Isolation and Characterization of Antioxidative Peptides from Gelatin Hydrolysate of Alaska Pollack Skin

Se-Kwon Kim,*,† Yong-Tae Kim,†,⊥ Hee-Guk Byun,† Kyung-Soo Nam,‡ Dong-Sik Joo,§ and F. Shahidi||

Department of Chemistry, Pukyong National University, Pusan 608-737, Korea, Laboratory of Pharmacology, College of Medicine, University of Dongguk, Kyongju, 780-714, Korea, East Sea Marine Bio Resources Research Center, Kangnung 210-702, Korea, and Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland A1A 3X9, Canada

Gelatin extracted from Alaska pollack skin was hydrolyzed with serial digestions in the order of Alcalase, Pronase E, and collagenase using a three-step recycling membrane reactor. The fraction from the second step, which was hydrolyzed with Pronase E, was composed of peptides ranging from 1.5 to 4.5 kDa and showed high antioxidative activity. Two different peptides showing strong antioxidative activity were isolated from the hydrolysate using consecutive chromatographic methods including gel filtration on a Sephadex G-25 column, ion-exchange chromatography on a SP-Sephadex C-25 column, and high-performance liquid chromatography on an ODS column. The isolated peptides, P1 and P2, were composed of 13 and 16 amino acid residues, respectively; and both peptides contained a Gly residue at the C-terminus and the repeating motif Gly-Pro-Hyp. The antioxidative activities of the purified peptides were measured using the thiobarbituric acid method, and the cell viability was measured with MTT assay. The results showed that P2 had potent antioxidative activity on peroxidation of linoleic acid. Moreover, the cell viability of cultured liver cells was significantly enhanced by addition of the peptide. These results indicate that the purified peptide, P2, from gelatin hydrolysate of Alaska pollack skin is a natural antioxidant which has potent antioxidative activity.

Keywords: *Characterization; antioxidative peptide; gelatin hydrolysate; Alaska pollack skin*

INTRODUCTION

Antioxidants are used to preserve food products by retarding discoloration and deterioration due to oxidation. Hence, antioxidants are increasingly used as a means to enhance the shelf-life and improve the stability of lipid and lipid-containing foods. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been commonly used by the food industry because they are effective and cheap when compared to natural antioxidants. However, synthetic antioxidants are suspected of causing some safety concerns (*1*−*3*) and have been restricted in their use as food additives. Natural antioxidants have been the focuse of considerable interest. α-Tocopherol is the most widely used natural antioxidant, and it is an effective agent in stabilization of lipid-containing foods, but it has limitations for food usage (*4*). Thus, there is motivation to search for safe and natural antioxidants from various sources.

It has been reported that many proteins, such as milk casein (*5*), soy protein (*6*), bovine serum albumin (*7*), ovalbumin (*8*), oilseed proteins (*9*), wheat gliadin (*10*), and maize zein (*11*), possess antioxidative activities against the peroxidation of lipids and fatty acids. Amino acids have also been reported to be antioxidants against linoleic acid in the freeze-dried emulsion condition (*12*, *13*). Yamaguchi et al. (*14*) have reported the antioxidative effect of soybean protein hydrolysate. Recently, six antioxidative peptides were isolated from the hydrolyzate of a soybean protein, *β*-conglycinin (*15*). These peptides were composed of 5 to 16 amino acid residues and included hydrophobic amino acids (Val or Leu) at the N-terminus, and Pro, His, or Tyr in sequences. However, little is known about the structure of antioxidative peptides from various food proteins.

In this study, we have described the isolation of antioxidative peptides from hydrolysates of gelatin extracts from Alaska pollack skin and have characterized the antioxidative activity of purified peptides in comparison with that of commercial antioxidants such as butylated hydroxytoluene (BHT) and α-tocopherol.

MATERIALS AND METHODS

Materials. Fresh samples of Alaska pollack skin were obtained from a local fisheries company and stored at −20 °C. Alcalase (0.6 L; sp gr, 1.25) was obtained from Novo Nordisk (Bagsvaerd, Denmark), and collagenase (335 units/mg) and Pronase E (from *Streptomyces griseus*, Type XIV, 4.4 U/mg of solid) were purchased from Sigma Chemical Co. (St. Louis, MO). Butylaed hydroxytoluene (BHT) and tetramethoxypropane (TMP) were obtained from Fluka. Sephadex G-25, SP-Sephadex C-25, 2-thiobarbituric acid (TBA), *tert*-butyl hydroperoxide, ammonium thiocyanate, and linoleic acid were purchased from Sigma Chemical Company (St. Louis, MO). All other reagents used were of the highest grade available commercially.

* To whom correspondence should be addressed. Tel: +82-51-620-6375. Fax: +82-51-628-8147. E-mail: sknkim@mail.pknu.ac.kr.

† Pukyong National University.

‡ University of Dongguk.

§ East Sea Marine Bio Resources Research Center.

|| Memorial University of Newfoundland.

⊥ Present address: Oklahoma Medical Research Foundation, 825 Northeast 13th Street, Oklahoma City, OK 73104.

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Preparation of Gelatin and Gelatin Hydrolysates. Gelatin from Alaska pollack skin was extracted by the method described previously (*16*). A three-step recycling membrane reactor was used for the enzymatic preparation of gelatin hydrolysates (*17*). One percent (w/v) gelatin was prepared in deionized water and digested with Alcalase (enzyme-to-substrate ratio, 1:50) at pH 8.0 and 50 °C in the first-step membrane reactor. The first hydrolysate was fractionated through a membrane (A/G Technology Co., model UFP-10-C-4; Needham, MA) with a 10 000 Da molecular-weight cutoff (MWCO), and then hydrolyzed with Pronase E (enzyme-to-substrate ratio, 1:33) at pH 8.0 and 50 °C in the second-step membrane reactor. The second hydrolysate was fractionated through a membrane (A/G Technology Co., model UFP-5-C-4) with a 5 000 Da MWCO and subsequently hydrolyzed with collagenase (enzyme-to-substrate ratio, 1:100) at pH 7.5 and 37 °C in the third-step membrane reactor. The third hydrolysate was fractionated through a membrane (Romicon, model UFP-1.0-43-PM1; Woburn, MA) with a 1000 Da MWCO. The fractions were then lyophilized and stored at −20 °C until used.

Measurement of Antioxidative Activity. For oxidation, samples (250 mg) dissolved in 4.87 mL of distilled water, 0.13 mL of linoleic acid, 10 mL of ethanol, and 10 mL of 50 mM phosphate buffer (pH 7.0) were mixed in glass flasks. The flasks were sealed tightly with silicone rubber caps and kept at 40 °C in the dark. At regular intervals, aliquots of the reaction mixtures were withdrawn with a microsyringe for measurements of the oxidation using the thiobarbituric acid (TBA) method (*18*) with a slight modification.

The reaction mixture (50 μL) was added to a mixture of 0.8 mL of distilled water, 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid (pH 3.5), and 1.5 mL of 0.8% 2-thiobarbituric acid (TBA) solution in water. The mixture was heated at 100 °C for 60 min. After the mixture was cooled in ice, it was centrifuged at 5 000*g* for 10 min. The absorbance of the upper layer was measured at 532 nm and the antioxidative activity of the samples was expressed as malondialdehyde (MDA) concentrations.

Purification of the Antioxidative Peptides. The hydrolysate showing antioxidative activity was dissolved in 10 mL of a 50 mM sodium phosphate buffer (pH 7.0) and loaded onto a Sephadex G-25 gel filtration column (2.5 × 90 cm) which was previously equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The column was then eluted with the same buffer, and the fractions showing antioxidative activity were pooled and lyophilized. This fraction was dissolved in a minimum volume of 20 mM sodium phosphate buffer (pH 4.0) and loaded onto an ion-exchange column (3.0 × 40 cm) with a SP-Sephadex C-25 (Sigma Chemical Co., St. Louis, MO) previously equilibrated with 20 mM sodium phosphate buffer (pH 4.0). The column was washed with the same buffer and eluted with a linear gradient of NaCl concentrations from 0 to 0.5 M. The fractions showing antioxidative activity were concentrated by ultrafiltration and dialyzed against distilled water. This fraction was separated by reversed-phase high performance liquid chromatography (HPLC) on an ODS column (5 μm, 10.0 × 250 mm) using a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.5 mL/min. Fractions showing antioxidative activity were rechromatographed on an ODS column (5 μm, 10.0 × 250 mm) using a linear gradient of acetonitrile in 10 mM ammonium acetate at a flow rate of 1.5 mL/min.

Determination of Cell Viability. Donryu rat liver cells (Ac2F) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The cells were cultured in Dulbecco's Modified Eagle Media (DMEM; Cibco Co., Grand Island, NY) with 10% heat-inactivated (55 °C for 15 min) fetal bovine serum (FBS), 58.4 μg/mL glutamine, 7.5% $NaHCO_3$, penicillin (100 units/mL), streptomycin (100 μg/mL), and fungizone (0.25 μg/mL). The cells were grown in a 75-cm^2 tissue-culture flask (Corning Co., Cambridge, MA) at concentrations ranging from 10^6 to 10^7 cells/mL and subcultured every 2 days, at 37 °C in 5% CO_2/95% air in a humidified incubator. Cells were harvested from 25-cm^2 tissue-culture flask by trypsinization, counted, and seeded in 24-well flat-bottomed plates with the density of 5×10^4 cells/well. After the cells grew to confluence, different concentrations of peptides in DMEM were added to the wells. Plates were incubated for 17 h at 37 °C in 5% CO_2. Following removal of FBS from wells, cells were washed with calcium magnesium free-phosphate buffered saline (CMF-PBS, pH 7.2) and then subjected to oxidant stress by incubation with 200 μL of 1 mM *tert*-butyl hydroperoxide (*t*-BHP) for 150 min. To determine the effect of antioxidative peptides on *t*-BHP-induced injury, MTT assay of cell viability was carried out as described by Sladowski et al. (*19*). After 2.5 h of incubation with *t*-BHP, cells in 24-well plates were rinsed with CMF-PBS. MTT (100 μL of a 0.4 mg/mL solution) was added to each well. Following an additional

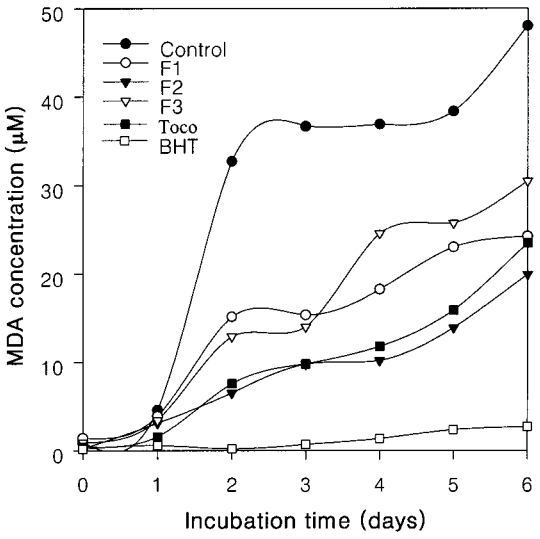

Figure 1. Antioxidative activities of gelatin hydrolysates from Alaska pollack skin. The activity was evaluated by the TBA method as described in the methods section. Control contained distilled water alone instead of sample. F1 denotes the first-step enzymatic hydrolysate; F2, the second-step enzymatic hydrolysate; F3, the third-step enzymatic hydrolysate; Toco, α-tocopherol; BHT, butylated hydroxytoluene. Values in this figure are the average of triplicates.

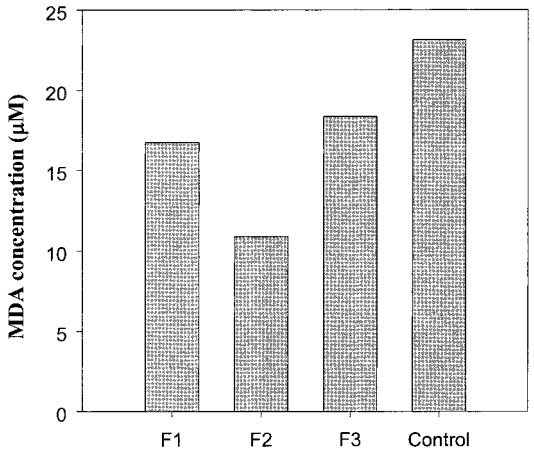

Figure 2. Synergistic effects of gelatin hydrolysates from Alaska pollack skin on the antioxidative activity of nonpeptidic antioxidant, α-tocopherol. Samples (25 mg) and α-tocopherol (25 mg) dissolved in 487 μL of distilled water, 13 μL of linoleic acid, 1 mL of ethanol, and 1 mL of 50 mM phosphate buffer (pH 7.0) were mixed in glass tube. The tubes were sealed tightly with silicon rubber caps and kept at 40 °C in the dark. After incubation for 6 days, the reaction mixtures (50 μL) were withdrawn and then the antioxidative activity was determined as described in the methods section. Control shows only the antioxidative activity of α-tocopherol. F1 denotes the first-step enzymatic hydrolysate; F2, the second-step enzymatic hydrolysate; F3, the third-step enzymatic hydrolysate.

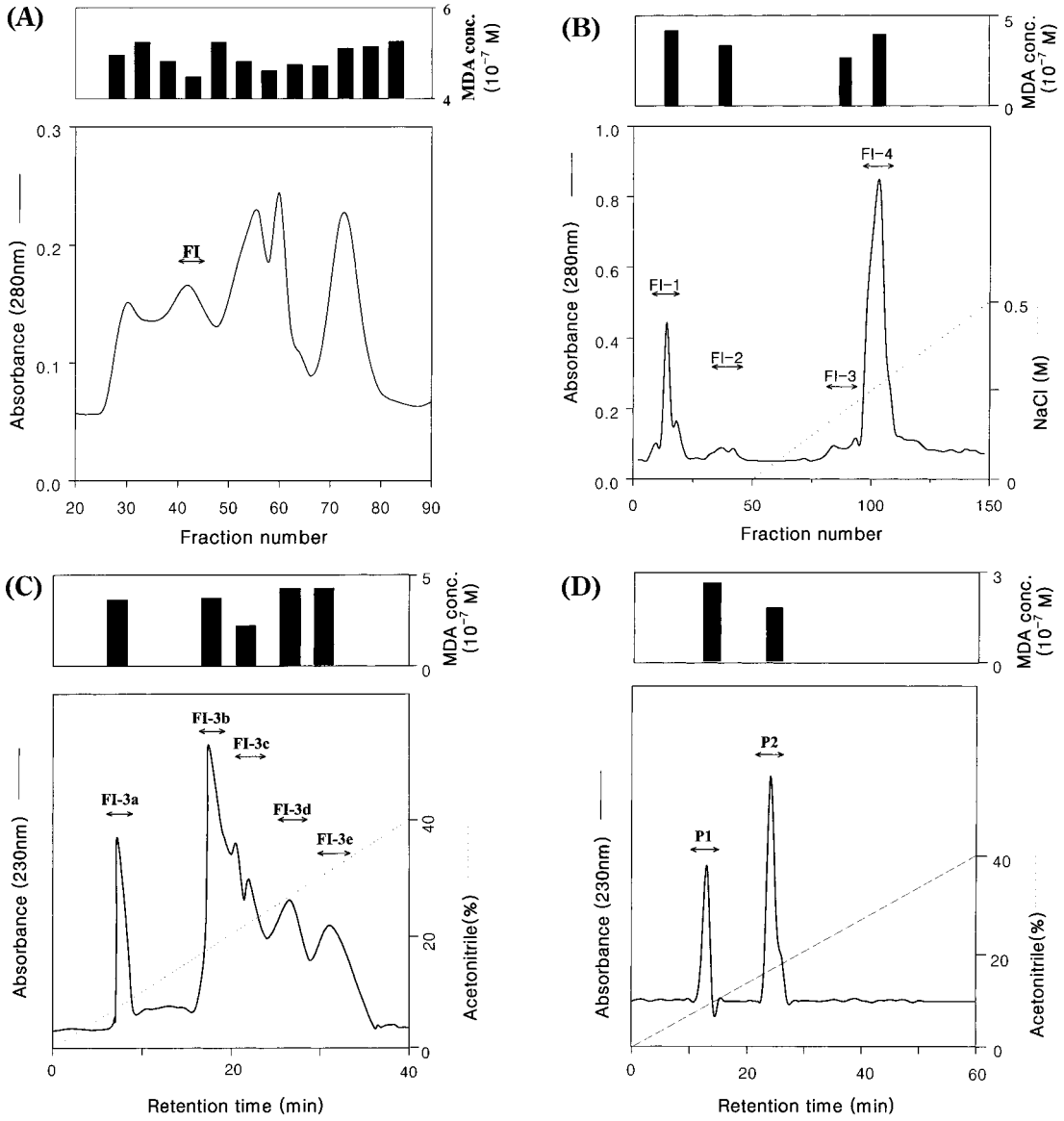

Figure 3. Purification of antioxidative peptides from the second hydrolysate of Alaska pollack skin gelatin. (A) Sephadex G-25 chromatography. The second hydrolysate was applied to a column (2.5 × 90 cm) of Sephadex G-25. The column was eluted with 50 mM sodium phosphate buffer (pH 7.0) at a flow rate of 30 mL/h. Fractions showing antioxidative activity were indicated by a solid bar and pooled. (B) SP−Sephadex C-25 chromatography. The active fractions from Sephadex G-25 were applied to a column (3 × 40 cm) of SP−Sephadex C-25 and washed with 20 mM sodium phosphate buffer (pH 4.0). The column was eluted with a linear gradient of NaCl concentration at a flow rate of 30 mL/h. Fractions indicated by solid line were pooled. (C) First HPLC chromatography. Active fractions from SP−Sephadex C-25 were applied to a C_{18}−HPLC column (1 × 25 cm) equilibrated with 0.1% TFA in H_2O and eluted with a linear gradient of 0.1% TFA in acetonitrile at a flow rate of 1 mL/min. The fractions under the bar were pooled. (D) Second HPLC chromatography. Pooled fractions from the first C_{18}−HPLC column were reapplied to a C_{18}−HPLC column (1 × 25 cm) and eluted with a linear gradient of acetonitrile containing 10 mM ammonium acetate at a flow rate of 1 mL/min. Each peak was assayed for antioxidative activity and designated as P1 or P2 according to the order of elution time of peaks. —, protein (lower panel); ■, antioxidative activity (upper panel).

4 h of incubation at 37 °C, dimethyl sulfoxide (DMSO)/ethanol (1:1) reagent (150 μL) was added to dissolve the formazan crystals. The plates were shaken at 37 °C for 20 min and the absorbance was then read at 570 nm using an ELISA reader (Behring Co., Marburg, Germany).

Other Analytical Methods. The antioxidative peptides were hydrolyzed in 6 N HCl containing 1% phenol and under a vacuum at 110 °C for 24 h. The amino acid analysis of the hydrolyzates was achieved using an automatic amino acid analyzer (PICO-TAG; Waters Co., Milford, MA). Edman degradation with an automated protein sequencer (Perkin-Elmer model 491, Branchburg, NJ) equipped with on-line HPLC was used for the analysis of the amino acid sequence of antioxidative peptides. The fast atom bombardment mass spectrometry (FABMS) was performed using a JEOL JMS-DX705 mass spectrometer (Shimadzu, Tokyo, Japan).

RESULTS AND DISCUSSION

Preparation and Characterization of Gelatin Hydrolysates. Gelatin hydrolysates from Alaska pollack skin were prepared with consecutive digestions with Alcalase, Pronase E, and collagenase, using a three-step recycling membrane reactor, as described in the previous section. Three different kinds of proteases hydrolyzed the gelatin at the optimum condition. The hydrolysates were fractionated on the basis of their molecular weight by ultrafiltration at each step. The molecular size distribution of gelatin hydrolysates was measured by HPLC on a GPC column (data not shown). The chromatograms obtained from the gel permeation column showed that major peaks of the first, second,

and third hydrolysates were located at 6−8, 2−4, and 0.5−2 kDa, respectively. Amino acid compositions of the gelatin and three different hydrolysates were also determined (data not shown). The amino acid composition of the gelatin from Alaska pollack skin was nearly identical with the that of the hydrolysates. They are rich in glycine (33%), alanine (11%), proline (13%), and hydroxyproline (10%), but poor in cysteine, histidine, methione, and tyrosine. The amino acid compositions were similar to those of other gelatins from various sources (*16*, *20*).

Antioxidative Activities of Gelatin Hydrolysates. The antioxidative activities of the gelatin hydrolysates from Alaska pollack skin were measured and compared with those of α-tocopherol and BHT. As shown in Figure 1, the oxidative activity of linoleic acid was markedly inhibited by the addition of the hydrolysates from the gelatin. Among the three hydrolysates, the highest antioxidative activity was observed in the second-step hydrolysate, which exhibited about 58% inhibition of linoleic acid peroxidation. The antioxidative activity of the second hydrolysate was similar to that of α-tocopherol. The first and third hydrolysates also inhibited to approximately 40% of the oxidation. These results, therefore, indicate that the hydolysates of Alaska pollack skin gelatin seemed to contain some antioxidative peptides.

Synergistic Antioxidative Effect of Gelatin Hydrolysates. The synergistic antioxidative effects of the gelatin hydrolysates from Alaska pollack skin with the nonpeptidic antioxidant α-tocopherol have been studied. As shown in Figure 2, all hydrolysates of the gelatin exhibited synergistic effects on combined use with α-tocopherol. The second hydrolysate that possessed high antioxidative activity showed high synergistic effect with α-tocopherol. The synergistic effects of nonpeptidic antioxidants on the antioxidative activity had been previously demonstrated with the hydrolysates of a vegetable protein, yeast protein, and bovine serum albumin (*21*, *22*). Soybean protein hydolysates were also shown to be synergistic with α-tocopherol. Although some hydrolysates of soybean protein had very litte antioxidative activity, the hydrolysates showed a strong synergistic effect with nonpeptidic antioxidants (*23*). However, the hydolysates of Alaska pollack skin have both antioxidative activity and synergistic effect with α-tocopherol using the linoleic acid in water/alcohol system. Therefore, we focused on isolation and structural characterization of potent antioxidative peptides from the second hydrolysate of Alaska pollack skin gelatin that had the highest antioxidative activity.

Identification of Purified Peptides. To identify the antioxidative peptides, the second-step hydrolysate of Alaska pollack skin with the highest antioxidative activity was separated by size-exclusion chromatography on a Sephadex G-25 column and fractionated to five fractions (Figure 3A). Fraction I (FI) exhibited the strongest antioxidative activity, although antioxidative activity was widely observed for all fractions. The lyophilized Fraction I was subjected to cation-exchange chromatography on a SP-Sephadex C-25 column and fractionated to four fractions (Figure 3B). When these fractions were tested for antioxidative activity, fraction FI-3 was found to possess strong activity, and this fraction was further separated by reversed-phase HPLC using 0.1% TFA−acetonitrile system and fractionated to five fractions (Figure 3C). The fraction (FI-3c) was further separated by reversed-phase HPLC using acetonitrile containing 10 mM ammonium acetate system (Figure 3D). Two antioxidative peptides (P1and P2) were finally obtained from the second-step hydrolysate of Alaska pollack skin gelatin.

Table 1. Amino Acid Compositions of Alaska Pollack Skin Gelatin and the Hydrolysates

amino acid	gelatin	1st hydrolysate	2nd hydrolysate	3rd hydrolysate
Asp	3.59	2.83	3.50	3.05
Ser	5.74	6.20	5.68	5.78
Glu	5.94	5.04	5.65	5.13
Gly	39.89	40.97	10.54	41.47
His	1.75	0.00	0.00	0.00
Arg	5.66	6.92	5.96	6.38
Thr	2.49	2.97	2.30	2.45
Ala	9.41	9.45	10.10	9.47
Pro	9.38	9.96	9.75	9.73
Tyr	0.00	0.00	0.00	0.00
Val	1.78	1.88	1.93	1.90
Met	1.43	0.36	1.69	1.72
Lys	1.96	1.56	1.87	1.55
Ile	1.20	1.23	1.02	1.01
Leu	1.99	2.03	1.99	1.98
Phe	1.36	1.69	1.76	2.05
Hyp	6.44	6.91	6.25	6.34

The amino acid sequences of the peptides (P1 and P2) are shown in Table 1. The two isolated antioxidative peptides were composed of 16 and 13 amino acid residues, respectively. The sequence of P1 (Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly) was found to be the same sequence as P2 (Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly), except for the additional three amino acid residues, Gly-Glu-Hyp, at the N-terminus of P1. The sequences of the peptides are in good agreement with the data from their amino acid compositions and molecular weights (data not shown). Furthermore, the isolated peptides contain the specific sequence (Gly-X-Hyp; X, some amino acid) for amino acid sequences of collagen.

Antioxidative Activities of Purified Peptides. The antioxidative activity of the purified peptides in a water/alcohol system was examined by the TBA method as shown in Figure 3D. P2 showed high antioxidative activity and was more effective than P1. The sequence of P2 was almost identical to that of P1 except for the three additional amino acid residues at the N-terminal. Therefore, the difference of antioxidative activity between the two peptides isolated in this study is thought to be attributable to only three amino acid residues (Gly-Glu-Hyp) at N-terninus of the peptides. On the other hand, several amino acids, such as Tyr, Met, His, Lys, and Trp, are generally accepted as antioxidants despite their pro-oxidative effects in some cases (*24*−*26*). It has been known that many antioxidative peptides included hydrophobic amino acid residue, Val, or Leu at the N-terminus of the peptides (*15*, *27*). Both of the peptides isolated in this study mainly constituted Gly, Pro, and Hyp (Table 1). These results indicate that the antioxidative activity of the peptides isolated from gelatin hydrolysate of Alaska pollack skin depends on their amino acid sequences.

The antioxidant effect of P2 isolated from gelatin hydrolysate was also investigated in vitro using Donryu rat liver cells (Figure 4). The cells were preincubated with different concentrations of P2 for 24 h, washed, and then exposed to *t*-BHP for 150 min. Measuring cell viability with methylthiazol tetrazolium (MTT) assay assessed cell injury. When the cells were pretreated

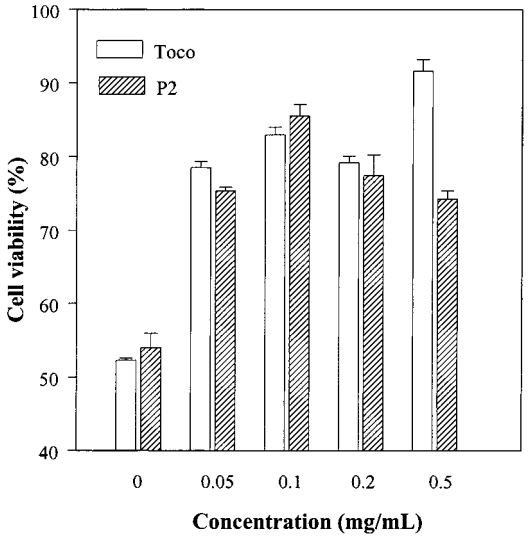

Figure 4. Effect of the purified peptide (P2) from gelatin hydrolysate of Alaska pollack skin on cell viability. Donryu rat liver cells were prepared and incubated with the purified peptide (P2) or α-tocopherol, respectively, at various concentrations. After 17 h, cells were placed in DMED medium without serum and then incubated with 1 mM *t*-BHP for 2.5 h. MTT assay was used to measure cell viability. Values are given as the mean ± standard error. P2, purified peptide from gelatine hydrolysate of Alaska pollack skin; Toco, α-tocopherol.

without the peptide, P2, 53% of the cells were killed within 150 min by 1 mM *t*-BHT (data not shown). Preincubation of the cells with P2 (0.5 mg/mL) before *t*-BHP exposure significantly increased the result to 25% cell viability. However, P2 exhibited its maximum antioxidative effect at 1 mg/mL dosage, which then decreased with increasing concentration. Although the protection of cell injury of P2 was similar to that of α-tocopherol, the viability of the cells was enhanced with increasing concentration of α-tocopherol. These results show that P2 can protect Donryu rat liver cells from oxidant injury by *t*-BHT. Therefore, it is concluded that the purified peptide, P2, of the gelatin hydrolysate from Alaska pollack skin is a natural antioxidant which has potent antioxidative activity.

ABBREVIATIONS USED

BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DPPH, diphenylpicrylhydrazyl; MTT, (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TBA, 2-thiobarbituric acid; TMP, tetramethoxypropane.

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