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Purification of bioactive peptides from digestive enzyme hydrolysates of cutlass fish muscle

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GRADUATE SCHOOL

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CONTENT

국문초록

갈치는 농어목 갈치과의 바닷물고기다. 칼처럼 긴 몸을 가지고 있다는 이유로 도어 또는 갈치, 칼치로 불린다. 몸길이 1m 정도로 몸은 가늘고 길며 납작하다. 꼬리의 끝부분이 길어서 끈과 같은 모양이며, 눈사이 간격은 평평하다. 입은 크며 아랫부분이 돌출해 있고, 양턱 앞부분의 이빨 끝은 갈고리 모양이다. 배지느러미, 꼬리지느러미, 허리뼈는 없으며, 등지느러미는 길어서 등표면을 모두 덮고 있다. 뒷지느러미는 작으며, 등지느러미는 길어서 등표면을 모두 덮고 있다. 뒷지느러미는 작은 돌기 모양이다. 비늘이 없으며 옆선은 가슴지느머리 위쪽으로 기울어져 있고, 몸빛깔은 은백색이다. 대륙붕의 모래진흙 바닥에 서식하며, 주로 밤에 활동하고 산란기는 봄이다. 갈치는 급한 경우를 제외하고는 머리를 세운 상태로 헤엄치며 가끔 머리를 아래위로 움직여 'W' 자 모양을 그린다. 산란기는 8-9월경이며 육식성으로 플랑크톤 및 정어리, 전어, 오징어 등을 먹는다. 갈치 조림, 갈치구이, 갈치배추국으로 요리되어 자주 섭취하는 어류 중 하나이다.

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 갈치는 넙치 다음으로 제주도에서 생산량이 높은 어종이며 매출액도 높은 어종이다. 갈치 외에 참치, 고등어, 명태, 대구, 넙치,

hoki, capelin 및 chum salmon 등으로부터 얻어진 가수분해물이 항산화 및 항고혈압 활성이 탁월하다는 효능이 밝혀져 있지만, 아직 갈치에 대한 연구는 진행 되어지지 않았다. 따라서 이 연구에서는 갈치를 우리가 섭취하였을 때, 인체 내 소화효소들의 가수분해 후 생리활성을 검정하기 위해 소화효소인 Pepsin 과 Trypsin을 사용한 가수분해물에 대한 생리활성을 평가하였다.

첫 번째 파트에서는 소화효소인 Pepsin과 Trypsin을 이용하여 갈치 근육으로부터 시간별 (5분, 15분, 30분, 1시간, 3시간, 6시간), 기질 대 효소비 (100:1)를 처리하여 얻은 가수분해물의 항산화 및 ACE 소거 활성을 측정하였다. 갈치 근육의 Pepsin, Trypsin 가수분해물의 활성은 시간별 큰 차이가 없이 모두 좋은 활성을 나타내었다. 따라서 인체 내에서 소화효소에 의해 소화되는 시간은 짧기 때문에 시간별 가수분해물의 수율, 단백질 함량, 가수분해 정도, 전기영동 결과의 비교를 통해 가수분해가 충분히 진행되고 수율도 높았던 Pepsin 3시간, Trypsin 1시간 가수분해물을 최적의 조건으로 선택하였다.

두 번째는, 최적의 조건으로 선택된 Pepsin 3시간, Trypsin 1시간 가수분해물을 통해 저분자 펩타이드를 얻어내기 위한 공정을 진행하였다. 처음으로 FPLC를 사용하여 이온교환 크로마토그래피를 통해 Pepsin 3시간 가수분해물에서 5개의 프랙션을 얻었고 Trypsin 1시간 가수분해물에서는 4개의 프랙션을 얻을 수 있었다. Pepsin 3시간 가수분해물에서 얻어진 프랙션 5개의 항산화 활성을 각각 비교하였을 때 프랙션 3번에서 아주 우수한 항산화 활성을 확인할 수 있었고, 프랙션 3번의 양을 충분히 수집하여 두번째 공정인 HPLC

를 통하여 프랙션 3번의 물질확인을 하였을 때 2개의 물질을 확인 할 수 있었으며, 이 2개의 물질을 각각 분취하여 활성을 다시 비교하였을 때 FPLC 프랙션 3번의 2번째 분취물에서 아주 우수한 활성이 있는 것을 확인하였다.

ESR을 이용해 DPPH 및 Alkyl 라디칼 소거 효과를 측정하여 아주 강한 라디칼 소거 효과를 확인 하였기 때문에 정제된 프랙션으로 일반 세포인 Vero 세포를 이용하여 in vitro 실험을 진행하여 AAPH 유도 산화 데미지 실험과 세포독성 실험을 통하여서도 산화를 억제하고 독성이 없는 좋은 결과를 얻을수 있었다.

위의 결과들을 종합하여 봤을때, Pepsin 과 Trypsin 에 의한 갈치 가수분해물로부터 저분자 물질은 우수한 항산화 효과와 항고혈압에 대한 기능성을 보여주었고, Pepsin과 Trypsin은 장내 효소이기 때문에 우리가 갈치를 섭취하였을 때, 신체 내에서 펩신과 트립신의 소화 작용 후 이와 같은 효능을 기대할 수 있을 것으로 사료 되어진다.

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Introduction

Most of the protein hydrolysates obtained from plant and animal sources have been reported to contain various biological activities. Many of these bioactive peptides have been used to develop novel drugs and healthy food material with numerous kinds of biological effects such as nutritional, antioxidative, antihypertensive, antimicrobial, anti-inflammatory, and immunomodulatory properties. Fish proteins can also be utilized for such applications with the use of enzymatic technology to produce products of medical value. Mostly among them, many fish protein hydrolysates possess antioxidant activity and angiotensin I converting enzyme (ACE) inhibitory activity. Free radicals generated in live tissues due to various kinds of metabolic activities are a major cause in many chronic diseases conditions such as diabetes, cardiovascular disease, neurodegenerative disorders and cancer (1). Protein hydrolysates prepared from various fish sources such as capelin, mackerel, yellowfin sole, alaska pollack, atlantic salmon, hoki, conger eel, and scad have been reported to contain peptides with antioxidant activity (2). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tbutylhydroquinone (TBHQ) and propyl gallate are widely used in preserving food products by delaying the discoloration and deterioration due to oxidation (3). But, the use of these synthetic antioxidants has been limited in some countries due to their potential health hazard (4). As recent studies indicated hypertension is another major problem poses a major worldwide threat to human health. Hypertension has been estimated to affect approximately 20% of the worlds adult population (5). ACE plays an important role in regulating the blood pressure by controlling the rennin-angiotensin system (RAS) and kallikrein kinnin system (KKS) (5, 6). Therefore inhibition of ACE activity is considered to be a pivotal therapeutic approach for controlling hypertension. Several

synthetic ACE inhibitors have been developed, including alacepril, captopril, benazepril, enalapril, fosinopril, ramipril, and zofenopril, all of which are currently extensively used in the treatment of hypertension and heart failure in humans (11,12). Although they contains anti hypertension activity, synthetic ACE inhibitors have several side effect, including cough, taste disturbances, and skin rashes (13,14). Recently, many ACE inhibitory peptides were reported as natural alternative bioactive peptides that are safer than synthetic ACE inhibitors.

The cutlass fish can be identified as one of the most representative fish species in Eastern Sea and most abundant of the fish species in Korea. In Korea, cutlassfish is used as a traditional food that is a rich source of calcium, n-3 polyunsaturated fatty acids, and vitamins. But reliable studies have not been carried out using cutlass fish tissues to evaluate the antioxidant and ACE inhibitor activity of cutlass fish proteins and their hydolysates. Thus it is of utmost importance to study the health-related activities of fish muscle hydrolysates and purify low molecular weight peptides from cutlassfish hydrolysates using proteases such as pepsin and trypsin. The objective of this study was to analyze the antioxidant activity and ACE inhibitory activity of cutlassfish protein hydrolysates obtained by pepsin and trypsin treatment of the cutlassfish muscles.

Purification of bioactive peptides from digestive enzyme hydrolysates of cutlass fish muscle

Abstract

Fish can be identified as a rich source of protein, which can be used to obtain bioactive peptieds through enzyme hydrolysis process. During this study pepsin and trypsin was used as digestive enzymes in order to study, the protein hydrolysates obtained from the muscles of cutlass fish. Two protein hydrolysates obtained from the muscle of cutlass fish using pepsin and trypsin were evaluated separately for their bioactive ability. Both hydrolysates indicated a strong in vitro antioxidant activity and angiotensin I converting enzyme inhibition activity. Antioxidant activity of enzyme hydrolysates was evaluated using electron spin resonance (ESR) spectrometer for DPPH radical scavenging activity and Alkyl radical scavenging activity. According to the results, both hydrolysates indicated significantly high DPPH radical scavenging activity and Alkyl radical scavenging activity (for pepsin hydrolysates IC_{50} value 0.19 mg/ml and 0.21 mg/ml and for trypsin hydrolysates IC_{50} values 0.17 mg/ml and 0.16 mg/ml, respectively). In addition, both hydrolysates exhibited significant ACE inhibition activity at concentrations lower than 0.0625 mg/ml. The purification of bioactive peptides were performed using pepsin 3 hour hydrolysate obtained from the cutlass fish. It showed a higher yield, a higher protein content and a higher degree of hydrolysis. Fast protein liquid chromatography (FPLC) connected to diethyl amino ethyl (DEAE) anion exchange column followed by sephadex G-25 size exclusion column and high performance liquid chromatography (HPLC) using an ODS column were used to purify and isolate the peptides. The isolated peptide exhibited a higher DPPH and alkyl radical scavenging activity (IC_{50}

value 0.03 mg/ml and 0.022 mg/ml, repectively).

The results of this study suggest that the pepsin and trypsin enzymatic hydrolysates from muscles of cutlass fish have antioxidant and antihypertension properties, which enables them to be used as functional food and material of medicinal and commercial value. It further suggests that these isolated peptides from cutlass fish proteins could be used as potential cadidates to produce natural antioxidative and antihypertensive peptides.

Materials and methods

2.1. Materials

Cutlass fish, *Trichiurus lepturus*, were cultivated in Jeju island. After harvesting, the muscles were collected and stored in frozen condition at -80 ℃. The frozen muscles were lyophilized at -70 ℃ using a freeze dryer. The lyophilized sample was then homogenized using a grinder and the powder was stored at -20 ℃ until the hydrolysis being carried out.

2.2. Chemicals and reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2-azocis-(2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma Chemical Co. (USA). Proteases such as pepsin, trypsin and Albumin were purchased from Sigma Chemical Co. (USA). Acrylamide/Bis solution (29:1) was purchased from Bio-rad Laboratories, Inc. (USA). Ammonium persulfate (APS) and TEMED were purchased from Amresco Inc. (USA). Angiotensin I converting enzyme (from rabbit lung) was purchased from Sigma chemical Co. (USA).

2.3. Proximate composition

The approximate composition of dried cutlass fish muscle powder was determined according to the AOAC methods. Moisture content was determined by keeping the powdered sample in a dry oven at 105 ℃ for 24h. Ash content of the dried muscle powder was determined by calcinations in a furnace at 550 ℃. Crude protein content was determined by Kjeldahl method and the crude lipid content was determined by Soxhlet method.

2.4. Preparation of cutlassfish protein hydrolysate

A volume of 100 ml of distilled water was added to 5 grams of the dried cutlass fish powder and the PH was adjusted according to the optimum conditions of the used enzymes as given in Table 1. Proteases (pepsin and trypsin) were added to the aforementioned mixture so that the substrate to enzyme ratio will be 100:1. Hydrolysis was carried out at optimum temperature for each time (5min, 15min, 30min, 1h, 3h, 6h). At the end of the reaction, the hydrolusates were adjusted to PH 7.0 and the enzymes were inactivated by incubating under 100 ℃ for 10 min. The hydrolysates were then centrifuged at 10,000 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.

2.5. Measurement of protein content

The protein content of the soluble fraction was determined by the Lowry method. One milliliter of alkaline-copper solution and 3 ml of 50% Folin-Ciocalteu's phenol reagent was mixed with 1 ml of the hydrolysate. The mixtures were allowed to react for 45 min. The absorbance was measured at a wavelength of 540 nm using a spectrophotometer. Bovine serum albumin was used as the calibration standard (7).

2.6. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 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 5min prior to the electrophoresis. 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. Degree of hydrolysis (DH) of the digestive enzymatic hydrolysates from cutlassfish muscle

The effect of hydrolysis time on DH was monitored using the method described by Hoyle and Merritt (16), with slight modification. At the end of each hydrolysis time period (5min, 15min, 30min, 1h, 3h and 6h), hydrolysis was stopped and an aliquot of 2 ml was taken and mixed with 2ml of 20% trichloroacetic acid (TCA) and then centrifuged at 4500 rpm for 10min. The supernatant was decanted and analyzed for nitrogen content by BCA™ protein assay kit. The degree of hydrolysis DH (%) was calculated as:

Degree of hydrolysis (%)

= 10% TCA soluble nitrogen in sample / Total nitrogen in sample x 100

2.8. Free radical scavenging capacities using ESR spectrometer

The different radicals tested here were generated according to the previously described procedure described by Heo SJ et al. [8], and the spin adducts were recorded using JES-FA electron spin resonance (ESR) spectrometer (JES-FA ESR, JEOL, Tokyo, Japan).

2.8.1. DPPH radical scavenging activity

DPPH radical scavenging activity was measured using an ESR spectrometer in accordance

with the method described by [9]. A distilled water solution of 60 μl of each sample (or distilled water solution itself as a control) was added 60 μl DPPH (60 μM) in methanol solvent, and the sample was mixed vigorously. After 2 min, the solution was transferred to a capillary tube, and the spectrum was recorded with an ESR spectrometer (JES-FA machine, JEOL, Tokyo, Japan). The experimental conditions were as follows: magnetic field, 336.5 ± 5mT ; power, 1mW, modulation frequency, 100kHz; amplitude, 10 x 100; modulation width, 0.8 mT; sweep width, 10 mT; sweep time, 30s; and time constant, 0.03 s. The extent of scavenging activity was calculated as follows.

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

Where HC is the relative peak height of the radical signals without sample, and HS is the relative peak height of the radical signals with sample.

2.8.2. Peroxyl radical scavenging activity

Peroxyl radicals were generated by AAPH and their scavenging effects were investigated by the method described by [10]. Sample 20 μl of various concentrations were mixed with distilled water 20 μl, 40mM AAPH 20 μl and 40mM 4-POBN 20 μl were mixed with PBS. The solution was incubated for 30 min at 37 ℃ in a water bath, and then transferred to capillary tube. The experimental conditions were as followed; magnetic field, 336.5 ± 5mT ; power, 1mW, modulation frequency, 100kHz; amplitude, 1 x 1000; modulation width, 0.2 mT; sweep width, 10 mT; sweep time, 30s; and time constant, 0.03 s.

2.9. Measurement of ACE inhibitory activity

ACE inhibitory activity was measured by colorimetric method using ACE kit-WST (Dojindo inc., Japan), determining the amount of 3-hyroxybutylic acid generated from 3-hydroxybutyryl-Gly-Gly-Gly with the enzyme methods (Zghonda et al., 2012). In brief, the substrate buffer was preprocessed without or with the test compound in 96-well plates and the enzyme solution was added in the wells treated with the test compound. After the incubation, the indicator solution was added in the all of the wells. The amount of 3-hyroxybutylic acid was evaluated by measuring absorbance at 450 nm.

2.10. Purification of the antioxidant peptide

2.10.1. Ion exchange chromatography

The lyophilized cutlass fish protein (20mg/ml) was dissolved in 20 mM sodium acetate buffer (PH 4.0), and loaded onto FPLC on a FF 16/10 DEAE anion exchange column equilibrated with 20mM sodium acetate buffer (PH 4.0), and eluted with a linear gradient of NaCl (0-1 M) in the same buffer at a flow rate of 3ml/min. Each fraction collected at a volume 10 ml was monitored at 280 nm, pooled fractions were then frozen under -80 ℃ followed by lyophilization. The antioxidant activities were investigated by ESR. The fraction having strong antioxidant properties were subjected to further separation.

2.10.2. Size exclusion chromatography

The fraction 3 showing higher free radical scavenging activity was dissolved in distilled water and loaded onto a Sephadex G-25 gel filteration column (2.5 x 100 cm), equilibrated

with distilled water. The column was eluted with distilled water at a flow rate of 2.0 ml/min. The absorbance values of the fractions were recorded at 280 nm. This was lyophilized and stored at -20 ℃ until used.

2.10.3. Preparative RP-HPLC

The fraction with the strongest antioxidant activity after FPLC separation was further purified using preparative reversed-phase high-performance liquid chromatography on a Sunfire C18 (5 μm, 4.6 × 250 mm) column, at a flow rate of 1 ml/min. The composition of the mobile phase solvent system was adjusted using, Eluent A: acetonitrile; Eluent B: water. The chromatographic column was initially conditioned with 100% eluent B. After injecting 10 μl of the sample (5 mg/ml) into the C18 column, concentration of eluent A was increasing as: 0-40 min, 0-50% (v/v); 40-50 min, 50-100% (v/v). The UV absorbance of the above eluent was monitored at 280 nm (Waters 2487 Detector). The fractions with the desire peaks were pooled, concentrated and lyophilized for antioxidant activity test.

Table 1. Optimal hydrolysation conditions of digestive enzyme.

Enzyme	sources	Optimal	
		PH	Temperature (℃)
Pepsin	**Porcine gastric mucosa**	**6.2**	**37**
Trypsin	**Bovine pancreases**	**7.6**	**37**

Results

3.1. Proximate composition

Proximate composition of moisture, ash, crude protein, and crude lipid content in lyophilised cutlass fish muscles were determined according to the AOAC methods (1990) and result are presented as percentages in (Table 2).

3.2. Preparation of digestive enzymatic hydrolysates from cutlass fish

Hydrolysys was carried out for different time periods (5min, 15min, 30min, 1h, 3h, 6h) keeping the substrate to enzyme ratio constant (100 : 1) in order to find the optimal hydrolysis time period which gives the highest yield, degree of hydrolysis, antioxidant and ACE inhibitory activities. Hydrolysates exhibited wide variety of larger, medium and smaller peptides depending on the different of hydrolysis time periods. The obtained yields of enzymatic hydrolysates by digestive proteinases pepsin and trypsin, and the distilled water extracts are given in Fig. 1 a and b. The enzymatic hydrolysates resulted in a higher yield than distilled water extract and the values are given in Table. 3 a.

3.3. Protein content of digestive enzymatic hydrolysate from cutlass fish

The obtained yields of the protein contents are summarized in Fig. 1 a and b. When comparing with the yield of distilled water extract, enzymatic hydrolysates showed higher protein content. These results indicated that the pepsin and trypsin digests had the higher protein contents at each time (Table. 3 b).

Table 2. Proximate composition of dried flounder fish muscles.

Composition	Sources (%)
Moisture	**1.6 ± 0.043**
Ash	**5.4 ± 0.2**
protein	**83.07 ± 1.7**
lipid	**9.93 ± 1.4**

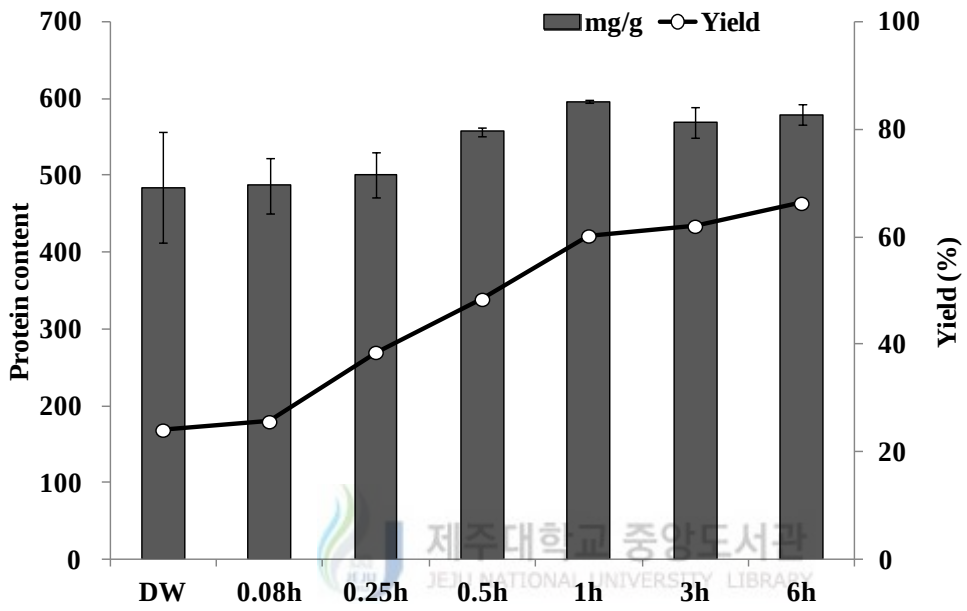

Fig 1a. Yield and protein content of pepsin hydrolysates with each time. Mean ± SD from triplicate determinations. Significant differences at $p<0.05$ indicated with different letters.

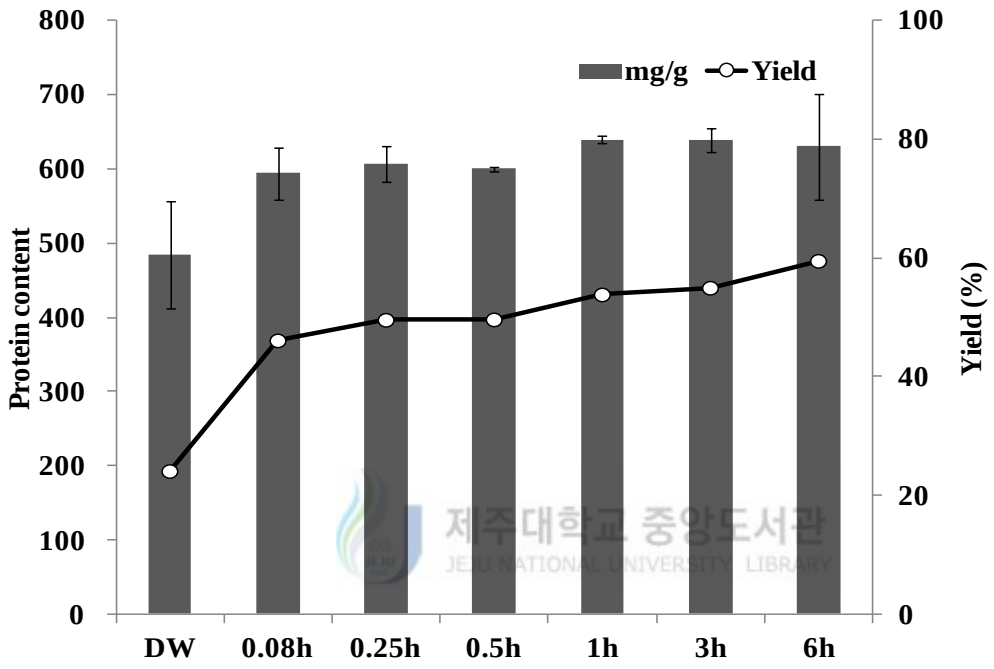

Fig 1b. Yield and protein content of trypsin hydrolysates with each time. Mean ± SD from triplicate determinations. Significant differences at $p<0.05$ indicated with different letters.

Table 3a. Yield of digestive enzyme hydrolysates from cutlass fish, according to each hydrolysis time period.

Enzymatic digests	Yield (%)					
	5min	15min	30min	1h	3h	6h
Pepsin	25.7 ± 1.4	38.6 ± 3.5	48.5 ± 1.0	60.3± 1.1	62.1 ± 1.2	66.3 ± 0.5
Trypsin	46.1 ± 0.5	49.6 ± 0.8	49.7 ± 1.1	53.9 ± 0.8	55.0 ± 0.7	59.5 ± 1.8
Distilled water	24.1 ± 0.6					

Table 3b. Protein content of digestive enzyme hydrolysates from cutlass fish, according to each hydrolysis time period.

Enzymatic digests	Protein content (mg/g)					
	5min	15min	30min	1h	3h	6h
Pepsin	487 ± 36	501 ± 30	557 ± 5	596 ± 2	569 ± 20	579 ± 13
Trypsin	594 ± 35	606 ± 24	600 ± 30	639 ± 5	638 ± 16	630 ± 71
Distilled water	484 ± 72					

3.4. Characterization of cutlass fish hydrolysates in SDS-PAGE

SDS-PAGE was used to characterize the molecular weight of cutlass fish protein hydrolysates (fig 2 a and b). The electrophoresis was carried out using a gel matrix containing 15% polyacrylamide. The results represents hydrolyzed myofibrillar proteins belonging to a variety of molecular weights. Distilled water extract band indicated an intact state. This brings to a conclusion that pepsin and trypsin enzymes significantly increase the amount of lower molecular weight proteins and there by resulted in a various band patterns based on the hydrolysis time. According these results, it is possible to identify the optimal hydrolysis time periods for pepsin and trypsin enzymes.

3.5. Preparation of digestive enzymatic hydrolysate from cutlass fish

Above results indicated that hydrolysates obtained from cutlass fish by pepsin (3 h) and trypsin (1 h) exhibited a higher yield, degree of hydrolysis, protein contents and similar antioxidative and ACE inhibitiory activity among other each hydrolysis time hydrolysates. Therefore, pepsin (3 h) and trypsin (1 h) hydrolysates were chosen for the isolation and identification of smaller molecular weight peptides. During hydrolysis, a wide variety of larger, medium and smaller peptides were generated, depending on different of hydrolysis time periods.

3.6. Determination of free radicals scavenging capacity by ESR spectrometer

3.6.1. DPPH radical scavenging activity of digestive enzymatic hydrolysate from cutlass fish

The DPPH radical is one of the few stable radical sources. Thus the DPPH radical is widely used to investigate the scavenging activity of some natural compounds. DPPH radical

displays a maximum absorbance at 517 nm in methanol (15). The DPPH radical scavenging activities of the enzymatic hydrolysates are graphically summarized Fig4. According to the result the antioxidative activities of enzymatic hydrolysates indicates a dose dependent increase with the increasing concentration from 0.125 mg/ml to 1 mg/ml. All the other hydrolysates and distilled water extracts indicated similar scavenging activity (Table 4).

3.6.2. Peroxyl radical scavenging activity of digestive enzymatic hydrolysate from cutlass fish

Alkyl radical were generated by the decomposition of AAPH incubated with spin traping 4-POBN at 37℃ for 30 min. Intensity of the energy absorption was measured using the ESR spectrometer. The scavenging activities of the enzymatic hydrolysates on alkyl radicals are shown on fig5. According to the results the antioxidative activities of enzymatic hydrolysates increases with increasing concentration from 0.0625 mg/ml to 1mg/ml. Other hydrolysates and distilled water extracts had similar scavenging activity (Table 4).

3.7. ACE inhibition activity of cutlass fish hydrolysates

Antihypertensive treatment with ACE inhibitors can improve endothelium-dependent vasorelaxation (Clozel et al., 1990). The antihypertensive effect of the enzymatic hydrolysates of cutlassfish was evaluated by measuring ACE inhibitory activity using ACE kit-WST (Dojindo inc., Japan). As shown in Fig. 6, enzymatic hydrolysates of cutlassfish showed ACE inhibitory activity in a concentration-dependent manner the concentrations of lower than 0.0625 mg/ml, and its IC_{50} values are shown on Table 6.

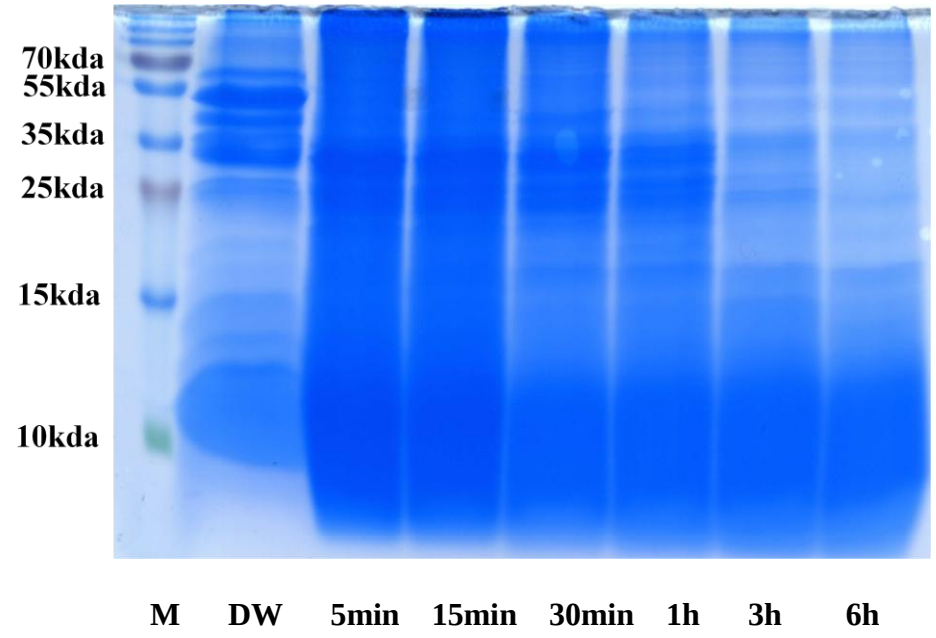

Fig. 2a. Hydrolysis pattern of pepsin hydrolysates from cutlass fish with each hydrolysis time on 15% SDS-PAGE.

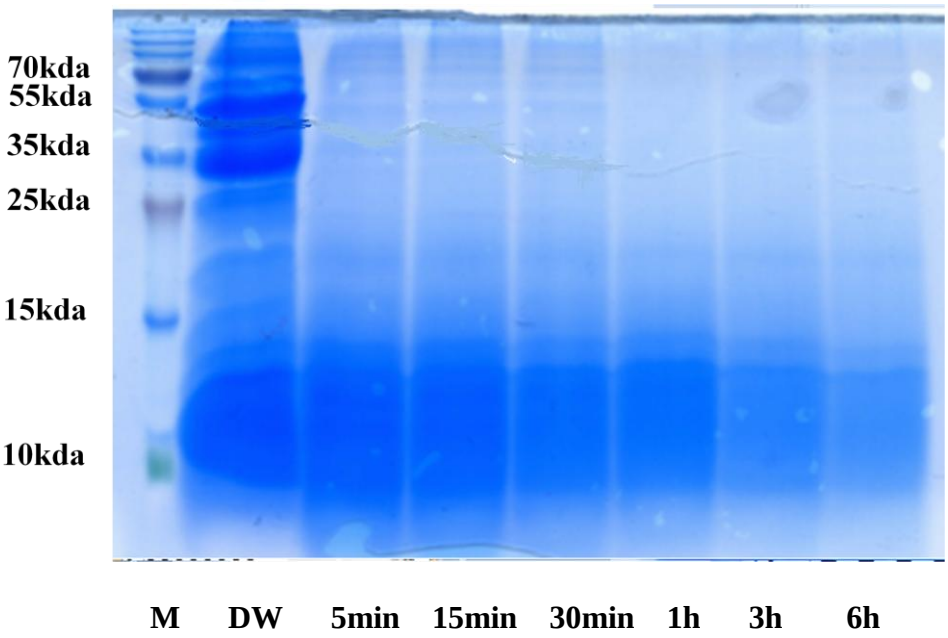

Fig. 2b. Hydrolysis pattern of trypsin hydrolysates from cutlass fish with each hydrolysis time on 15% SDS-PAGE.

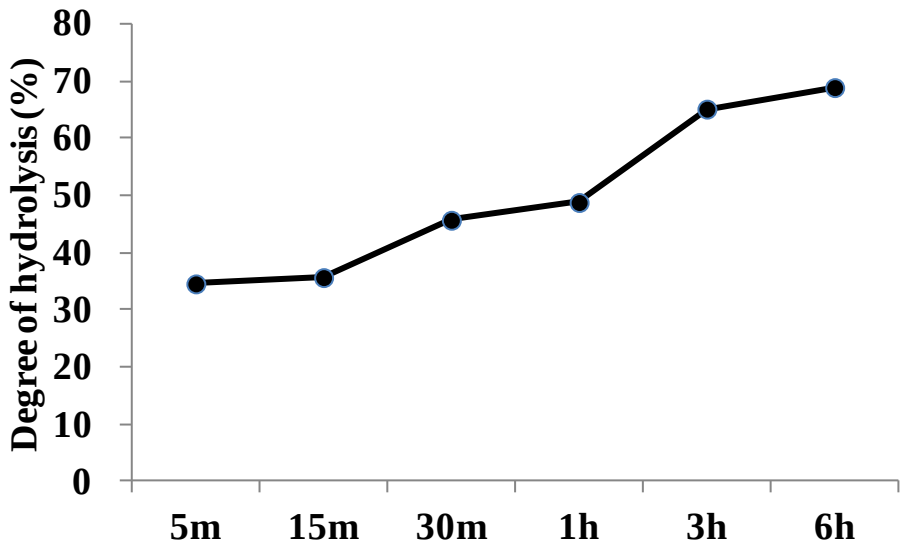

Fig 3a. Degree of hydrolysis of pepsin hydrolysates from cutlass fish, according to hydrolysis time.

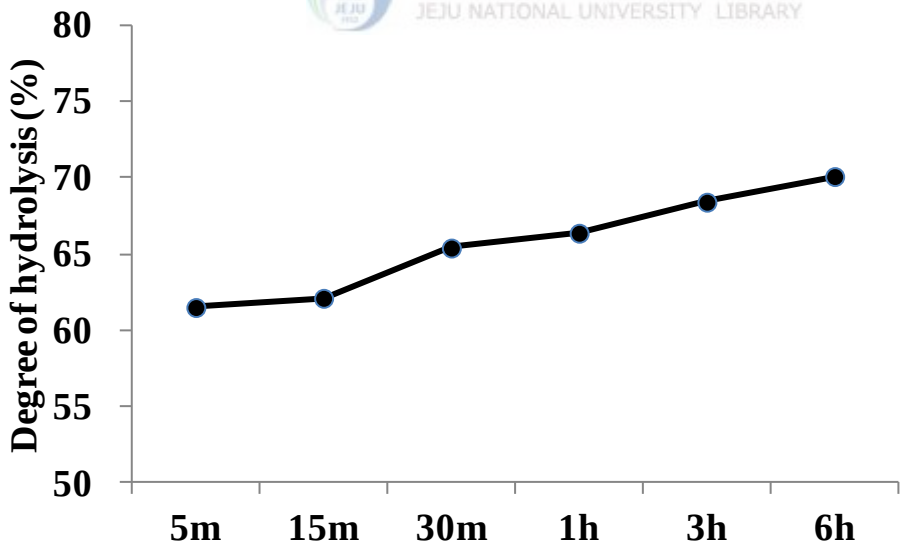

Fig 3b. Degree of hydrolysis of trypsin hydrolysates from cutlass fish, according to hydrolysis time.

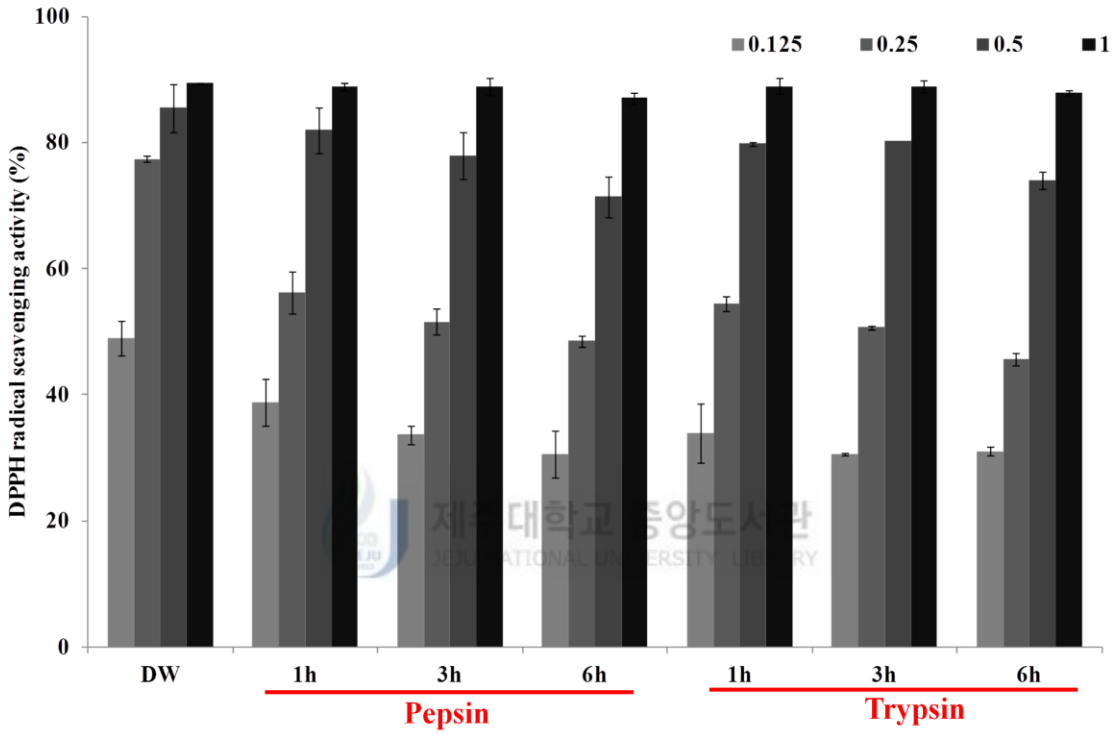

Fig 4. DPPH radical scavenging activity of digestive enzyme hydrolysates from cutlass fish, according to each hydrolysis time. Mean ± SD from triplicate determinations.

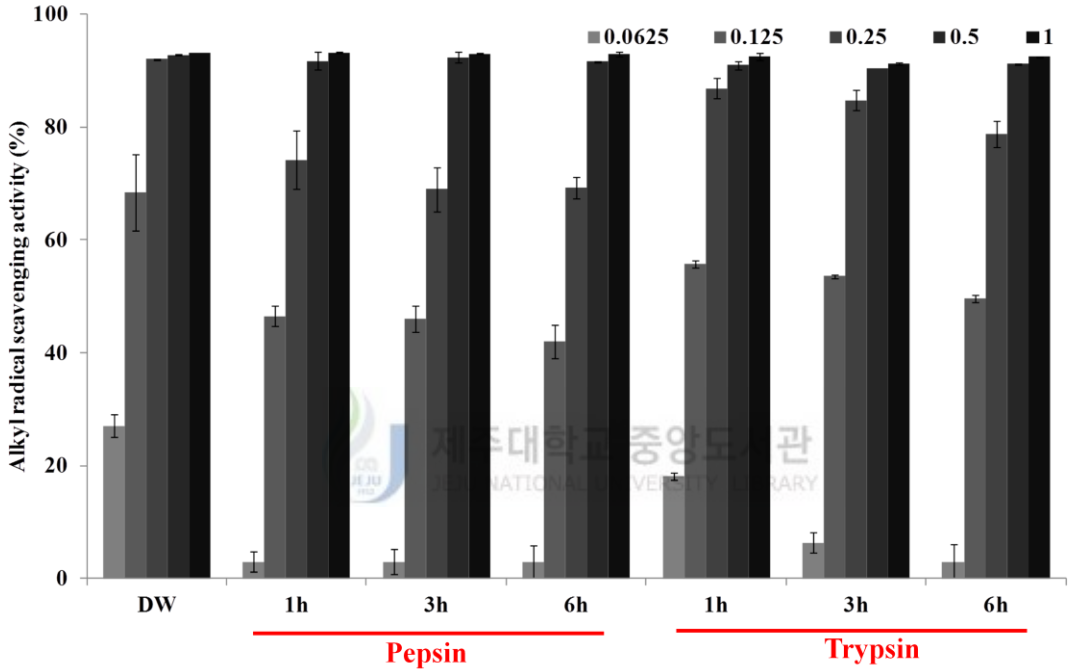

Fig 5. Peroxyl radical scavenging activity of digestive enzyme hydrolysates from cutlass fish, according to each hydrolysis time. Mean ± SD from triplicate determinations.

Table 4. Radicals scavenging activities of digestive enzyme hydrolysates from cutlass fish, according to hydrolysis times.

Radicals	Enzymatic digests	Hydrolysis times (IC_{50} mg/ml)		
		1h	3h	6h
DPPH	Pepsin	0.19 ± 0.01	0.22 ± 0.003	0.31 ± 0.01
	Trypsin	0.22 ± 0.002	0.27 ± 0.002	0.32 ± 0.01
Peroxyl	Pepsin	0.14 ± 0.01	0.18 ± 0.02	0.18 ± 0.01
	Trypsin	0.12 ± 0.03	0.12 ± 0.02	0.13 ± 0.01

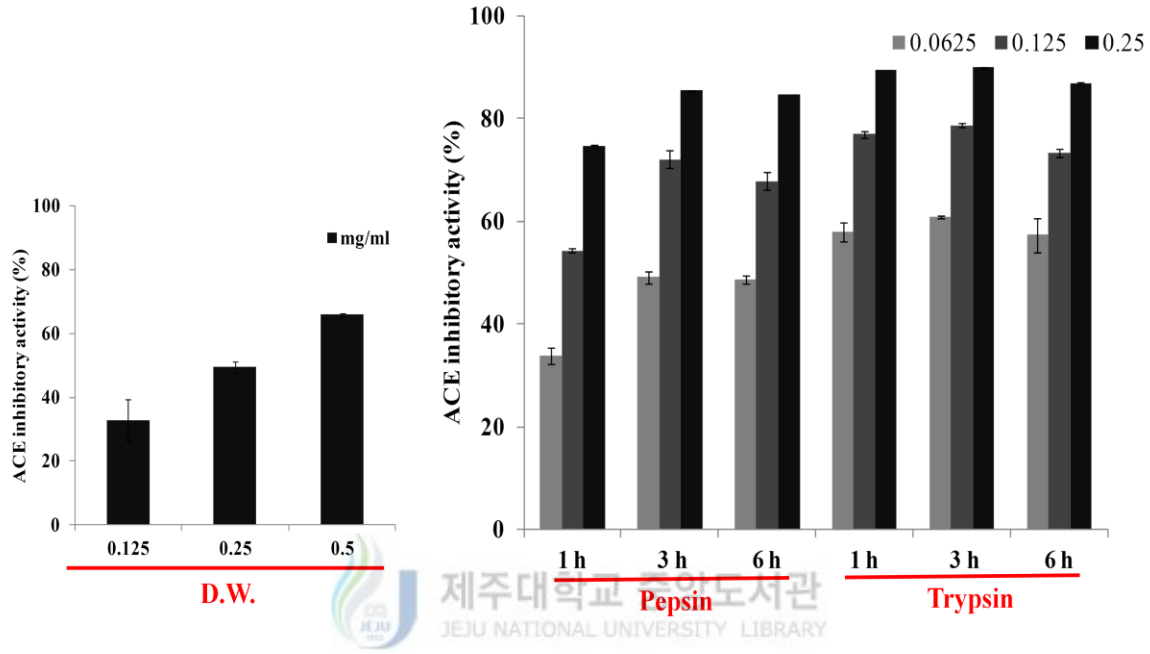

Fig 6. ACE inhibition activity (%) of digestive enzyme hydrolysates from cutlass fish, according to hydrolysis time.

Table 5. ACE inhibition activity (%) of digestive enzyme hydrolysates from cutlass fish, according to hydrolysis time.

	Enzymatic digests	Hydrolysis times (IC_{50} mg/ml)		
		1h	3h	6h
ACE inhibitory activity	Pepsin	0.13	0.04	0.05
	Trypsin	< 0.0625	< 0.0625	< 0.0625

3.8. Isolation of the antioxidant peptide by FPLC

The lyophilized cutlass fish protein was dissolved in 20 mM sodium acetate buffer (PH 4.0), and loaded into a FF 16/10 DEAE anion exchange column with the linear gradient of NaCl (0-1M). Elution peaks were monitored at 280 nm, and each fraction was collected as 10ml and pepsin 3h hydrolysate fractionated into one unbound portion and four bound portion (Fig. 7a). Each fraction was labeled (as I, II, III, Ⅳ & Ⅴ), pooled, lyophilized, and measured for antioxidative activity against DPPH and alkyl radical scavenging effects using ESR spectroscopy. Among 5 different fractions, the fraction III of pepsin 3h hydrolysate had both strong DPPH and alkyl radical scavenging activity (Fig. 7b,c).

3.9. Preparative RP-HPLC

The active fraction III was further separated by RP-HPLC using the Sunfire C18 (5 μm, 4.6 x 250 mm), and the fractions were divided into two portions (Fig. 8a). Elution peaks were monitored at 280 nm. Fraction III-2 showed the highest potent DPPH and alkyl radical scavenging activities (IC_{50} value 0.03 mg/ml and 0.022 mg/ml, respectively). The pure peptide of the superior fraction from the pepsin 3h hydrolysate was separated using Sephadex G-25 column. Then the pure peptide was identified using RP-HPLC (Fig. 8b).

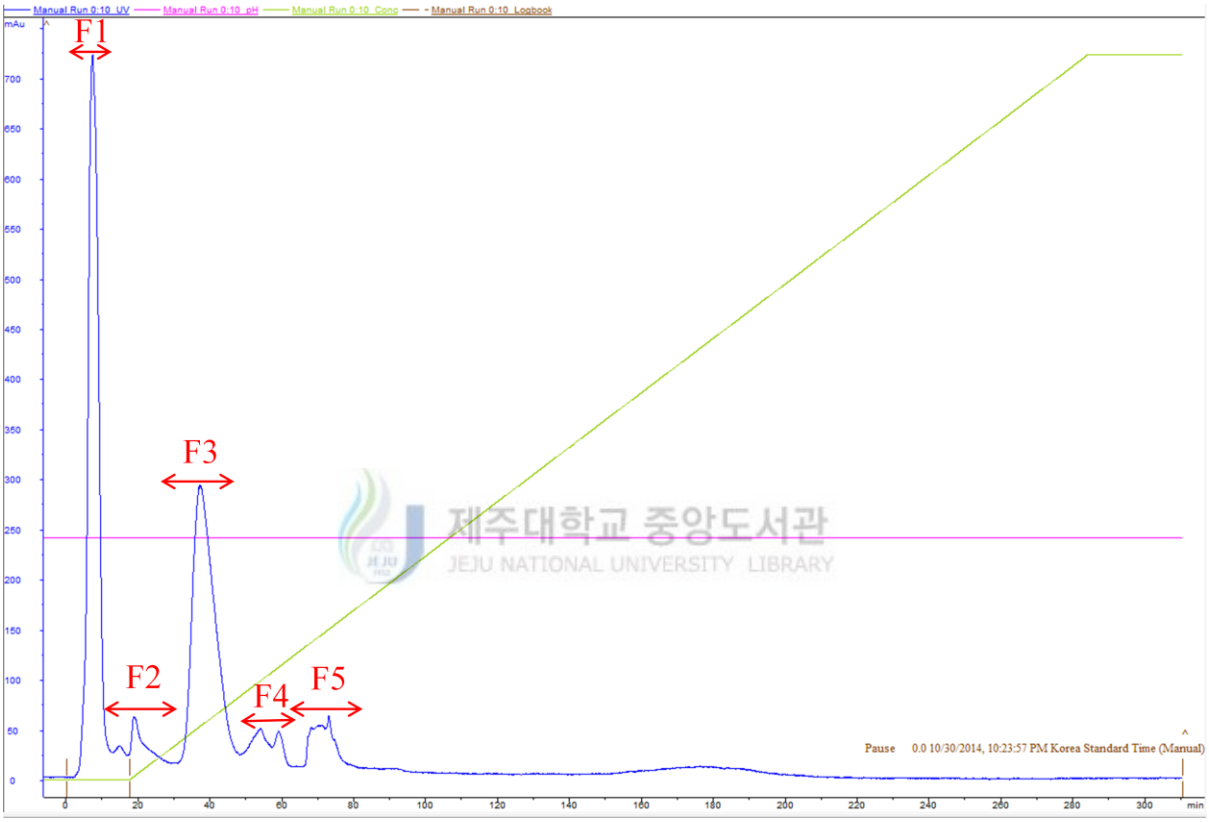

Fig 7a. Purification of the antioxidant peptide from cutlass fish protein separation of antioxidant peptides by FF 16/10 DEAE anion exchange chromatography.

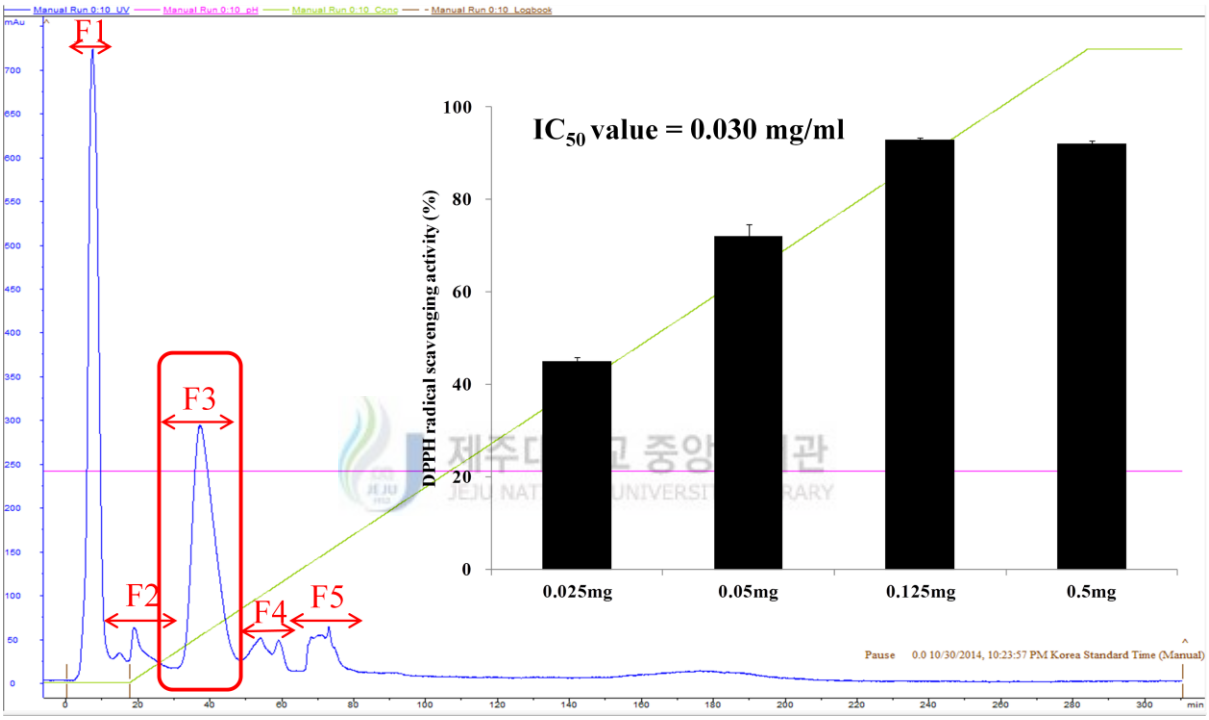

Fig 7b. DPPH radical scavenging activity of the FPLC F3 from pepsin 3h hydrolysate of cutlass fish.

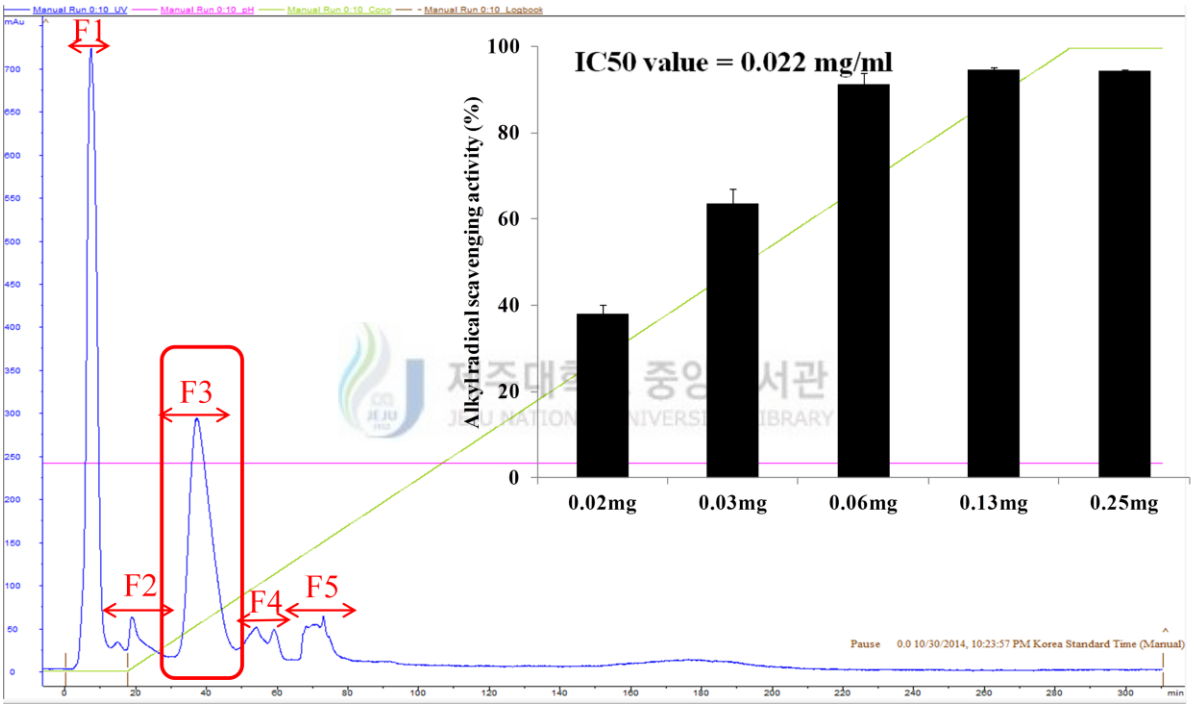

Fig 7c. Peroxyl radical scavenging activity of the FPLC F3 from pepsin 3h hydrolysate of cutlass fish.

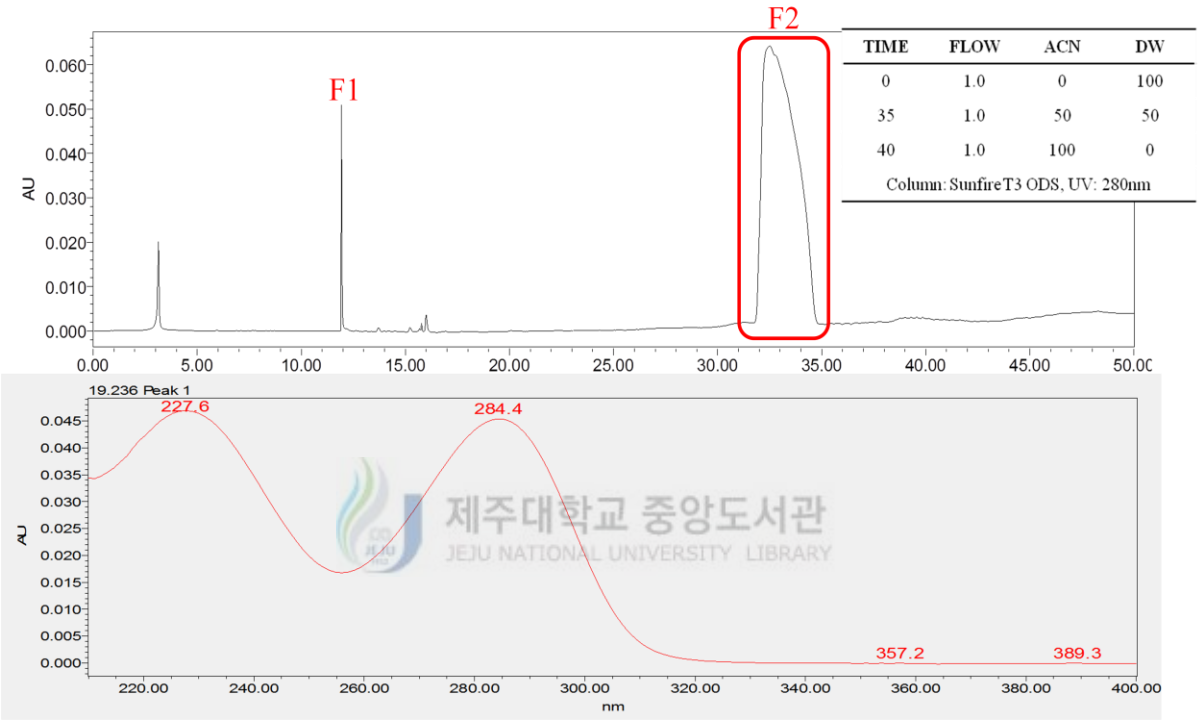

Fig 8a. HPLC chromatogram of the FPLC F3 from pepsin 3h hydrolysate of cutlass fish.

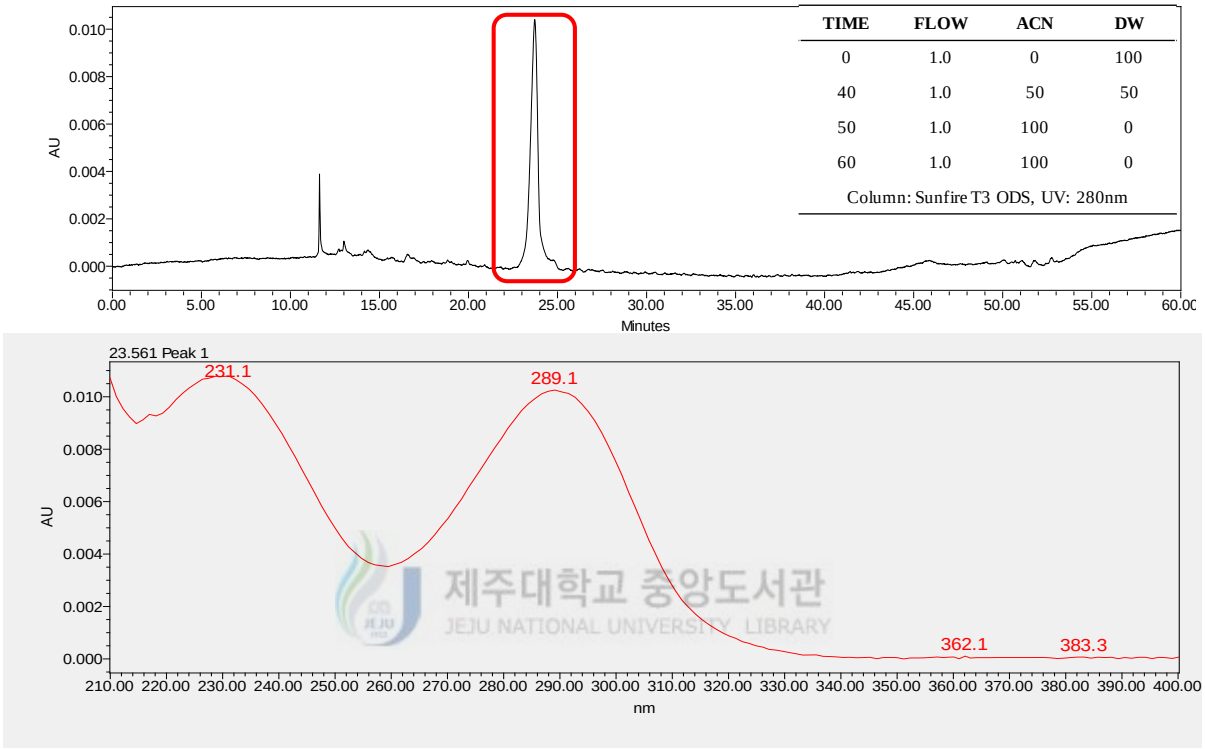

Fig 8b. HPLC chromatogram of the pure peptide from FPLC F3 of pepsin 3h hydrolysate of cutlass fish.

3.10. Effect of the antioxidative peptide on cell viability in normal cells

In this study, normal cells were treated with different concentrations of the antioxidative peptide to determine non-cytotoxic effects for further experiments. The cell viability data confirmed that the antioxidative peptide was non-cytotoxic in normal cells (Fig. 8). Therefore, it was determined that the antioxidative peptide could be used in further experiments.

3.11. Protective effects of the antioxidative peptide against AAPH-induced cell damage

The effect of the antioxidative peptide on cell viability in AAPH-induced Vero cells was measured via MTT assay. As shown in Fig 9, AAPH treatment without the antioxidative peptide decreased cell viability to 56.7 %, while the antioxidative peptide prevented cells from AAPH-induced cell damage, restoring cell survival to 53.2 %, 66.1 %, 70.6 % and 72.4 % at the concentration of 3, 6, 12.5 and 25 μg/ml, respectively.

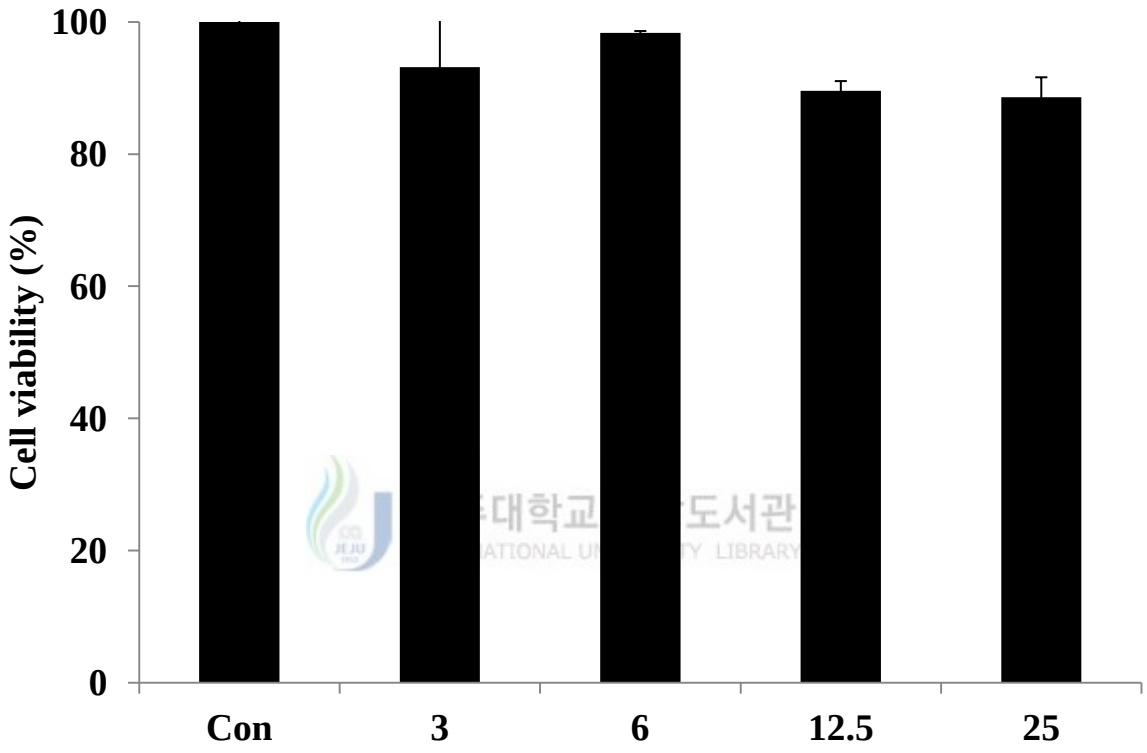

Fig 8. The cytotoxic effect of the F3 of pepsin 3h hydrolysate on viability in normal cells. Cells were treated with the refined fraction at the indicated concentrations (3, 6, 12.5 and 25 μg/ml).

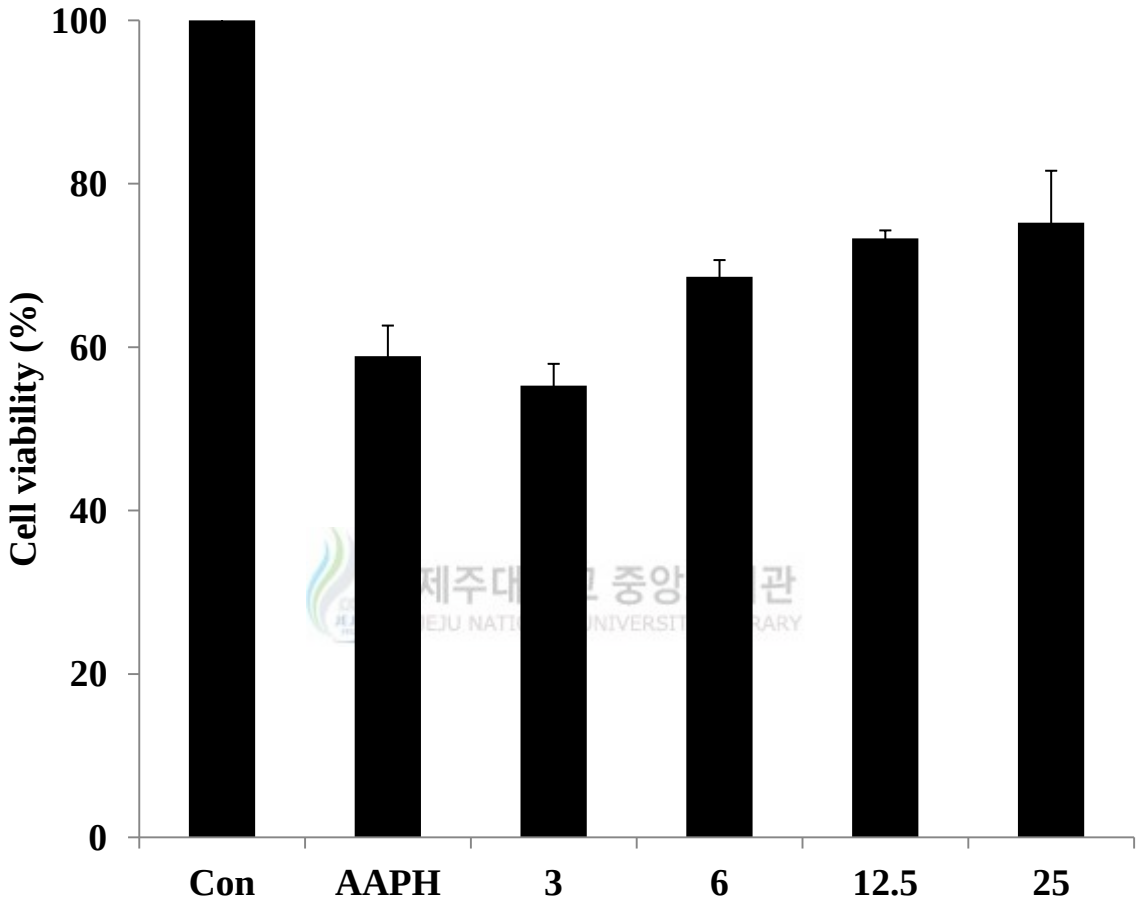

Fig 9. Protective effect of the F3 of pepsin 3h hydrolysate on AAPH-induced oxidative damage in normal cells. The viability of cells on AAPH treatment was assessed by MTT assay.

Discussion

The cutlass fish is the most abundant of the fish species in Korea. In this study, we evaluated the antioxidant activity and ACE inhibitory activity of the pepsin and trypsin hydrolysates of cutlass fish muscles. According to the results, pepsin and trypsin hydrolysates indicated a strong antioxidant activity against DPPH and Alkyl radicals and a significant ACE inhibitory activity. So far no records are available for antioxidant and ACE inhibitory biological activity of cutlass fish hydolysates. On the basis of these results, this study suggests that the cutlass fish hydrolysates can be used as a potential marine source for producing biologically active natural products and more studies are in need for purification of low molecular weight peptides of cutlass fish hydrolysates. Normally low molecular weight peptides have good biological activity and can be easily absorbed by the body. Thus hydrolysates of cutlass fish protein could be used as natural antioxidant and antihypertensive to enhance the antioxidant and antihypertensive properties of functional food. In addition, it is expected that this will contribute to a developing interest in basic research in the potential application of bioactive peptides. In this study, we evaluated the antioxidant and ACE inhibitory activity of the purified peptide from digestive enzymatic hydrolysates of cutlass fish muscles. Via consecutive chromatographic methods, the antioxidant and ACE inhibitory peptides were found to exhibit potent inhibitory activity. During hydrolysis, a wide variety of smaller peptides and free amino acids were generated, depending on the specificity of the enzymes. Changes in size, level and composition of free amino acids and small peptides affects the antioxidant activity of these peptides (17). To isolate the active antioxidant peptide from the crude protein hydrolysate the sample was loaded into a FPLC connected to a DEAE anion exchange column and the active peak was further purified using RP-HPLC. The antioxidant activity was found to be increased after the purification and successfully scavenged the

DPPH and peroxyl radicals. During hydrolysis, peptide bond cleavage allows the release of active peptides capable of sequestering oxygen radicals, chelating prooxidant metal ions and inhibiting lipid peroxidation in food systems (21). The amino acid sequence of the peptide is playing an important role on its activity. Bioactive peptides usually contain 2-20 amino acid residues per molecule (18), and lower its molecular weight, the higher their chance to cross the intestinal barrier and exert biological effects (19). Li et al. (22) reported that the antioxidative activity of histidine containing peptides was attributed to the proton-donation ability of the imidazole group in histidine. As well, histidine and proline participate in the antioxidative activity of designed peptides tests, among which Pro-His-His sequence has exhibited the greatest antioxidative activity (23). Davalos et al. (24) reported that, tyrosine, tryptophan and methionine showed the highest antioxidant activity among the other α-amino acids, followed by histidine, cysteine and phenylalanine. The antioxidant activity of histidine containing peptides has been attributed to the chelating and lipid radical-trapping ability of the imidazole ring (25, 26). Then structure-activity relationships among different peptide inhibitors of ACE indicates that binding to ACE is strongly affected by the C-terminal tripeptide sequence of the substrate, and it is proposed that peptides, which include hydrophobic amino acids at these positions, are potent inhibitors (20). Therefore, it is possible to produce natural antioxidant peptides from cutlass fish protein by digestive enzymatic hydrolysis and purification. Further research should be carried out to sequence and synthesize the purified peptide and evaluate its antioxidant activity.

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