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Isolation and Charaterization of Bioactive Peptides from *Hwangtae* (yellowish dried Alaska pollack) Protein Hydrolysate

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Abstract

***Hwangtae*, dried Alaska pollack, is a major storage product in the fish processing industry. *Hwangtae* is prepared by removing the internal organs and drying outdoors during the cold witner months by allowing it to thaw during the daytime and re-freeze at night under sub-zero (-10°C) conditions and gradually dry from December until the next April for around 5 months from *Myungtae*. In this study, ground *Hwangtae* was hydrolyzed using two proteolytic enzymes (pepsin and alcalase) which produced five soluble active peptides from *Hwangtae* (yellowish dried Pollack, *Theragra chalcogramma*) protein. Two different peptides with strong antioxidative activity were isolated from the hydrolysate using consecutive chromatographic methods of Sephadex G-25 gel, ion-exchange chromatography on a Sepharose-Sephadex C-25 gel, and high-performance liquid chromatography. The isolated peptides, APO1 and APO2, were composed of 16 and 13 amino acid residues, respectively. Both peptides contained a Gly residue at the C-terminus and the repeating motif Gly-Pro-Hyp. The peptide with a molecular weight less than 1,000 Daltons (APACE) obtained from enzymatic hydrolysates of *Hwangtae* exhibited the highest ACE inhibitory activity. The APACE peptides was composed of 4 amino acid residues (Gly-Leu-Leu-Pro). These results suggest that *Hwangtae* hydrolysates could be a good source of peptides with ACE inhibitory activity. Biochemical analysis indicated that two 70 kDa peptides (APG1 and APG2) isolated from the hydrolysate had gelatinoytic activity, which was shown to be a calcium dependent protease type as showed by gelatin SDS PAGE.**

Key words: *Hwangtae* (yellowish dried pollack), bioactive peptide, antioxidative activity, ACE inhibitory activity, gelatinolytic activity

INTRODUCTION

The market for functional foods and beverages is rapidly and dynamically developing. Biological activities of many naturally occurring food components have been the subject of many scientific and industrial investigations. Nutrients and other bioactive substance from fish can be used as ingredients for functional foods. For example, there are established roles of vitamin D and calcium for bone health promotion, and fatty acids for reducing the risk of cardiovascular disease. Because of its high digestibility, fish protein could have beneficial effects on the colon by reducing protein fermentation (1). Also, the preparation of bioactive peptides derived from fish protein should also be further explored. Many fishery components from fish (including taurine, chitosan, glucosamine and phospholipids) have gained interest as potential bioactive ingredients for functional foods (1). Producing products for human consumption can achieve a better profitability; especially by producing bioactive compounds such as enzymes, bioactive peptides and biopolymers for biotechnological or pharmaceutical applications. Bioactive peptides are relesed during the structural and chemical changes that occurr during the protein processing. Their multifunctional activity is based on their structure and other factors including e.g. hydrophobicity, charge, or microelements binding properties (2). The isolated peptides from fish hydrolysate exert physiological and functional activities such as antithromobotic, antioxidative, antibacterial and antifungal, sensory, and improving the nutritional values of food, but the evidence for positive effects is limited and as yet largely insufficient for health claims. Depending on the amino acid sequence, biopeptides may exert a number of different activities *in vivo*, including effects on the cardiovascular, endocrine, immune and nervous systems in addition to nutrient utilization (3).

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Antioxidants are increasingly used as a means to enhance the shelf-life and improve the stability of lipid and lipid-containing foods. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been commonly used by the food industry, but there are also safety concerns about synthetic antioxidants (4) that have been used as food additives. α-Tocopherol is the most widely used as a natural antioxidant, and it is an effective agent for stabilizing lipid-containing foods, but it has limitations for food usage (5). Thus, there is motivation to search for safe and natural antioxidants from various sources originating from sea foods. Recently, oyster hydrolysate and peptides isolated from the hydrolysate (6), yellow sole frame protein (7), by-product of fresh fish such as Alaska pollack skin (8) and Alaska pollack frame proteins (9) are reported to possess antioxidative activities against the peroxidation of lipids and fatty acids. However, little is known about the structure of antioxidative peptides from various food proteins.

Hypertension is the most common serious chronic health problem because it carries high risk factors for arteriosclerosis, stroke, myocardial infraction and end-stage renal disease (10). Many synthetic ACE inhibitors such as captopril, enalapril, lisinopril and alacepril are available as clinical interventions in hypertension or cardiovascular diseases (11). However, most ACE inhibitors have undesirable side effects such as cough, loss of taste, renal impairment and angioneurotic oedema (12). Therefore, a search for ACE inhibitors from natural materials has become a major field of research. In recent years, many ACE inhibitory peptides have been isolated from various seafood proteins such as carrageenan (13), and sardine (14) as well as from some fermented foods such as tuna sauce (15). In addition, peptide products which have ACE inhibitory effect are currently on the market and some of them are at the stage of being tested, due to the strict requirements for demonstrating the efficacy of these bioactive peptides prior to their widespread utilization as physiologically beneficial functional foods/ food ingredients (16).

In this study, we have described the isolation of bioactive peptides from hydrolysates of *Hwangtae* and have characterized the biofunctional activity of purified peptides in comparison with that of commercial drug or other sources. The aim of the present study was to investigate *Hwangtae* protein as sources of new peptides exhibiting biological activities and develop methods to apply then in food systems and to enable their optimum utilization in the body.

MATERIALS AND METHODS

Materials

Hwangtae was obtained from the village of Yongdaeri, (Inje country, Korea), and stored at -40°C until further processed. Alcalse (2.4 AU/g), pepsin (570 unit/mg) Sephadex G-25, Sepharose-Sephadex C-25, angiotensin I-converting enzyme (from rabbit lung), a substrate peptide (Hip-His-Leu; HHL), ammonium thiocyanate and linoleic acid were purchased from Sigma Chemical Company (St. Louis, MO). Butylated hydroxytoluene (BHT), and α-Tocopherol were obtained from Fluka Buchs, Switzerland. All other reagents used were of the highest grade available commercially.

Preparation of fish protein hydrolysate

Soluble *Hwangtae* protein powder was made by the method described previously (17). A 500 g portion of each ground sample was mixed with an equal volume of distilled water (23°C) and homogenized for 2 min. The mixture was continuously stirred for 60 min at 85°C. The heated suspension was centrifuged at 2560×*g* for 15 min, resulting in three separate phases the semisolid phase at the bottom containing insoluble protein, the heavier liquid phase in the middle containing soluble proteins, and the lighter liquid phase at the top containing crude lipids. The heavier middle-layer liquid was separated, collected, and freeze-dried.

0.5 percent (w/v) freeze-dried soluble protein from *Hwangtae* was prepared in buffer solution and digested with pepsin (enzyme-to-substrate ratio, 1:50) at pH 2.0 and 37°C in the first-step membrane reactor for 24 hr. The first hydrolysate was fractionated through a membrane (A/G Technology Co., model UFP-10-C-4; Needham, MA) with a 10 kDa molecular-weight cut off (MWCO), and then hydrolyzed with Alcalase (enzyme-to-substrate ratio, 1:50) at pH 8.0 and 55°C in the second-step membrane reactor. The second hydrolysate was fractionated through a membrane (A/G Technology Co., model UFP-5-C-4) with a 5000 Da MWCO. The fractions were then lyophilized and stored at -20°C until used.

Molecular characterization of the hydrolysates by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on all peptide samples from *Hwangtae* hydrolysate using a 4% stacking gel and 15% acrylamide gel (18). Protein content of the samples was determined by the Bradford method (19) using bovine serum albumin as a standard. The electrophoresis was performed in a water-cooled electrophoresis appara-

tus (Bio-Rad® instruments, USA) followed by coomassie blue staining. The protein standard (Sigma Aldrich®, USA) consisted of apotin 6.5 kDa; cytochrom 12.4 kDa, carbonic anhydrate 29 kDa, bovine serum albumin 68 kDa.

The protease activities of 70 kDa proteins, separated in an SDS-polyacrylamide mini gel, were detected by copolymerizing with 0.2% gelatin in the polyacrylamide matrix (20). Minigels were electrophoresed at 15 mA per gel, washed twice in 100 mL of 2.5% Triton X-100 (Sigma) for a total of 2 hr to remove SDS, and then incubated in 0.1 M Tris-2.5 mM $CaCl_2$ (pH 8.3) for 16 hr at 37°C. Electrophoresed mini-gels were fixed and stained with 0.1% amido black in methanol-acetic acid-water (30:60:60). After the gels were destained with 10% acetic acid-40% methanol, protease activity was detected by measuring clear zones in the SDS-gelatin-polyacrylamide gel. To determine the characteristic of protease to which the 70-kDa proteins belongs, two samples were electrophoresed on 0.2% gelatin SDS-polyacrylamide gel, in which, $CaCl_2$ was omitted from the incubation buffer in the sample. The gels were then stained for one hour with Coomassie blue staining solution (0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid) and destained for 1.5 hours in destaining solution (40% methanol and 10% glacial acetic acid) on an orbital shaker.

Purification of the active peptides

The hydrolysate was dissolved in a minimum volume of 20 mM sodium acetate buffer (pH 4.0) and loaded onto an ion-exchange column (4.0×40 cm) with Sepharose-Sephadex C-25 (Sigma Chemical Co., St. Louis, MO) previously equilibrated with 20 mM sodium acetate buffer (pH 4.0). The column was washed with the same buffer and eluted with a linear gradient of NaCl concentrations from 0 to 1 mol/L in the same buffer at a flow rate of 60 mL/h. The obtained fractions were dissolved in 10 mL of a 50 mM sodium phosphate buffer (pH 4.0) and loaded onto a Sephadex G-25 gel filtration column (2.5×90 cm) which was previously equilibrated with 50 mmol/L sodium phosphate buffer (pH 7.0). The column was eluted with distilled water and 5.0 mL of fractions were collected at a flow rate of 60 mL/h. The fractions showing biofunctional activity were concentrated by ultrafiltration and dialyzed against distilled water. This fraction was separated by reversed-phase high performance liquid chromatography (HPLC) on a Primesphere 10 C_{18} (20 mm×250 mm) column using a linear gradient of acetonitrile (0～35% in 30 min) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 2.0 mL/min. The active peak was concentrated using a centrifugal evaporator. The peak representing the active peptide was rechromatographed on a ODS (10 mm ×250 mm) column using a linear gradient of acetonitrile (0～15% in 30 min) containing 0.1% TFA at a flow rate of 2.0 mL/min. Finally, the sequence of an isolated active peptide was determined by automated Edman degradation with a Perkin-Elmer 491 protein sequencer (Branchburg, NJ, USA).

Measurement of antioxidative acitivty

The antioxidative activities of the purified peptides from *Hwangtae* hydrolysate were measured in a linoleic acid model system according to the methods of Osawa and Namiki (21). Briefly, a sample (1.3 mg) was dissolved in 10 mL of 50 mM phosphate buffer (pH 7.0), and added to a solution of 0.13 mL of linoleic acid and 10 mL of 99.5% ethanol. The total volume was adjusted to 25 mL with distilled water. The mixture was incubated in a conical flask with a screw cap at 40±1°C in a dark room and the degree of oxidation was evaluated by measuring the ferric thiocyanate values. The reaction solution (100 μL) incubated in the linoleic acid model system described above (Osawa and Namiki) (14) was mixed with 4.7 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 2×10^2 M ferrous chloride solution in 3.5% HCl. After 3 min, the thiocyanate value was measured by reading the absorbance at 500 nm following color development with $FeCl_2$ and thiocyanate at different intervals during the incubation period at 40±1°C.

Measurement of ACE inhibitory activity

ACE inhibitory activity was measured by the method of Cushman and Cheung (22) with slight modifications implemented by Karawita et al. (23). Purified peptides from *Hwangtae* hydrolysate (20～2000 μg) were dissolved in 100 mM sodium borate buffer (pH 8.3) containing 300 mM NaCl. A 200 μL volume of 5 mM peptide solution was mixed with 80 μL of sample solution followed by pre-incubation for 3 min at 37°C. The reaction was started by adding of 20 μL of ACE solution in distilled water (100 mU/mL), and the reaction mixture was incubated for 30 min at 37°C. The reaction was stopped by adding 1.0 M HCl (250 μL), and the liberated hippuric acid was extracted with 1.7 mL of ethyl acetate. After centrifugation (800×*g*, 15 min), 1.0 mL of the upper layer was transferred into a test tube and evaporated at room temperature for 2 hr in a vacuum evaporator. The hippuric acid was dissolved in 1.0 mL of distilled water, and the absorbance was measured at 228 nm using an UV/VIS spectrophotometer (CBM-20A, Shimadzu Co., Ltd., Japan). The IC_{50} value was defined as the concentration of inhibitor required to in-

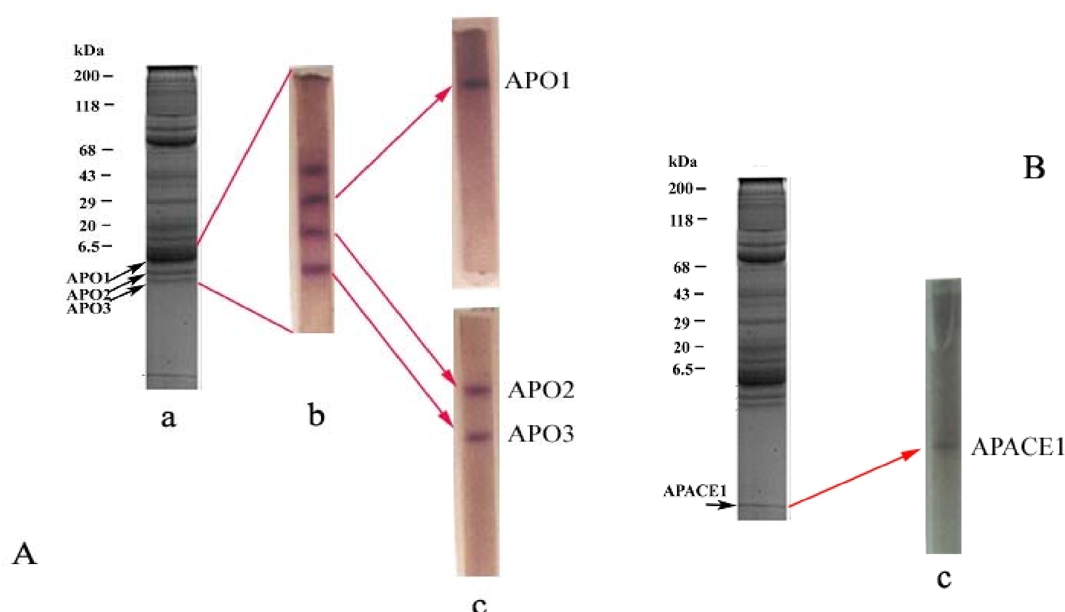

Fig. 1. SDS-PAGE profile of isolated peptides from *Hwangtae* hydrolysate were fractionated on the basis of their molecular weight and polarity by chromatography at each step. A, Antioxidative peptides; B, ACE inhibitory peptide; a, Enzymatic hydrolysate obtained from two-step recycling membrane reactor (pepsin and alcalase); b, The fractions obtained through an ion-exchange Sepharose-Sephadex C-25 column; c, Isolated peptide was separated by reversed-phase high performance liquid chromatography (HPLC) on a Primesphere 10 C_{18} column. APO1, 2, 3: potentially antioxidative peptides; APACE: low weight molecular size and potentially angiotensin I converting enzyme (ACE) inhibited peptide.

hibit 50% of the ACE activity.

RESULTS AND DISCUSSION

Preparation and characterization of *Hwangtae* hydrolysates

The hydrolysates from *Hwangtae* were prepared by consecutive digestions with pepsin and alcalase using a two-step recycling membrane reactor, as described in the previous section. Two different kinds of endoproteases with different optimal pH conditions were used to hydrolyze the protein in *Hwangtae*. The acid-dependent proteolytic enzymes, such as pepsin has been most successful in solubilizing fish protein (24) with low lipid content but very poor nutritional value (25). Alcalase-treated hydrolysates also had the lowest lipid content (0.18%) and excellent functional properties. Its hydrolysates made with alcalase at higher degrees of hydrolysis showed a decrease in high molecular-weight fractions, and increased solubility (26). In food protein hydrolysis, endoproteinases are always used, but occasionally endoproteinases are combined with exopeptidases and two endopeptidases to achieve a more complete degradation (27).

SDS-PAGE

The electrophoretic patterns of the hydrolysates and isolated peptides were fractionated on the basis of their molecular weight and polarity by chromatography at each step using SDS-PAGE (Fig. 1). The isolated peptide from *Hwangtae* hydrolysate showed the presence of four bands with a molecular mass of less than 4.5 kDa, which had smaller molecular size and is more soluble than the other hydrolysates and peptides. The two isolated hydrolysates also showed two bands in the range of 68 and 118 kDa, which may be the result of larger proteins from the *Hwangtae* hydrolysate containing gelatinolytic acitivies.

Detection of proteolytic activity

Gelatin SDS PAGE has been shown to be a valuable tool for investigating the presence of MMPs (metalloproteinases) and other proteinases with gelatinolytic activity (28-30). The isolated peptides from *Hwangtae* hydrolysate were subjected to gelatin minigel SDS-PAGE to determine the presence of proteolytic activity. Any proteolytic activity was evident as clear areas within the gel. Both the purified peptides from *Hwangtae* hydrolysate (APG1 and APG2) had proteolytic activity. The strongest activity was observed with gelatin as a substrate in the 70 kDa band (Fig. 2A). Adjacent peptides (APG1 and APG) were isolated using a chromatography procedure, the fractions from 68 to 118 containing proteolytic activity. The 70 kDa peptide (APG2) was still

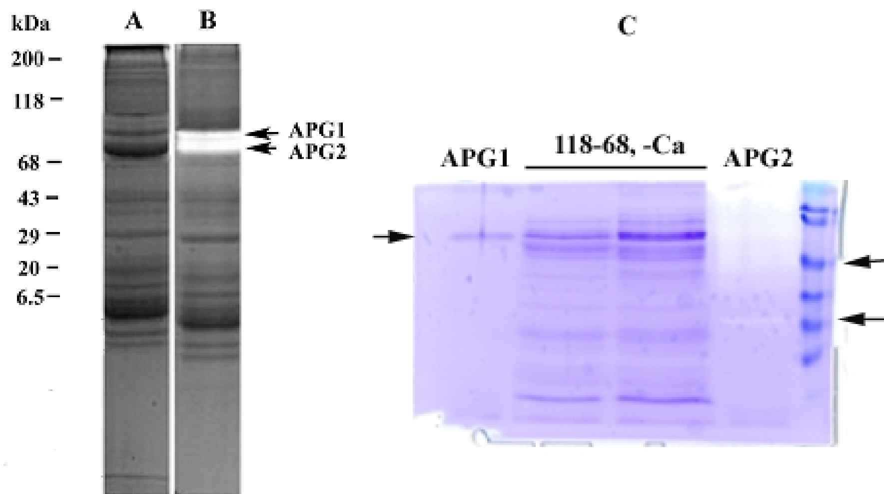

Fig. 2. SDS-PAGE of hydrolysate (A), gelationolytic SDS PAGE of peptides poteintially possesing proteolytic activity (B) and characteristics of protease activity of isolated peptides and fractions under calcium-free conditions (C) in *Hwangtae*.

active in the absence of other adjacent peptide (APG1, Mw 70 kDa). However, the similar size of peptide (APG1) showed loss of protease activity in the absence of other adjacent peptide (APG2). These results suggested that APG1 and APG2 peptides appeared to show proteolytic activities with synergistic and associated manner. In addition, the proteolytic activity mainly depended on major role of APG2 in these peptides from *Hwangtae*. Also, APG1 and APG2 had proteolytic activity was shown to be calcium dependent protease type. The proteolytic activities were lost in the absence of calcium in the incubation buffer of gelatinolytic SDS-PAGE (Fig. 2B).

Identification of purified peptides

To identify the biofunctional peptides, the second-step hydrolysate of Alaska pollack skin with the highest antioxidative activity was separated by ion-exchange, size exclusion chromatography and HPLC. The *Hwangtae* hydrolysate was dissolved in sodium acetate buffer (pH 4.0), and loaded onto a Sepharose-Sephadex C-25 column with the linear gradient of NaCl (0～1.0 M), and fractionated into eight portions (Fig. 3A). Each fraction was pooled and lyophilized. The lyophilized active fraction was subjected to size exclusion chromatography on Sephadex G-25, and fractionated (Fig. 3B). Each fraction was pooled and lyophilized. This active fraction was further separated by Reversed Phase-HPLC on a Primesphere 10 C_{18} (20 mm×250 mm) column using a linear gradient of acetonitrile (0～35%) containing 0.1% TFA, and the fractions were divided into six portions (Fig. 3C).

The amino acid sequences of two isolated antioxidative peptides were composed of 16 and 13 amino acid residues, respectively. The sequence of APO1 (Gly-Glu-Hyp-Gly-Pro-His-Gly-Pro-Ser-Gly-Pro-Hyp-Gly-Pro-Hyp-Thr, 1362.48 Da) was found to be the same sequence as APO2 (13 amino acid, Gly-Pro-Hyp-Gly-Pro-His-Gly-Pro-Ser-Gly-Pro-Tyr-Gly, 1079.20 Da). The sequences of the peptides are in good agreement with the data from antioxidative peptides from hydrolysate of Alaska Pollack skin (8). Furthermore, the isolated peptides contained Gly, His residue and the rich specific repeating motif sequence (Gly-Pro-Hyp) consistent with amino acid sequences of collagen.

Recently, the antioxidative activity of histidine containing peptides (31-33) especially, Alaska pollack frame protein (9), has been reported. This activity may be attributed to the chelating and lipid radical-trapping ability of the imidazole ring. In this study, the antioxidative peptide contained a histidine residue. In addition, this peptide contained a tyrosine residue, which is a potent hydrogen donor in its sequence. These results suggest that antioxidative activity of the isolated peptide was depended on their amino acid residues and molecular weights.

Fig. 3A shows the elution profile of the Sepharose-Sephadex C-25 chromatogram of the hydroysate fractionated through a membrane. The fractions of the active peak (No. 1～11) were collected and lyphophilized and then purified by Primesphere 10 C_{18} column. The purified peptide (APACE) was analyzed for amino acid sequence by automated Edaman procedure. The peptide was determined to be a four residue peptide, Gly-Leu-Leu-Pro which corresponds to the amino acid sequence 394-396 of rabbit muscle glycogen phosphorylase (34) except for the additional one amino acid residue, Gly

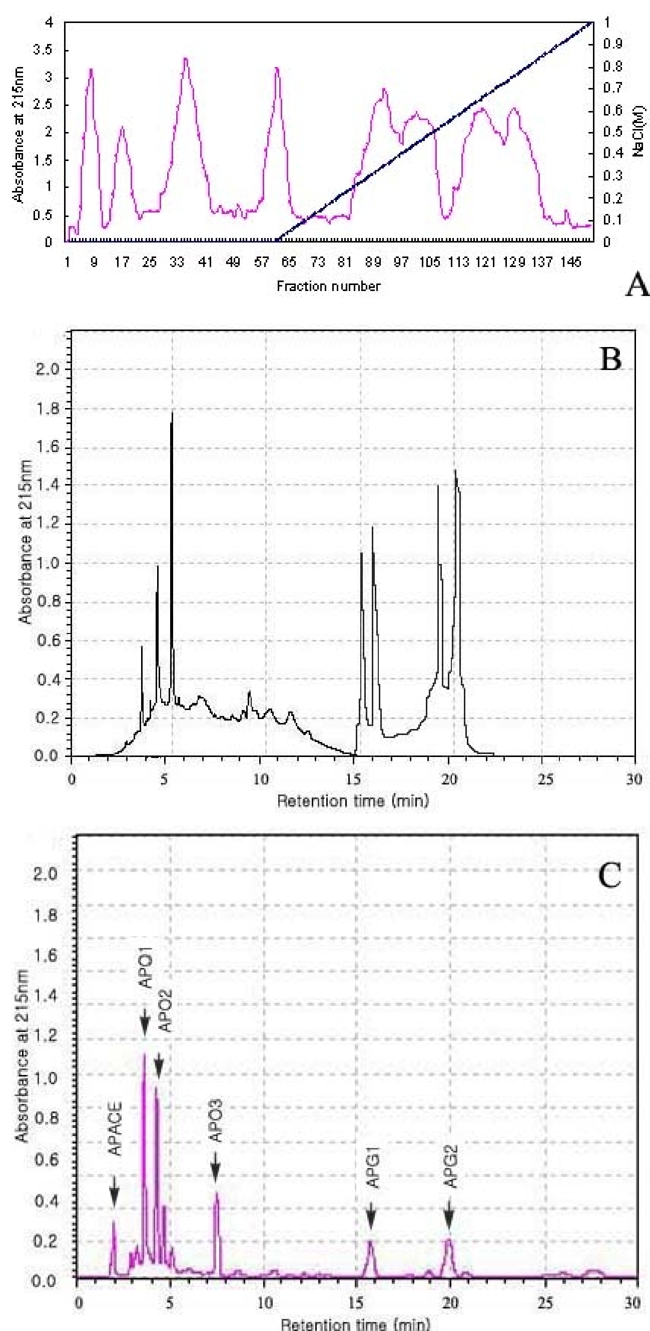

Fig. 3. Purification of bioactive peptides from hydrolysate of *Hwangtae*. (A) Sepharose-Sephadex C-25 chromatography. Elution was performed at 60 mL/h of flow rate with a linear gradient of NaCl (0～1 M) in 20 mM sodium acetate buffer, pH 4.0. (B) Rechromatography of fraction from Fig. 3A on Sephadex G-25 gel chromatography Elution was done at 60 mL/h with distilled water. (C) Reversed-phase HPLC pattern on a Primesphere 10 C_{18} column of active fraction from Fig 3b. HPLC was carried out with 35% acetonitrile as the mobile phase at a flow rate of 2 mL/min using UV detection at 215 nm.

at N-terminus. Lee et al. (35) reported that isolation and identification of an ACE inhibitory peptide from hydrolysate of Manila clam, in which the amino acid sequence of this inhibitory peptide activity was determined to be Leu-Leu-Pro.

Table 1. Antioxidative activities of isolated peptides from *Hwangtae* hydrolysate using the linoleic acid auto-oxidation system measured by the ferric thiocyanate method

Fractionation of hydrolysates		Absorbance at 500 nm
	APO1	0.198±0.012
	APO2	0.132±0.024
	APO3	0.689±0.036
Positive control	BHT (Butylated hydroxytoluene)	0.134±0.010
Negative control	BSA (Bovine serum albumin)	0.754±0.021

Value present means±SE (n=3).

Antioxidative activities of purified peptide

The antioxidative activities of the isolated peptides from *Hwangtae* hydrolysate were measured and compared with those of bovine serum albumin and BHT. The oxidative activity of linoleic acid was markedly inhibited by the addition of the isolated peptides from *Hwangtae* hydrolysate (Table 1). Among the three hydrolysates, the highest antioxidative activity was observed in APO2 with molecular weight of below 1 kDa, which exhibited about 82.5% inhibition of linoleic acid peroxidation compared to control protein (BSA). The antioxidative activity of the APO2 peptide was similar to that of BHT. The APO1 peptide has similar to peptide size, and also inhibited to approximately 73% of the oxidation, but APO3 had no detectable antioxdative activity. The sequence of APO2 was similar to that of APO1. Therefore, the difference in antioxidative activity between the two peptides isolated was thought to be attributable to only by three amino acid residues (Gly-Glu-Hyp). On the other hand, several amino acids, such as Tyr, Gly and His are generally accepted as antioxidants despite their pro-oxidative effects in some cases (36-38). These results indicate that the antioxidative activity of the peptides isolated from gelatin hydrolysate of Alaska pollack skin depends on their amino acid sequences.

ACE inhbitory activity of purified peptide

The isolated peptides (APACE) below 1 kDa was identified to be a four residue peptide, Gly-Leu-Leu,-Pro which showed ACE inhibitory activity (Table 2). The ACE inhibitory activity of the fragmented hydrolysate from fresh fish of Alaska pollack (*Theragra chalcogramma*) skin was markedly increased with decreasing molecular weights (39). The results of ACE inhibitory activities of hydrolysates in Alaska pollack indicate that peptides with low molecular weights may be responsible for their antihypertensive effects. Therefore, the hydro-

Table 2. Angiotensin I converting enzyme (ACE) inhibitory activity (IC_{50}) of enzymatic hydrolysates from *Hwangtae* with pepsin enzymes.

Sample	ACE inhibition concentration (IC50 μg/mL)
APACE1	0.230±0.070
Captopril	0.085±0.016

Value present means±SE (n=3).

lysate of *Hwangtae* has potential as a source of anti-ACE agents for the controlling hypertension, a known complication of long-term diabetes and/or hyperglycemia.

Cleavage of amide linkage in the protein chain leads to the formation of peptides with different number of amino acids. The peptides produced from the action of a specific enzyme may be subjected to further hydrolysis by other enzymes. The *Hwangtae* peptides so obtained may be subjected to chromatographic separation and then evaluated for their amino acid sequence as well as for their antioxidant and other activities. Therefore, it is concluded that the purified peptides from *Hwangtae* have the potential to be used in the food industry because of their desirable biological properties. It may be possible to produce a broad spectrum of food ingredients or industrial products for a wide range of application.

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