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Peptide Isolated from Japanese Flounder Skin Gelatin Protects against Cellular Oxidative Damage

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ABSTRACT: Gelatin was extracted from the skin of Japanese flounder (*Palatichtys olivaceus*) and was subjected to enzymatic hydrolysis. The peptic hydrolysate resulted in a potent antioxidative peptide Gly-Gly-Phe-Asp-Met-Gly (582 Da), which bears +12.61 kcal/mol hydrophobicity. The antioxidative potential of the peptide was characterized by analyzing the protective effect of the peptide on reactive oxygen species (ROS)-mediated intracellular macromolecule damage. It was found that the peptide is a potent scavenger of intracellular ROS, thereby protecting the radical-mediated damage of membrane lipids, proteins, and DNA. Moreover, the peptide is capable of upregulating the expression of inherent antioxidative enzymes, superoxide dismutase-1, glutathione, and catalase. Collectively, it can be concluded that Japanese flounder skin, a processing byproduct of filleting, can be effectively used to produce a bioactive peptide with potent antioxidant capacity.

KEYWORDS: *Peptide, antioxidant, Palatichtys olivaceus, ROS, macromolecular oxidation, RAW264.7 cells*

INTRODUCTION

Reactive oxygen species (ROS) are resultant products of oxygen metabolism, which include a broad range of undesirable radicals, including superoxide radicals, hydrogen peroxide, hydroxyl radicals, peroxyl radicals, alkoxyl radicals, and singlet oxygen species.[1] These ROS are capable of oxidizing cellular macromolecules, such as membrane lipids, nucleic acids, and proteins, which results in detrimental oxidative damage to the cells.[2] Under normal physiological conditions, the cell environment is at a redox balance. The cells are protected by number of protective mechanisms, including intracellular antioxidant enzymes, such as superoxide dismutase (SOD), glutathione (GSH), and catalase.[3] However, at an incidence of unregulated production of oxidants, the redox balance is disturbed, leading to oxidative stress conditions. The consequences of unregulated ROS production have been implicated in many of the pathophysiological conditions occurring in the human body, such as cancer,[4] cardiovascular diseases,[5] neurodegenerative diseases,[6] arthritis,[7] and diabetes.[8] Therefore, supplementation with potent antioxidants and boosters of intracellular antioxidant mechanisms has gained much attention.

A large number of studies has been conducted to find potent radical scavengers to protect against oxidative stress. However, undesirable factors, such as toxicity, unsustainable production, and lower activity at the cellular level, have pushed the scientists to explore candidates that do not fall into these categories. In this regard, bioactive peptides isolated from marine byproduct have earned much attention because they provide a cheap protein source to produce value-added bioactive peptides. These bioactive peptides are capable of exerting physiological hormone-like effects on humans to protect against adverse pathological conditions.[9] Several potent antioxidant peptides have been isolated from marine fish processing by products, such as hoki frame protein,[10] Nile tilapia skin gelatin,[11] tuna backbone protein,[12] and Pacific cod skin gelatin.[13] A large quantity of fish skin is discarded during fish filleting. Fish skin is composed of high amounts of collagen, which could be readily converted into gelatin. This gelatin can be enzymatically hydrolyzed to obtain bioactive peptides. Generally, gelatin-derived peptides contain mainly hydrophobic amino acids, and an abundance of these amino acids favor higher antioxidant effects. Japanese flounder (*Palatichtys olivaceus*) is the most common flatfish species raised in Korea, and the skin is discarded as a processing waste. Therefore, this study was planned to isolate active peptides from the skin of Japanese flounder to use this processing waste effectively.

MATERIALS AND METHODS

Materials. Fresh samples of Japanese flounder (*P. olivaceus*) skin were obtained from the Jakalchi Fish Market, Busan, South Korea. Pepsin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were purchased from Sigma Chemical Co. (St. Louis, MO). The fluorescence probes, such as 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and diphenyl-1-pyrenylphosphine (DPPP), were obtained from Molecular Probes, Inc. (Eugene, OR). The mouse macrophage (RAW264.7) cell line was obtained from American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other cell culture materials were purchased from Gibco BRL, Life Technologies (Gaithersburg, MD). RNA was isolated using Trizol reagent (Invitrogen Co., Carlsbad, CA), and specific primers for the revese transcription polymerase chain reaction (RT-PCR) were obtained from Bioneer, Inc. (Alameda, CA). All other chemicals were of the highest grade available commercially.

Gelatin Extraction and Enzyme Hydrolysis. The Japanese flounder skin was washed to remove surface slime, cut into small pieces (1 × 2 cm), and soaked in 1% $Ca(OH)_2$ at a skin/solution ratio of 1:4 (w/v) for 4 days. The solution was changed 3 times in every 24 h to remove noncollagenous proteins. Alkaline-treated skin particles

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were then thoroughly washed with tap water and soaked in distilled water at a skin/water ratio of 1:4 (w/v) with pH 6 at 60 °C for another 30 h with a continuous stirring to extract gelatin from the skin matter. Next, the mixture was centrifuged at 3000 rpm for 10 min; the supernatant was immediately collected and freeze-dried. The dry matter was collected as gelatin powder. The collected gelatin was hydrolyzed with pepsin enzyme using the following procedure: Gelatin was initially dissolved in glycine-HCl buffer (0.1 M, pH 2) and hydrolyzed with pepsin [enzyme/substrate (E/S) ratio of 1:100] for 4 h while stirring. The reaction was stopped by heating the mixture in a boiling water bath for 10 min. Lyophilized hydrolysate was stored at −80 °C until used.

Fast-Protein Liquid Chromatography (FPLC). The obtained hydrolysate was further purified using FPLC (AKTA, Amersham Bioscience Co., Uppsala, Sweden) on a HiPrep 16/10 diethylaminoethyl fast flow (DEAE FF) ion-exchange column. The hydrolysate was loaded onto a HiPrep 16/10 DEAE FF ion-exchange column equilibrated with a 20 mM sodium acetate buffer (pH 4.0) and eluted with a linear gradient of NaCl (0−2 M) in the same buffer at a flow rate of 2 mL/min. Each fraction (4 mL) was monitored at 280 nm, and fractions corresponding to the peaks were collected and pooled accordingly. Then, the pooled fractions were lyophilized, and hydroxyl and DPPH radical scavenging activities were analyzed. The fraction with the highest radical scavenging activity was further purified by high-performance liquid chromatography (HPLC) to obtain a pure peptide.

HPLC. The fraction exhibiting the highest radical scavenging activity (hydroxyl and DPPH radicals) was further purified using reverse-phase HPLC (RP-HPLC, Dionex Korea, Ltd., Sunnyvale, CA) on a Primesphere 10 C_{18} (20 × 250 mm, Phenomenex, Cheshire, U.K.) column with a linear gradient of acetonitrile (0−30% in 50 min) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 mL/min. Elution peaks were detected at 215 nm, and collected elute was concentrated using a rotary evaporator. The collected fractions were analyzed for their radical scavenging potential to screen the active fraction to analyze the molecular weight and amino acid sequence.

Determination of the Amino Acid Sequence. An accurate molecular mass and amino acid sequence of the purified peptide was determined using a hybrid quadrupole time-of-flight (Q-TOF) LC/MS/MS mass spectrometer (AB Sciex Instruments, Foster City, CA) coupled with an electrospray ionization (ESI) source. The purified peptide was separately infused into the electrospray source after being dissolved in methanol/water (1:1, v/v), and its molecular mass was determined by the doubly charged $(M + 2H)^{2+}$ state in the mass spectrum. After molecular mass determination, the peptide was fragmented and the parameters for the analysis were set as ion spray voltage (IS) at 5.5 kV and curtain gas (CUR) at 30 units. Finally, the sequence of the peptide was determined using the Denovo sequencing program (AB Sciex Instruments, Foster City, CA).

Determination of Hydroxyl Radical Scavenging Activity. Hydroxyl radical scavenging activity was assessed as described previously.[14] Hydroxyl radicals were generated by the Fenton reaction[15] and trapped using a DMPO nitrone spin trap. The fractions (50 μL) were mixed with 50 μL of 0.3 M DMPO (in 0.1 M phosphate buffer at pH 7.4) and vortexed for 10 s. After 2 min, the mixture was transferred into a capillary tube, and the DMPO−OH adduct was detected using a JESFA ESR spectrometer (JEOL, Japan). Spectrometer settings were as follows: modulation frequency, 100 kHz; magnetic fields, 336.35 mT; modulation amplitude, 200; microwave power, 0.998 mW; and response, 0.3 s. Radical scavenging activity of fractions was calculated as the scavenging percentage (S) compared to the radical alone injected control group using the equation $S = \{(h_0 - h_1)/h_0\} \times 100$, where h_0 is the electron spin resonance (ESR) signal intensity of the control and h_1 is the ESR signal intensity in the presence of the fractions.

Determination of the DPPH Radical Scavenging Activity. DPPH radical scavenging activity was analyzed according to the method described previously[16] using a JESFA ESR spectrometer (JEOL, Japan). A 30 μL sample solution was added to 30 μL of DPPH (60 μM in methanol) solution and mixed vigorously for 10 s. The solution was then transferred into a 50 μL quartz capillary tube, and within 2 min, the ESR spectrum was recorded at 5 mW microwave power and 1000 amplitude. Radical scavenging activity of fractions was calculated as the scavenging percentage (S) compared to the radical alone injected control group using the equation $S = \{(h_0 - h_1)/h_0\} \times 100$, where h_0 is the ESR signal intensity of the control and h_1 is the ESR signal intensity in the presence of the fractions. Because of the shown potencies in *in vitro* radical scavenging, the isolated peptide was further analyzed in cellular systems as an antioxidative agent against oxidative-stress-mediated cell damage.

Cell Culture and Cell Viability Determination. Mouse monocyte cells (RAW264.7) were cultured in DMEM, containing 100 μg/mL penicillin−streptomycin and 10% fetal bovine serum (FBS), and maintained at 37 °C under a humidified atmosphere with 5% CO_2. The cytotoxicity level of the peptide on RAW264.7 cells was measured using the 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as documented previously.[17] Cells were cultured in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were washed with fresh medium and treated with different concentrations of peptide, following 24 h of incubation. Then, cells were washed with phosphate-buffered saline (PBS), and 100 μL of MTT solution (1 mg/mL) was added to each well and kept for 4 h. MTT solution in each well was removed, and 100 μL of dimethyl sulfoxide (DMSO) was added to solubilize the formed formazan crystals. The optical density was measured at 540 nm using an ultraviolet (UV) microplate reader (Tecan Austria GmbH, Grödig, Austria). Relative cell viability was calculated in comparison to the nontreated blank group. The data were expressed as the mean of at least three independent experiments. Furthermore, the effect of H_2O_2 treatment on the viability of RAW264.7 cells was also analyzed by the MTT assay, where the reading was taken at different time intervals.

Membrane Lipid Peroxidation. Intracellular lipid hydroxyperoxide levels were determined by the fluorescence probe DPPP as described previously.[18] Cells grown in culture dishes were washed 3 times with PBS and labeled with 13 μM DPPP (dissolved in DMSO) for 30 min at 37 °C in the dark. Cells were washed 3 times with PBS and seeded into fluorescence microtiter 96-well plates at a density of 1×10^8 cells/mL in serum-free media. After complete attachment, cells were treated with various concentrations of the test sample and incubated for 1 h. After incubation, 3 mM 2,2′-azobis(2-methylpropanimidamide) dihydrochloride (AAPH) in PBS was added and DPPP-oxide fluorescence intensity was measured after 8 h at the excitation wavelength of 361 nm and the emission wavelength of 380 nm using a GENios fluorescence microplate reader (Tecan Austria GmbH, Grödig, Austria).

Membrane Protein Oxidation. The amount of carbonyl groups produced was used to assess the cellular protein oxidation level as described previusly.[19] Cells were grown in culture dishes and washed 3 times with PBS before they were lysed with lysis buffer having no reducing agents [25 mM Tris-HCl at pH 7.8, 2 mM ethylenediaminetetraacetic acid (EDTA), 180 mM NaCl, and 1% Triton X-100]. Aliquots of cell lysates were separately incubated with different concentrations of test sample for 30 min at 37 °C. Protein oxidation was initiated by adding 0.1 M $FeSO_4$ and 2 mM H_2O_2 to the mixture, and incubation was continued for another 1 h. Trichloroacetic acid (400 μL from 20% solution) was added to each reaction mixture, and solubilized proteins were precipitated by centrifugation at 3000 rpm. The precipitated protein was resuspended in 0.2% 2,4-dinitrophenylhydrazine (in 2 mM HCl) and incubated at room temperature for 40 min. The protein was precipitated again with 20% trichloroacetic acid, and the pellet was washed 3 times with ethanol/ethyl acetate (1:1, v/v) solution. The pellet was then dissolved in 6 M guanidin hydrochloride (500 μL) and incubated for 15 min at 37 °C. After centrifugation at 1500 rpm for 5 min, absorbance of the supernatant was recorded against a complementary blank at 370 nm using an ultraviolet−visible (UV−vis) microplate reader (Tecan Austria GmbH, Grödig, Austria). A blank was prepared with a parallel procedure using 2 mM HCl alone, instead of the 2,4-dinitrophenylhydrazine reagent.

Genomic DNA Isolation and Determination of Radical-Mediated DNA Damage. Genomic DNA was isolated from

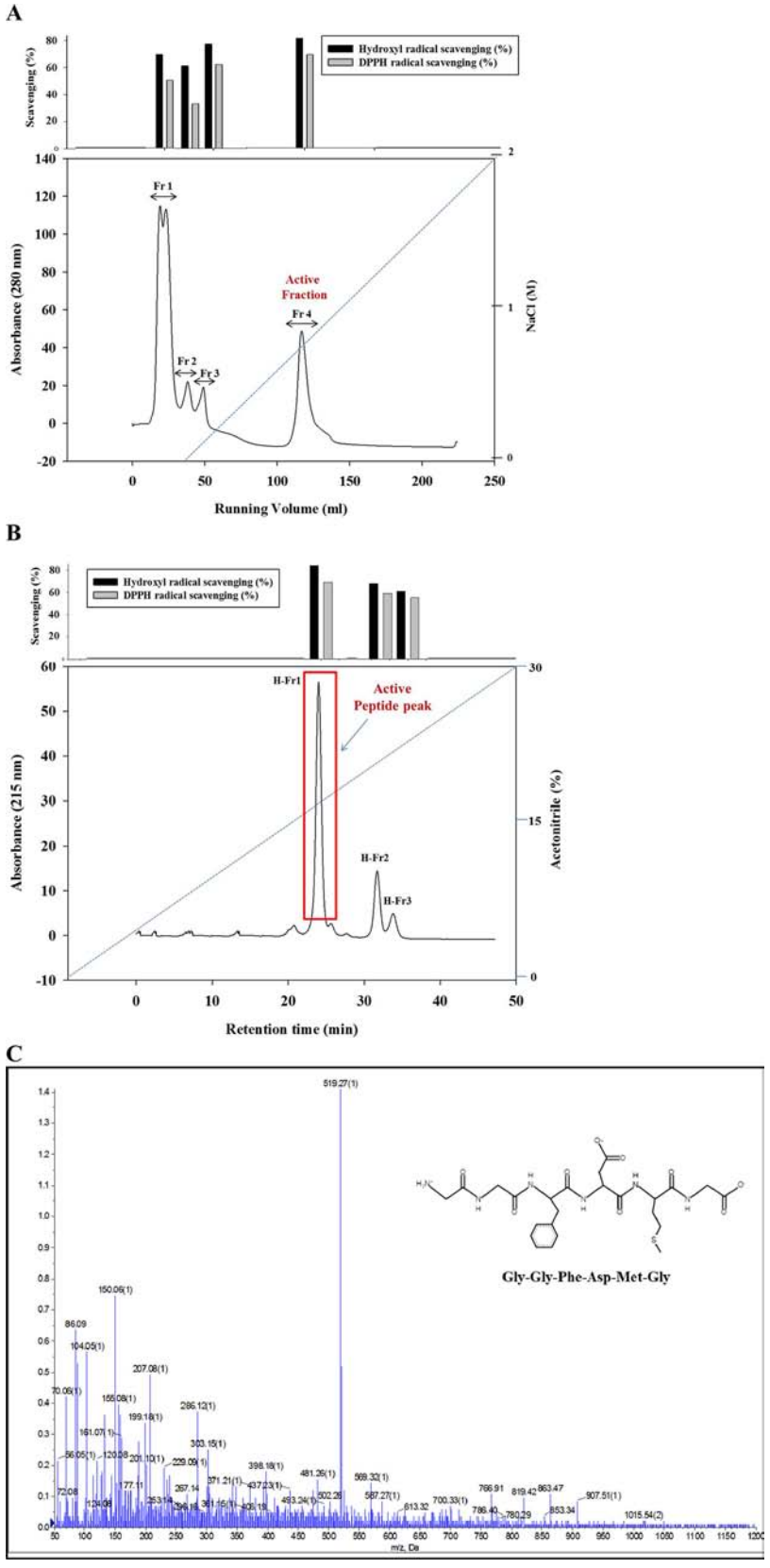

Figure 1. Purification steps and identification of Japanese flounder skin gelatin-derived peptide. (A) Anion-exchange FPLC chromatogram of the peptic hydrolysate of Japanese flounder skin gelatin (lower panel). Hydroxyl and DPPH radical scavenging effect of the purified FPLC fractions, analyzed by the ESR technique (upper panel). (B) RP-HPLC chromatogram of the active fraction 4 (lower panel). Hydroxyl and DPPH radical scavenging abilities of each RP-HPLC fractions (upper panel). (C) Q-TOF ESI mass spectrum and the structure of the purified peptide.

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RAW264.7 cells using a standard phenol/proteinase K procedure with slight modifications.[20] Briefly, cells were washed twice with PBS and aliquot into 1 mL of PBS containing 10 mM EDTA. After centrifugation, the cells were dissolved in RNase (0.03 mg/mL), NaOAC (0.175 M), proteinase K (0.25 mg/mL), and sodium dodecyl sulfate (SDS) (0.6%). The mixture was then incubated for 30 min at 37 °C and 1 h at 55 °C. Then, phenol/chloroform/isoamyl alcohol (25:24:1) was added at a 1:1 ratio, and the mixture was centrifuged at 6000 rpm for 5 min at 4 °C. After centrifugation, the supernatant was mixed with 100% ice-cold ethanol at 1:1.5 ratios and kept for 15 min at −20 °C. After centrifugation at 12 000 rpm for 5 min at 4 °C, the pellet was dissolved in TE buffer and the purity of DNA was spectrophotometrically determined at 260/280 nm.

H_2O_2-mediated DNA oxidation was determined using a previously documented method.[21] Briefly, 100 μL of DNA reaction mixture was prepared by adding predetermined concentrations of the test sample (or same volume of distilled water as a control), 200 μM final concentration of $FeSO_4$, 2 mM final concentration of H_2O_2, and 50 μg/mL final concentration of genomic DNA in the same order. Then, the mixture was incubated at room temperature for 10 min, and the reaction was terminated by adding 10 mM final concentration of EDTA. An aliquot (20 μL) of the reaction mixture containing about 1 μg of DNA was electrophoresed on a 1% agarose gel for 30 min at 100 V. Gels were then stained with 1 mg/mL ethidium bromide and visualized by UV light using AlphaEase gel image analysis software (Alpha Innotech, San Leandro, CA).

Determination of Intracellular ROS Formation. Intracellular formation of ROS was assessed as described previously using DCFH-DA as the fluorescing substrate.[22] RAW264.7 cells growing in fluorescence 96-well plates were loaded with 20 μM DCFH-DA in Hank's balanced salt solution (HBSS) and incubated for 30 min in the dark. Nonfluorescent DCFH-DA dye is freely penetrated into cells, becomes hydrolyzed by intracellular esterases to 2′,7′-dichlorodihydrofluororescein (DCFH), and is trapped inside the cells. After DCFH-DA staining, cells were washed with PBS 3 times to remove the nonpenetrated fluorescent probe and then treated with test concentrations of peptide for another 1 h. The cells were washed again, and 500 μM H_2O_2 dissolved in HBSS was added to the cells to stimulate ROS generation. The formation of 2′,7′-dichlorofluorescein (DCF) as a result of the oxidation of DCFH by cellular ROS in the presence or absence of the test concentrations of peptide was read every 20 min for 2 h at the excitation wavelength of 485 nm and the emission wavelength of 528 nm using a fluorescence microplate reader (Tecan Austria GmbH, Grödig, Austria). Dose- and time-dependent effects of the peptide-treated groups were plotted and compared to the fluorescence intensity of the control and blank groups, which are only treated with and without H_2O_2, respectively.

RNA Isolation and RT-PCR Analysis. For the isolation of RNA, cells were lysed with Trizol (Invitrogen, CA) and centrifuged at 12 000 rpm for 15 min at 25 °C, following the addition of chloroform. The supernatant was separated, and isopropanol was added to it at a 1:1 ratio. From centrifugation for 10 min at 10 000 rpm, the RNA pellet was obtained. After washing with ethanol, extracted RNA was solubilized in diethyl pyrocarbonate-treated RNase-free water and quantified by measuring the absorbance at 260 nm using the GENios microplate reader (Tecan Austria GmbH, Grödig, Austria). Total RNA (2 μg) was converted to single-stranded cDNA using a reverse transcription system (Promega, Madison, WI). Then, the changes in the steady-state concentration of antioxidant enzyme expression were assessed by polymerase chain reaction (PCR) in an automatic Whatman thermocycler (Biometra, Kent, U.K.). The target cDNA was amplified using the following primers: for superoxide dismutase-1 (SOD-1), forward 5′-AGG-GCA-TCA-TCA-ATT-TCG-AG-3′ and reverse 5′-TGC-CTC-TCT-TCA-TCC-TTT-GG-3′; for GSH, forward 5′-AGC-CCA-ACT-TCA-TGC-TCT-TC-3′ and reverse 5′-CCC-ACC-AGG-AAC-TTC-TCA-AA-3′; and for β-actin, forward 5′-GCC-ACC-CAG-AAG-ACT-GTG-GAT-3′ and reverse 5′-TGG-TCC-AGG-GTT-TCT-TAC-TCC-3′. The amplification cycles were carried out at 95 °C for 45 s, 60 °C for 1 min, and 72 °C for 45 s. After 35 cycles, the PCR products were separated by electrophoresis on 1.5% agarose gel for 15 min at 100 V. Gels were then stained with 1 mg/mL ethidium bromide and visualized by UV light using AlphaEase gel image analysis software (Alpha Innotech, San Leandro, CA). The expression levels were quantified by Multi Gauge V3.0 software (Fujifilm Life Science, Tokyo, Japan).

Statistical Analysis. All of the data were presented as the mean ± standard deviation (SD) from three independent experiments, unless stated otherwise. Statistical comparisons between different treatments were performed by one-way analysis of variation (ANOVA) with Student Newman Keul's post-hoc tests using the SPSS program (version 12.0). Differences with a value of $p < 0.05$ were considered to be statistically significant.

RESULTS

Assay-Guided Purification of the Papain Hydrolysate. The extracted gelatin from Japanese flounder skin was hydrolyzed with pepsin at optimal conditions as described earlier. The hydrolysate was tested for its potential to scavenge hydroxyl and DPPH radicals before subjecting into further purification. It was found that the hydrolysate was able to scavenge 71% hydroxyl and 59% DPPH radicals formed. The peptic hydrolysate was purified with FPLC, following the confirmation of its potent radical scavenging effect. Four fractions were obtained by the FPLC purification, and fraction 4 showed the highest radical scavenging abilities (Figure 1A). The highest active fraction 4 was further purified with RP-HPLC to obtain the active peptide. Three fractions were obtained after RP-HPLC analysis, and among them, fraction 1 was identified as the active peptide with the highest potency to scavenge hydroxyl and DPPH radicals (Figure 1B).

Identification of the Amino Acid Sequence and Molecular Weight of the Peptide. The HPLC fraction 1 was analyzed by Q-TOF ESI mass spectroscopy to elucidate the amino acid sequence. The purity of fraction 1 was over 99% according to RP-HPLC and N-terminal sequence analysis. The amino acid sequence of the peptide was identified as Gly-Gly-Phe-Asp-Met-Gly, and the structure of the peptide is shown in Figure 1C. The observed molecular mass was in agreement with the calculated molecular mass of the peptide. Using this obtained molecular weight (582 Da = 582 g/mol), the concentration of the peptide was calculated in micromoles for further experiments.

Effect of the Peptide and H_2O_2 on the Cell Viability. The effect of the peptide on the viability of RAW264.7 cells were analyzed by the MTT assay as described earlier. As shown in Figure 2A, the peptide did not exert any toxicity toward the cells because no significant changes in the cell viability was observed. However, a non-significant increase in the viability was observed at the high concentrations of the peptide, indicating its potential in enhancing the cell survival. Further, the cytotoxic effect of oxidative stress stimulator H_2O_2 was analyzed in RAW264.7 cells. Time- and dose-dependent results demonstrate the reduction in the cell number because of created oxidative stress conditions in the cells. No significant toxicity was observed by H_2O_2 concentrations up to 2 mM until 6 h of treatment. However, after that, the cell viability was significantly decreased, where at 24 h, only 64% viability was observed by 50 μM H_2O_2 treatment (Figure 2B). Interestingly, with the addition of the peptide (50 μM), the cell viability was markedly increased up to 89% at 50 μM H_2O_2 concentration after 24 h of treatment. These results indicate that the peptide is not only nontoxic to the RAW264.7 cells but also protects the cells from H_2O_2-mediated cell damage.

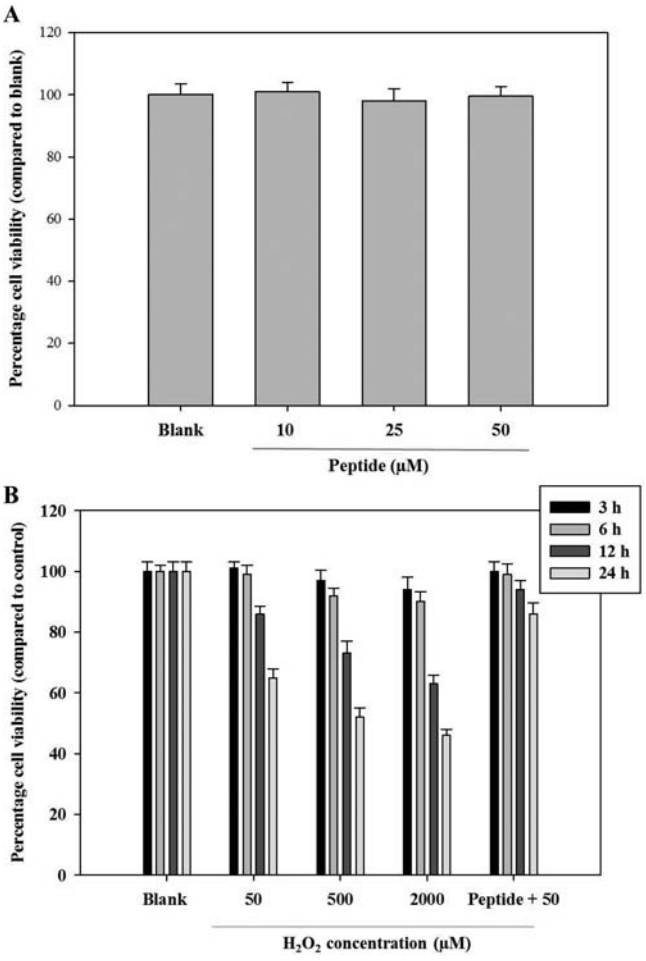

Figure 2. (A) Effect of the isolated peptide on the viability of RAW264.7 cells. (B) Cytocompatibility of H_2O_2 at different concentrations and times. The cell viability was assessed using the MTT assay after treating with different concentrations of the peptide or H_2O_2 for different time periods. Results are the mean ± SD of three independent experiments.

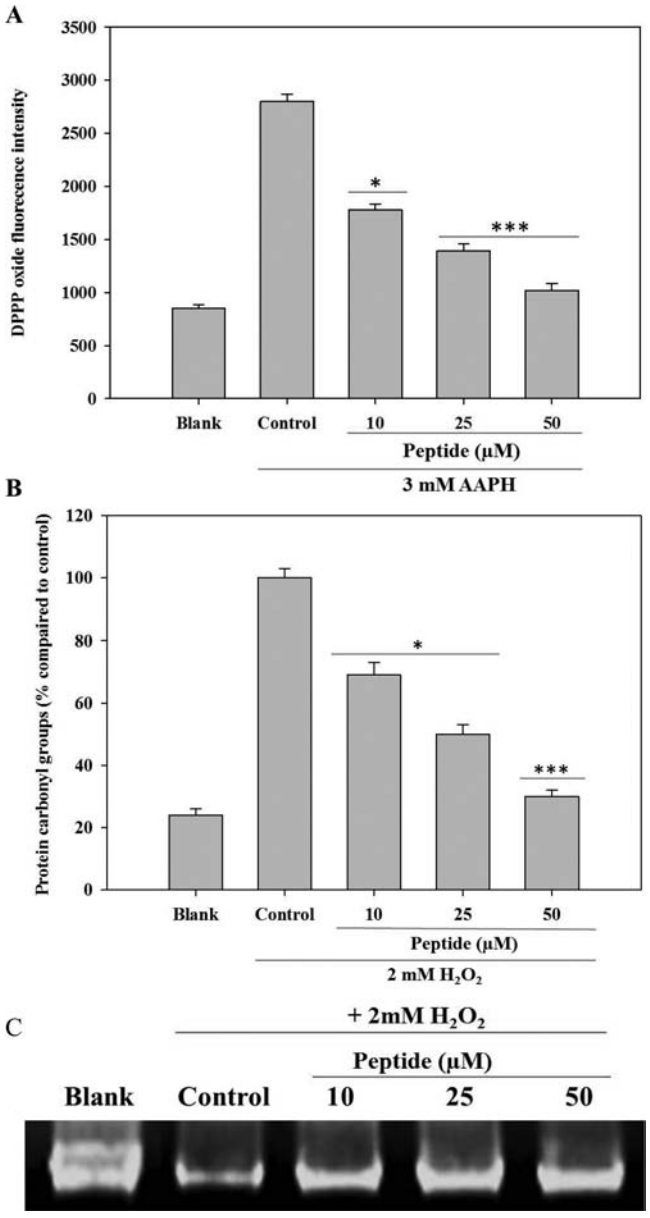

Figure 3. Peptide-mediated protective effect of cellular macromolecule oxidation. (A) Effect of the peptide on lipid peroxidation in H_2O_2-induced AAPH-induced RAW264.7 cells. (B) Effect of the peptide on the protein oxidation in H_2O_2-induced RAW264.7 cells. (C) Protective effect of the peptide on H_2O_2-induced DNA damage in RAW264.7 cells. Results are the mean ± SD of three independent experiments, and significance was determined at (*) $p < 0.05$ and (***) $p < 0.001$.

Protective Effect of the Peptide on Membrane Lipid Peroxidation. Lipid peroxidation of RAW264.7 cells was induced by exposure to the strong alkyl radical generator AAPH. The isolated peptide showed a promising dose-dependent protective effect against AAPH-radical-mediated membrane lipid peroxidation, where it showed a 71% inhibition at the highest treatment level (50 μM) compared to the control group (Figure 3A). DPPP fluorescence intensity was directly proportional to lipid oxidation levels, and the results demonstrate that the peptide is capable of protecting membrane lipids from oxidative radicals.

Protective Effect of the Peptide on Cellular Protein Oxidation. The reaction of protein carbonyls with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenylhydrazones (DNP) was used to assess the protein oxidation level. As depicted in Figure 3B, the peptide treatment significantly ($p < 0.05$) reduced the protein oxidation in a dose-dependent manner. At the highest treatment level (50 μM), the protein oxidation was reduced by 70% compared to H_2O_2-alone-treated control group. This reduction in the carbonyl groups indicates the peptide-mediated suppression of cellular protein oxidation.

Protective Effect of the Peptide on DNA Oxidation. The isolated DNA from RAW264.7 cells was treated with different concentrations of the peptide, and the DNA damage was induced by hydroxyl radicals. It was found that the DNA was significantly oxidized with Fe^{II}–H_2O_2 treatment and the addition of the peptide has suppressed the DNA oxidation in a dose-dependent manner (Figure 3C). Interestingly, even at the lowest concentration of the peptide treatment, DNA oxidation was clearly inhibited in comparison to that of the control group. Therefore, it was clearly identified that the peptide protects the DNA from hydroxyl-radical-mediated damage.

ROS Scavenging Ability of the Peptide. To understand whether the peptide protects cellular macromolecules by scavenging the cellular ROS, the effect of the peptide on the ROS production levels in H_2O_2-stimulated RAW264.7 cells

were analyzed using the fluorescent probe DCFH-DA. The oxidation level of DCFH by intracellular ROS was measured in every 20 min for 2 h. As shown in Figure 4, peptide treatment

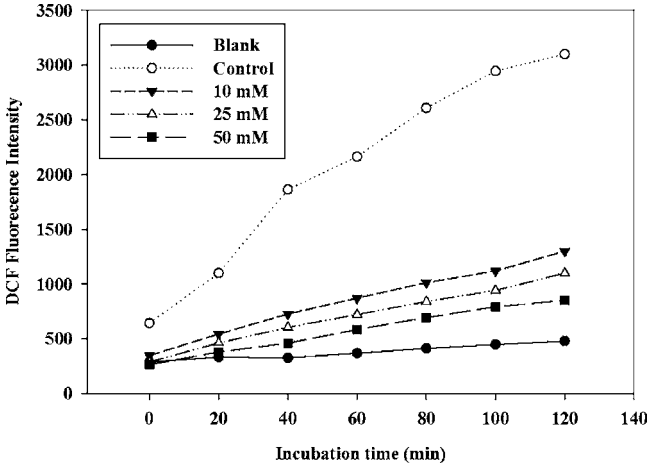

Figure 4. ROS scavenging effect of the peptide in H_2O_2-induced RAW264.7 cells at different concentrations over a time course of 2 h. A DCFH-DA fluorescent probe was used to detect the levels of intracellular ROS.

reduced the fluorescence intensity dose-dependently. DCFH-DA is esterified into DCFH inside the cytosol, and intracellular ROS oxidize DCFH into fluorescing DCF. The lower florescence intensity indicates the suppression of DCFH-DA oxidation, which corresponds to the reduction in intracellular ROS production. At 50 μM treatment, the peptide reduced the fluorescence intensity by 4-fold compared to the H_2O_2-alone-treated control group, which indicates the significant radical scavenging capacity of the peptide.

Effect of the Peptide on the Expression of Antioxidative Enzymes. The effect of the peptide on the mRNA expression levels of antioxidative enzymes SOD-1, GSH, and catalase was analyzed by RT-PCR. As depicted in Figure 5, the mRNA expression levels of antioxidative enzymes were significantly elevated with the treatment of the peptide. The elevation in the mRNA expression increases with the increasing dose of the peptide. These data suggest that the peptide

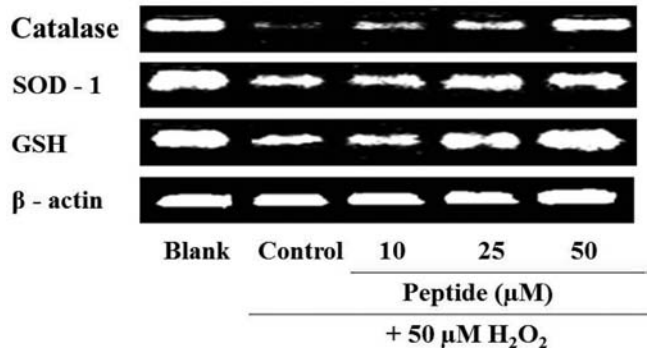

Figure 5. Effect of the peptide on the intracellular antioxidant enzymes. The mRNA expression levels were analyzed by RT-PCR using the RNA isolated from H_2O_2-stimulated RAW264.7 cells treated with different concentrations of the peptide. β-Actin expressions were used as an internal control.

upregulates the expression of antioxidative enzymes, which can dilute the oxidative stress conditions in the cells.

DISCUSSION

In this study, gelatin was extracted from the skin of Japanese flounder and the extracted gelatin was hydrolyzed with pepsin. A series of assay-guided purification steps was conducted to obtain an antioxidant peptide. The protective effect of the peptide against macromolecular oxidation was examined using hydroxyl-radical-induced RAW264.7 cells as an *in vitro* model. The membrane lipids are predominantly susceptible to peroxidation by oxidative radicals,[23] which result in highly reactive peroxides.[24] In this experiment, the effect of the isolated peptide on membrane lipid peroxidation was determined using a lipid hydroperoxide-sensitive fluorescent probe, DPPP. When oxidized by lipid hydroperoxides, DPPP will form a fluorescing DPPP-oxide, and the fluorescence level can be measured as a parameter describing the amount of lipid peroxides formed. As indicated in the Results, the peptide has significantly reduced the DPPP fluorescence levels (71%), indicating low levels of lipid peroxidation. The reduction of lipid peroxidation by the isolated peptide showed a much higher level compared to that of reported marine-derived antioxidants, such as glucosamine (42% inhibition),[25] sulfated glucosamine (65%),[25] and gallic-acid-conjugated chitosan oligosaccharides (67%),[26] which were tested similarly using 3 mM stimulated AAPH RAW264.7 cells.

Cellular proteins are also readily oxidized by ROS and produce an array of new reactive groups, such as carbonyls, hydroperoxides, and 3,4-dihydroxyphenylalanine.[27] Among them, the formation of carbonyl groups was used as a marker of ROS-mediated protein oxidation to calculate the level of protein oxidation in this study. Protein carbonyls are the most employed marker to determine protein oxidation, and it can be formed by oxidation of the side chains of several amino acids, such as lysine, arginine, proline, and histidine, by the reaction of proteins with oxidative radicals or lipid oxidation products.[28] The percentage of protein carbonyl groups is reduced dose-dependently with the presence of peptide, which can be directly correlated to the reduction in protein oxidation.

Cellular DNA is also a prime target of hydroxyl radicals.[29] The peptide has markedly reduced the DNA oxidation levels in this experimental model. All of this collected evidence supports the fact that the isolated peptide effectively reduced the oxidation of cellular macromolecules. To find out the underlying causes of this significant protective effect, the ability of the peptide to scavenge intercellular ROS was analyzed. From the results, it was found that the peptide significantly reduced the production of intracellular ROS. This reduction could have resulted from peptide-mediated scavenging of ROS and upregulation of antioxidative systems. It was found in the early stage of this study that the peptide is capable of scavenging reactive radical species, such as hydroxyl radicals and DPPH radicals *in vitro*.

Furthermore, RT-PCR analysis confirmed that the peptide is inducing the expression of inherent antioxidant enzymes in stimulated RAW264.7 cells. In regulated conditions, the antioxidative enzymes present in the cell defend against oxidative stress. The most common endogenous enzymatic antioxidants are catalase, SOD, and GSH.[30] However, unregulated production of ROS leads to downregulation in antioxidative enzyme expression.[31] Collectively, the results indicate that the peptide is not only acting as an exogenous

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antioxidant but also inducing the activation of the endogenous antioxidant system and thereby protecting the cells effectively against oxidative damage.

The ability of the peptide to scavenge ROS radicals could be directly correlated to its structural features. The size, amino acid composition, and sequence of the peptide are determining factors of its activity. It was reported that the antioxidant activity has a direct relation to its molecular weight, where the antioxidant peptides with 500−1500 Da molecular weight are more potent antioxidants.[32] The presence of hydrophobic amino acids is preferred over the others because they will facilitate reaching hydrophobic targets, such as the cell membrane, and thereby enhance availability.[33] Furthermore, hydrophobic peptides can protect macromolecule oxidation by donating photons to reactive radicals.[34] The presence of aromatic amino acids in the structure of a peptide is an advantage in this regard because they can donate protons easily to electron-deficient radicals and, at the same time, maintain their stability via resonance structures.[35] Not only the presence of functional amino acids but also their positioning in the peptide sequence is affecting the antioxidant activity. The amino acids present in the carboxyl and amino terminals of a peptide are important factors for its bioactivity. The presence of hydrophobic amino acids, preferably aromatic amino acids, at carboxyl and amino terminals of a peptide enhances the antioxidant capacity of the peptide. The isolated peptide is a highly hydrophobic peptide (+12.61 kcal/mol) with a molecular weight of 582 Da. Moreover, hydrophobic glycine, methionine, and phenylalanine are present at the end terminals of the peptide. These interesting structural features of the peptide may have given a large contribution to its antioxidant activity.

In conclusion, seafood processing by the product Japanese flounder skin was used to extract gelatin, which was hydrolyzed by pepsin to produce an antioxidant peptide. The isolated peptide has shown potent radical scavenging activities. Furthermore, the peptide acts effectively to reduce oxidative-stress-induced damage in RAW264.7 cells. The peptide has upregulated the expression of endogenous antioxidative enzymes. These results indicate that this isolated peptide is capable of not only scavenging the ROS but also boosting the inherent antioxidative system in the cells.

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ABBREVIATIONS USED

DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DMEM, Dulbecco's modified Eagle's medium; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DPPP, diphenyl-1-pyrenylphosphine; FBS, fetal bovine serum; FPLC, fast-performance liquid chromatography; GSH, glutathione; H_2O_2, hydrogen peroxide; HBSS, Hank's balanced salt solution; MTT, 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PR-HPLC, reverse-phase high-performance liquid chromatography; Q-TOF, quadrupole time-of-flight; ROS, reactive oxygen species; RT-PCR, revese transcription polymerase chain reaction; SOD-1, superoxide dismutase-1

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