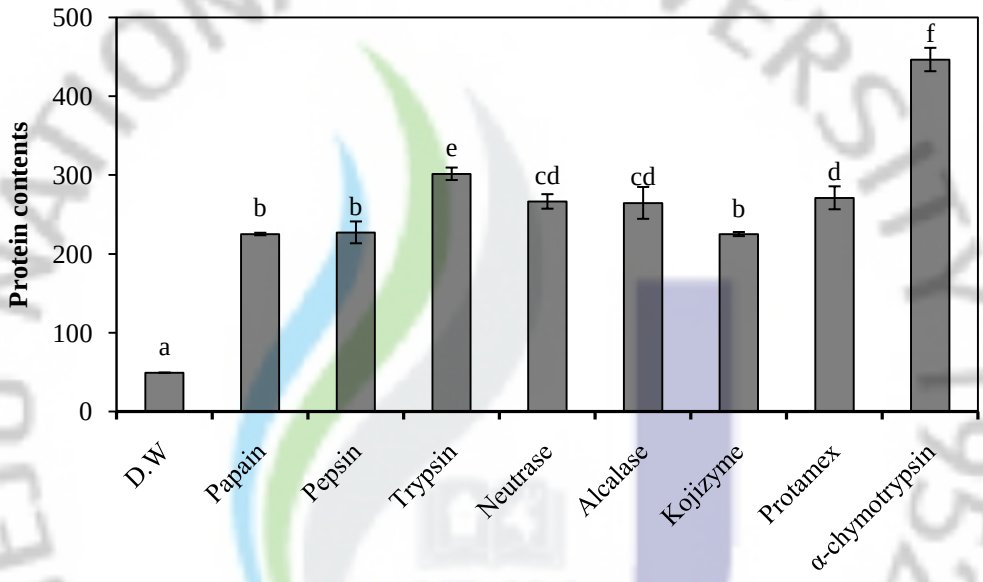

Fig. 1-4. Protein contents of enzymatic hydrolysates wiht eight proteinase enzyme. Mean ± SD from triplicate determinations. Significant differences at $p<0.05$ indicated with different letters.

3. 2. 1. Characterization of flounder fish hydrolysates in SDS-PAGE

SDS-PAGE was used to characterize the molecular weight of flounder fish protein hydrolysates (Fig. 1-5). And then SDS-PAGE was carried out using 15% polyacrylamide gel. These may represented various hydrolyzed myofibrillar proteins. Distilled water extract's band showed remain intact state. Using various proteinase significantly increased the amount of low molecular weight proteins and showed various band patterns. The band patterns of all enzymatic hydrolysates indicated less than 6 kDa. Among them, especially neutrase, alcalase and α-chymotrypsin hydrolysates showed smaller molecular materials than the rest hydrolysates.

3. 2. 2. Free radicals scavenging capacities using ESR spectrometer and ACE inhibition activity

The DPPH radical was one of the few stable radical sources. Thus the DPPH radical was widely used to investigate the scavenging activity of some natural compounds. DPPH radical displays a maximum absorbance at 517 nm in methanol. When DPPH encounters a proton-donating substance, the radical would be scavenged, as visualized by changing its colour from purple to yellow, and the absorbance is reduced (Shimada et al., 1992). The scavenging activities of the enzymatic hydrolysates on DPPH radical scavenging activity are shown on Fig 1-6. Antioxidant components in the enzymatic hydrolysates react with DPPH radical, which is a nitrogen-centered radical and convert to 1,1-diphenyl-2-picryl hydrazine due to its hydrogen donating ability at very rapid rate (Jayaprakasha et al., 2004). Antioxidative activitys of enzymatic hydrolysates were increased with increasing concentration from 1 mg/ml to 10 mg/ml. And then, there are showed all hydrolysates had higher scavenging activity than distilled water extract. Also we were determined IC_{50} value. The lower IC_{50} values indicate higher free radical scavenging ability. Among them, pepsin, neutrase and alcalase hydrolysates had low scavenging activity (IC_{50} value 10 mg/ml <),

whereas kojizyme and α-chymotrypsin hydrolysates had higher scavenging activity (IC_{50} value 3.227 mg/ml and 2.775 mg/ml, respectively) than other enzymatic hydrolysates (Table 1-3).

Hydroxyl radicals generated in Fe^{2+}/H_2O_2 system were trapped by DMPO forming spin adduct, which could be detected by an ESR spectrometer. The typical 1:2:2:1 ESR signal of the DMPO-OH adduct was observed (Je et al., 2005). The scavenging activities of the enzymatic hydrolysates on hydroxyl radical scavenging activity are shown on Fig 1-7. Increased concentrations of all enzymatic hydrolysates exhibited dose-dependent scavenging activities with varying capacity. And then the hydroxyl radicals scavenging effect of the enzymatic hydrolysates showed IC_{50} values in Table 1-3. In generally, most hydrolysates showed good scavenging capacity except trypsin hydrolysate (IC_{50} value 1.459 mg/ml). Among them, pepsin hydrolysate had the lowest IC_{50} values (0.124 mg/ml).

Alkyl radicals were generated by the decomposition of AAPH incubated with spin trap 4-POBN at 37℃ for 30 min were measured using the ESR spectrometer. The scavenging activities of the enzymatic hydrolysates on alkyl radical scavenging activity are shown on Fig 1-8. Antioxidative activitys of enzymatic hydrolysates were increased with increasing concentration from 0.125 mg/ml to 1 mg/ml. And then, there are showed all hydrolysates had higher scavenging activity than distilled water extract. Among them, alcalase had low scavenging activity (IC_{50} value 0.339 mg/ml), whereas α-chymotrypsin hydrolysate had higher scavenging activity (IC_{50} value 0.156 mg/ml) than other enzymatic hydrolysates (Table 1-3).

ABTS is commonly used as a substrate with hydrogen peroxide for a peroxidase enzyme. In this reaction, there are produced water molecular and oxidized ABTS (H_2O_2 + ABTS → $2H_2O$ + Oxidized ABTS). Hydrogen peroxide scavenging activity indicates to decrease production of oxidized ABTS by Antioxidant materials. The scavenging activities of the enzymatic hydrolysates on hydrogen peroxide scavenging activity are shown on Fig 1-9. Antioxidative activities of enzymatic hydrolysates were increased with increasing concentration from 0.25 mg/ml to 4 mg/ml. And then, there are shown all hydrolysates had higher scavenging activity than distilled water extract. Among them, pepsin and kojizyme hydrolysates had low scavenging activity (IC_{50} value 0.964 mg/ml and 1.039 mg/ml, respectively), whereas papain and α-chymotrypsin hydrolysates had higher scavenging activity (IC_{50} value 0.675 mg/ml and 0.711 mg/ml, respectively) than other enzymatic hydrolysates (Table 1-3).

The ACE inhibition activities of the enzymatic hydrolysates are shown on Fig 1-10. There are showed all hydrolysates had higher ACE inhibition activity than distilled water extract. Among them, papain and kojizyme hydrolysates had low scavenging activity (IC_{50} value 2.141 mg/ml and 2.849 mg/ml, respectively), whereas alcalase, protamex and α-chymotrypsin hydrolysates had higher inhibition activity (IC_{50} value 0.881 mg/ml, 0.857 mg/ml and 0.859 mg/ml, respectively) than other enzymatic hydrolysates (Table 1-4).

In conclusion, alcalase and protamex hydrolysates had high yields, but α-chymotrypsin hydrolysate had highly two times protein contents compare with other hydrolysates. And α-chymotrypsin hydrolysate had the highest scavenging activity among enzymatic hydrolysates on DPPH and alkyl radicals scavenging activities. Although pepsin, papain and papain hydrolysates exhibited the highest hydroxyl radical (IC_{50} value 0.124mg/ml), hydrogen peroxide scavenging activity (IC_{50} value 0.675mg/ml) and ACE inhibition activity (IC_{50} value 0.857mg/ml), respectively, the hydroxyl radical, hydrogen peroxide scavenging

activity and ACE inhibition activity of the α-chymotrypsin hydrolysate are also shown the second high activities. Therefore we selected α-chymotrypsin hydrolysate and measured free radicals and ACE inhibition activities to determine optimum conditions assay of the active enzymatic hydrolysates and confirmed their protein weight pattern by SDS-phase.

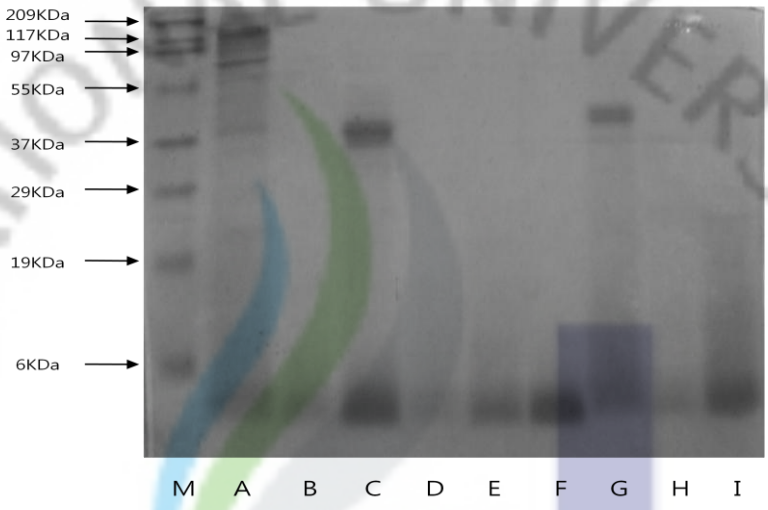

Fig. 1-5. Hydrolysis pattern of enzymatic hydrolysates from flounder fish with eight proteinase on 15% SDS-PAGE. M: Marker, A: D.W, B: Papain, C:Pepsin, D:Trypsin, E:Neutrase, F:Alcalase, G:Kojizyme, H:Protamex, I:α-chymotrypsin.

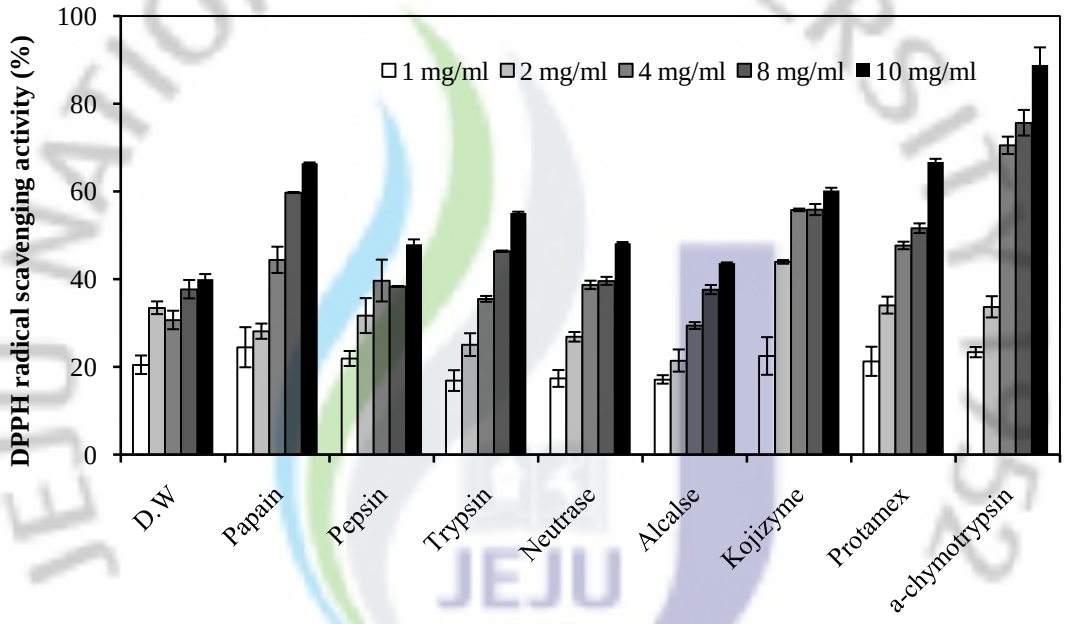

Fig. 1-6. DPPH radical scavenging activity of enzymatic hydrolysates with eight proteinase from flounder fish. Mean ± SD from triplicate determinations.

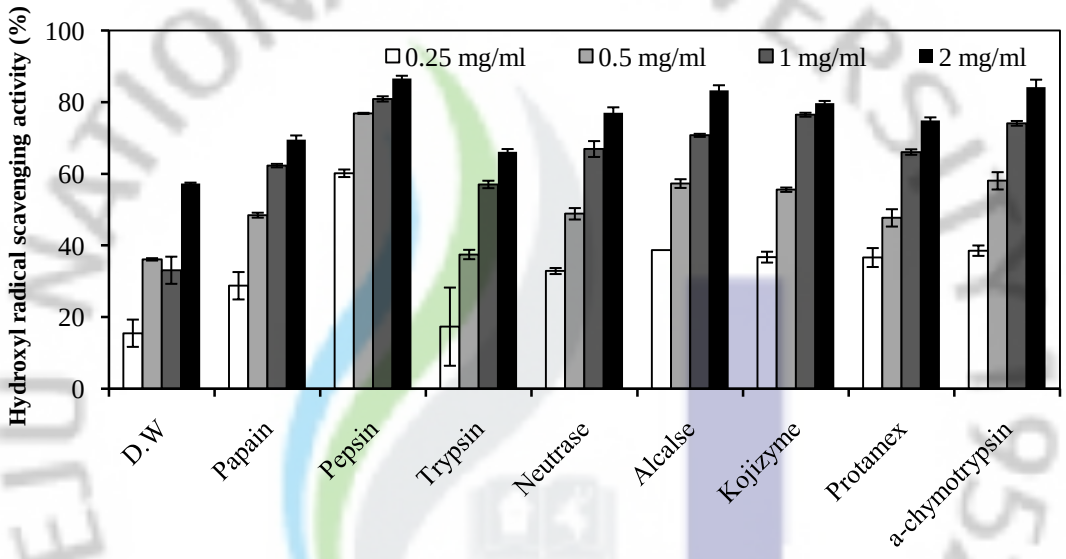

Fig. 1-7. Hydroxyl radical scavenging activity of enzymatic hydrolysates with eight proteinase from flounder fish. Mean ± SD from triplicate determinations.

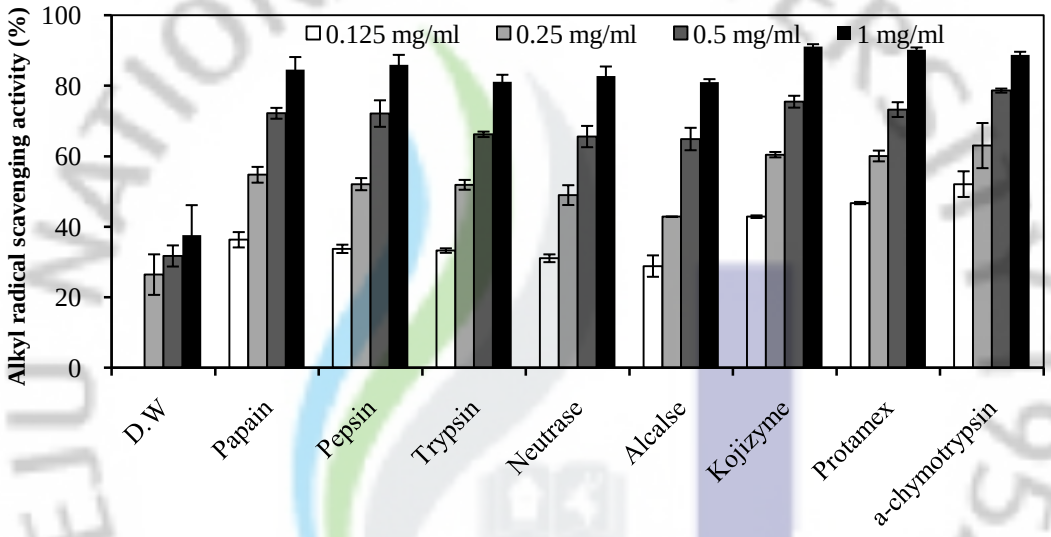

Fig. 1-8. Alkyl radical scavenging activity of enzymatic hydrolysates with eight proteinase from flounder fish. Mean ± SD from triplicate determinations.

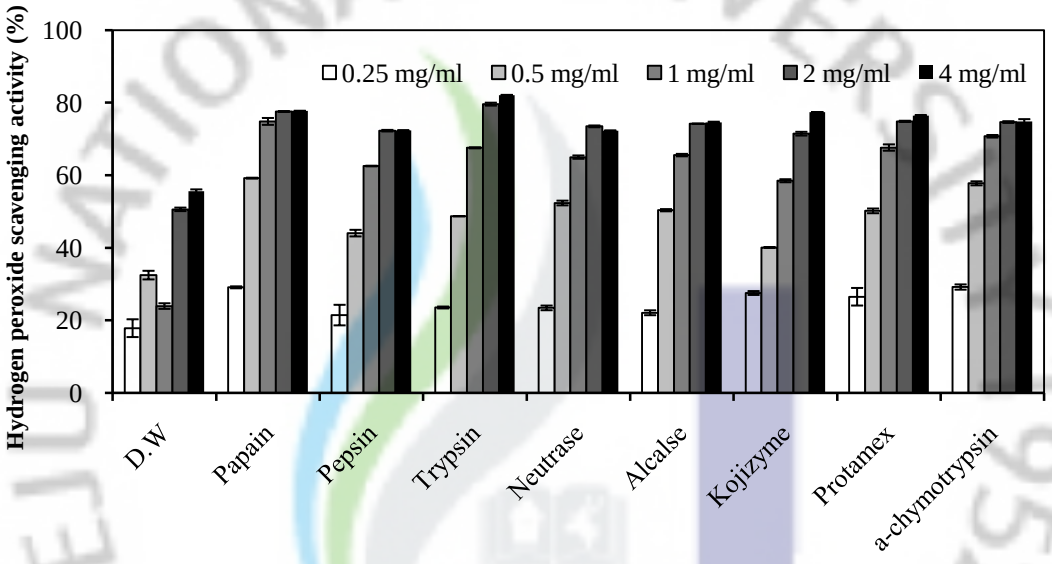

Fig. 1-9. Hydrogen peroxide scavenging activity of enzymatic hydrolysates with eight proteinase from flounder fish. Mean ± SD from triplicate determinations.

Table 1-3. IC_{50} value of enzymatic hydrolysates with eight protein proteinase from Flounder fish.

Enzymatic digests	IC_{50} values (mg/ml)			
	DPPH radical	Hydroxyl radical	Alkyl radical	Hydrogen peroxide
Distilled water	10 <	1.653	1.423	2.814
Papain	5.850	0.666	0.244	0.675
Pepsin	10 <	0.124	0.248	0.964
Trypsin	8.685	1.459	0.286	0.859
Neutrase	10 <	0.594	0.308	0.858
Alcalase	10 <	0.446	0.339	0.876
Kojizyme	3.227	0.461	0.184	1.039
Protamex	6.642	0.580	0.181	0.826
α-chymotrypsin	2.775	0.403	0.156	0.711

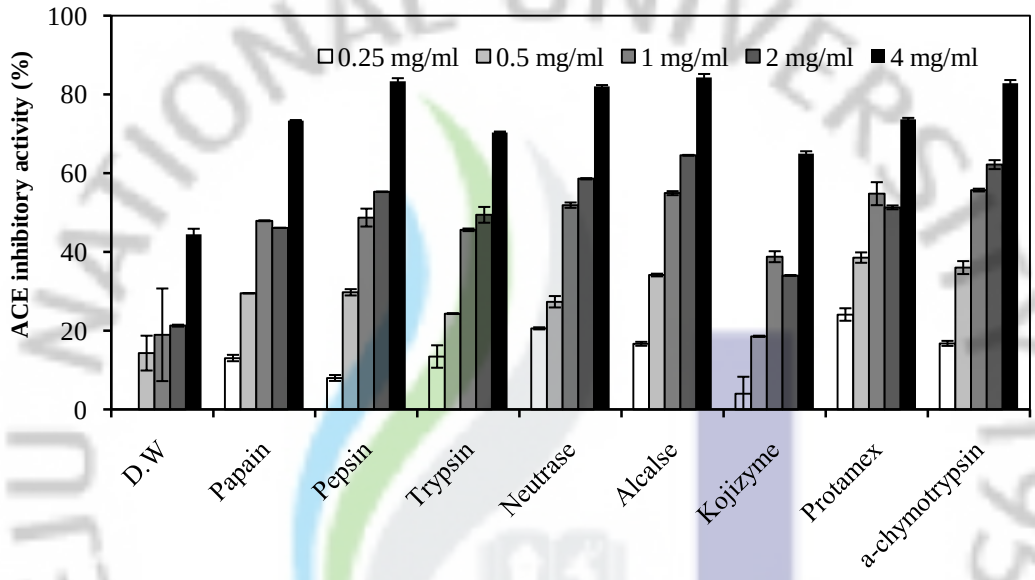

Fig. 1-10. ACE inhibition activity of enzymatic hydrolysates with eight proteinase from flounder fish. Mean ± SD from triplicate determinations.

Table 1-4. ACE inhibition activity IC_{50} value of enzymatic hydrolysates with eight protein proteinase from Flounder fish.

Enzymatic digests	IC_{50} values (mg/ml)
	ACE inhibition activity
Distilled water	4<
Papain	2.141
Pepsin	1.262
Trypsin	1.470
Neutrase	0.975
Alcalase	0.881
Kojizyme	2.849
Protamex	0.857
α-chymotrypsin	0.859

3. 3. Optimum conditions assay for the active enzymatic hydrolysate

The above results showed that hydrolysates obtained from flounder fish by α-chymotrypsin among commercial enzyme exhibited high antioxidantive and ACE inhibition activity. Therefore, α-chymotrypsin hydrolysate was chosen for the isolation and identification of small molecular.

It was investigated in the different of hydrolysis times (6h, 12h, 18h and 24h) and various substrate to enzyme ratio (1000:1, 500:1 and 100:1) to find optimal hydrolysis condition which has the higher antioxidative and ACE inhibition activities than previous hydrolysis time and substrates to enzyme ratio (24h and 500:1). During hydrolysis, a wide variety of larger, medium and smaller peptides are generated, depending on different of hydrolysis time and various substrate to enzyme ratio.

As the hydrolysis time and substrate to enzyme ratio were increased, the yield of α-chymotrypsin hydrolysate was increased (Table 1-5). The α-chymotrypsin hydrolysate was obtained the lowest yield (39.80%) through the hydrolysis time 6h and substrate to enzyme ratio 1000:1, whereas the highest yield (74.37%) was obtained through hydrolysis time 24hr and substrate to enzyme ratio 100:1.

Protein contents are also shown the increased protein contents of α-chymotrypsin hydrolysate, when hydrolysis time and substrate to enzyme ratio were increased (Table 1-6). The lowest protein contents (304.24 mg/g) were obtained when the hydrolysate was performed hydrolysis time 6h and substrate to enzyme ratio 1000:1, whereas the highest protein content (446.51 mg/g) was shown the by substrate to enzyme 500:1 for 24h.

Table 1-5. Yield of α-chymotrypsin hydrolysate from flounder fish, according to hydrolysis time and substrate to enzyme ratio.

Enzymatic hydrolysate	Substrate : Enzyme (S:E)	Hydrolysis time (h)			
		6h	12h	18h	24h
α-chymotrypsin	1000:1	39.80±0.10^{a}	43.52±0.45^{b}	43.83±0.00^{b}	54.48±0.60^{cd}
	500:1	44.51±0.44^{b}	45.44±0.34^{b}	53.64±1.27^{c}	56.60±2.38^{d}
	100:1	59.51±0.24^{e}	62.00±0.11^{f}	66.79±0.94^{g}	74.37±0.14^{h}

Mean ± SD from triplicate determinations.

Significant differences at $p<0.05$ indicated with different letters.

Table 1-6. Protein contents of α-chymotrypsin hydrolysate from flounder fish, according to hydrolysis time and substrate to enzyme ratio (mg/g).

Enzymatic hydrolysate	Substrate :Enzyme (S:E)	Hydrolysis time (h)			
		6h	12h	18h	24h
α-chymotrypsin	1000:1	304.24±7.64[a]	309.40±4.19[a]	306.86±4.21[a]	427.66±27.86[e]
	500:1	347.60±4.28[c]	329.37±5.81[b]	402.45±10.29[d]	446.51±14.85[f]
	100:1	403.38±4.73[d]	399.56±17.86[d]	411.14±23.52[d]	443.84±19.02[f]

Mean ± SD from triplicate determinations.

Significant differences at $p<0.05$ indicated with different letters.

3. 3. 1. Characterization of α-chymotrypsin hydrolysate SDS-PAGE

α-chymotrypsin hydrolysate according to hydrolysis time and substrate to enzyme ratio were characterized using SDS-PAGE (Fig 1-11). Using various hydrolysis time and substrate to enzyme ratio significantly increased the amount of low molecular weight proteins and showed various band patterns. The band patterns of all enzymatic hydrolysates indicated less than 6 kDa.

3. 3. 2. Free radicals scavenging capacities using ESR spectrometer and ACE inhibition activity

The scavenging activities of the α-chymotrypsin hydrolysate according to hydrolysis time and substrate to enzyme ratio on DPPH radical scavenging activity are shown on Table 1-7, 1-8. Antioxidative activity of α-chymotrypsin hydrolysate were increased with decreasing substrate to enzyme ratio from 100:1 to 1000:1. Among them, the lowest DPPH scavenging activity (IC_{50} value 7.945 mg/ml) of α-chymotrypsin hydrolysate was obtained when the hydrolysate was performed hydrolysis time 12h and substrate to enzyme ratio 100:1, whereas the highest DPPH scavenging activity (IC_{50} value 1.963 mg/ml and 1.968 mg/ml, respectively) was shown the by substrate to enzyme 1000:1 for 6h and 18h.

The scavenging activities of the α-chymotrypsin hydrolysate according to hydrolysis time and substrate to enzyme ratio on hydroxyl radical scavenging activity are shown on Table 1-7, 1-8. Increased concentrations of all enzymatic hydrolysates exhibited dose-dependent scavenging activities with varying capacity. And then hydroxyl radical scavenging activities were increased, when decreasing hydrolysis time and substrate to enzyme ratio from 100:1 to 1000:1. In generally, most hydrolysates showed good scavenging capacity. Among them, the lowest hydroxyl radical scavenging activity (IC_{50} value 0.793 mg/ml) of α-chymotrypsin hydrolysate was obtained when the hydrolysate was performed hydrolysis time 24h and substrate to enzyme ratio 100:1, whereas the highest hydroxyl radical

scavenging activity (IC_{50} value 0.212 mg/ml) was shown the by substrate to enzyme 1000:1 for 6h .

Alkyl radical scavenging activity is shown on Table 1-7, 1-8. Increased concentrations of all enzymatic hydrolysates exhibited dose-dependent scavenging activities with varying the same capacity in hydroxyl radical scavenging activity. In generally, most hydrolysates showed good scavenging capacity and among them, substrate to enzyme ratio 1000:1 for 18h had the highest activity (IC_{50} value 0.148 mg/ml).

The scavenging activities of the enzymatic hydrolysates on hydrogen peroxide scavenging activity are shown on Table 1-7, 1-8. In case of hydrogen peroxide scavenging activity, generally most hydrolysates showed the same scavenging capacity.

The ACE inhibition activities of the enzymatic hydrolysates are shown on Table 1-9, 1-10. Among them, hydrolysates of substrate to enzyme ratio 100:1 and according to hydrolysis time (6h, 12h, 18h and 24h) had high inhibition activity (IC_{50} value 0.638 mg/ml, 0.584 mg/ml, 0.572 mg/ml and 2.849 mg/ml, respectively).

In conclusion, yield and protein contents are shown increased yield and protein contents of α-chymotrypsin hydrolysate as the hydrolysis time and substrate to enzyme ratio were increased. And then, α-chymotrypsin hydrolysate by substrate to enzyme 1000:1 had high scavenging activity on DPPH and hydroxyl radical, whereas alkyl radical and hydrogen peroxide scavenging activities were similar activities in all α-chymotrypsin hydrolysates. In ACE inhibition activity, α-chymotrypsin hydrolysate by substrate to enzyme 100:1 had higher ACE inhibition activity than other hydrolysates.

So, this established optimal condition (substrate to enzyme 1000:1 for 18h) based on previous results. The results demonstrated that the α-chymotrypsin hydrolysate possessed higher antioxidant activity and ACE inhibition activity, therefore, it was chosen as sample for

analyzing molecular weight distribution and antioxidant and ACE inhibition activities of the separated fractions.

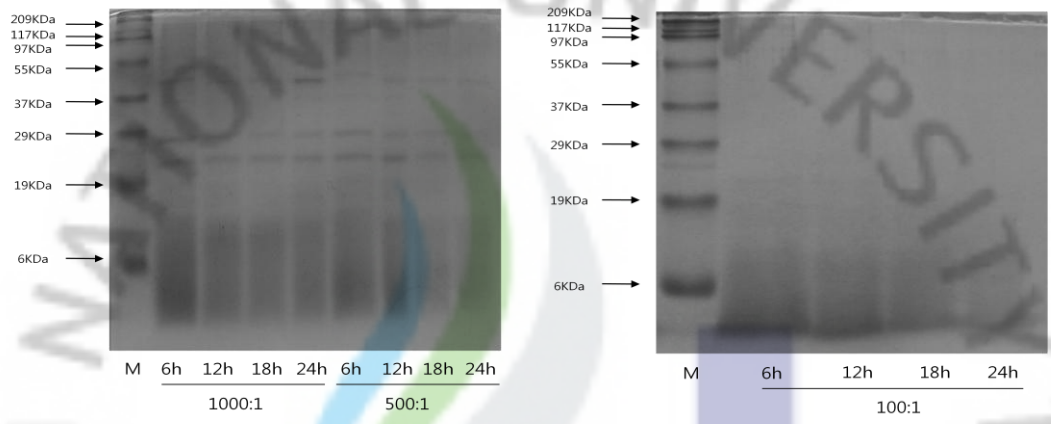

Fig 1-11. Hydrolysis pattern of α-chymotrypsin hydrolysate from flounder fish with hydrolysis time and substrate to enzyme ratio on 15% SDS-PAGE.

M:Marker

Table 1-7. ROS scavenging activity (%) of α-chymotrypsin hydrolysate from flounder fish, according to hydrolysis time and substrate to enzyme ratio.

ROS species	1000:1 (S:E)				500:1 (S:E)				100:1 (S:E)			
	6h	12h	18h	24h	6h	12h	18h	24h	6h	12h	18h	24h
DPPH radical	58.13 ±0.00	41.48 ±0.78	53.33 ±0.06	35.03 ±0.03	43.87 ±0.00	36.20 ±2.18	38.96 ±0.63	33.65 ±2.42	37.74 ±3.74	30.03 ±0.02	22.22 ±2.68	43.81 ±0.14
Hydroxyl radical	61.41 ±3.03	64.18 ±2.15	63.54 ±1.67	67.66 ±0.10	61.78 ±0.75	61.48 ±1.03	63.40 ±2.20	58.05 ±2.42	65.38 ±1.27	57.57 ±0.81	46.43 ±2.22	37.50 ±0.31
Alkyl radical	63.46 ±4.06	53.67 ±1.92	72.26 ±1.14	59.73 ±0.40	63.81 ±1.43	58.39 ±1.26	59.21 ±0.00	63.04 ±6.40	62.48 ±0.83	47.19 ±2.34	64.58 ±0.48	51.84 ±1.44
Hydrogen peroxide	54.40 ±0.34	53.80 ±0.52	56.12 ±0.06	54.81 ±0.52	59.48 ±0.00	54.70 ±0.21	55.80 ±0.47	57.75 ±0.78	61.53 ±0.14	58.49 ±0.42	57.59 ±0.70	54.81 ±0.04

The sample concentration : DPPH - 2 mg/ml, HO^- - 0.5 mg/ml, ROO^- - 0.25 mg/ml, H_2O_2 - 0.5 mg/ml.

Mean ± SD from triplicate determinations.

Table 1-8. IC_{50} values of α-chymotrypsin hydrolysate from Flounder fish, according to hydrolysis time and substrate to enzyme ratio (IC_{50} : mg/ml).

ROS species	Substrate : Enzyme (S:E)	Hydrolysis time (h)			
		6h	12h	18h	24h
DPPH Radical	1000:1	1.963	2.890	1.968	3.803
	500:1	3.760	3.778	2.548	2.775
	100:1	3.379	7.945	7.668	2.919
Hydroxyl Radical	1000:1	0.212	0.301	0.347	0.313
	500:1	0.366	0.324	0.353	0.403
	100:1	0.326	0.416	0.672	0.793
Alkyl radical	1000:1	0.172	0.228	0.148	0.200
	500:1	0.152	0.208	0.158	0.156
	100:1	0.177	0.326	0.156	0.209
Hydrogen peroxide	1000:1	0.794	0.750	0.678	0.677
	500:1	0.684	0.714	0.694	0.711
	100:1	0.668	0.628	0.613	0.783

Table 1-9. ACE inhibition activity (%) of α-chymotrypsin hydrolysate from flounder fish, according to hydrolysis time and substrate to enzyme ratio.

	1000:1 (S:E)				500:1 (S:E)				100:1 (S:E)			
	6h	12h	18h	24h	6h	12h	18h	24h	6h	12h	18h	24h
ACE Inhibition activity	58.46 ±1.39	61.65 ±1.34	60.53 ±1.76	52.88 ±2.38	64.37 ±2.07	59.71 ±0.46	68.46 ±3.27	55.70 ±0.35	70.03 ±1.54	68.61 ±1.44	66.98 ±0.21	63.17 ±0.64

The sample concentration was 1 mg/ml.

Mean ± SD from triplicate determinations.

Table 1-10. IC_{50} values of α-chymotrypsin hydrolysate ACE inhibition activity from Flounder fish, according to hydrolysis time and substrate to enzyme ratio.

	Substrate : Enzyme (S:E)	Hydrolysis time (h)			
		6h	12h	18h	24h
IC_{50} (mg/ml)	1000:1	0.814	0.731	0.780	0.858
	500:1	0.771	0.768	0.800	0.859
	100:1	0.638	0.584	0.572	0.702

3. 4. Molecular weight fractionation of α-chymotrypsin hydrolysate by substrate to enzyme ratio 1000:1 for 18h

The demonstrated hydrolysate analyzed molecular weight distribution and antioxidative and antihypertension activities of the separated fractions. The hydrolysates were ultra-filtered using 5 kDa and 10 kDa membranes. When a 10 kDa membrane was used, > 10 kDa retentate fraction and < 10 kDa filtrate were obtained. Also when a 5 kDa membrane was used, > 5 kDa retentate fraction and < 5 kDa filtrate were obtained. Ultrafiltration is a fast and easy technique for separating peptides based on their molecular weight (MW), it would require numerous washings and prolonged separation times to completely get rid of low MW fractions (i.e. < 5 kDa fractions) from the > 10 kDa retentate. However, as the majority of < 5 kDa fractions are removed during ultrafiltration, the > 10 kDa retentate will be concentrated in high MW fractions rather than with low MW fractions (Sivakumar, R. and Hordur, G.K. 2009).

The yield of fractions using ultrafiltration showed in Table 1-11. According to molecular size, yields presented above 10 kDa fraction 54.07%, 5-10 kDa fraction 15.07% and below 5 kDa 22.13%. protein contents of fractions are shown above 10 kDa 418.30 mg/g, 5-10 kDa 98.93 mg/g and below 5 kDa 111.08mg/g.

3. 4. 1. Characterization of α-chymotrypsin hydrolysate fractions using ultrafiltration membrane SDS-PAGE

In the SDS-PAGE gel, this was showed various band pattern according to molecular size (Fig. 1-12). Molecular size of fractions became smaller, band showed low extent as compared with molecular weight marker.

Table 1-11. Yield and Total protein contents of molecular weight fractions from α-chymotrypsin hydrolysate.

Molecular size	Yield (%)	protein contents (mg/g)
> 10 kDa	54.07±0.05[c]	418.30±5.52[c]
5-10 kDa	15.07±0.09[a]	98.93±8.95[a]
< 5 kDa	22.13±0.14[b]	111.08±27.13[b]

α-chymotrypsin are substrate to enzyme 1000:1 and hydrolysis time 18hr..

Mean ± SD from triplicate determinations.

Significant differences at $p<0.05$ indicated with different letters.

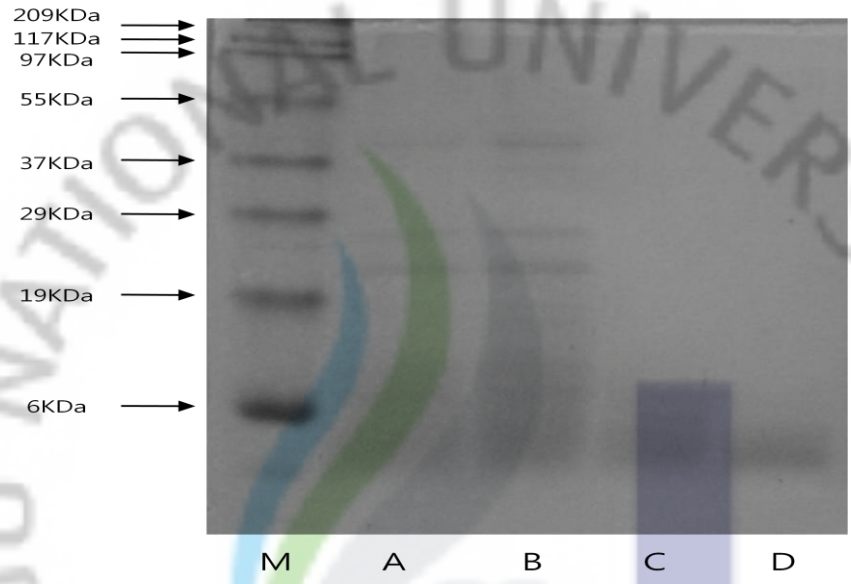

Fig. 1-12. Hydrolysis pattern of molecular weight fractions from α-chymotrypsin hydrolysate on 15% SDS-PAGE. M:Marker, A:α-chymotrypsin (S:E 1000:1, 18hr), B:Above 10kDa, C:5-10kDa, D:Below 5kDa.

3. 4. 2. Free radicals scavenging capacities using ESR spectrometer and ACE inhibition activity

The free radicals scavenging activities of the fractions using ultrafiltration membranes are shown on Table 1-12, 1-13. All fractions exhibited dose-dependent scavenging activities with varying capacity. Molecular size of below 5 kDa fraction had higher free radical scavenging activities (alkyl radical scavenging activity IC_{50} value 0.130 mg/ml, hydrogen peroxide scavenging activity IC_{50} value 0.632 mg/ml and ACE inhibition activity IC_{50} 0.732 mg/ml) than other molecular size fractions. Whereas, scavenging activity on hydroxyl radical had molecular size of above 10 kDa fraction (IC_{50} value 0.165 mg/ml).

In conclusion, yield and protein contents showed higher yield and total protein contents at above 10 kDa fraction than 5-10 kDa and below 5 kDa fractions. Molecular size of below 5 kDa fraction had the highest free radical scavenging activities on alkyl radical, hydrogen peroxide and ACE inhibition activity, except hydroxyl radical scavenging activity.

Previous many works reported also that low molecular weight peptides showed higher antioxidant activity (Hernandez-Ledesma et al., 2005). Also a similar increase in the ACE inhibitory activity of ultra filtered low MW fractions was also reported for whey protein digests (Pihlanto-Leppala et al., 2000) and for hydrolysates prepared from the protein of yellowfin sole frames (Jung et al., 2006). In this study, α-chymotrypsin contributed to a high degree of hydrolysate due to their different enzymatic actions, and the ultrafiltration was an successful method to enhance its antioxidant activity and ACE inhibition activity of enzymatic hydrolysate from flounder fish. Therefore, we selected bellow 5 kDa molecular fraction for use in further experiments.

Table 1-12. ROS scavenging activity (%) and ACE inhibition activity (%) of molecular weight fractions.

	Molecular size		
	> 10 kDa	5-10 kDa	< 5 kDa
Hydroxyl radical	62.17±0.09	38.87±4.33	45.42±2.37
Alkyl radical	47.83±1.55	62.13±0.83	69.77±0.92
Hydrogen peroxide	46.06±0.76	57.43±0.63	62.31±1.00
ACE inhibition	32.16±2.62	53.06±1.39	62.97±0.00

The sample concentration : HO^- - 0.25 mg/ml, ROO^- - 0.25 mg/ml, H_2O_2 - 0.5 mg/ml, ACE inhibition – 1 mg/ml.

Mean ± SD from triplicate determinations.

Table 1-13. IC_{50} value of molecular weight fractions from α-chymotrypsin digest.

Molecular size	IC_{50} values (mg/ml)			
	Hydroxyl Radical	Alkyl Radical	Hydrogen Peroxide	ACE inhibition
> 10 kDa	0.165	0.296	0.938	1.462
5-10 kDa	0.413	0.250	0.727	0.915
< 5 kDa	0.332	0.130	0.632	0.732

3. 5. Size exclusion chromatography

To clarify the active fractions of the fish muscle hydrolysates, partial purification of the hydrolysates using a size exclusion column was performed. Previously ultrafiltration using a membrane with molecular weight cut off at 5,000 Da, the filtrate of the fraction from the α-chymotrypsin hydrolysate (Substrate to enzyme ratio 1000:1 for 18h) was loaded on a size exclusion column on Sephadex G-25. Sephadex G-25 had an optimal molecular weight range of 1,000-5,000 Da and separated with distilled water. The size exclusion chromatograms of the below 5 kDa from α-chymotrypsin hydrolysate are shown in Fig. 1-13. Eluted fractions were monitored peptide bond at 220 nm. We are obtained through size exclusion chromatography four fractions and each fractions (7.5 ml) were collected, freeze dried and alkyl radical scavenging activities were determined. All fractions had alkyl radical scavenging activity when concentration is 1 mg/ml. Alkyl radical scavenging activity was 38.29%, 66.82%, 60.04% and 90.54% for Fr. 1, Fr. 2, Fr. 3 and Fr. 4, respectively and Fr. 4 had the highest alkyl radical scavenging activity (Table 1-14). The results about amino acids analysis of Fr. 4 showed in Table 1-15. There were rich phenylalanine, histidine and lysine which accounted for 10.12%, 19.93% and 13.15% of the total amino acids in Fr.4, respectively.

In conclusion, we have known that small molecular weight material from flounder fish has strong antioxidant activity and ACE inhibition activity. And then Da'valos et al. (2004) reported that among the amino acids, Trptophan, Tyrosine and Methionine showed the highest antioxidant activity, followed by Cystein, Histidine and Phenylalanine. The antioxidant activity of histidine-containing peptides has been reported and attributed to the chelating and lipid radical-trapping ability of the imidazole ring (Murase et al., 1993; Park et al., 2001; Uchida and Kawakishi., 1992). Therefore, these results suggested that

antioxidative activity of the Fr. 4 was dependent on their amino acid compositions and molecular weight.

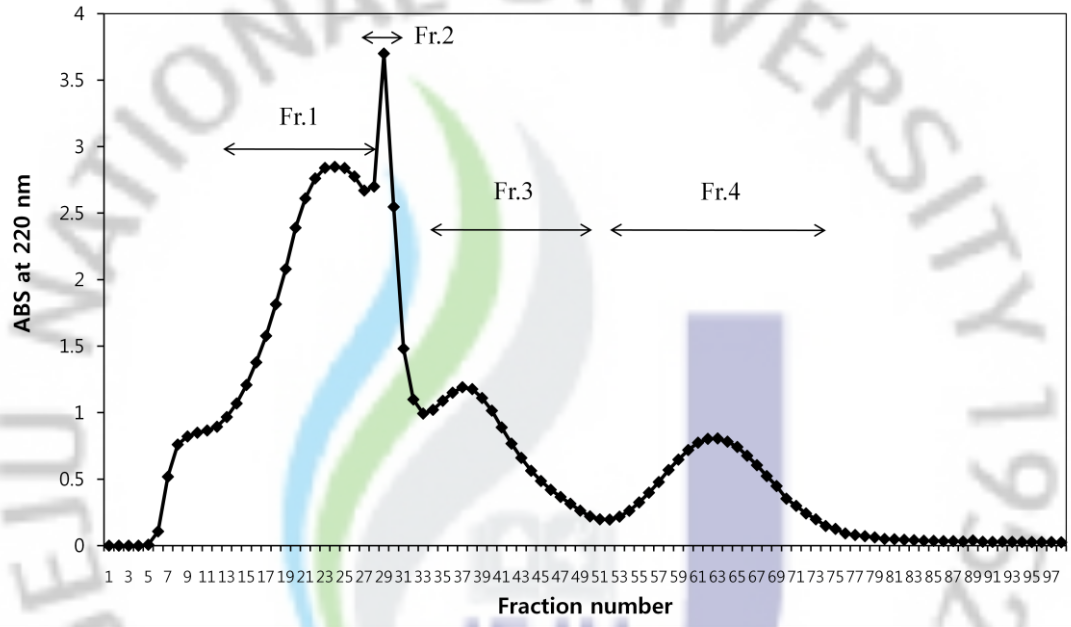

Fig. 1-13. Size exclusion chromatography on sephadex G-25 gel of below 5kDa from α-chymotrypsin hydrolysate. Separation was collected at a peak volume (7.5ml). The peaks isolated by Sephadex G-25 gel column were designated F1-F4. Elution was monitored at 220 nm.

Table 1-14. Alkyl radical scavenging activity (%) of gel chromatography fractions.

Fractions	Alkyl radical Scavenging activity (%)
Fr. 1	38.29±1.84
Fr. 2	66.82±1.56
Fr. 3	60.04±0.57
Fr. 4	90.54±0.08

The sample concentration was 1 mg/ml.

Mean ± SD from triplicate determinations

Table 1-15. Amino acids analysis of the fraction 4.

Amino acids	Content (%)
Aspartic acid	8.40
Threonine	3.07
Serine	4.02
Glutamic acid	7.76
Glycine	6.96
Alanine	7.58
Valine	2.09
Methionine	0.31
Isoleucine	0.75
Leucine	3.58
Tyrosine	1.03
Phenylalanine	**10.12**
Histidine	**19.93**
Lysine	**13.15**
Arginine	9.99
Proline	1.26

Part II

Biological activity of enzymatic digests from flounder fish muscle by proteolytic enzymes in human body

ABSTRACT

This study investigated the effect of digests from flounder fish muscle by complex enzyme (pepsin, α-chymotrypsin) on antioxidant activity and angiotensin converting enzyme (ACE) inhibition activity. The digests were hydrolyzed by α-chymotrypsin after being hydrolyzed by pepsin protease. The antioxidant activity was determined by measuring free radical such as alkyl radical, hydroxyl radical using electron spin resonance (ESR). Hydrogen peroxide, ACE inhibition activity was determined by colorimetric assay. In results of antioxidant activity and ACE inhibition activity of digests preparing as hydrolysis times (0.5hr, 1hr, 3hr, 6hr, 12hr) in 500:1 substrate to enzyme ratio, digest which was hydrolyzed by α-chymotrypsin for 12hr after being hydrolyzed by pepsin for 1hr, was exhibited higher level of antioxidant activity (Alkyl radical IC_{50} value 0.246 mg/ml, Hydroxyl radical IC_{50} value 0.392 mg/ml and Hydrogne peroxide IC_{50} value 1.051 mg/ml) and ACE inhibition activity (ACE inhibition IC_{50} value 0.982 mg/ml) compared with hydrolysates of other condition. Ultrafilteration was performed for separating of pesin (1hr) - α-chymotrypsin (12hr) digest as molecular weight (above MW 10 kDa, MW 5-10 kDa, below MW 5 kDa). Although pesin (1hr) - α-chymotrypsin (12hr) digest of above MW 10 kDa exhibited the highest hydroxyl radical scavenging activity (IC_{50} value 0.354 mg/ml), alkyl radical scavenging activity (IC_{50} value 0.188 mg/ml) and ACE inhibition activity (IC_{50} value < 0.500 mg/ml) were exhibited the strongest activity at pesin (1hr) - α-chymotrypsin (12hr) digest of below MW 5 kDa. In conclusion, we have been known that flounder fish has strong antioxidant activity and anti-hypertension activity when we eat flounder fish.

MATERIALS and METHODS

2. 1. Materials

Flounder fish (*Paralichthys olivaceus*) were cultivated in Jeju island. Fish after capture were filleted and muscle was collected. It was washed twice with freshwater, then immediately frozen and stored at -20℃ until used. The frozen sample was lyophilized and homogenized with a grinder before hydrolysis.

2. 2. Chemicals and reagent

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 5,5-Dimethyl-1-pyrrolin N-oxide (DMPO), α-(4-pyridyl-1-oxide)-N-t-butylnitrone (4-POBN), Folin-Ciocalteu reagent, peroxidase, 2,2-Azino-bis(3-ethylbenz-thiazolin)-6-sulfonic acid (ABTS), 2,2-azobis-(2-amidinopropane) hydrochloride (AAPH) and Albumin were purchased from Sigma Chemical Co. (USA). $FeSO_4 \cdot 7H_2O$, Hydrogen peroxide were purchased from Dae Jung Chemical Co. (Korea). 30% Acrylamide/Bis solution (29:1) were purchased from Bio-rad Laboratories, Inc. (USA). Ammonium persulfate (APS) and TEMED were purchased from Amresco Inc. (USA). N-Hippuryl-His-Leu tetrahydrate (HHL) and Angiotensin I converting enzyme (from rabbit lung) were purchased from Sigma chemical Co. (USA). Protein protease such as Pepsin and α-chymotrypsin were purchased from Sigma chemical Co. (USA). All the other chemicals used were analytical grade.

2. 3. Preparation of enzymatic digests by proteolytic enzyme in human body

2. 3. 1. Preparation of pepsin digests and optimum conditions assay for the active enzymatic digest

It was carried out optimum conditions assay (Ko, 2009) for antioxidant and ACE

inhibition digests from flounder fish. Freeze dried flounder fish muscle was homogenized with a grinder. Five gram of the dried flounder fish powder put into distilled water 100 ml and was adjusted pH 2.0. And then various substrates to enzyme ratio (1000:1, 500:1 and 100:1) was added. Hydrolysis time was carried out various times (6h, 12h, 18h and 24h). At the end of the reaction, the digests was adjusted to pH 7.0 and heating at 90℃ for 10 min in order to inactivate the enzyme. The digests were centrifuged at 3,500 rpm for 20 min to separate insoluble and soluble fractions. The soluble phase was freeze dried using freeze dryer and stored at -20℃ for further use.

2. 3. 2. Preparation of complex enzyme digest and optimum conditions assay for the active enzymatic digest

It was carried out optimum conditions assay (Ko, 2009) for antioxidant and ACE inhibition digest from flounder fish and preparation of complex enzyme digest used pepsin and α-chymotrypsin protease. Freeze dried flounder fish muscle was homogenized with a grinder. Five gram of the dried flounder fish powder put into distilled water 100 ml and was adjusted pH 2.0. And then pepsin protease of substrate to enzyme ratio 500:1 was added. Hydrolysis time was carried out various times (0.5h, 1h, 3h, 6h and 12h). At the end of the reaction, the digests was adjusted to pH 7.0 and heating at 97℃ for 10 min in order to inactivate the enzyme and the digests cool down temperature. Once again protease was added in the cooling digests. Added substrate to enzyme ratio of α-chymotrypsin protease is 500:1. Hydrolysis time was carried out 12h. At the end of the reaction, the digests was adjusted to pH 7.0 and heating at 97℃ for 10 min in order to inactivate the enzyme. The digests were centrifuged at 3,500 rpm for 20 min to separate insoluble and soluble fractions. The soluble phase was freeze dried using freeze dryer and determined antioxidant activity and ACE inhibition activity. It selected digests which was the highest activities, and then it is performed to hydrolysis as hydrolysis time (0.5h, 1h, 3h, 6h and 12h) by α-chymotrypsin

(500:1).

2. 4. Molecular weight fractionation of active enzymatic hydrolysate

In all the enzymatic digests tested antioxidant and ACE inhibition activities. The superior enzymatic digest were selected. And then selected digest separated molecular weight by Ultra filtration membranes using Millipore's Lab scale TFF system (Millipore Corporation, Bedford, Massachusetts, USA) at 4℃. The collected molecular weight fractions are above 10 kDa, 5-10 kDa and below 5 kDa. This was lyophilized and stored at -20℃ for use.

2. 5. Measurement of protein content

The soluble protein content was determined by the Lowry method. One milliliter of the digests, 1 ml of alkaline-copper working solution and 3 ml of 50% Folin-Ciocalteu's phenol reagent were mixed. The mixtures were allowed to react for 45 min and absorbance at 540 nm was measured with a spectrophotometer. Calibration standard is used bovine serum albumin (Lowry et al., 1951).

2. 6. SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the protein digests using a 15% Tris/Hcl gel to characterize the digests based on their molecular weights (MW). The MW of the digest was calculated with reference to the migration of SDS-PAGE wide range molecular weight standard. Samples were heated at 100℃ for 5 min prior to the electrophoresis run. After electrophoresis, the gels were stained with Bio-Rad Coomassie Blue R-250. The bands in the samples were compared with known bands of protein standards.

2. 7. Free radical scavenging capacities using ESR spectrometer

2. 7. 1. Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by the iron-catalyzed Haber-Weiss reaction (Fenton-driven Haber-Weiss reaction; $Fe^{2+} + H_2O_2 \rightarrow {}^{\cdot}OH + OH^-$) and the hydroxyl radicals rapidly reacted with nitrone spin trap DMPO (Rosen and Rauckman, 1980). The resultant DMPO-OH adducts was detectable with the ESR spectrometer. Sample 20 ul of various concentrations were mixed with 0.3 M DMPO 20ul in phosphate buffer solution (PBS; pH 7.4), 10 mM $FeSO_4$ 20 ul and 10 mM H_2O_2 20 ul. And then after 2.5 minutes transferred into a capillary tube and recorded spectrum by ESR. The experimental conditions were as followed; magnetic field 336.5±5 mT, power 1 mW, modulation frequency 100 kHz, amplitude 1 X 200, modulation width 0.1 mT, sweep width 10 mT, sweep time 30 sec, time constant 0.03 sec.

Scavenging activity % = (HC –HS) / HC X 100

HC : Relative peak heights of the radical signals without sample

HS : Relative peak heights of the radical signals with sample

2. 7. 2. Alkyl radical scavenging activity

Alkyl radicals were generated by AAPH and their scavenging effects were investigated by the method described by Hiramoto et al. (1993) Sample 20 ul of various concentrations were mixed with distilled water 20 ul, 40 mM AAPH 20 ul and 40 mM 4-POBN 20ul in the PBS (pH 7.4). And then after 30 minutes incubated at 37℃ in a water bath. So transferred into a capillary tube and recorded spectrum by ESR. The experimental conditions were as followed; magnetic field 336.5±5 mT, power 1 mW, modulation frequency 100 kHz, amplitude 1 X 1000, modulation width 0.2 mT, sweep width 10 mT, sweep time 30 sec, time constant 0.03 sec.

2. 7. 3. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was carried out according to the method of Muller (1995). Sample 100 ul was mixed with 0.1 M phosphate buffer (pH 5.0) 100 ul and 10 mM hydrogen peroxide 20 ul in a 96 microwell plate and incubated at 37℃ for 5 minutes. Thereafter, 1.25 mM ABTS 30 ul and peroxidase (1 U/ml) 30 ul were mixed and incubated at 37℃ for 10 minutes. The absorbance was measured at 405 nm.

2. 8. ACE inhibition activity

The ACE inhibition activity assay was performed according to the method of Cushman and Cheung (1971) with slight modification. The amount of hippuric acid (HA) formed from HHL by is determined by a spectrophotometric assay. A sample solution 50ul with 50 ul of ACE solution (25 mU/ml) was pre-incubated at 37℃ for 10 minutes, and then incubated with 100 ul 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 minutes. The reaction stopped by added 250 ul of 1 N HCl. Hippuric acid was extracted with 350 ul of ethyl acetate. After centrifugation (4,000 rpm, 10 minutes), 200 ul of the upper phase was transferred into a ependorf tube and evaporated in dry-oven at 80℃. The hippuric acid was dissolved in 1 ml distilled water, and the absorbance was measured at 228 nm. The extent of inhibition was calculated as followed.

Inhibition activity % = (AC – AS) / (AC – AB) X 100

AC : Absorbance of control

AS : Absorbance of reaction sample solution

AB : Absorbance of blank solution

2. 9. Statistical analysis

All experiments were conducted in triplicate (n=3) and an ANOVA test (using SPSS 11.5 statistical software) was used to analyze the data. Significant differences between the means of parameters were determined by using the Duncan's test ($p < 0.05$).

RESULTS and DISCUSSION

3. Preparation of enzymatic digests by proteolytic enzyme in human body

3. 1. Preparation of pepsin digests and optimum conditions assay for the active enzymatic digest

3. 1. 1. Determined optimum conditions for pepsin digests

The optimal hydrolysis conditions for production of antioxidative and antihypertensive peptides from flounder fish muscle were investigated in the different of hydrolysis times (6h, 12h, 18h and 24h) and various substrate to enzyme ratio (1000:1, 500:1 and 100:1). In the results, yield of pepsin digests were influenced, according to substrate to enzyme ratio (Table 2-1), whereas different hydrolysis times didn't influenced in yield. The lowest yield 33.21% was obtained through substrate to enzyme ratio 1000:1 for 6h and pepsin digest by Substrate to enzyme ratio 100:1 for 18h had the highest yield 58.60%.

Protein contents are also shown the increased protein contents of pepsin digests, when substrate to enzyme ratio was increased (Table 2-2). In generally, when hydrolysis times were increased, protein contents were decreased. The lowest protein contents (220.04 mg/g) were obtained by substrate to enzyme 1000:1 for 24h, whereas the highest protein contents (351.07mg/g) were obtained by substrate to enzyme 100:1 for 6h.

Table 2-1. Yield of pepsin digests from flounder fish, according to different hydrolysis times and substrate to enzyme ratio.

Enzymatic digest	Substrate : Enzyme (S:E)	Incubation time (h)			
		6h	12h	18h	24h
Pepsin	1000:1	33.21±0.30^{a}	43.08±1.01^{d}	39.34±0.19^{bc}	38.54±0.00^{b}
	500:1	40.61±0.22^{c}	49.34±0.00^{f}	40.00±0.75^{bc}	44.87±0.60^{e}
	100:1	48.34±0.86^{f}	52.01±0.62^{g}	58.60±1.20^{i}	53.73±0.62^{h}

Mean ± SD from triplicate determinations

Significant differences at $p<0.05$ indicated with different letters.

Table 2-2. Protein of pepsin digests from flounder fish, according to hydrolysis time and substrate to enzyme ratio.

Enzymatic digest	Substrate : Enzyme (S:E)	Incubation time (h)			
		6h	12h	18h	24h
Pepsin	1000:1	230.52±14.79[a]	294.63±19.16[b]	227.08±33.51[a]	220.04±28.14[a]
	500:1	289.58±26.31[b]	310.19±10.00[c]	232.60±51.84[a]	227.16±13.87[a]
	100:1	351.07±37.33[d]	350.28±24.85[d]	313.56±0.15[c]	310.94±16.96[c]

Mean ± SD from triplicate determinations

Significant differences at $p<0.05$ indicated with different letters.

3. 1. 1. 1. Characterization of pepsin digests SDS-PAGE

Pepsin digests according to different hydrolysis times and substrate to enzyme ratio were characterized using SDS-PAGE (Fig 2-1). In the results, pepsin digests showed various band pattern. Especially , pepsin digests of substrate to enzyme ratio 1000:1 and 500:1 showed significantly high number of strong bands between 29 kDa and 37 kDa, whereas substrate to enzyme ratio 100:1 hydroylsate showed high number of strong bands between 37 kDa and 55 kDa. And then, as increase hydrolysis time, number of bands of below 6 kDa of all Pepsin digests was increased.

3. 1. 1. 2. Free radicals scavenging capacities using ESR spectrometer and ACE inhibition activity

The scavenging activities of the pepsin digests according to different hydrolysis time and substrate to enzyme ratio on hydroxyl radical scavenging activity are shown in Table 2-3, 2-4. Overall, all digests showed good scavenging activity excepted digest of substrate to enzyme ratio condition is 100:1. When the hydrolysis condition had substrate to enzyme ratio 500:1 for 18h, it had the highest scavenging activity (IC_{50} value 0.114 mg/ml).

Results of Alkyl radical scavenging capacity showed in Table 2-3, 2-4. The all hydrolysates also had good alkyl radical scavenging activity, however, hydrolysis conditions such as different hydrolysis and substrate to enzyme ratio didn’t influence in scavenging capacity. When hydrolysis condition is substrate to enzyme ratio 1000:1 for 6h and 18h, and 100:1 for 18h, they had the highest activities (IC_{50} values 0.210 mg/ml, 0.209 mg/ml and 0.210 mg/ml, respectively).

Hydrogen peroxide scavenging activity results showed in Table 2-3, 2-4. Overall, hydrogen peroxide scavenging activity of all digests had lower scavenging activity than other radical scavenging activity. The lowest activity showed IC_{50} value of above 4 mg/ml when the hydrolysis condition had substrate to nzyme ratio 1000:1 for 6h, and highest

activity is IC_{50} value 0.964 mg/ml when the hydrolysis condition had substrate to enzyme ratio 500:1 for 24h.

ACE inhibition activities of the pepsin digests are shown in Table 2-5, 2-6. Among them, digests of substrate to enzyme ratio 100:1 and according to hydrolysis time (6h, 12h, 18h and 24h) had overall higher inhibition activity than other digests (Substrate to enzyme ratio 1000:1 and 500:1). The lowest activity is IC_{50} value 1.297 mg/ml when hydrolysis condition is substrate to enzyme ratio 1000:1 for 18h, whereas the highest activity is IC_{50} value 0.447 mg/ml when hydrolysis condition was substrate to enzyme ratio 100:1 for 18h.

In conclusion, yield and protein contents of pepsin digests were increased when substrate to enzyme ratio was increased. In case of ROS scavenging activity, when hydrolysis condition was substrate to enzyme ratio 500:1 for 18h, it had the highest scavenging activity on hydroxyl radical and digest with substrate to enzyme ratio 500:1 for 24h had higher activity than other digests on hydrogen peroxide. On alkyl radical, hydrolysis condition 1000:1 for 6h and 18h, and 100:1 for 18h had the highest scavenging activity. On ACE inhibition activity, pepsin digest hydrolyzed by substrate to enzyme ratio 100:1 for 18h had the superior inhibition activity.

On the whole, we established optimal condition (substrate to enzyme 100:1 for 18h) based on previous results. The results were demonstrated that the pepsin digest possessed higher antioxidant activity and ACE inhibition activity. Therefore, it was chosen as sample for analyzing molecular weight distribution and antioxidant and ACE inhibition activities of the separated fractions.

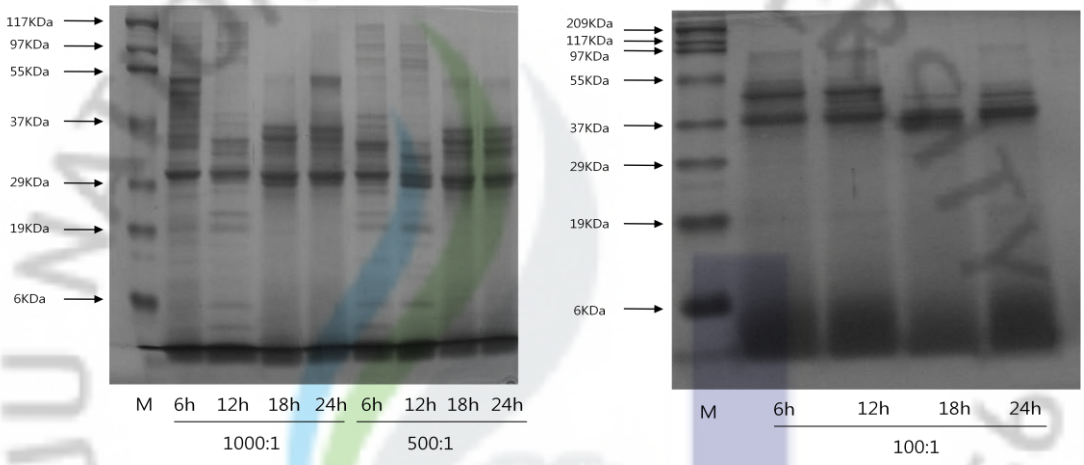

Fig 2-1. Hydrolysis pattern of pepsin digests from flounder fish with hydrolysis time and substrate to enzyme ratio on 15% SDS-PAGE. M: Marker

Table 2-3. ROS scavenging activity (%) of pepsin digests from flounder fish, according to hydrolysis time and substrate to enzyme ratio.

ROS species	1000:1 (S:E)				500:1 (S:E)				100:1 (S:E)			
	6h	12h	18h	24h	6h	12h	18h	24h	6h	12h	18h	24h
Hydroxyl radical	75.49 ±2.33	73.16 ±2.39	73.71 ±2.44	73.72 ±3.13	75.04 ±0.61	74.73 ±1.22	78.26 ±1.42	76.88 ±0.16	73.61 ±0.98	68.67 ±0.26	64.34 ±0.93	69.81 ±2.33
Alkyl radical	58.12 ±3.44	45.27 ±1.83	60.16 ±1.42	48.22 ±2.71	52.35 ±6.31	48.46 ±7.09	55.68 ±0.80	50.51 ±3.17	52.86 ±0.00	41.24 ±8.97	54.87 ±5.13	44.68 ±1.15
Hydrogen peroxide	30.24 ±1.80	30.46 ±0.40	30.45 ±1.39	31.05 ±2.67	29.90 ±0.06	33.44 ±0.69	36.60 ±1.91	44.05 ±0.91	38.64 ±0.44	38.58 ±1.63	37.99 ±0.86	41.30 ±1.29

The sample concentration : $HO^{\cdot}$ - 0.5 mg/ml, $ROO^{\cdot}$ - 0.25 mg/ml, H_2O_2 – 0.5 mg/ml.

Mean ± SD from triplicate determinations

Table 2-4. IC_{50} value of pepsin digests from Flounder fish, according to hydrolysis time and substrate to enzyme ratio. (IC_{50} : mg/ml)

ROS species	Substance : Enzyme (S:E)	Incubation time (h)			
		6h	12h	18h	24h
Hydroxyl radical	1000:1	0.121	0.124	0.180	0.180
	500:1	0.123	0.121	0.114	0.124
	100:1	0.167	0.191	0.200	0.184
Alkyl Radical	1000:1	0.210	0.317	0.209	0.312
	500:1	0.235	0.282	0.226	0.248
	100:1	0.229	0.335	0.210	0.328
Hydrogen peroxide	1000:1	4 <	1.165	1.305	2.204
	500:1	1.900	1.173	1.769	0.964
	100:1	1.039	1.099	1.161	1.072

Table 2-5. ACE inhibition activity (%) of pepsin digests from flounder fish, according to hydrolysis time and substrate to enzyme ratio.

	1000:1 (S:E)				500:1 (S:E)				100:1 (S:E)			
	6h	12h	18h	24h	6h	12h	18h	24h	6h	12h	18h	24h
ACE inhibition	26.87 ±2.75	48.11 ±0.64	42.88 ±0.49	43.57 ±1.59	43.47 ±0.67	34.33 ±0.00	53.47 ±1.37	48.84 ±1.37	48.58 ±0.08	57.88 ±0.42	69.39 ±0.30	62.81 ±0.33

The sample concentration was 1 mg/ml.

Mean ± SD from triplicate determinations

Table 2-6. IC_{50} value of pepsin digests ACE inhibition activity from Flounder fish, according to hydrolysis time and substrate to enzyme ratio.

	Substance : Enzyme (S:E)	Incubation time (h)			
		6h	12h	18h	24h
ACE Inhibition	1000:1	1.849	1.221	1.297	1.195
	500:1	1.290	1.287	0.896	1.107
	100:1	1.014	0.829	0.447	0.703

3. 1. 2. Molecular weight fraction of pepsin digest by substrate to enzyme ratio 100:1 for 18h

Pepsin digest by substrate to enzyme ratio 100:1 for 18h was ultra-filtered using 5 kDa and 10 kDa membranes. Their yield showed in Table 2-7. According to molecular size, yields presented above 10 kDa fraction 38.81%, 5-10 kDa fraction 21.68% and below 5 kDa fraction 25.53%, respectively. Protein contents of fractions are shown above 10 kDa 283.29 mg/g, 5-10 kDa 123.23 mg/g and below 5 kDa 102.95 mg/g, respectively.

3. 1. 2. 1. Characterization of pepsin digest fractions using ultrafiltration membrane SDS-PAGE

Pepsin digest fractions according to molecular size were characterized using SDS-PAGE (Fig 2-2). As molecular weight became lower, it was shown molecular weight band of below 6kDa became stronger. We could confirm to distribute well as molecular weight by ultrafilteration.

3. 1. 2. 2. Free radicals scavenging capacities using ESR spectrometer and ACE inhibition activity

The free radicals scavenging activities of the fractions by ultrafiltration membranes are shown in Table 2-8, 2-9. Molecular size of below 5 kDa fraction had higher scavenging activity and ACE inhibition activity than other fractions except of hydroxyl radical scavenging activity (Above 10 kDa IC_{50} value 0.311 mg/ml, 5-10 kDa 0.388 mg/ml and below 5 kDa 0.397 mg/ml, respectively). And also, in case of ACE inhibition activity 5 kDa fraction had superior inhibition activity (IC_{50} value 0.377 mg/ml). Wu *et al*. (2003) and Je *et al*. reported that low molecular weight peptides showed higher scavenging activities than large molecular weight peptides. Whereas, free radical activities of fractions of below 5kDa from flounder fish showed similar activities compare with previously digests which was

substrate to enzyme ratio 100:1 for 18h.

Table 2-7. Yield and protein contents of molecular weight fractions from pepsin digests.

Molecular size	Yield (%)	Total protein contents (mg/g)
> 10 kDa	38.81±0.22^{c}	283.29±49.20^{c}
5-10 kDa	21.68±0.07^{a}	123.23±43.77^{b}
< 5 kDa	25.53±0.15^{b}	102.95±12.96^{a}

Mean ± SD from triplicate determinations

Significant differences at $p<0.05$ indicated with different letters.

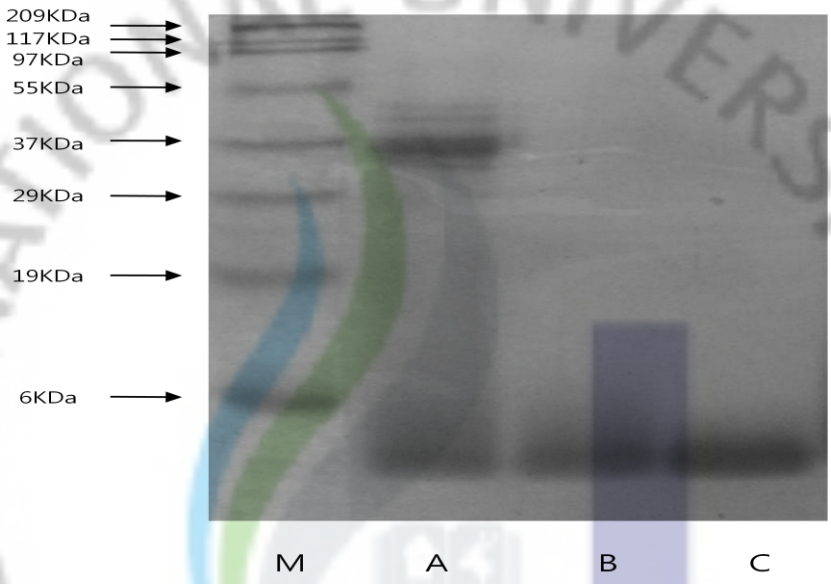

Fig. 2-2. Hydrolysis pattern of molecular weight fractions from pepsin digests on 15% SDS-PAGE. M:Marker, A: Above 10kDa, B:5-10kDa, C:Below 5kDa.

Table 2-8. ROS scavenging activity (%) and ACE inhibition activity(%) of molecular weight fractions.

	Molecular size		
	> 10 kDa	5-10 kDa	< 5 kDa
Hydroxyl radical	46.03±2.16	41.74±0.64	39.48±1.51
Alkyl radical	27.77±3.09	37.09±4.07	51.10±1.41
Hydrogen peroxide	18.53±0.47	29.36±0.25	33.70±0.20
ACE inhibition	31.21±3.33	45.57±2.85	84.14±0.52

The sample concentration : $HO^{\cdot}$ - 0.25 mg/ml, $ROO^{\cdot}$ - 0.25 mg/ml, H_2O_2 - 0.5 mg/ml, ACE inhibition – 1 mg/ml.

Mean ± SD from triplicate determinations.

Table 2-9. IC_{50} value of molecular weight fractions from pepsin digest.

Molecular size	IC_{50} values (mg/ml)			
	Hydroxyl Radical	Alkyl Radical	Hydrogen Peroxide	ACE inhibition
> 10 kDa	0.311	0.434	2 <	1.659
5-10 kDa	0.388	0.387	1.239	1.126
< 5 kDa	0.397	0.244	1.152	0.377

3. 2. Preparation of complex enzyme digest and optimum conditions assay for the active enzymatic digests

3. 2. 1. Determined optimum conditions for complex enzyme using pepsin and α-chymotrypsin protease.

The optimal digest conditions for complex enzyme were investigated in the different of hydrolysis times (0.5h, 1h, 3h, 6h and 12h). Yield is shown in Table 2-10. When hydrolysis time was increased in complex hydrolysis (It hydrolyzed α-chymotrypsin for 12h after hydrolyzing pepsin as different hydrolysis time), yield and protein contents were increased except hydrolysis time 6h. In case of it hydrolyzed α-chymotrypsin as different hydrolysis time after hydrolyzing pepsin for 1h, yield and protein contents were increased according to hydrolysis time is increased.

3. 2. 1. 1. Free radicals scavenging capacities using ESR spectrometer and ACE inhibition activity

The scavenging activities of the complex enzyme digests according to different hydrolysis time (0.5h, 1h, 3h, 6h and 12h) are shown in Table 2-11, 2-12. Results of hydroxyl radical scavenging capacity showed the lowest activity (IC_{50} 533 mg/ml) in digest hydrolyzed α-chymotrypsin for 3h after hydrolyzing pepsin for 1h. It was shown high hydroxyl radical scavenging activities (IC_{50} value 0.375 mg/ml and 0.392 mg/ml) when hydrolyzed α-chymotrypsin for 1h and 12h after hydrolyzing pepsin for 1h, respectively.

Alkyl radical scavenging activity results showed good scavenging activity overall. Among them, it was shown low alkyl radical scavenging activity (IC_{50} value 0.323 mg/ml and 0.321 mg/ml) when hydrolyzed α-chymotrypsin for 12h after hydrolyzing pepsin for 3h and 6h, respectively. Whereas, it was shown high scavenging activity (IC_{50} value 0.211mg/ml and 0.230 mg/ml) at digests hydrolyzed α-chymotrypsin for 12h and 0.5h after hydrolyzing pepsin for 0.5h and 1h, respectively.

Hydrogen peroxide scavenging activity showed in Table 2-11, 2-12. Complex enzymatic digests hydrolyzed α-chymotrypsin for 6h and 12h after hydrolyzing pepsin for 1h, respectively, had high activity (IC_{50} value 0.942 mg/ml and 1.051 mg/ml, respectively).

ACE inhibition activity showed the lowest activity IC_{50} value 1.895 mg/ml when hydrolyzed α-chymotrypsin for 3h after hydrolyzing pepsin for 1h, whereas, the highest activity IC_{50} value 0.982 mg/ml when hydrolyzed α-chymotrypsin for 12h after hydrolyzing pepsin for 1h.

In conclusion, yield and protein contents generally are increased when hydrolysis time is increased. Complex enzymatic digests hydrolyzed α-chymotrypsin for 12h after hydrolyzing pepsin for 1h digest had as a whole high free radical scavenging activities and ACE inhibition activity. Therefore, we selected this hydrolysis condition. It was chosen as sample for analyzing molecular weight distribution and antioxidant and ACE inhibition activities of the separated fractions.

Table 2-10. Yield and total protein contents of complex enzymatic digests.

Hydrolysis	Pepsin→α-chymotrypsin (12h)					Pepsin (1h)→α-chymotrypsin				
Time (h)	0.5 hr	1 hr	3 hr	6 hr	12 hr	0.5 hr	1 hr	3 hr	6 hr	12 hr
Yield	49.33 ±0.37^{b}	44.62 ±0.82^{a}	56.30 ±2.12^{d}	53.15 ±0.82^{c}	64.48 ±0.76^{e}	42.05 ±0.12^{a}	43.74 ±0.23^{b}	47.06 ±0.11^{c}	51.83 ±0.12^{d}	44.62 ±0.82^{b}
protein	305.03 ±12.11^{b}	327.46 ±25.20^{c}	326.05 ±13.27^{c}	287.09 ±3.96^{a}	365.91 ±7.78^{d}	280.16 ±12.64^{ab}	272.75 ±16.43^{a}	290.00 ±21.56^{ab}	302.15 ±13.28^{bc}	327.46 ±25.20^{c}

Substrate to enzyme ratio is 500:1.

Mean ± SD from triplicate determinations

Table 2-11. ROS scavenging activity (%) and ACE inhibition activity of complex enzymatic digests.

	Pepsin→α-chymotrypsin (12h)					Pepsin (1h)→α-chymotrypsin				
	0.5h	1h	3h	6h	12h	0.5h	1h	3h	6h	12h
Hydroxyl radical^{-}	52.21 ±0.98	54.15 ±0.48	52.47 ±0.50	54.09 ±0.70	52.39 ±0.24	54.53 ±2.38	57.99 ±3.40	49.05 ±2.68	52.54 ±2.53	54.15 ±0.48
Alkyl radical	56.83 ±3.44	56.25 ±4.88	48.21 ±3.07	45.93 ±1.61	52.05 ±2.82	52.45 ±0.06	47.50 ±2.22	45.79 ±1.88	49.07 ±0.84	56.25 ±4.88
Hydrogen peroxide	33.29 ±0.08	33.10 ±0.35	30.32 ±0.21	31.28 ±1.08	34.34 ±0.52	34.20 ±0.42	36.62 ±1.27	38.73 ±0.07	41.18 ±0.09	33.10 ±0.35
ACE inhibition	40.11 ±0.98	51.39 ±0.11	39.32 ±0.12	34.56 ±0.23	35.50 ±0.88	26.81 ±1.67	33.53 ±0.94	28.99 ±1.99	35.97 ±1.86	51.39 ±0.11

Substrate to enzyme ratio is 500:1.

Mean ± SD from triplicate determinations.

The sample concentration : HO^{-} - 0.5 mg/ml, ROO^{-} - 0.25 mg/ml, H_2O_2 – 0.5 mg/ml, ACE inhibition − 1 mg/ml

Table 2-12. IC_{50} value of complex enzymatic digests. (IC_{50} : mg/ml)

	Pepsin→α-chymotrypsin (12h)					Pepsin (1h)→α-chymotrypsin				
	0.5h	1h	3h	6h	12h	0.5h	1h	3h	6h	12h
Hydroxyl radical	0.441	0.392	0.427	0.402	0.429	0.437	0.375	0.533	0.436	0.392
Alkyl radical	0.211	0.246	0.323	0.321	0.235	0.230	0.281	0.307	0.269	0.246
Hydrogen peroxide	1.029	1.051	1.130	1.093	1.059	1.185	1.057	1.017	0.942	1.051
ACE inhibition	1.474	0.982	1.707	1.833	1.744	1.719	1.615	1.895	1.508	0.982

3. 2. 2. Molecular weight fraction of complex enzyme digests by hydrolyzed α-chymotrypsin for 12h after hyzrolyzing pepsin for 1h

Complex enzymatic digest by hydrolyzed α-chymotrypsin for 12h after hydrolyzing pepsin for 1h were ultra-filtered using 5 kDa and 10 kDa membranes. Their yield showed in Table 2-13. According to molecular weight, yields presented above 10 kDa fraction 36.06%, 5-10 kDa fraction 35.59% and 5 kDa fraction 20.70%. Protein contents of fractions are shown above 10 kDa 315.95 mg/ml, 5-10 kDa 266.09 mg/ml and below 5 kDa 98.17 mg/ml.

3. 2. 2. 1. Characterization of complex enzyme digest fractions using ultrafiltration membrane SDS-PAGE

Complex enzymatic digest fractions according to molecular weight were characterized using SDS-PAGE (Fig 2-3). As molecular size became lower, it was shown molecular weight band of below 6kDa became stronger. We could confirm to distribute well as molecular weight by ultrafilteration.

3. 2. 2. 2. Free radicals scavenging capacities using ESR spectrometer and ACE inhibition activity

The free radicals scavenging activities of the fractions using ultrafiltraction membranes are shown in Table 2-14, 2-15. Molecular weight of below 5 kDa fraction had higher scavenging activity and ACE inhibition activity than other fractions except hydroxyl radical scavenging activity (Above 10 kDa IC_{50} value 0.354 mg/ml, 5-10 kDa 0.391 mg/ml and below 5 kDa 0.401 mg/ml, respectively). And also, in case of ACE inhibition activity of below 5 kDa fraction had superior inhibition activity (IC_{50} value < 0.500 mg/ml).

Different molecular weight size led to different peptide chain lengths and exposure of terminal amino groups, which greatly influenced the antioxidant activity and ACE inhibition activity of the hydrolysates. Wu et al. (2003) reported that the peptides with a molecular

weight of approximately 1400 Da showed a stronger in vitro antioxidant activity than those of the 900 and 200 Da peptides, while Je et al. (2005) found that purified peptides with a molecular weight of less than 1000 Da from Alaska pollack frames proteins showed the stronger antioxidant activity among all the hydrolysate fractions.

Table 2-13. Yield and protein contents of molecular weight fractions from complex enzmye digests.

Molecular size	Yield (%)	Total protein contents (mg/15g)
> 10 kDa	36.06±0.00[c]	315.95±16.92[c]
5-10 kDa	35.59±0.20[b]	266.09±12.11[b]
< 5 kDa	20.70±0.00[a]	98.17±14.12[a]

Substrate to enzyme ratio is 500:1.

Mean ± SD from triplicate determinations

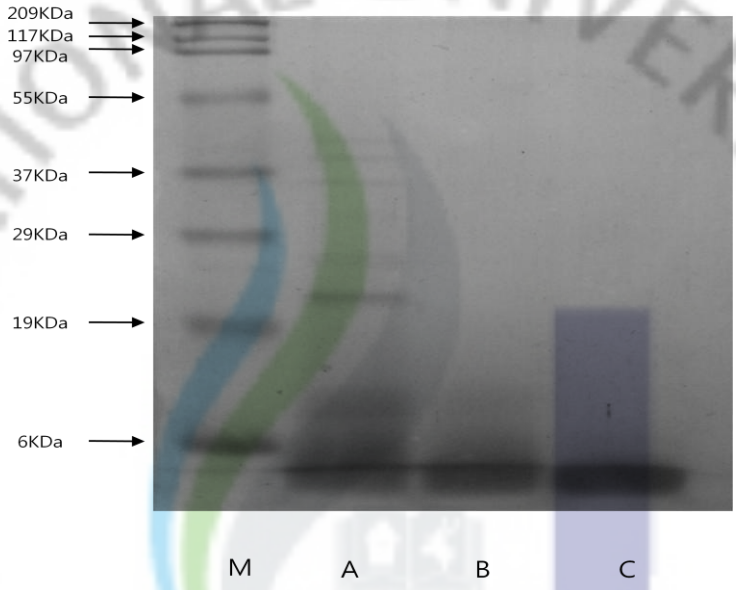

Fig. 2-3. Hydrolysis pattern of molecular weight fractions from complex enzyme digest on 15% SDS-PAGE. M:Marker, A:Above 10kDa, B:5-10kDa, C:Below 5kDa.

Table 2-14. ROS scavenging activity (%) and ACE inhibition activity of molecular weight fractions from complex enzyme digest.

	Molecular size		
	> 10 kDa	5-10 kDa	< 5 kDa
Hydroxyl radical	39.88±0.06	40.66±3.30	35.34±0.01
Alkyl radical	48.87±5.88	52.58±1.37	58.99±1.32
Hydrogen peroxide	33.06±1.00	39.37±0.46	41.68±1.02
ACE inhibition	33.53±0.95	32.36±2.22	83.89±0.20

Substrate to enzyme ratio is 500:1.

Mean ± SD from triplicate determinations.

The sample concentration : HO^- - 0.5 mg/ml, ROO^- - 0.25 mg/ml, H_2O_2 – 0.5 mg/ml, ACE inhibition − 1 mg/ml

Table 2-15. IC_{50} value of molecular weight fractions from complex enzyme digest.

Molecular size	IC_{50} values (mg/ml)			
	Hydroxyl radical	Alkyl radical	Hydrogen peroxide	ACE inhibition
> 10 kDa	0.354	0.274	1.345	1.613
5-10 kDa	0.391	0.234	1.029	1.675
< 5 kDa	0.401	0.188	0.994	< 0.5

CONCLUSIONS

Flounder fish (*Paralichthys olivaceus*) is one of the most important industrial factors and major cultivated fish species in Korea. But it hasn't been reported about bioactive peptides study of flounder fish until now. In this study, we found hydrolysates of flounder fish obtained by various commercial enzyme and proteolytic enzyme in human body possessed antioxidant activity and ACE inhibition activity. α-chymotrypsin hydrolysats had the most antioxidant and ACE inhibition activity compare with other enzymes. The antioxidant activity and ACE inhibition activity of flounder fish hydrolysates were related to its substrate to enzyme ratio, hydrolysis time and molecular weight. Both Flounder fish obtained by α-chymotrypsin and complex enzyme were found to possess antioxidant and ACE inhibition activity at below 5 kDa fraction. Fr. 4 of α-chymotrypsin hydrolysate of isolated by size exclusion chromatography had the highest alkyl radical scavenging activity. Based on this study, these are potential useful peptides that can keep our body in healthy condition about antioxidant and possibility of anti-hypertension. We need further work is being carried out to confirm the peptide sequence and study about anti-hypertension *in vivo*.

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