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An Antihypertensive Peptide from Tilapia Gelatin Diminishes Free Radical Formation in Murine Microglial Cells

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ABSTRACT: A peptide possessing antihypertensive activity was purified from Nile tilapia (*Oreochromis niloticus*) gelatin using alcalase, Pronase E, pepsin, and trypsin. Among them, the alcalase hydrolysate exhibited the highest angiotensin converting enzyme (ACE) inhibitory activity. Therefore, it was further analyzed, and a potent ACE inhibitory peptide of DPALATEPDPMPF (1382 Da) was separated and purified. In addition, the protective effect of the purified peptide against free radical-induced cellular and DNA damage in murine microglial cells (BV-2) was determined. These results suggest that the peptide isolated from Nile tilapia (*O. niloticus*) gelatin acts as a candidate against hypertension and oxidative stress and could be used in health-functional foods.

KEYWORDS: Nile tilapia gelatin, bioactive peptide, antihypertension, antioxidant, murine microglial cells

INTRODUCTION

The peptides regulating blood pressure are potent inhibitors of angiotensin-I converting enzyme (EC 3.4.15.1, ACE). ACE plays an important role in the regulation of blood pressure as it promotes the conversion of angiotensin-I to the potent vasoconstrictor angiotensin-II. ACE belongs to a class of zinc proteases that require both zinc and chloride for its enzymatic activity. Therefore, in the development of drugs to control high blood pressure, inhibition of ACE is considered to be a useful therapeutic approach in the treatment of hypertension.[1]

Reactive oxygen species (ROS) and other free radicals attack macromolecules such as membrane lipids, proteins, and DNA, leading to many health disorders such as hypertension, cardiovascular, cancer, diabetes mellitus, and neurodegenerative and inflammatory diseases with severe tissue injuries. Antioxidants may have a positive effect on human health as they can protect the human body against damage by ROS.[2,3]

A large amount of low-priced skin byproducts can be obtained from the fish surimi and filleting production. Up to now, many studies have shown that the enzymatic digestion of fish byproduct is an efficient means of producing peptides with enhanced bioactivity. Among these, large numbers of bioactive peptides with antihypertensive and antioxidant activities derived from various marine organisms such as tuna,[4] Alaska pollack,[5] shark,[6] bullfrog,[7] blue mussel,[8] oyster,[9] sea cucumber,[10] and algae[11] have widely been researched. In this study, we investigate the production of an ACE inhibitory peptide derived from enzymatic digestion of Nile tilapia (*Oreochromis niloticus*) gelatin and evaluate its antioxidant activity using the murine microglial BV-2 cell line as an in vitro model.

MATERIALS AND METHODS

Materials. Nile tilapia (*O. niloticus*) gelatin was obtained from Geltech Co., Busan, South Korea. The fluorescence probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was obtained from Molecular Probes Inc. (Eugene, OR). MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide), agarose, fetal bovine serum (FBS), Pronase E, pepsin, trypsin, ACE (from rabbit lung), hippuryl-histidyl-leucine (HHL) as a substrate of ACE, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO). Alcalase was purchased from Novozymes Co. (Bagsvaerd, Denmark).

Murine microglial cells (BV-2 cells) were obtained from American Type Culture Collection (Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin, and the other materials required for culturing of cells were purchased from Gibco BRL, Life Technologies (Grand Island, NY). All other reagents used in this study were of the highest grade available commercially.

Preparation of Enzymatic Hydrolysates. Gelatin was separately hydrolyzed with four different enzymes (alcalase, Pronase E, pepsin, and trypsin) under optimal conditions. At an enzyme to substrate ratio of 1:100 (w/w), 1% substrate and enzyme were mixed. The mixture was incubated for 4 h at each optimal pH and temperature with stirring and then heated in a boiling water bath for 10 min to inactivate the enzyme. Lyophilized hydrolysates were stored at −80 °C until used.

Purification of Peptide. *Ion Exchange Chromatography.* The peptide was purified from enzymatic hydrolysates using fast protein liquid chromatography (FPLC, AKTA, Amersham Bioscience Co., Uppsala, Sweden) on a HiPrep 16/10 diethylaminoethyl fast flow (DEAE FF) ion exchange column (1.6 × 10 cm, Amersham Biosciences, Piscataway, NJ). The hydrolysate showing the highest ACE inhibition was loaded (2 mL) onto a HiPrep 16/10 DEAE FF anion 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 was monitored at 280 nm, collected at a volume of 4 mL, and concentrated using a rotary evaporator. Each fraction was analyzed for ACE inhibitory activity, and the strongest ACE inhibitory active fraction was lyophilized and used as the next step.

High-Performance Liquid Chromatography (HPLC). The fraction exhibiting the strongest ACE inhibitory activity was further purified using reversed-phase HPLC (RP-HPLC, Dionex Korea Ltd., Sunnyvale, CA) on a Primesphere 10 C_{18} (10 mm × 250 mm, Phenomenex,

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Cheshire, U.K.) column with a linear gradient of acetonitrile (0−40%) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 2 mL/min. Elution peaks were detected at 215 nm, and the active peak was concentrated using a rotary evaporator. Potent peaks were collected, lyophilized, and evaluated for ACE inhibitory activity. Finally, the purified peptide from the alcalase digest of Nile tilapia gelatin was analyzed for its amino acid sequence.

Determination of Amino Acid Sequence. An accurate molecular mass and amino acid sequence of the purified peptide was determined using a Q-TOF mass spectrometer (Micromass, Altrincham, U.K.) 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. Following molecular mass determination, the peptide was automatically selected for fragmentation, and sequence information was obtained by tandem mass spectrometry analysis.

Assay of ACE Inhibitory Activity. The ACE inhibitory activity assay was performed using the method of Cushman and Cheung[12] with slight modifications. Briefly, a sample solution (50 μL) with 50 μL of ACE solution (25 milliunits/mL) was incubated at 37 °C for 10 min, and the mixture was preincubated with 150 μL of substrate (8.3 mM HHL in a 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3) at 37 °C for 30 min. Finally, the reaction was terminated by the addition of 1.0 M HCl (250 μL), and the resulting hippuric acid was extracted by the addition of 0.5 mL of ethyl acetate. After centrifugation (3000*g* for 10 min), 0.2 mL of the upper layer was transferred into a glass tube and evaporated at room temperature for 2 h in a vacuum. The hippuric acid was dissolved in 1.0 mL of distilled water, and the absorbance was measured at 228 nm using an UV spectrophotometer (Tecan Austria GmbH, Grodig/Salzburg, Austria). The IC_{50} value defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

The extent of inhibition was calculated as

$$\text{inhibitory activity } (\%) = (C - S)/(C - B) \times 100$$

where C = absorbance of control solution (no sample or ACE inhibitor), S = absorbance of test sample, and B= absorbance of blank solution.

Cell Cytotoxicity, Cellular ROS, and DNA Oxidation Determination. BV-2 cells were cultured and maintained in DMEM containing 5% (v/v) FBS, 100 μg/mL penicillin/streptomycin, and 5% CO_2 at 37 °C. Cytotoxicity levels of samples on cells were measured using the MTT method as described by Vo et al.[13] with slight modifications.

Intracellular formation of ROS was assessed according to a method described previously by employing oxidation-sensitive dye DCFH-DA as the substrate.[14]

Genomic DNA was extracted from BV-2 cells using an AccuPrep genomic DNA extraction kit (Bioneer, Daejeon, South Korea) according to the manufacturer's protocol. Hydrogen peroxide mediated DNA oxidation was determined according to the method of Ngo et al.[15]

Statistical Analysis. All statistical analyses were performed with independent experiments, and values are expressed as the mean ± standard deviation of three independent determinations. The significance of differences between two samples was analyzed using the Student *t* test, and a *p* value of <0.05 was considered to be significant.

RESULTS AND DISCUSSION

Preparation of Gelatin Hydrolysates and Their ACE Inhibitory Activity. Enzymatic hydrolysis is widely used in the food industry; this technique does not adopt any toxic chemicals compared to solvent extraction. Therefore, gelatin was separately hydrolyzed using different commercial enzymes such as alcalase, Pronase E, pepsin, and trypsin to release ACE inhibitory peptides. Among the hydrolysates tested, the alcalase hydrolysate showed the highest ACE inhibitory activity of about 52% at 500 μg/mL (Figure 1). Alcalase produces shorter peptide sequences as well as terminal amino acid sequences responsible for various health-beneficial bioactivities including ACE inhibition. Furthermore, bioactive peptides from alcalase hydrolysates are resistant to trypsin, but not resistant to pepsin, which could allow for absorption of peptides contained in the hydrolysate.[16] Moreover, alcalase affords the highest ACE inhibitory activity in the production of antihypertensive peptides when compared with other specific (pepsin and trypsin) and nonspecific (Pronase E) proteases. Previous studies have reported that alcalase is capable

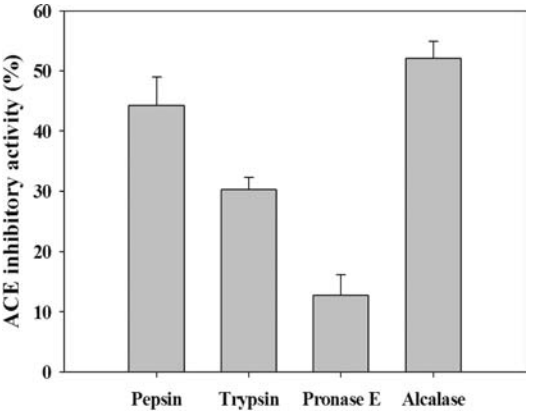

Figure 1. ACE inhibitory activities of enzymatic hydrolysates from Nile tilapia (*O. niloticus*) gelatin.

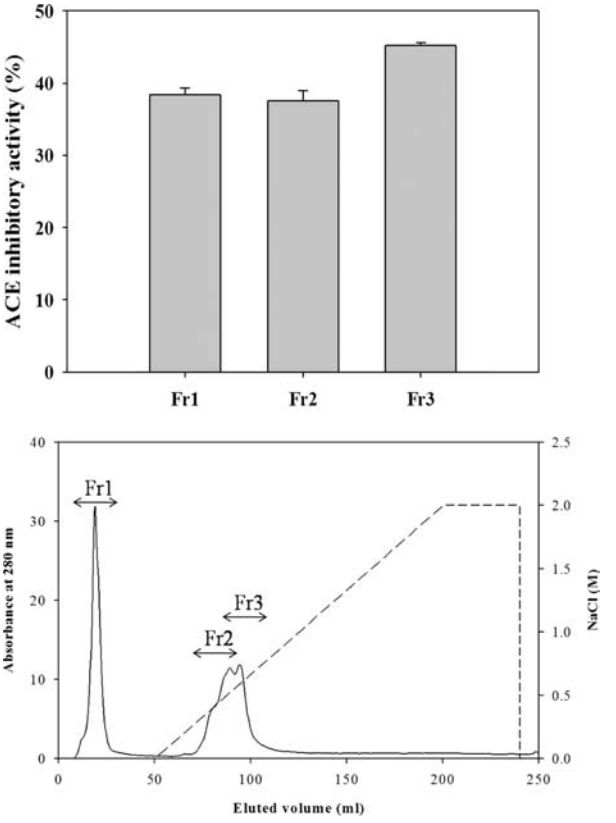

Figure 2. FPLC chromatogram of alcalase hydrolysate by HiPrep 16/10 DEAE FF anion exchange column (top) and its ACE inhibitory activity (bottom).

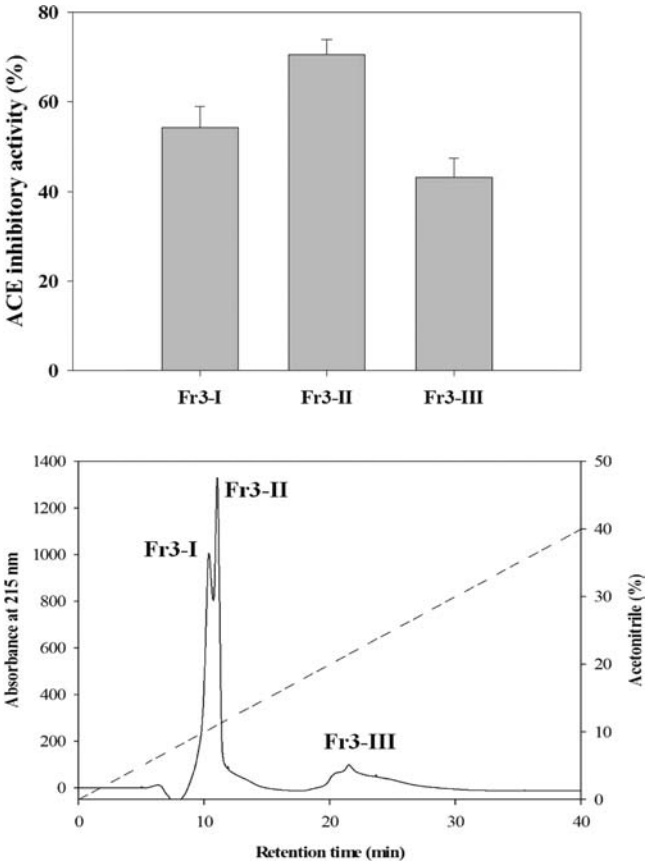

Figure 3. Reverse-phase HPLC chromatogram for further purification of Fr3 active fraction from FPLC (bottom) and its ACE inhibitory activity (top).

of producing bioactive peptides when it is incorporated to hydrolyze food proteins.[17] The alcalase proteolytic hydrolysate was chosen to purify and isolate ACE inhibitory peptides from tilapia gelatin.

Purification of ACE Peptides and Their ACE Inhibitory Activity. The lyophilized alcalase proteolytic hydrolysate was dissolved in 20 mM sodium acetate buffer (pH 4.0) and loaded onto a Hiprep 16/10 DEAE FF anion exchange column with a linear gradient of NaCl (0−2.0 M). All eluents were monitored at 215 nm and then fractionated into one nonadsorptive portion and two adsorptive portions. Each fraction was pooled, lyophilized, and measured for ACE inhibitory activity. The third fraction (Fr3) was found to possess the highest ACE inhibition of about 45% at 250 μg/mL (Figure 2), and this fraction was lyophilized and further separated by RP-HPLC on a Primesphere 10 C_{18} (10 mm × 250 mm) column with a linear gradient of acetonitrile (0−40%) and was divided into three further fractions. Fr3-II showed the most potent ACE inhibitory activity of about 70% at 125 μg/mL (Figure 3). Amino acid sequence analysis by Q-TOF mass spectrometer revealed that Fr3-II was composed of 13 amino acid residues, DPALATEPDPMPF (1382 Da), and showed potent ACE inhibition with an IC_{50} of 62.2 μM. Bioactive peptides usually contain 3−20 amino acid residues, and low molecular weight peptides are more potent as bioactive peptides than high molecular weight peptides.[18] With regard to the relationship between structure and activity of ACE inhibitory peptides, those peptides having proline, phenylalanine, or tyrosine

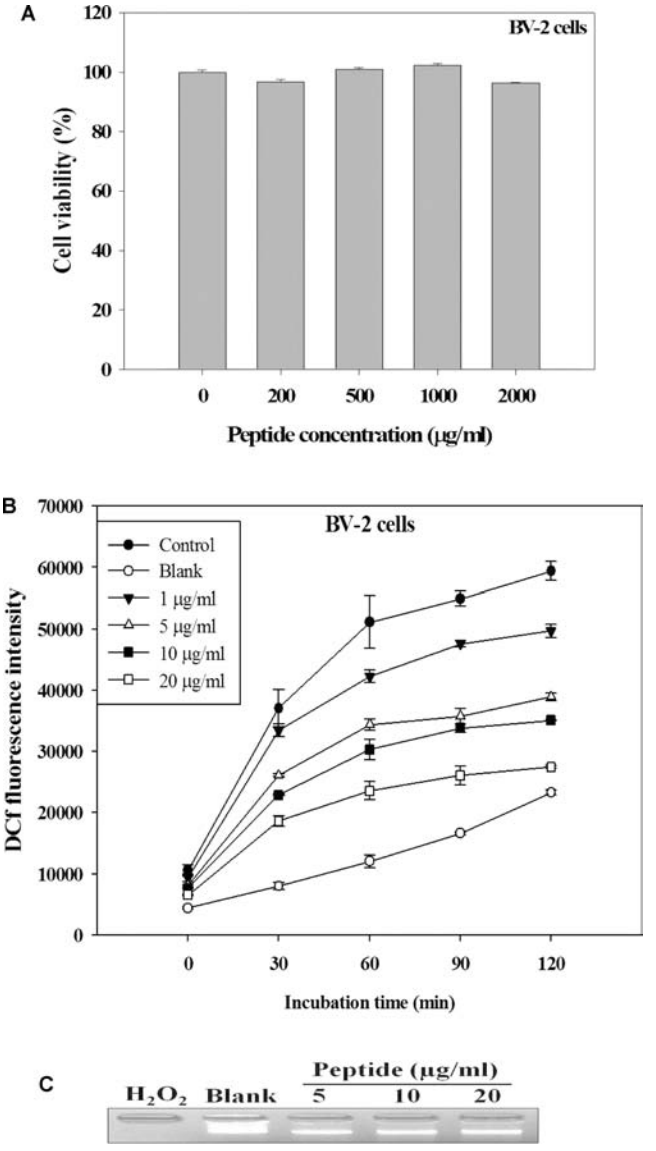

Figure 4. Cell viability assessed by the MTT assay (A), intracellular radical scavenging activity (B), and DNA oxidative protection (C) of the purified peptide in BV-2 cells.

at the C terminus and isoleucine and valine at the N terminus showed highly potent inhibitory activity.[19] Most of the reported ACE inhibitory peptides are usually short peptides with a proline residue at the C-terminal end.[20,21] Proline is known to prevent digestion of enzymes and may pass from the capillary into the circulation of blood in the sequence of short peptides.[22,23] Although the structure−activity relationship of ACE inhibitory peptides has not yet been fully established, the purified peptide showed some common features.

Cell Viability, Cellular Radical Scavenging Effect, and DNA Protection of the Purified Peptide. BV-2 cells were treated with different concentrations of the purified peptide to determine noncytotoxic concentration for further experiments. The MTT assay showed no cytotoxicity of the purified peptide on BV-2 cells (Figure 4A).

To determine the scavenging effect of the purified peptide on cellular radicals, BV-2 cells were labeled with DCFH-DA, a fluorescence probe. DCFH-DA easily diffuses through cell

membranes and is hydrolyzed by esterase to become DCFH, which reacts with ROS to form DCF, a fluorescent product. The monitoring of DCF fluorescence intensities every 30 min for 2 h revealed that H_2O_2-mediated oxidation increased with incubation time. However, treatment with the purified peptide decreased the DCF fluorescence time dependently. The purified peptide exhibited a free radical scavenging effect even only after 30 min of incubation compared to the control (H_2O_2 alone-treated) and blank (H_2O_2 nontreated) as shown in Figure 4B. These results showed that its antioxidant effect varies with the concentration of the purified peptide, and the free radical scavenging effect of the purified peptide at 20 μg/mL concentration was higher than other tested concentrations. We could confirm that the purified peptide scavenged free radicals in a dose- and time-dependent manner in BV-2 cells. Several amino acids, such as tyrosine, methionine, lysine, and tryptophan, are generally accepted as antioxidants despite their pro-oxidative effects in some cases.[24] It has been known that many antioxidative peptides include hydrophobic amino acid residues, valine or leucine, at the N terminus of the peptides.[25] The peptide from Nile tilapia (*O. niloticus*) gelatin composed of 13 amino acid residues, DPALATEPDPMPF (1382 Da), was rich in hydrophobic amino acids (>69%) such as proline. Some hydrophobic amino acids such as proline, alanine, and leucine have been reported to contribute to scavenging of free radicals.[26−28] Proline is involved in scavenging of not only hydroxyl radicals but also superoxide radicals, which triggers the chain of oxidative reactions in the cells.[29] This peptide has an aromatic amino acid (phenylalanine) at the C-terminal, and an aspartic acid residue at the N-terminal may cause its potent antioxidant activity. Therefore, the purified peptide scavenged free radicals and inhibited radical-mediated oxidation in BV-2 cells.

Hydroxyl radicals ($^{\bullet}$OH) generated by the Fenton reaction are known to cause oxidative breaks in DNA strands to yield its open circular or relaxed forms. Genomic DNA was isolated from BV-2 cells to study the DNA protective effect of the purified peptide. In this study, oxidation of DNA was determined by combining the effects of 200 μM $FeSO_4$ and 2 mM H_2O_2 on the integrity of genomic DNA. The DNA protective effect was assessed by DNA electrophoresis in the presence or absence of the purified peptide. After 10 min of reaction, all DNA was degraded in the control group treated only with Fe(II)−H_2O_2. It was observed that the purified peptide significantly inhibited the oxidative damage of DNA (Figure 4C). The DNA damage was inhibited at all tested concentrations of the purified peptide by about 75% on the basis of the intensity of DNA bands. The results of this study clearly explain that the purified peptide can prevent oxidative damage to DNA when DNA is exposed to $^{\bullet}$OH generated by Fe(II)−H_2O_2. Fe^{2+} catalyzes the conversion of H_2O_2, which is a major route to the synthesis of $^{\bullet}$OH in biological systems. The $^{\bullet}$OH reacts with all components of the DNA molecule, including base modification (purine and pyrimidine) and also deoxyribose units, and damages DNA. This lesion increases with increment of free radical attack on cellular DNA, which is involved in mutagenesis, cancer, and aging.[30,31] However, the purified peptide can contribute to preventing these diseases by its inhibitory effect against DNA oxidation in BV-2 cells.

Conclusion. In this study, an antihypertensive and antioxidant peptide was obtained from Nile tilapia (*O. niloticus*) gelatin by alcalase enzymatic hydrolysis. The purified peptide, DPALATEPDPMPF (1382 Da), was shown to exhibit potent ACE inhibitory and radical scavenging activities. The results of this study suggested that the ACE inhibitory and antioxidant peptide from Nile tilapia gelatin could be useful as a potential functional ingredient in food and pharmaceutical industries against hypertension, free radicals, and related diseases.

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Author Contributions

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