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Antioxidant Properties of a Radical-Scavenging Peptide Purified from Enzymatically Prepared Fish Skin Gelatin Hydrolysate

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Hoki (*Johnius belengerii*) skin gelatin was hydrolyzed with three commercial enzymes to identify radical-scavenging potencies of derived peptides. Peptides derived from tryptic hydrolysate exhibited the highest scavenging activities on superoxide, carbon-centered 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals assessed by ESR spectroscopy. Following consecutive chromatographic separations of tryptic hydroolysate, the peptide sequence His-Gly-Pro-Leu-Gly-Pro-Leu (797 Da) acted as a strong radical scavenger under studied conditions. Further, this peptide could act as an antioxidant against linoleic acid peroxidation and the activity was closer to the highly active synthetic antioxidant butylated hydroxytoluene (BHT). In addition, antioxidative enzyme levels in cultured human hepatoma cells were increased in the presence of this peptide and it was presumed to be the peptide involved in maintaining the redox balance in the cell environment. Present data indicate that free-radical-scavenging activities of hoki skin gelatin peptides substantially contribute to their antioxidant properties measured in different oxidative systems.

KEYWORDS: Free radicals; radical-scavenging peptide; fish gelatin; lipid peroxidation; hepatoma cells; antioxidant enzymes

INTRODUCTION

Oxidation of biomolecules has been identified as a free-radical-mediated process, which causes many unfavorable impacts on food and biological systems. In aerobic organisms, harmful radicals that inevitably form during the metabolism of oxygen are associated with the occurrence of several disease conditions including atherosclerosis, inflammation, and cancer (*1*). Cellular antioxidative enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and some food-derived nutritional antioxidants protect tissues from free-radical-mediated oxidative injuries (*2*). In foods, development of rancid flavor and undesirable chemical compounds are the results of free-radical-mediated oxidation of fatty acids and lipids. Further, oxidation of food lipids leads the deterioration of quality and shortens the shelf life of foods. Even though the use of some synthetic antioxidants to overcome these problems in food industry is common, it is under strict regulations because of potential health hazards. Therefore, there is a growing interest to identify antioxidative properties in many natural sources including some dietary protein compounds. Chen et al. has reported that enzymatically derived soybean peptides can act as potent antioxidants against lipid peroxidation and the primary structure of those peptides were critical for their activity (*3*, *4*). Recently, other potent antioxidative and radical-scavenging peptide sequences have been identified from porcine mussel and milk casein hydrolysates, and some amino acids are important for antioxidation (*5*, *6*).

Gelatin, a heterogeneous mixture of high molecular weight water-soluble proteins derived from collagen, contains relatively high amounts of some amino acids such as Gly, Pro, and Ala and is extensively used as a food additive to increase the texture, water-holding capacity, and stability of several food products (*7*). Even though byproducts from fish processing are a potential source for gelatin, less studies have been performed to identify the potential uses of fish skin gelatin than that derived from mammalian origin. Our previous research revealed that fish skin gelatin can be modified into biologically active peptides by protease treatments and such purified peptides exhibited a potential to act as inhibitors of angiotensin I converting enzyme (*8*) and as antioxidants against peroxidation of linoleic acid (*9*). Further, our studies demonstrated that gelatin peptides could inhibit lipid peroxidation at higher efficiencies compared to the antioxidative peptides derived from many other protein sources (*10*).

In the present study, we investigated the radical-scavenging activity of enzymatically prepared hoki fish (*Johnius belengerii*) skin gelatin hydrolysate and isolated a potent radical-scavenging peptide. Furthermore, the antioxidative activity of the peptide against lipid peroxidation was tested in a linoleic acid model system. In addition, we assessed the impact of purified peptide on cellular antioxidative enzyme levels in cultured human hepatoma cells. We hope that understanding of radical-scavenging potency and other antioxidative properties of gelatin peptides may lead to utilize fish gelatin as a potent natural antioxidant.

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MATERIALS AND METHODS

Materials and Reagents. Hoki (*Johnius belengerii*) skin discarded after filleting process was obtained from Daerim Co. (Busan, South Korea) and stored under −80 °C. Antioxidative enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and other digestive proteases used were purchased from Sigma Chemical Co. (St. Louis, MO). Chemicals required for the electron-spin-trapping techniques including 1,1-diphenyl-2-picrylhydrazyl (DPPH), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) were also obtained from Sigma Chemical Co. (St. Louis, MO). Human hepatoma cell line, Hep3B (ATCC HB-8064), was purchased from American Type Culture Collection (Manassas, VA). Cell culture media and all the other materials required for culturing were purchased from Gibco BRL, Life Technologies (USA). Other chemicals and reagents used were of analytical grade and commercially available.

Extraction and Hydrolysis of Gelatin from Fish Skin. Gelatin in the hoki skin was extracted with hot water following the method of Kim et al. (*11*). The gelatin extract was tested for its protein content by the method of Lowry et al. (*12*) and hydrolyzed for 4 h separately with three different enzymes, trypsin, α-chymotrypsin, and pepsin, giving optimum conditions for the digestion. Hydrolysates were lyophilized and stored at −80 °C until use.

Measurement of Superoxide Radical-Scavenging Activity. Superoxide radicals were generated by UV irradiated riboflavin/EDTA system (*13*). The reaction mixture containing 0.3 mM riboflavin, 5.0 mM EDTA, 0.1 M DMPO, and an indicated concentration of peptide fraction was irradiated for 1 min under UV lamp at 365 nm. The mixture was transferred into the cavity of JES−FA ESR spectrometer (JEOL, Japan) in a Teflon capillary tube and the spin adduct was recorded. Radical-scavenging activity of peptides was calculated as scavenging percentage by $S = (h_o - h_1)/h_o \times 100\%$, where h_1 and h_o were ESR signal intensities in the presence and the absence of peptides, respectively. Spectrometer settings were central field, 3475 G; modulation frequency, 100 kHz; microwave power, 4 mW; modulation amplitude, 2 G; magnetic field, 336.50 mT (±10 mT); and gain, 6.3×10^5.

Measurement of Carbon-Centered Radical-Scavenging Activity. According to the method of Hiramoto et al. (*14*), a reaction mixture containing 20 μL of 0.1 M phosphate buffer saline (pH 7.4), 40 mM AAPH, 40 mM 4-POBN, and an indicated concentration of peptide fraction was vortexed and incubated at 37 °C in a water bath. After 30 min, it was transferred into a Teflon capillary tube and fitted in the cavity of ESR spectrometer. Spin adduct was recorded with the same spectrometer settings as described above.

Measurement of DPPH Radical-Scavenging Activity. As described by Nanjo et al. (*15*), 60 μL of DPPH dissolved in ethanol was mixed with the same volume of peptide solution in ethanol and vortexed for 10 s. The reaction mixture was transferred to the cavity of ESR spectrometer in a Teflon capillary tube and the spin adduct was recorded after 60 s. Except for the microwave power (5 mW), spectrometer settings were the same as described above.

Purification of Radical-Scavenging Peptide. The lyophilized tryptic gelatin hydrolysate was dissolved in 50 mM sodium acetate buffer (pH 4) and separated using an SP−Sephadex C-25 cationic exchange column (ø 35 × 350 mm) with the same buffer. Bound peptides were eluted using a linear gradient of 0−2 M NaCl, and 5-mL fractions under a single elution peak were pooled monitoring optical density (OD) at 215 nm. Adsorbed fractions eluted with NaCl were desalted using a microacylizer, model G3 (Asahi Kasei Inc., Kanagawa, Japan), and pooled fractions were assayed for radical-scavenging activities. The potent fraction was further purified by size exclusion chromatography on a Sephadex G-25 gel filtration column (ø 20 × 750 mm) equilibrated with distilled water. Resulted fractions were pooled after OD measurements and assayed for radical-scavenging activities. The highest active fraction was then injected into a Capcell pack C_{18} UG120 (ø 20 × 250 mm) semipreparative reverse phase HPLC column (Shiseido Fine Chemicals, Tokyo, Japan) and separated using a linear gradient of acetonitrile (0−40% v/v) containing 0.1% trifluoroacetic acid (TFA). The potent peak was finally purified to a single peptide on a Zorbax SB C_{18} (ø 4.6 × 250 mm) reversed phase HPLC analytical column (Agilent Technologies, USA) using a linear gradient of acetonitrile (0−20% v/v) in 0.1% TFA and named as gelatin-derived radical-scavenging peptide (GRSP).

Molecular Mass and Amino Acid Sequence of the Purified Peptide. The molecular mass of the purified peptide was determined by electrospray ionization mass spectrometer (LCQ, Thermo Finnigan, San Joes, Japan). Purified peptide was infused into the electrospray source following dissolving in methanol/water (1:1, v/v), and molecular mass was determined by $(M + H)^+$ ions of the mass spectrum.

Automated Edman sequencing was performed by standard procedures using an automated protein sequencer (Perkin-Elmer model 491, Branchburg, NJ) equipped with online HPLC to determine the amino acid sequence of the purified peptide.

Measurement of Lipid Peroxidation in Linoleic Acid Model System. Linoleic acid was oxidized in a linoleic acid model system to measure the antioxidative activity following the method of Osawa et al. (*16*) with some modifications. The sample was dissolved in 2.5 mL of 50 mM phosphate buffer (pH 7.0) and added into a 99.5% ethanol (2.5 mL) and linoleic acid (32.5 μL) mixture. The final volume was adjusted to 6.25 mL with distilled water. In a single experiment, the sample was replaced with α-tocopherol to compare the antioxidative activity. The mixed solution in lightly sealed screw cap conical tube was incubated at 40 °C in dark and air-circulating conditions. The degree of linoleic acid oxidation was measured at 24-h intervals by ferric thiocyanate method according to Mitsuda et al. (*17*). An aliquot (0.1 mL) of the reaction mixture was mixed with 75% ethanol (4.7 mL) followed by the addition of 30% ammonium thiocyanate (0.1 mL) and 2×10^{-2} M ferrous chloride solution (0.1 mL) in 3.5% HCl. After 3 min, the degree of color development, which represents the linoleic acid oxidation, was measured spectrophotometrically at 500 nm.

Culture of Human Hepatoma Cells and Preparation of Cell Extracts for Antioxidative Enzyme Activity Assays. Hep3B, human hepatoma cells, were cultured as a monolayer at 37 °C in a humidified incubator under 5% CO_2 and 95% air. The cells were routinely passaged by dissociation in 0.05% (w/v) trypsin-EDTA and maintained in minimal essential medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine. The cells in the subconfluent growth phase were treated with 150 μg/mL of purified peptide (GRSP) concentration and treated and controlled cells were harvested at different time intervals (6, 12, and 24 h). Then, the cells were suspended at the concentration of 10^7 cells/mL in 50 mM phosphate buffer, pH 7.0 (for CAT assay), 10 mM phosphate buffer (pH 7.0) with 1.0 mM dithiothreitol (for GPx assay), and 50 mM sodium carbonate buffer pH 10.0 (for SOD assay). Suspended cells were lysed on ice by sonication (550 Sonic Dismembrator, Fisher Scientific, Itasca, IL) and centrifuged at 20 000*g* for 30 min. Supernatant was saved for the analysis of antioxidative enzyme activities and the protein content.

Superoxide Dismutase Assay. The nitroblue tetrazolium (NBT) method of Beauchamp and Fridovich (*18*), which is based on the inhibition of NBT reduction by SOD (EC 1.15.1.1), was used for the determination of SOD activities. Briefly, 2.5 mL of 0.05 M sodium carbonate buffer (pH 10) was mixed with 0.1 mL of 3 mM EDTA, 3 mM xanthine, 1.5 mg/mL bovine serum albumin, 0.75 mM NBT, and the cell lysate containing SOD. Reaction was initiated by adding 0.1 mL of 56 mU/mL xanthine oxidase. After 30 min of incubation, the reaction was terminated by mixing 6 mM $CuCl_2$ and was centrifuged at 350*g* for 10 min. Absorbance of blue formazan was recorded at 560 nm and 25 °C. The relative absorbance was then converted into units of SOD activity per mg protein, where one unit of SOD activity is equivalent to the quantity of SOD that caused a 50% reduction in the background rate of NBT reduction.

Glutathione Peroxidase Assay. The method of Wendel (*19*), with some modifications, was employed to quantitate the activity of GPx (EC 1.11.1.9). Into 1 mg of NADPH, 9.2 mL of 1 mM sodium azide solution (in 50 mM sodium phosphate buffer with 0.4 mM EDTA), 0.1 mL of glutathione reductase enzyme solution (100 U/mL), and 0.05 mL of glutathione reduced (GSH) were added and mixed by inversion. Then, into 3 mL of the mixture 0.05 mL of cell lysate containing Gpx was added. It was vortexed and incubated for 5 min at room

temperature. After the incubation, 0.05 mL of H_2O_2 was immediately mixed by inversion and the spectrophotometric measurements were recorded kinetically at 340 nm after every 30 s over a period of 5 min. GPx activity was calculated from the change in optical density per minute in the maximum linear rate range using a molar extinction coefficient for NADPH of $6.22 \times 10^3/\mu$mol and assuming 2 mol of GSH formed for each mole of NADPH consumed. One unit activity was defined as 1 μmol NADPH oxidized per minute.

Catalase Assay. Catalase (EC 1.11.1.6) activity was determined according to the method of Beers et al. (*20*), by following the decomposition of H_2O_2 at 240 nm and 25 °C. Hydrogen peroxide solution (10 mM) in 50 mM potassium phosphate buffer (pH 7) with 0.1 mL of cell lysate containing catalase was mixed with inversion, and decrease of absorbance every 30 s over a period of 3 min was recorded kinetically using a spectrophotometer. Changes in the rate of absorbance were converted into units of catalase/mg protein using a conversion factor (3.45), which corresponds to the decomposition of 3.45 micromoles of hydrogen peroxide in a reaction mixture producing a decrease in the absorbance from 0.45 to 0.40 units.

Other Analytical Methods. The amino acid composition of the enzymatically derived crude hydrolysate was determined by an automatic amino acid analyzer (PICO-TAG; Waters Co., Milford, MA) following hydrolysis of the sample in 6 N HCl under a vacuum at 110 °C for 24 h. Protein concentrations of the cell homogenates were determined by the standard method of Lowry et al. (*12*), using bovine serum albumin as the standard.

Statistical Analysis. Each data point represents the mean of three samples ± SE. Data were subjected to analysis of variance (ANOVA) followed by Duncan's multiple range posthoc test, and the significance level of $P < 0.05$ was employed.

RESULTS AND DISCUSSION

Enzymatic Preparation of Gelatin Peptides for Radical-Scavenging Potency. The functional properties of bioactive peptides are highly influenced by the molecular structure, molecular mass, as well as conditions of processing. Enzymatic hydrolysis has become the most important tool for modifying the functionality of dietary proteins to identify different bioactivities (*21*). Therefore, gelatin extracted from hoki skin was separately hydrolyzed by trypsin, α-chymotrypsin, and pepsin; the most commonly reported enzymes for the production of bioactive peptides. The resultant hydrolysates were tested for their scavenging activities on three different radicals, superoxide, carbon-centered, and DPPH using ESR spin-trapping techniques. This method provides a sensitive, direct, and accurate means of monitoring radical species at room temperature. Superoxide and carbon-centered radicals were generated from the irradiated riboflavin/EDTA system and AAPH, respectively, as described in the assay methods. Under specified conditions, radical scavenging was estimated as a percentage by measuring the relative intensity of the observed radical spin adduct signal. We could observe that gelatin hydrolysates exert potential scavenging effects on three radicals in different capacities. **Figure 1** represents the scavenging activities of superoxide, carbon-centered, and DPPH radicals recorded in the presence of the above gelatin peptide hydrolysates. All three hydrolysates exhibited a significant ($P < 0.01$) potency to scavenge superoxide and carbon-centered radicals relatively at a low concentration of 250 μg/mL. However, DPPH radical-scavenging activity of hydrolysates at the same concentration was lower. As a whole, trypsin-derived peptides exhibited the highest radical-scavenging activity in the order of superoxide (48.6 ± 0.58%), carbon-centered (43.6 ± 0.73%), and DPPH radicals (30.2 ± 0.55%). In addition, all hydrolysates were tested for their antioxidative activity against the peroxidation of linoleic acid in a linoleic acid model system. Trypsin-derived hydrolysate exhibited a significantly ($P < 0.01$) higher peroxidation inhibition compared to that of natural antioxidant, α-tocopherol **(Figure 2)**. The other two hydrolysates had a lower inhibition of lipid peroxidation compared to tryptic hydrolysate. Therefore, tryptic hydrolysate of hoki skin gelatin was utilized as a potential source to isolate and characterize radical-scavenging peptides.

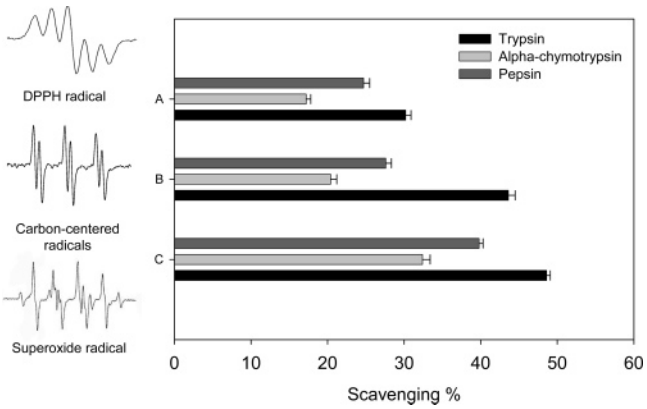

Figure 1. Scavenging effects of hoki skin gelatin hydrolysates prepared with trypsin, alpha-chymotrypsin, and pepsin on (**A**) DPPH, (**B**) carbon-centered, and (**C**) superoxide radicals. The typical ESR spectrum of each radical generated under the assay conditions described in the text is illustrated at the left, and horizontal bars represent the percentage scavenging of each radical by different enzyme hydrolysates.

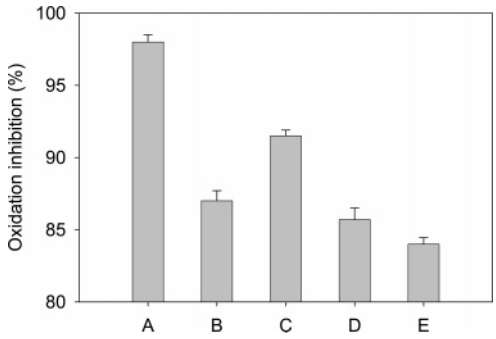

Figure 2. Antioxidant activities of different hoki skin gelatin hydrolysates. Hoki skin gelatin hydrolyzed with trypsin (**C**), α-chymotrypsin (**D**), and pepsin (**E**) were tested for antioxidant activity against linoleic acid oxidation after 8 days of incubation. Antioxidants butylated hydroxytoluene (**A**) and α-tocopherol (**B**) were used as positive controls.

The amino acid composition of the tryptic gelatin hydrolysate was analyzed, and it was rich in Gly (37.5%), Pro (9.3%), Glu (8.8%), Ala (8.6%), Arg (7.1%), and Hyp (5.98%) (data not shown). These data were comparable with the amino acid compositions of gelatin hydrolysates reported from other fish sources (*9*, *22*).

Purification of Gelatin-Derived Radical-Scavenging Peptide (GRSP). To purify radical-scavenging peptides from tryptic hydrolysate of hoki skin gelatin, different chromatographic techniques were utilized. We considered the scavenging potency of superoxide radical as the selection criteria of radical-scavenging peptides during purification. **Figure 3** illustrates the chromatographic profiles obtained during different purification steps of gelatin-derived radical-scavenging peptide (GRSP) and their scavenging effects on superoxide radical. Trypsin hydrolysate was initially separated into eight fractions (A−H) on SP−Sephadex C-25 cation exchange column as shown in **Figure 3A**. A−D represent the unbound fractions and E−H indicate the bound fractions, which were eluted with a 0−2 M NaCl gradient. All bound fractions resulted in a higher scavenging activity on the three radicals than that of unbound fractions. Among the bound fractions, fraction F had the highest scaveng-

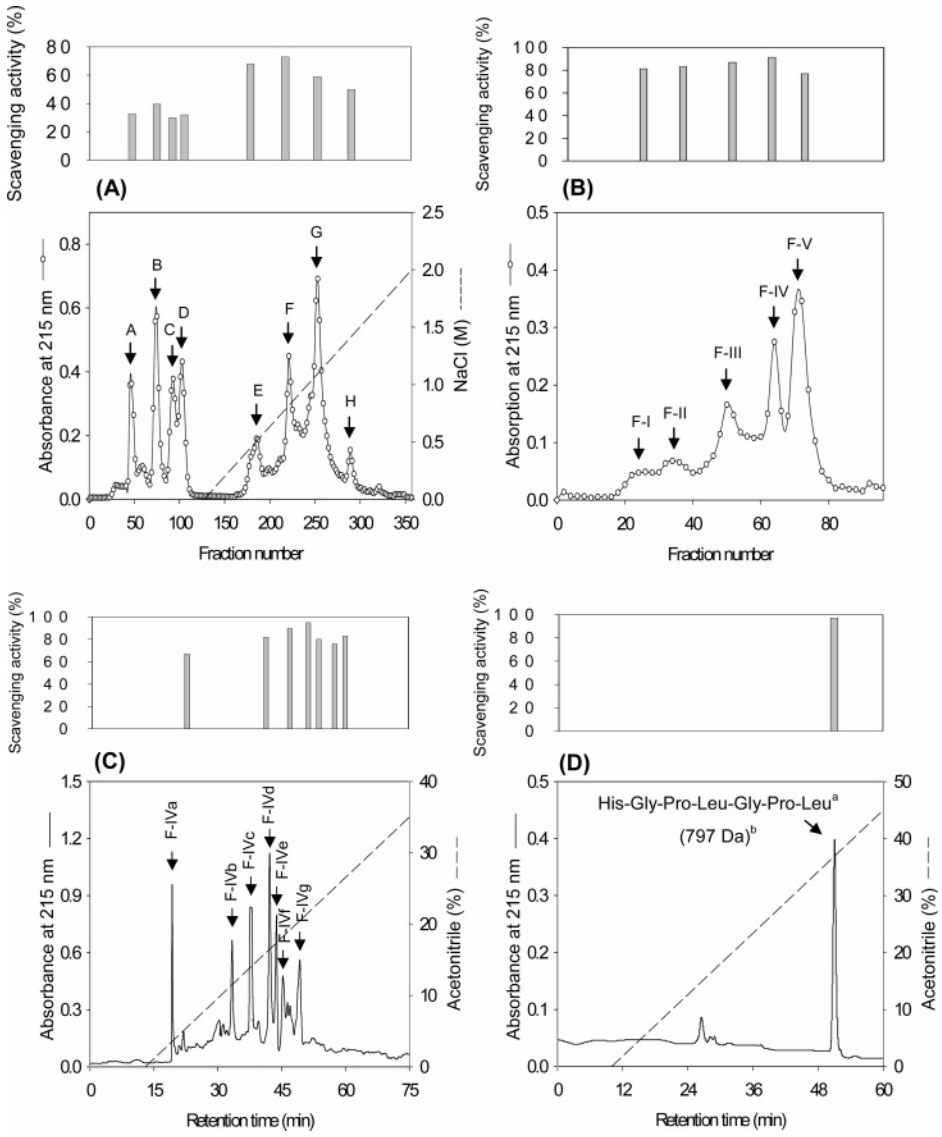

Figure 3. Purification scheme of gelatin-derived radical-scavenging peptide (GRSP) obtained from tryptic hydrolysate of hoki skin gelatin. (**A**) Ion-exchange chromatography on an SP–Sephadex C-25 column. Lyophilized hydrolysate was dissolved in sodium acetate buffer (pH 4) and loaded onto the ion-exchange column. Unbound (**A**–**D**) and bound (**E**–**H**) fractions were tested for the scavenging activity of superoxide radical and are illustrated as a percentage in the upper panel. (**B**). Gel filtration chromatography on a Sephadex G-50 column. Potent fraction (**F**) obtained from ion-exchange chromatography was separated into five fractions and tested for radical-scavenging activities. (**C**) The potent fraction (F-IV) obtained from gel filtration chromatography was purified on a Capcell pack C_{18} UG120 HPLC column with a linear gradient of 0–45% acetonitrile. (**D**) The highest active peak (F-IVd) was finally purified into GRSP by Zorbax C_{18} RP-HPLC analytical column. [a]Amino acid sequence of GRSP obtained from tandem mass spectroscopy. [b]Accurate molecular weight of GRSP.

ing activity on superoxide radical (72.68 ± 0.53%) with a 2.7-fold purification. Further separation of fraction F on Sephadex G-25 gel filtration column resulted in five distinct fractions (**Figure 3B**). Fraction F-IV was responsible for the highest superoxide radical-quenching activity (91.41% ± 0.73). Following further separation of F-IV on C_{18} HPLC column, eight different scavenging activities were obtained (**Figure 3C**). A clear scavenging difference was not observed among the activities of eight fractions and fraction F-IVd was more potent. Therefore, F-IVd was fully characterized following purification into a clear single peak with an 11.3-fold overall purification, by RP-HPLC C_{18} analytical column (**Figure 3D**). In addition, potent peptide fractions responsible for the highest superoxide radical-scavenging activity obtained from each chromatographic purification were further tested for their scavenging effects on carbon-centered and DPPH radicals (**Table 1**). The purification fold of the purified peptide (GRSP) at each chromatographic separation was calculated on the basis of the scavenging activities of the superoxide radical (**Table 2**).

Table 1. Radical-Scavenging Activities of Potent Fractions Obtained from Each Chromatographic Purification

potent peptide fraction	radical scavenging (%)		
	superoxide radical	carbon-centered radical	DPPH radical
F	72.68 ± 0.53	69.56 ± 0.54	43.01 ± 0.46
F-IV	91.41 ± 0.73	89.24 ± 0.64	72.07 ± 1.01
F-IVd	95.45 ± 0.92	93.26 ± 0.45	79.09 ± 0.31
GRSP	97.65 ± 0.43	95.12 ± 0.85	80.09 ± 0.31

Amino Acid Sequence and Radical-Scavenging Activities of GRSP. The molecular mass of GRSP (797 Da) determined by ESI mass spectroscopy agrees with its amino acid sequence (His-Gly-Pro-Leu-Gly-Pro-Leu) to be composed of seven amino

Table 2. Purification of GRSP from Tryptic Hydrolysate of Hoki Skin Gelatin

purification stage	total protein (mg)	specific activity[a] (μg/ mL)	purification fold
crude extract	7500.45	260	1.0
SP-Sephadex C-25	250.69	95	2.7
Sephadex G-50	17.58	50	4.8
Capcell pack C_{18} UG120	1.31	30	8.0
Zorbax SB C_{18}	0.99	23	11.3

[a] Concentration of peptide fraction required to inhibit 50% of superoxide radical ESR signal intensity.

acid residues. As a fact, gelatin possesses a specifically arranged amino acid sequence where glycine strictly represents every third amino acid residue. Frequently, proline and hydroxyproline follow each other and the Gly-Pro-Hyp sequences make up about 10% of the molecule (*23*). In our previous research, we could isolate gelatin-derived antioxidative peptides from marine fish skin (*9*) and bovine skin (*10*) with the above sequence repetition. However, in agreement with typical gelatin sequence, GRSP represents two repeating amino acid residues (Gly-Pro) placing leucine or histidine at the other position.

Propagation of the free-radical chain reaction in a system could be suppressed by adding substances capable of competing for the existing radicals. Therefore, the extent to which the radicals are suppressed by GRSP was tested using spin-trapping techniques and compared with a known natural antioxidant α-tocopherol. Superoxide anion was measured using the relative ESR signal intensity of the DMPO−OOH spin adduct and GRSP was responsible for a strong scavenging effect (97.65 ± 0.43%) (**Table 1**). This activity was 2-times the observed α-tocopherol activity in the same concentration. Spin adduct intensity of 4-POBN/carbon-centered radicals was suppressed by 95.12 ± 0.85% (1.8-times the α-tocopherol activity) with a final peptide concentration of 3.1×10^{-4} M, representing the same order of radical scavenging observed in the tryptic crude hydrolysate. However, a much larger and more stable DPPH radical was poorly scavenged (80.09 ± 0.31%) by GRSP at the same concentration and this was 40% of the observed α-tocopherol activity. The IC_{50} values of the peptide to scavenge superoxide, carbon-centered, and DPPH radical signals generated as described were 28.8 μM, 94.1 μM, and 156.8 μM, respectively. Generally, the quenching of free radicals by natural antioxidants has been reported through donation of hydrogen. Some amino acids such as His, Leu, Tyr, and Met enhance radical-scavenging activities of antioxidative peptides (*24*). Especially, histidine-containing peptides such as carnosine, anserine, and balenine (also known as ophidine) exhibit higher radical-scavenging activity and that is due to the proton-donation ability of imidazole group of histidine. Therefore, the N-terminus histidine residue of GRSP can be expected as a strong proton-donating residue in the sequence. Further, GRSP is rich in Leu, Gly, and Pro and repeats in the sequence. Up to date, very few peptides have been identified to exhibit radical-scavenging potencies and none of them are composed of higher percentages of Leu, Gly and Pro. Suetsuna et al. (*6*) have purified a radical-scavenging peptide from casein hydrolysate having Leu and Pro at the C-terminal end of the sequence. Furthermore, GRSP agrees with the fact that leucine is important for the antioxidant activity of a peptide when present at the C-terminus. However, the higher radical-scavenging activity of this peptide cannot be only due to the presence of His and Leu in the sequence.

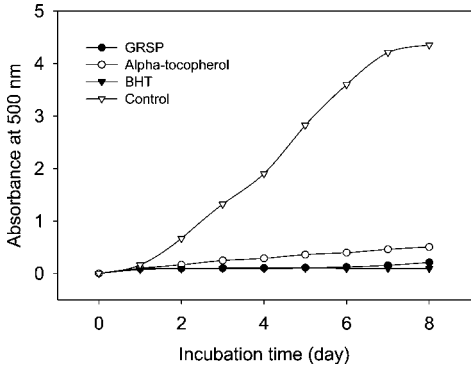

Figure 4. Antioxidant activity of GRSP. GRSP was incubated in a linoleic acid oxidation system for 8 days as described in the method. The degree of linoleic acid oxidation was assessed by measuring optical density at 500 nm at every 24-h interval. Butylated hydroxytoluene and alpha-tocopherol were used as positive controls.

Therefore, we can assume that Gly and Pro also play an important role to act GRSP as the potent radical scavenger.

Antioxidative Activity of GRSP Measured in Linoleic Acid Proxidation System. In addition to the radical-scavenging potency, antioxidative activity of GRSP was further tested in a linoleic acid peroxidation system. Interestingly, the addition of GRSP inhibited lipid peroxidation more significantly ($P < 0.05$) than in the presence of α-tocopherol and closer to inhibition activity of highly active synthetic antioxidant butylated hydroxy toluene (BHT) (**Figure 4**). This was presumed to be that GRSP reacts with radicals in the system including peroxyl radicals and thereby inhibits the propagation cycle of lipid peroxidation. Some peptides are reported to exhibit higher antioxidative activities against lipid peroxidation in the presence of specific amino acids such as Gly, Leu, Phe, and Pro (*5*). In the sequence of GRSP, more than 50% of the residues are composed of such hydrophobic amino acids, glycine, and leucine. Therefore, the higher antioxidative potency of this peptide can be expected because of the larger hydrophobicity, which may lead to a higher interaction between the peptide and fatty acids. In addition to the hydrophobicity, the specific positioning of amino acid residues in the peptide sequence plays an important role in the antioxidative activity. Chen et al. examined the residue−activity relationship of synthetic antioxidative peptide mimics by comparing their antioxidative activities in linoleic acid peroxidation system and observed loss of activity after deletion of terminal histidine residue (*3*). Therefore, we can speculate that the N-terminal histidine may contribute higher antioxidative activity to the GRSP sequence.

Effect of GRSP on the Antioxidative Enzyme Activities in Hep3B Cells. It is now becoming apparent that the redox balance inside cells is crucial for the correct functioning of many enzymes. Some important redox-sensitive molecules in this respect must be antioxidative enzymes, the first line of cellular defense against free radicals. Few evidences suggest that lowered antioxidative enzyme levels in cancer cells may be due to the imbalanced redox status generated by excessive free radicals (*25*). A growing body of evidence suggests that antioxidants help to maintain cellular defenses against free radicals by changing their redox state. In this research, GRSP confirmed its antioxidative activity as a radical scavenger and lipid peroxidation inhibitor in vitro. Therefore, we investigated further whether the presence of GRSP has any impact on cellular antioxidative enzyme levels. In this regard, Hep3B, human hepatoma cells, were treated with GRSP and the levels of three main antioxidative enzymes, superoxide dismutase (SOD),

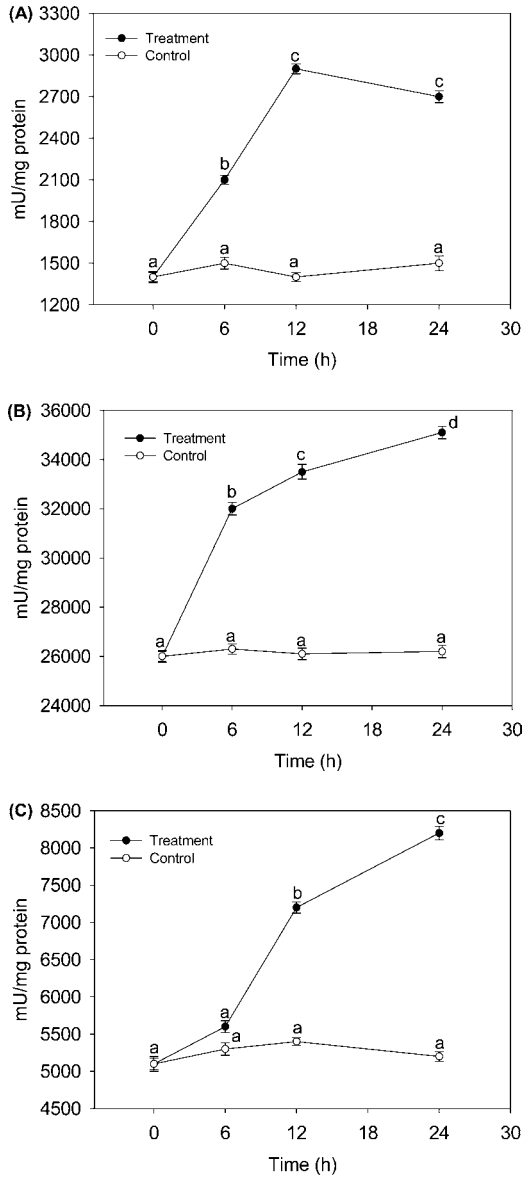

Figure 5. Effects of GRSP on antioxidative enzyme expression in Hep3B cells measured over time: (**A**) SOD; (**B**) GPx; (**C**) CAT. Values are means ± SE of triplicate experiments. Values with nonidentical superscripts within plots representing each enzyme expression over time are significantly different ($P < 0.05$) using ANOVA with Duncan's as a posthoc test.

catalase (CAT), and glutathione peroxidase (GPx), were measured. Time-course specific activity curves of SOD, CAT, and GPx in GRSP (1.8×10^{-4} M) treated Hep3B cells are depicted in **Figure 5**. An increment in expression of all three enzymes as compared to the respective control could be observed over the incubation time. SOD activity was 92.8% higher after incubation with GRSP for 24 h (**Figure 5A**). Relative increments observed after 24 h in activities of GPx and CAT were 60.78% and 35%, respectively (**Figure 5B** and **5C**). Even though the molecular mechanism underlying the induction of antioxidative enzymes by GRSP is not understood, similar research has observed an increment of antioxidative enzyme levels following the treatment of antioxidants. Dietary administration of genistein, an isoflavonoid, significantly elevated SOD, CAT, and GPx levels in murine skin and small intestine (*26*, *27*). Another recent research revealed that in the presence of isoflavonoid, antioxidants stimulate the production of nonenzymatic cellular antioxidants in human umbilical cord vein endothelial cells (*13*). However, none of these studies have elucidated a mechanism of antioxidants in promoting natural cellular antioxidant system. However, on the basis of the results of this research, we can presume that the GRSP may be involved in maintaining the redox balance because of its radical-scavenging properties observed.

Conclusions. The ability of antioxidants to act at different levels in an oxidative sequence is vital for their potential utilization against oxidation. From the standpoint of direct radical-scavenging activities of GRSP, the structural features in the primary sequence of gelatin favor antioxidative properties in different oxidative systems. Antioxidative activity of GRSP confirms our previous results that gelatin peptides have higher antioxidative activities under specified conditions. Therefore, results of this study can be aided in developing potential applications of fish gelatin peptides as antioxidants.

ABBREVIATIONS USED

ESR, electron spin resonance; SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; 4-POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; EDTA, ethylenediaminetetraaceticacid; NBT, nitroblue tetrazolium; GRSP, gelatin-derived radical-scavenging peptide.

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