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Purification and characterization of antioxidant peptide from hoki (*Johnius belengerii*) frame protein by gastrointestinal digestion

Soo-Yong Kim, Jae-Young Je, Se-Kwon Kim*

Department of Chemistry, Pukyong National University, Busan 608-737, Korea

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Abstract

To extract antioxidant peptide from hoki frame protein hydrolysate (APHPH), we employed six proteases (pepsin, trypsin, papain, α-chymotrypsin, Alcalase and Neutrase) for enzymatic hydrolysis, and the antioxidant activities of their hydrolysates were investigated using both lipid peroxidation inhibition assay and free radical scavenging assay by electron spin resonance spin-trapping technique. Among hydrolysates, peptic hydrolysate, having the highest antioxidant activity, further separated into four groups using ultrafiltration membranes and purified consecutive chromatographic methods. Finally, the purified peptide had a molecular mass of 1801 Da, and amino acid sequence was identified as Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn. APHPH inhibited lipid peroxidation higher than that of α-tocopherol as positive control and efficiently quenched different sources of free radical: 1,1-diphenyl-2-pycryl-hydrazyl (IC_{50}=41.37 μM), hydroxyl (IC_{50}=17.77 μM), peroxyl (IC_{50}=18.99 μM) and superoxide radicals (IC_{50}=172.10 μM). Furthermore, APHPH decreased *t*-butylhydroperoxide-induced cytotoxicity on human embryonic lung fibroblasts and efficiently protected free-radical-induced DNA damage.

Keywords: Antioxidant peptide; Free radical scavenger; DNA damage; Cytotoxicity

1. Introduction

Reactive oxygen species (ROS) and free radicals play an important role in many diseases [1]. Formation of free radicals, such as superoxide anion radical ($O_2^{\cdot-}$) and hydroxyl radical ($^{\cdot}OH$), is an unavoidable consequence in aerobic organisms during respiration. These radicals are very unstable and react rapidly with other groups or substances in the body, leading to cell or tissue injury. Under normal conditions, ROS is effectively eliminated by the antioxidant defense system, such as antioxidant enzymes and nonenzymatic factors. However, under pathological conditions, the balance between the generation and the elimination of ROS is broken; as a result of these events, biomacromolecules, including DNA, membrane lipids and proteins, are damaged by ROS-mediated oxidative stress.

Lipid oxidation is of great concern to the food industry and consumers because it leads to the development of undesirable off-flavors and potentially toxic reaction products [2]. Increasing evidences revealed that uncontrolled lipid peroxidation is involved in the occurrence of numerous chronic diseases [3,4]. Specially, lipid peroxidation in foods affects nutritive value and may cause disease conditions following consumption of potentially toxic reaction products. Therefore, during the last few decades, the research fields of human nutrition and biochemistry have focused on an antioxidant derived from a food ingredient that could retard lipid peroxidation.

Bioactive peptides can be released by enzymatic proteolysis of food proteins and may act as potential physiological modulators of metabolism during intestinal digestion. Bioactive peptides usually contain 3–20 amino acid residues, and their activity is based on their amino acid composition and sequence [5]. Possible regulatory effects of peptides relate to nutrient uptake, immune defense [6,7] and opioid [5], antioxidant [8] and antihypertensive activities [9,10].

Annually, over 100 million tons of fish are harvested worldwide, and approximately 30% of the total catch is used for fish meal and animal feed because of their poor functional properties. Furthermore, more than 50% of the total catch is discarded as processing waste or by-product.

* Corresponding author. Tel.: +82 51 620 6375; fax: +82 51 628 8147.
E-mail address: sknkim@pknu.ac.kr (S.-K. Kim).

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These by-products are important protein and mineral sources [11], and they can be converted to value-added products by enzymatic hydrolysis, which is widely applied to improve and upgrade the functional and nutritional properties of proteins. In our laboratory, for the efficient recovery of proteins from fish frames, enzymatic hydrolysis of cod frame protein was carried out using the crude proteinase extracted from the pyloric caeca of tuna [12]. We also investigated the antioxidant properties of hoki frame protein hydrolysates (HPH) prepared by ultrafiltration (UF) membrane in a previous study [13]. Therefore, the aim of this study was to isolate antioxidant peptide from hoki frame protein hydrolysate (APHPH) and to evaluate antioxidant properties using cellular and noncellular systems.

2. Materials and methods

2.1. Materials

Hoki frame was donated by Charmson Food Co. (Busan, Korea). Digestive proteases were products of Sigma (St. Louis, MO). All testing chemicals, including 1,1-diphenyl-2-pycryl-hydrazyl (DPPH), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH), *t*-butylhydroperoxide (*t*-BHP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and α-(4-pyridyl-1-oxide)-*N*-*t*-butyl nitrone (4-POBN), were also purchased from Sigma. Human embryonic lung fibroblasts (MRC-5 cell line; ATCC CCL-171) were obtained from the American Type Culture Collection (Manassas, VA). Cell culture medium and all the other materials required for culturing were obtained from Gibco (Grand Island, NY). Plasmid pBR 322 DNA was purchased from Takara Biomedicals (Tokyo, Japan). Other chemicals and reagents used were of the highest analytical grade commercially available.

2.2. Preparation of HPH by gastrointestinal digestion

The hydrolysis of hoki frame protein was performed. At an enzyme/substrate ratio of 1/100 (wt/wt), 1% substrate in 10 l of glycine–HCl buffer (pH 2.0) and pepsin were mixed. The mixture was incubated for 8 h at 37°C with stirring and then heated in a boiling water bath for 10 min to inactivate the enzyme. The resultant HPH were fractionated through UF membranes with a range of molecular weight cutoffs (M_{WCO}) of 10, 5, 3 and 1 kDa, respectively. Fractionates were designed as follows: HPH I with M_W distribution of 5–10 kDa, HPH II with M_W distribution of 3–5 kDa, HPH III with M_W distribution of 1–3 kDa and HPH IV with M_W distribution <1 kDa. All HPH recovered were lyophilized in a freeze drier for 5 days.

2.3. Lipid peroxidation inhibition assay

The lipid peroxidation inhibition activity of HPH was measured in a linoleic acid emulsion system according to the methods of Osawa and Namiki [14]. Briefly, a sample (1.3 mg) of HPH 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. Then 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 measured using the ferric thiocyanate (FTC) method of Mitsuda et al. [15]. The reaction solution (100 μl) incubated in the linoleic acid emulsion system described above 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, 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.

2.4. Electron spin resonance (ESR) measurement

2.4.1. Scavenging effect on DPPH radical

DPPH radical scavenging activity was measured using the method described by Nanjo et al. [16]. An ethanol solution of 60 μl of each peptide (or ethanol itself as control) was added to 60 μl of DPPH (60 μM) in ethanol solution. After mixing vigorously for 10 s, the solution was then transferred into a 100-μl quartz capillary tube, and the scavenging activity of peptides on DPPH radical was measured using a JES-FA ESR spectrometer (JEOL Ltd., Tokyo, Japan). A spin adduct was measured on an ESR spectrometer exactly 2 min later. Experimental conditions were as follows: magnetic field=336.5±5 mT, power=5 mW, modulation frequency=9.41 GHz, amplitude=1×1000 and sweep time=30 s.

2.4.2. Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by iron-catalyzed Haber–Weiss reaction (Fenton-driven Haber–Weiss reaction), and the generated hydroxyl radicals rapidly reacted with nitrone spin-trap DMPO [17]. The resultant DMPO–OH adduct was detectable with an ESR spectrometer. Peptide (0.2 ml) with various concentrations was mixed with DMPO (0.3 M, 0.2 ml), $FeSO_4$ (10 mM, 0.2 ml) and H_2O_2 (10 mM, 0.2 ml) in a phosphate buffer solution (pH 7.2) and then transferred into a 100-μl quartz capillary tube. After 2.5 min, ESR spectrum was recorded using an ESR spectrometer. Experimental conditions were as follows: magnetic field=336.5±5 mT, power=1 mW, modulation frequency=9.41 GHz, amplitude=1×200 and sweep time=4 min.

2.4.3. Assay for peroxyl radical

Peroxyl radicals were generated by AAPH. A phosphate-buffered saline reaction mixture containing 10 mM AAPH, 10 mM 4-POBN and peptide at various concentrations was incubated for 30 min at 37°C in a water bath [18] and then transferred to a 100-μl quartz capillary tube. The spin adduct was recorded using an ESR spectrometer. Experimental conditions were as follows: magnetic field=336.5±5 mT, power=10 mW, modulation frequency=9.41 GHz, amplitude=1×1000 and sweep time=1 min.

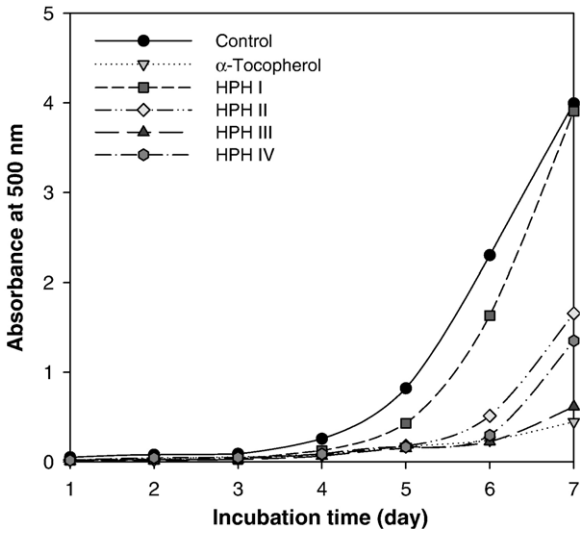

Fig. 1. Lipid peroxidation inhibition activity of peptic hydrolysate fractionated by UF membranes. The activity was measured in the linoleic acid emulsion system by the FTC method. Lower absorbance at 500 nm represents higher lipid peroxidation inhibition.

2.4.4. Superoxide radical scavenging activity

Superoxide radicals were generated by UV irradiation of a riboflavin/EDTA solution [19]. The reaction mixtures containing 0.8 mM riboflavin, 1.6 mM EDTA, 800 mM DMPO and various concentrations of peptide were irradiated for 1 min under a UV lamp at 365 nm. The mixtures were transferred to a 100-μl quartz capillary tube of the ESR spectrometer for measurement. Experimental conditions were as follows: magnetic field=336.5±5 mT, power=10 mW, modulation frequency=9.41 GHz, amplitude=1×1000 and sweep time=1 min.

2.5. Purification of antioxidant peptide

2.5.1. Ion exchange chromatography

Among the HPH of pepsin, HPH III, showing the highest antioxidant activity, was selected as purification material. The lyophilized HPH III (10 mg/ml) was loaded onto a HiPrep 16/10 CM FF ion exchange column equilibrated with 20 mM 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 62 ml/h. Each fraction was monitored at 280 nm, collected at a volume of 3 ml and concentrated using a rotary evaporator; antioxidant activity was also investigated. A strong antioxidant fraction was lyophilized, and chromatography was used as the next step.

2.5.2. High-performance liquid chromatography (HPLC)

The fraction exhibiting antioxidant activity was further purified using reverse-phase HPLC (RP-HPLC) on a Capcell Pak C_{18} UG-120 (20×250 mm) column with a linear gradient of acetonitrile (0–20% in 50 min) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 2.0 ml/min. Elution peaks were detected at 215 nm, and active peak was concentrated using a rotary evaporator. For further purification, the active peak from RP-HPLC was loaded onto a Synchropak RPP-100 RP-HPLC analytical column (4.6×250 mm) with a linear gradient of acetonitrile (0–10% in 20 min) containing 0.1% TFA at a flow rate of 2.0 ml/min. Potent peaks were collected, evaluated for antioxidant activity and then lyophilized. The active fraction from the analytical column was further applied onto a Synchropak RPP-100 RP-HPLC analytical column with a concentration of 2% acetonitrile containing 0.1% TFA at a flow rate of 1 ml/min. The final purified peptide was analyzed for amino acid sequence.

2.6. Determination of the amino acid sequence of the purified peptide

Accurate molecular mass and amino acid sequence of the purified peptide were determined with a Q-TOF mass spectrometer (Micromass, Altrincham, UK) coupled with an electrospray ionization (ESI) source. The purified peptide was separately infused into the electrospray source following dissolution in methanol/water (1:1, vol/vol), and molecular mass was determined by a doubly charged $(M+2H)^{+2}$ state in the mass spectrum. Following molecular mass determination, the peptide was automatically selected for fragmentation, and sequence information was obtained by tandem mass spectroscopy (MS) analysis.

2.7. Cell culture study of the purified peptide

2.7.1. Cell culture

MRC-5 (ATCC CCL-171) cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS) and maintained at 37°C under a humidified atmosphere with 5% CO_2.

2.7.2. Cytotoxic assessment using MTT assay

Cells were seeded at 1.3×10^4 cells/well in 96-well microtiter plates in complete medium DMEM with 10% FBS for MRC-5 cell. After 24 h of incubation in a humidified 5% (vol/vol) CO_2/air environment at 37°C, 20 μl of the purified peptide solution in sterile distilled water was transferred to the well to give a final concentration ranging from 5.55 to 278 μM. Following 24 h of incubation with the peptide, culture media were aspirated, and 200 μl of

Table 1
Free radical scavenging effects of fractionated HPH

Fractions	Free radical scavenging effects (%)			
	DPPH[a] radical	Hydroxyl[a] radical	Peroxyl[b] radical	Superoxide[b] radical
HPH I	75.04±1.23	75.45±1.23	70.10±2.31	15.34±1.23
HPH II	78.22±2.34	72.60±1.45	80.52±1.78	21.88±2.43
HPH III	83.39±1.64	84.93±1.87	85.79±1.54	28.97±2.67
HPH IV	60.41±2.43	69.18±2.32	31.01±2.31	23.97±2.38

[a] Scavenging effects were tested at a concentration of 0.5 mg/ml.
[b] Scavenging effects were tested at a concentration of 1.0 mg/ml.

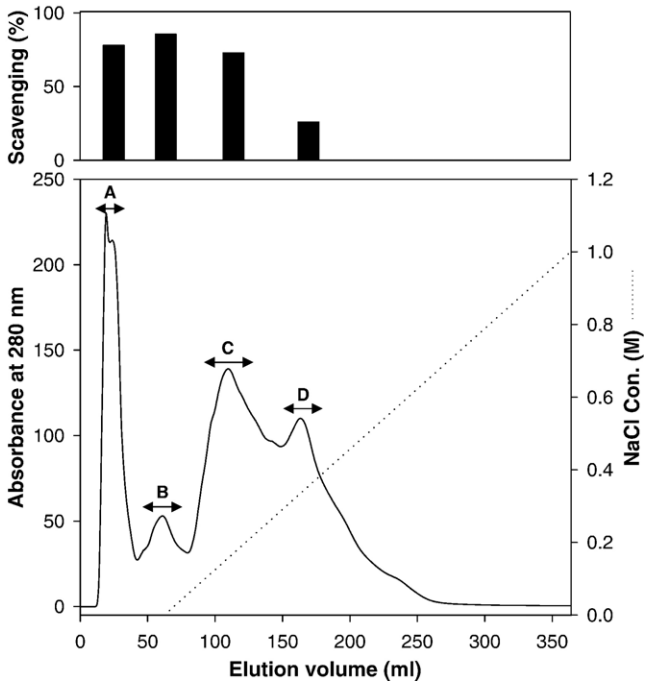

Fig. 2. FPLC chromatogram of antioxidant peptides from HPH III by a HiPrep 16/10 CM FF ion column. Adsorption peptides were eluted with a linear gradient of 0–1 M NaCl. The antioxidant activity of the eluted peaks was measured by hydroxyl radical scavenging effect using an ESR spectrometer.

MTT dye solution (0.5 mg/ml) was added to each well. After 4 h of incubation, media were aspirated and purple color crystals were dissolved with DMSO. Absorbance in each well was measured at 540 nm using a Genios Multifunction microplate reader (Tecan, Manchester, UK).

2.7.3. t-BHP-induced cytotoxicity with the purified peptide

Cells were treated with various concentrations of the purified peptide and incubated for 10 h. Cellular oxidation was accelerated by exposing cells to 200 μM *t*-BHP; after 2 h, cell viability was measured using the MTT method. For each well, 200 μl of MTT was added and incubated at 37°C in the dark for 1 h. Formed formazan crystals were solubilized in DMSO, and optical density was measured at 540 nm using a Genios Multifunction microplate reader (Tecan). Relative cell viability was determined using the amount of MTT converted into formazan salt, and data were expressed as mean percentages of viable cells compared to the respective control culture.

2.8. Protective effect of the purified peptide against hydroxyl-radical-induced DNA damage

To study the protection effects of the purified peptide on DNA damage by hydroxyl radical, the reaction was conducted in an Eppendorf tube at a total volume of 12 μl containing 0.5 μg of pBR 322 DNA in 3 μl of 50 mM phosphate buffer (pH 7.4), 3 μl of 2 mM $FeSO_4$ and 2 μl of the purified peptide at various concentrations. Then, 4 μl of 30% H_2O_2 was added, and the mixture was incubated at 37°C for 1 h [20]. The mixture was subjected to 0.8% agarose gel electrophoresis. DNA bands (supercoiled, linear and open circular) were stained with ethidium bromide.

2.9. Statistical analysis

Data were expressed as mean±standard error of the mean ($n=3$). Student's *t* test was used to determine the level of significance ($P<.05$).

3. Results

3.1. Enzymatic hydrolysis of hoki frame protein

Hoki frame proteins were separately hydrolyzed with six enzymes (pepsin, α-chymotrypsin, trypsin, papain, Alcalase, and Neutrase) at optimal conditions. The antioxidant activities of the hydrolysates were evaluated using lipid peroxidation inhibition assay and free radical scavenging activity by ESR spin-trapping technique. After 7 days of lipid autoxidative reaction, pepsin and papain hydrolysates prevented 54% and 45%, respectively, of lipid peroxidation in the linoleic acid emulsion system. Other hydrolysates showed lipid peroxidation inhibition lower than that of pepsin and papain (data not shown). In free radical scavenging activities against DPPH, hydroxyl, superoxide and peroxyl radical, pepsin hydrolysate revealed the most potent free radical scavenging ability (data not shown).

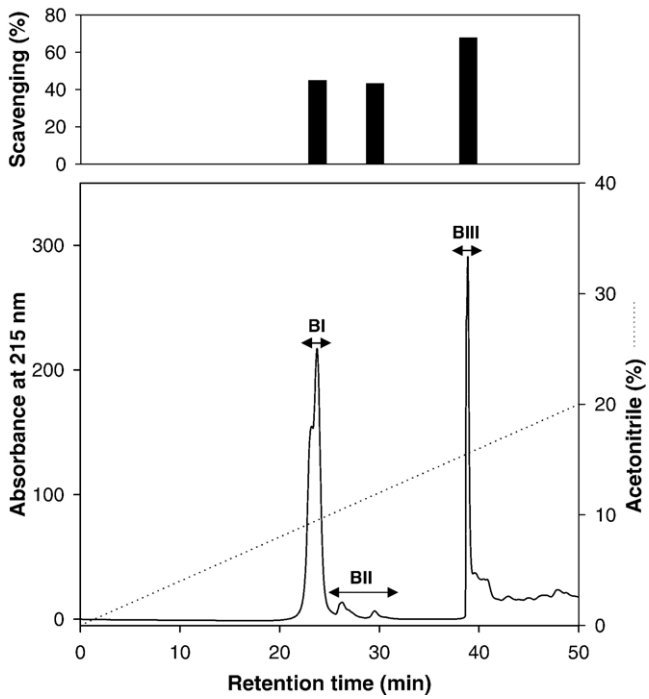

Fig. 3. A chromatogram of RP-HPLC (Capcell Pak C_{18} UG-120 column) for the purification of antioxidant peptides. Elution was performed with the linear gradient of acetonitrile (0–20%) containing 0.1% TFA. The antioxidant activity of the eluted peaks was measured by hydroxyl radical scavenging effect using an ESR spectrometer.

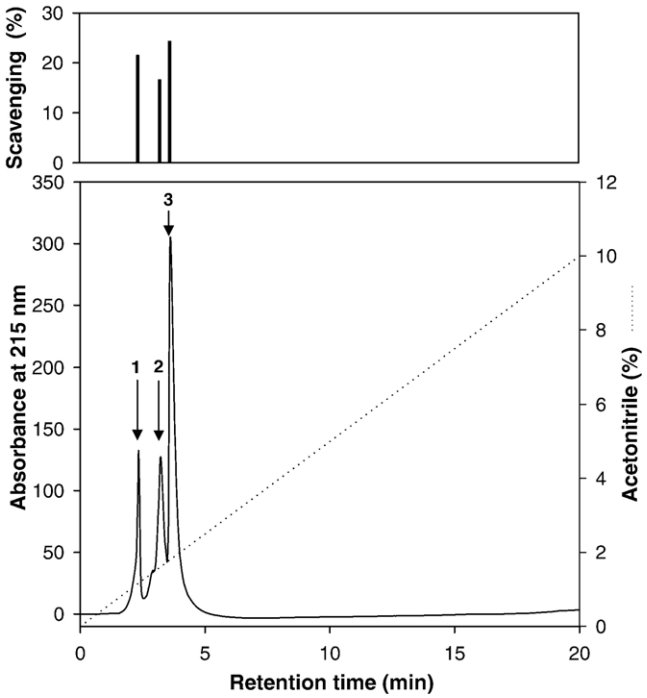

Fig. 4. A rechromatogram of RP-HPLC on a Synchropak RPP-100 analytical column. Elution was performed with the linear gradient of acetonitrile (0–10%) containing 0.1% TFA. The antioxidant activity of the eluted peaks was measured by hydroxyl radical scavenging effect using an ESR spectrometer.

Peptic hydrolysate, having the highest antioxidant activity, further separated four M_W groups using UF membranes (M_{WCO}=10, 5, 3 and 1 kDa) into HPH I (M_W=5–10 kDa), HPH II (M_W=3–5 kDa), HPH III (M_W=1–3 kDa) and HPH IV (M_W<1 kDa). The four groups were investigated by both lipid peroxidation inhibition assay and free radical scavenging activity. As shown in Fig. 1, the HPH III group effectively inhibited lipid peroxidation compared to other groups; the inhibition ratio was similar than that of α-tocopherol, which is used as a positive control. Table 1 shows the free radical scavenging activities of the four groups against different radical sources. The results showed that HPH III with an M_W=1–3 kDa exhibited free radical scavenging effects higher than those of other groups. Therefore, we selected HPH III for the identification of the antioxidant peptide.

3.2. Purification and identification of antioxidant peptide

Considering the inhibitory effects on both lipid peroxidation inhibition and free radical scavenging activities, HPH III was employed for the purification and identification of the antioxidant peptide. Firstly, HPH III was dissolved in sodium acetate buffer (20 mM, pH 4.0) and loaded onto a HiPrep 16/10 CM FF column using fast protein liquid chromatography (FPLC) with a linear gradient of NaCl (0–1 M). Elution peaks were monitored at 280 nm, and each fraction was collected as 3 ml and fractionated into four portions (Fig. 2). Each fraction was pooled, lyophilized and measured for antioxidant activity using the potency to suppress hydroxyl radicals. The results showed that fraction B has the highest hydroxyl radical scavenging activity. For further purification, the lyophilized active fraction B was subjected to RP-HPLC on a Capcell Pak C_{18} column using a linear gradient of acetonitrile (0–20%), and the fractions were divided into three portions (Fig. 3). Each fraction was pooled, lyophilized and measured for antioxidant activity. This active fraction was further separated by RP-HPLC on a Synchropak RPP-100 using a linear gradient of 0–10% acetonitrile (Fig. 4). Fraction 3, which has a strong antioxidant activity, further purified on a Synchropak RPP-100. Finally, we obtained the purified peptide. APHPH was analyzed for molecular mass with amino acid sequence using ESI/MS. APHPH was identified as Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn (M_W=1801 Da).

3.3. Antioxidant activities of APHPH

To evaluate the antioxidant activities of APHPH, both lipid peroxidation inhibition activity and direct free radical scavenging effects were investigated. As shown in Fig. 5, APHPH effectively inhibited lipid peroxidation in the linoleic acid emulsion system, as described above. The activity of APHPH for lipid peroxidation inhibition was higher than that of α-tocopherol as positive control after 7 days.

The direct free radical scavenging effects of APHPH were investigated using the ESR spin-trapping technique. Four free radicals were generated in in vitro systems. Table 2 shows IC_{50} values of APHPH against DPPH, hydroxyl, peroxyl and superoxide radicals. APHPH was effectively quenched in the order of hydroxyl, peroxyl, DPPH and superoxide radical, and IC_{50} values were 17.77, 18.99, 41.37 and 172.1 μM, respectively.

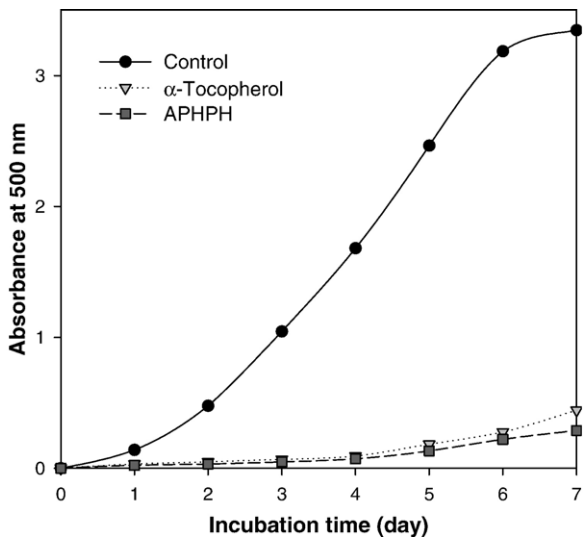

Fig. 5. Lipid peroxidation inhibition activity of APHPH. The activity was measured in the linoleic acid emulsion system by FTC method using α-tocopherol as positive control.

Table 2
IC_{50} values of APHPH

Peptide	IC_{50} (μM)			
	DPPH radical	Hydroxyl radical	Peroxyl radical	Superoxide radical
APHPH	41.37	17.77	18.99	172.10

3.4. Effect of APHPH on t-BHP-induced cytotoxicity

The cytotoxicity and protective effect of APHPH on radical-mediated cellular injuries and death were investigated using human embryonic lung fibroblasts (MRC-5 cell line). In order to avoid the cytotoxic interference of APHPH at high concentrations, the influence of APHPH on the cell viability of MRC-5 was determined using MTT assay. According to the result of the MTT assay, APHPH did not have any cytotoxic effect on MRC-5 cells at the tested concentrations. Therefore, nontoxic concentrations of APHPH (2.78–55.5 μM) were used for the experiment. Organic *t*-BHP is a useful model compound used to study the mechanism of oxidative cell injury. Cells were exposed to *t*-BHP for 2 h, and cell viability was determined using MTT assay. As shown in Fig. 6, MRC-5 cell viability increased with increasing concentrations of APHPH and enhanced the viability of t-BHP-induced cytotoxicity up to 91.1% at a concentration of 55.5 μM.

3.5. Protective effect of APHPH against hydroxyl-radical-induced DNA damage

The antioxidant effect of APHPH was evaluated using a protection effect on free-radical-induced plasmid pBR 322 DNA damage in vitro. DNA was broken into three forms — supercoiled (SC), open circular (OC) and linear form (Linear) — when exposed to hydroxyl radical derived from a Fenton reaction. The effect of APHPH on free-radical-induced DNA damage was investigated as shown in Fig. 7. The SC form in DNA was completely converted to the OC form under the treatment of hydroxyl radical generated from a Fenton reaction (Lane 2), and the SC form in the DNA treated with 2 mM Fe^{2+} was changed to the OC form (Lane 3), compared with plasmid DNA control (Lane 1). However, the SC form in DNA treated with H_2O_2 alone (Lane 4) was decreased by about 50% compared with the control lane. According to the result, DNA treated with APHPH at concentrations in the range of 2.78–55.5 μM protected hydroxyl-radical-induced dose-dependent DNA damage (Lanes 5–8), indicating an antioxidant effect.

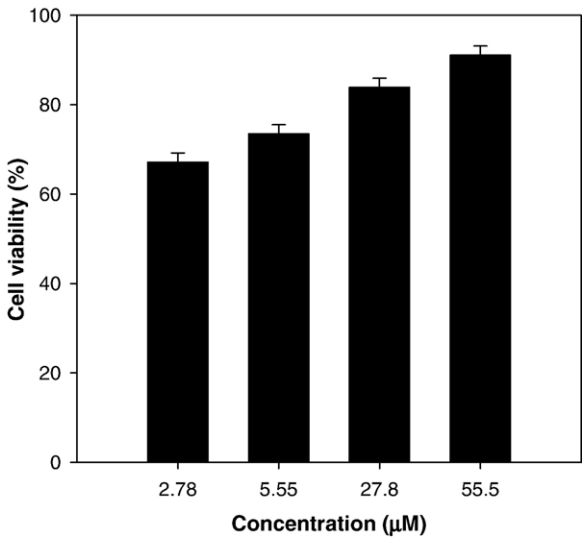

Fig. 6. Effect of APHPH on *t*-BHP-induced cytotoxicity to MRC-5 cells. MRC-5 cells were cultured in DMEM medium, and cellular oxidative stress was artificially induced. Viable cells were determined by MTT assay ($n=3$).

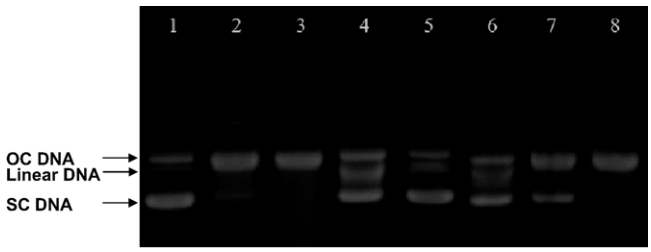

Fig. 7. Agarose gel electrophoretic patterns of plasmid DNA breaks by $^{\cdot}OH$ generated from a Fenton reaction in the presence of APHPH. An amount of 0.5 μg of pBR 322 DNA was incubated at 37°C for 1 h in 2 mM $FeSO_4$ and 30% H_2O_2 with the following additive combinations: Lane 1, no addition (plasmid DNA); Lane 2, $FeSO_4$ and H_2O_2 (DNA damage control); Lane 3, $FeSO_4$ treatment alone; Lane 4, H_2O_2 treatment alone; Lanes 5–8, $FeSO_4$ and H_2O_2 in the presence of APHPH with concentrations of 55.5, 27.8, 5.55 and 2.78 μM, respectively.

4. Discussion

Free radicals with major species of ROS are unstable and react readily with other groups or substances in the body, resulting in cell damage and, thus, human disease [21]. Therefore, removal of free radicals and ROS is probably one of the most effective defenses of a living body against various diseases. The beneficial effects of antioxidants are well known in scavenging free radicals and ROS or in preventing oxidative damage by interrupting the radical chain reaction of lipid peroxidation [22]. It is generally considered that the inhibition of lipid peroxidation by an antioxidant may be due to free radical scavenging activity. Lipids of biological membranes, especially those in the spinal cord and brain, containing highly oxidizable polyunsaturated fatty acids are particularly affected [23]. Moreover, the brain contains considerable amounts of prooxidant transition metal ions and utilizes much oxygen. These properties set the stage for the generation of free radicals and ROS and for subsequent acute cellular injury.

Several studies have already observed that some peptides derived from different protein sources possess antioxidant activities [24–28]. Many antioxidant peptides were extracted by enzymatic hydrolysis using various enzymes. One of the approaches for the effective release of bioactive peptides from protein sources is enzymatic hydrolysis, which is widely applied to improve and upgrade the

functional and nutritional properties of proteins. In the present study, we also utilized six proteases to extract antioxidant peptides, and we evaluated their antioxidant activities using both lipid peroxidation inhibition ability and free radical scavenging capacity, as described previously. Among six proteases, the gastrointestinal protease pepsin was more effective than other proteases in extracting bioactive peptides. Therefore, we separated peptic hydrolysate with different M_W distributions using UF membranes because many researchers reported that low M_W peptides are more potent as bioactive peptides [6,9]. Our results also revealed that low M_W peptides (1–3 kDa) showed the highest antioxidant activities (Fig. 1 and Table 1). Following different chromatographic methods, we purified the potent antioxidant peptide as the sequence of Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn (M_W= 1801 Da).

To evaluate the antioxidant activities of the purified peptide (APHPH), we employed two in vitro experiments. Firstly, linoleic acid was oxidized in a water/ethanol emulsion at 40±1°C in a dark room to evaluate lipid peroxidation inhibition ability. In this model system, peroxyl ($ROO^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals derived from the preexisting lipid peroxide were employed directly to initiate lipid peroxidation in the emulsified linoleic acid system [29]. Cheng et al. [29] reported that phenolic compounds afforded their protective actions in lipid peroxidation by scavenging lipid-derived radicals ($R^{\cdot}$, $RO^{\cdot}$ or $ROO^{\cdot}$) to stop chain reactions in a heterogeneous lipid phase. In the present study, APHPH effectively inhibited lipid peroxidation in the linoleic acid emulsion system after 7 days (Fig. 5), and the activity was higher than that of α-tocopherol. In the sequence of APHPH, 44% hydrophobic amino acids exert a high affinity to linoleic acid. Due to this property, APHPH could interact with lipid molecules and could scavenge by donating protons to lipid-derived radicals.

We also investigated the direct scavenging effects of APHPH against different free radical sources using the ESR spin-trapping technique. DPPH is a stable free radical and accepts an electron or a hydrogen radical to become a stable diamagnetic molecule. Therefore, DPPH is often used as a substrate to evaluate the antioxidant activity of an antioxidant. Hydroxyl radicals were generated in a Fenton reaction and were visualized by an ESR spectrometer. The ESR signal is inhibited by the presence of $^{\cdot}OH$ scavengers, which compete with DMPO for $^{\cdot}OH$. Superoxide radicals were generated by UV irradiation of a riboflavin/EDTA solution. AAPH can decompose to form carbon-centered radicals that can react swiftly with O_2 to yield peroxyl radicals to stimulate lipid peroxidation [30]. Our results showed that APHPH efficiently quenched toward four free radical sources (Table 2). Especially, APHPH was more potent for scavenging hydroxyl and peroxyl radicals than for DPPH and superoxide radicals. The chemical activity of hydroxyl radical is the strongest among ROS. It easily reacts with biomolecules, such as amino acids, proteins and DNA [31]. Therefore, the removal of hydroxyl radical is probably one of the most effective defenses of a living body against various diseases. Carbon-centered radicals that represent $R^{\cdot}$, $RO^{\cdot}$ and $ROO^{\cdot}$ could be quenched by APHPH at a low concentration. This result agreed with a lipid peroxidation inhibition assay finding that APHPH inhibited lipid peroxidation by scavenging lipid-derived radicals.

In the cellular system, we employed the organic hydroperoxide *t*-BHP to generate lipid-derived radicals by exposing human embryonic lung fibroblasts. Organic hydroperoxide is a model compound that is useful to the study of the mechanisms of oxidative cell injury. It is well known that *t*-BHP rapidly penetrates into mammalian cells. The proposed mechanisms of *t*-BHP-induced toxicity include the finding that cytochrome *P*450 catalyzes the hemolytic scission of *t*-BHP to the initially produced reactive alkoxyl radical and, as a result of catalysis, initiates peroxidation of membrane lipids [32]. The ability of APHPH to overcome *t*-BHP-induced cytotoxicity involved dose-dependent increments (Fig. 6). Furthermore, in the direct scavenging effect of APHPH toward peroxyl radicals, APHPH displayed a low IC_{50} value (18.99 μM). These results clearly demonstrated that APHPH prevented *t*-BHP-induced cytotoxicity by scavenging lipid-derived radicals.

DNA is another sensitive biotarget for ROS-mediated oxidative damage [33]. DNA damage by ROS can initiate carcinogenesis or affect the pathogenesis of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease; among ROS, the hydroxyl radical is recognized as a DNA-damaging agent of physiological significance [34]. In the present investigation, we investigated the protective effect of APHPH on hydroxyl-radical-induced DNA damage by agarose gel electrophoresis method. APHPH inhibited the dose-dependent hydroxyl-radical-induced DNA damage. Moreover, APHPH efficiently scavenged hydroxyl radical with IC_{50}=17.77 μM. This result further strengthened the ability of APHPH to protect hydroxyl-radical-induced DNA damage.

5. Conclusion

Herein we purified APHPH by enzymatic hydrolysis using gastrointestinal protease. The purified peptide efficiently inhibited lipid peroxidation and was a potent free radical scavenger. Furthermore, the peptide decreased radical-mediated cytotoxicity on MRC-5 cells and protected free-radical-induced DNA damage. However, further detailed studies on APHPH in regard to antioxidant activities in vivo are needed.

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References

[1] Je JY, Park PJ, Kim SK. Free radical scavenging properties of hetero-chitooligosaccharides using an ESR spectroscopy. Food Chem Toxicol 2004;42:381–7.

[2] Park PJ, Jung WK, Nam KS, Shahidi F, Kim SK. Purification and characterization of antioxidative peptides from lecithin-free egg yolk protein. J Am Oil Chem Soc 2001;78:651–6.

[3] Pryor WA. Free radical biology: xenobiotics, cancer, and aging. Ann N Y Acad Sci 1982;393:1–22.

[4] Butterfield DA, Castenga A, Pocernich CB, Drake J, Scapagnini G, Calabrese V. Nutritional approaches to combat oxidative stress in Alzheimer's disease. J Nutr Biochem 2002;13:444–61.

[5] Pihlanto-Leppälä A. Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory. Trends Food Sci Technol 2000;11: 347–56.

[6] Chen J, Suetsuna K, Yamauchi F. Isolation and characterization of immunostimulative peptides from soybean. J Nutr Biochem 1995; 6:310–3.

[7] Tsuruki T, Kishi K, Takahashi M, Tanaka M, Matsukawa T, Yoshikawa M. Soymetide, an immunostimulating peptide derived from soybean β-conglycinin, is an fMLP agonist. FEBS Lett 2003; 540:206–10.

[8] Mendis E, Rajapakse N, Kim SK. Antioxidant properties of a radicals scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. J Agric Food Chem 2005;53:581–7.

[9] Suetsuna K, Maekawa K, Chen J. Antihypertensive effects of *Undaria pinnatifida* (wakame) peptide on blood pressure in spontaneously hypertensive rats. J Nutr Biochem 2004;15:267–72.

[10] Suetsuna K. Isolation and characterization of angiotensin I-converting enzyme inhibitor dipeptides derived from *Allium sativum* L. (garlic). J Nutr Biochem 1998;9:415–9.

[11] Jeon YJ, Kim SK. Improvement of functional properties of cod frame protein hydrolysates using ultrafiltration membranes. Proc Biochem 1999;35:471–8.

[12] Kim SK, Jeon YJ, Byun HG, Kim YT, Lee CK. Enzymatic recovery of cod frame proteins with crude proteinase from tuna pyloric caeca. Fish Sci 1997;63:421–7.

[13] Je JY, Kim SY, Kim SK. Preparation and antioxidative activity of hoki frame protein hydrolysates using ultrafiltration membrane. Eur Food Res Technol 2005;221:157–62.

[14] Osawa T, Namiki M. Natural antioxidants isolated from eucalyptus leaf waxes. J Agric Food Chem 1985;33:777–80.

[15] Mitsuda H, Yasumoto K, Iwami K. Antioxidative action of indole compounds during the autoxidation of linoleic acid. Eiyo To Shokuryo 1996;19:210–4.

[16] Nanjo F, Goto K, Seto R, Suzuki M, Sakai M, Hara Y. Scavenging effects of tea catechins and their derivatives on 1,1,-diphenyl-2-picrylydrazyl radical. Free Radic Biol Med 1996;21:895–902.

[17] Rosen GM, Rauckman EJ. Spin trapping of superoxide and hydroxyl radicals. Methods Enzymol 1984;105:198–209.

[18] Hiramoto K, Johkoh H, Sako KI, Kikugawa K. DNA breaking activity of the carbon-centered radical generated from 2,2-azobis-(2-amidino-propane)-hydrochloride (AAPH). Free Radic Res Commun 1993; 19:323–32.

[19] Zhao BL, Li XJ, He R, Cheng SJ, Xin WJ. Scavenging effect of extracts of green tea and natural antioxidants on active oxygen radicals. Cell Biophys 1989;14:175–85.

[20] Yeung SY, Lan WH, Huang CS, Lin CP, Chan CP, Chang MC. Scavenging property of three cresol isomers against H_2O_2, hypochlorite, superoxide and hydroxyl radicals. Food Chem Toxicol 2002;40: 1403–13.

[21] Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 2nd ed. Oxford: Oxford University Press; 1989.

[22] Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 3rd ed. Oxford: Oxford University Press; 1999.

[23] Braughler JM, Hall ED. Central nervous system trauma and stroke: I. Biochemical considerations for oxygen radical formation and lipid peroxidation. Free Radic Biol Med 1989;6:289–301.

[24] Kim SK, Lee HC, Byun HG, Jeon YJ. Isolation and characterization of antioxidative peptides from enzymatic hydrolysates of Yellowfin sole skin gelatin. J Korean Fish Soc 1996;29:246–55.

[25] Suetsuna K, Ukeda H, Ochi H. Isolation and characterization of free radical scavenging activities peptides derived from casein. J Nutr Biochem 2000;11:128–31.

[26] Kim SK, Kim YT, Byun HG, Nam KS, Joo DS, Shahidi F. Isolation and characterization of antioxidative peptides from gelatin hydrolysate of Alaska pollack skin. J Agric Food Chem 2001;49:1984–9.

[27] Jun SY, Park PJ, Jung WK, Kim SK. Purification and characterization of an antioxidant from enzymatic hydrolysates of yellowfin sole (*Limanda aspera*) frame protein. Eur Food Res Technol 2004;219:20–6.

[28] Je JY, Park PJ, Kim SK. Antioxidant activity of a peptide isolated from Alaska Pollack (*Theragra chalcogramma*) frame protein hydrolysate. Food Res Int 2004;38:45–50.

[29] Cheng Z, Ren J, Li Y, Chang W, Chen Z. Establishment of a quantitative structure–activity relationship model for evaluating and predicting the protecitive potentials of phenolic antioxidants on lipid peroxidation. J Pharm Sci 2003;92:475–84.

[30] Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 1990;186:1–85.

[31] Cacciuttolo MA, Trinh L, Lumpkin JA, Rao G. Hyperoxia induces DNA damage in mammalian cells. Free Radic Biol Med 1993;14: 267–76.

[32] Lapshina EA, Zavodnik IB, Labieniec M, Rekawiecka K, Bryszewska M. Cytotoxic and genotoxic effects of *tert*-butyl hydroperoxide on Chinese hamster B14 cells. Mutat Res 2005;583:189–97.

[33] Martinez GR, Loureiro AP, Marques SA, Miyamoto S, Yamaguchi LF, Onuki J, et al. Oxidative and alkylating damage in DNA. Mutat Res 2003;544:115–27.

[34] You HJ, Oh DH, Choi CY, Lee DG, Hahm KS, Moon AR, et al. Protective effect of metallothionein-III on DNA damage in response to reactive oxygen species. Biochim Biophys Acta 2002;1573:33–8.