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Purification and Characterization of Antioxidative Peptides from Bovine Skin

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To identify the antioxidative peptides in the gelatin hydrolysate of bovine skin, the gelatin was hydrolyzed with serial digestions in the order of Alcalase, pronase E, and collagenase using a three-step recycling membrane reactor. The second enzymatic hydrolysate (hydrolyzed with pronase E) was composed of peptides ranging from 1.5 to 4.5 kDa, and showed the highest antioxidative activity, as determined by the thiobarbituric acid method. Three different peptides were purified from the second hydrolysate using consecutive chromatographic methods. This included 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 octadecylsilane chloride column. The isolated peptides were composed of 9 or 10 amino acid residues. They are: Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Ala-Hyp (PI), Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly (PII), and Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp (PIII), as characterized by Edman degradation and fast-atom bombardment mass spectrometry. The antioxidative activities of the purified peptides were measured using the thiobarbituric acid method, and the cell viability with a methylthiazol tetrazolium assay. The results showed that PII had potent antioxidative activity on peroxidation of linoleic acid. Moreover, the cell viability of cultured liver cells was significantly enhanced by the addition of the peptide. These results suggest that the purified peptide, PII, from the gelatin hydrolysate of bovine skin is a natural antioxidant, which has potent antioxidative activity.

Keywords: Antioxidant peptide, Bovine skin, Characterization, Purification.

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Introduction

The antioxidant is defined as any substrate that significantly delays, or inhibits oxidation of that substrate, when present at low concentrations, compared to that of an oxidizable substrate (Halliwell *et al.*, 1995). Food manufacturers have used food-grade antioxidants to prevent quality deterioration of products and to maintain their nutritional value. Antioxidants have also been of interest to biochemists and health professionals, because they may help the body protect itself against damage caused by reactive oxygen species and degenerative diseases.

Many synthetic antioxidants and natural antioxidants from various sources have been reported. However, synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are suspected of causing some safety concerns (Imaida *et al.*, 1983; Becker, 1993). Therefore, research has focused on the development and utilization of antioxidants from natural sources. There have been a number of studies on the antioxidative activity of many proteins, such as alumin (Halliwell and Gutteridge, 1990a, b), transferrins and ferritins (Law *et al.*, 1992; Gutteridge and Quinlan, 1993), metallothioneins (Halliwell and Gutteridge, 1990a), ceruloplasmin (Samolyszyn *et al.*, 1989), heat shock proteins (Schlesinger, 1990; Donnelly *et al.*, 1992), and antioxidant enzymes (Ahmad *et al.*, 1990; Koh *et al.*, 1999; Baek *et al.*, 2000). In addition, some amino acids were also reported to be antioxidant against linoleic acid oxidation in a freeze-dried emulsion (Gopala and Prabhakar, 1994). Chen *et al.* (1995) reported on antioxidative peptides composed of 5 to 16 amino acid residues from a soybean protein hydrolysate. Recently, we also reported that the enzymatic hydrolysate from the skin of yellowfin sole has potent antioxidative activity (Kim *et al.*, 1996). However, little is known about the structural information on antioxidative peptides from various food proteins.

In this study, we investigated the antioxidative effects of enzymatic hydrolysates from bovine skin gelatin with three

different proteases, using a three-step recycling membrane reactor. Three antioxidative peptides were isolated from the hydolysate obtained with pronase E, and their amino acid sequences were determined. We also investigated the antioxidative activity of purified peptides in comparison with commercial antioxidant, α-tocopherol, by determination of cell viability with a methylthiazol tetrazolium (MTT) assay.

Materials and Methods

Materials The bovine skin gelatin was purchased from the Kyunggi Gelatin Co. (Yong-In, Korea). Alcalase (0.6 L, s.g. = 1.25) was obtained from the Novo Co. (Novo Nordisk, Bagsvaerd, Denmark), and collagenase (335 units/mg) and pronase E (from *Streptomyces griseus*, Type XIV, 4.4 U/mg-solid) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). BHT and tetramethoxypropane (TMP) were from the Fluka Co. (Fluka Chimie AG, Switzerland). Sephadex G-25, SP-Sephadex C-25, 2-thiobarbituric acid (TBA), *t*-butyl hydroperoxide, ammonium thiocyanate, and linoleic acid were purchased from the Sigma Chemical Co.. All other reagents used were of the highest grade available commercially.

Preparation of hydrolysates and Measurement of antioxidative activity The preparation of hydrolysates from bovine skin gelatin was performed according to a previous report (Kim and Byun, 1994), and the hydrolysates were then lyophilized and stored at -20°C until used. Each sample (2 mg) was dissolved in distilled water (4.87 ml) and mixed with linoleic acid (0.13 ml), ethanol (10 ml), and a 50 mM phosphate buffer (pH 7.0, 10 ml). The α-tocopherol and BHT (2 mg) were dissolved in ethanol. The mixed solution in a conical flask was placed in darkness at 40°C to accelerate oxidation. The peroxide value was measured using a modified version of the method of Ohkawa *et al.* (1979). 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 a 0.8% TBA solution in water. The mixture was incubated at 5°C for 1 h, and then heated at 95°C for 1 h. The absorbance of the mixture was read at 532 nm and the antioxidant activity was expressed as malondialdehyde (MDA) concentrations using TMP as the standard.

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 a 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 5 ml of a 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, USA) previously equilibrated with a 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 reverse-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; Sigma Chemical Co., St. Louis, MO, USA) 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 (Falcon Co., Paigton, Devon, UK) 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 a 75-cm^2 tissue culture flask by trypsinization, counted, and planted 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 the wells, cells were washed with a 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, a MTT assay of cell viability was carried out as described by Sladowski *et al.* (1993). After a 2.5 h incubation with *t*-BHP, the cells in the 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 4 h incubation at 37°C, a dimethyl sulfoxide/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 Edman degradation with an automated protein sequencer (Perkin-Elmer Model 491, Applied Biosystem Division, Branchburg, NJ, USA) equipped with on-line HPLC was used for the analysis of the amino acid sequence of antioxidative peptides. The fast atom bombardment mass spectrometry (FAB-MS) was performed using a JEOL JMS-DX705 mass spectrometer (Shimadzu, Tokyo, Japan).

Results and Discussion

Preparation and characterization of gelatin hydrolysates Gelatin hydrolysates from bovine skin were prepared with consecutive digestions with Alcalase, pronase E, and collagenase, using a three-step recycling membrane reactor. 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. The chromatograms obtained from

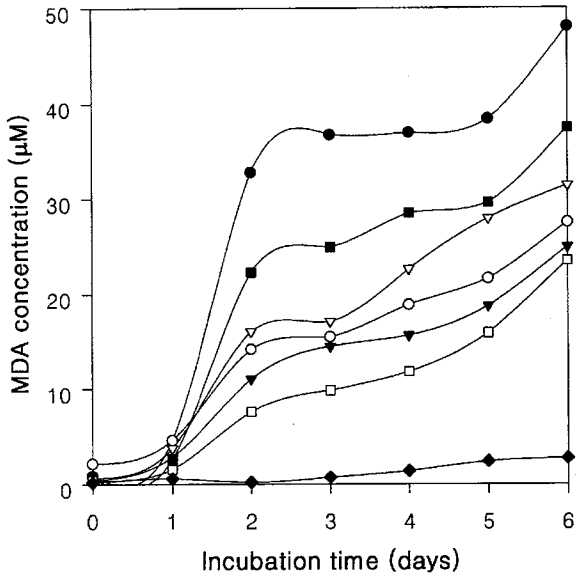

Fig. 1. Antioxidative activities of hydrolysates from bovine skin gelatin. The activity was evaluated by the TBA method, as described in the Methods Section. Control contained distilled water alone instead of sample. ●, control; ○, the first-step enzymatic hydrolysate; ❑, the second-step enzymatic hydrolysate; ▽, the third-step enzymatic hydrolysate; ■, gelatin; ▼, α-tocopherol; ◆, butylated hydroxytoluene (BHT). Values in this figure are the average of triplicates.

the gel permeation column showed that major peaks of the first, second, and third hydrolysates were located at 6.5-7.5, 1.5-4.5, and 0.5-2.0 kDa, respectively (data not shown).

The antioxidative activities of the hydrolysates from bovine skin on the peroxidation of linoleic acid are shown in Fig. 1. In general, the oxidative activity of linoleic acid was markedly inhibited by the addition of the hydrolysates of the bovine skin gelatin compared with the control assay. Among the three hydrolysates, the highest antioxidant activity was observed in the second hydolysate, which exhibited about a 50% inhibition of linoleic acid peroxidation. The antioxidative activity of the 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 the bovine skin gelatin seemed to contain some antioxidative peptides.

Identification of purified peptides To identify the antioxidative peptides, the second-step hydrolysate of bovine gelatin, having the highest antioxidative activity, was separated by size exclusion chromatography on a Sephadex G-25 column and fractionated. Fig. 2a shows the elution profile of the hydrolysate on a Sephade G-25 column, indicating four peaks. When these fractions were tested for antioxidant activity, fraction FI exhibited the strongest antioxidative activity, although the activity scattered all of the fractions. The lyophilized FI was subjected to cation exchange chromatography on a SP-Sephadex C-25 column and fractionated to five fractions (Fig. 2b). When these fractions were tested for activity, the third fraction (FI-3) possessed strong activity; this fraction was further separated by C_{18} -reversed phase HPLC using 0.1% TFA-acetonitrile system. As illustrated in Fig. 2c, the elutes containing each major peak were divided into five fractions, FI-3a~FI-3e. Fraction FI-3c possessed the highest antioxidative activity. It was further separated by C_{18} -reversed phase HPLC column using acetonitrile containing 10 mM ammonium acetate system, and three fractions (PI, PII, and PIII) were obtained as shown in Fig. 2d. Considering their relative amounts, PII and PIII are considered to be major antioxidative peptides, and PI to be a minor one.

The amino acid sequences of the peptides (PI, PII and PIII) are shown in Table 1. The three purified antioxidative peptides were composed of 9 or 10 amino acid residues, respectively. The sequences of the purified peptides were as follow: PI, Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Ala-Hyp; PII, Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly; PIII, Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp. The sequences of the antioxidative peptides agree with the data from their amino acid compositions and mass spectrometry (Table 1). Furthermore, the isolated peptides contain the specific sequence (Gly-X-Hyp; X, some amino acid) in the 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 test (Fig. 2d.) The PII showed the highest antioxidative activity, and was more effective than PI and PIII. The antioxidative activity of the three purified peptides from bovine hydrolysate decreased in the order PII>PI>PIII. The sequence of PII was similar to those of PI and PIII, except for a few amino acid residues (Table 1.) PII had the same sequence as PIII, except for the addition of the C-terminal glycine. PI also had a sequence common to the sequence of PIII, except for two amino acid residues (Glu and Ala). Therefore, the most important difference of antioxidative activity between the peptides (PI, PII and PIII) is thought to be attributable to an amino acid residue, Gly, at the C-terminus of PII. On the other hand, all purified antioxidative peptides contain three hydroxyproline and three proline residues that are partly responsible for the antioxidant activity, although it was reported that prolyl polypeptides are sensitive to oxidation (Uchida *et al.*, 1992). Previously, many proteins were reported to have strong antioxidative activity against the peroxidation of lipid or fatty acids upon hydrolysis (Yamaguchi *et al.*, 1975; Bishov and Henick, 1972, 1975). Six antioxidative peptide fragments (5-16 amino acid residues) were isolated from the hydrolysates of soybean protein, β-conglycinin (Chen *et al.*, 1995).

The antioxidant effect of PII, isolated from gelatin hydrolysate, was further studied by using an *in vivo* model. The cell was preincubated with different concentrations of PII for 24 h, washed, and then exposed to *t*-BHP for 150 min.

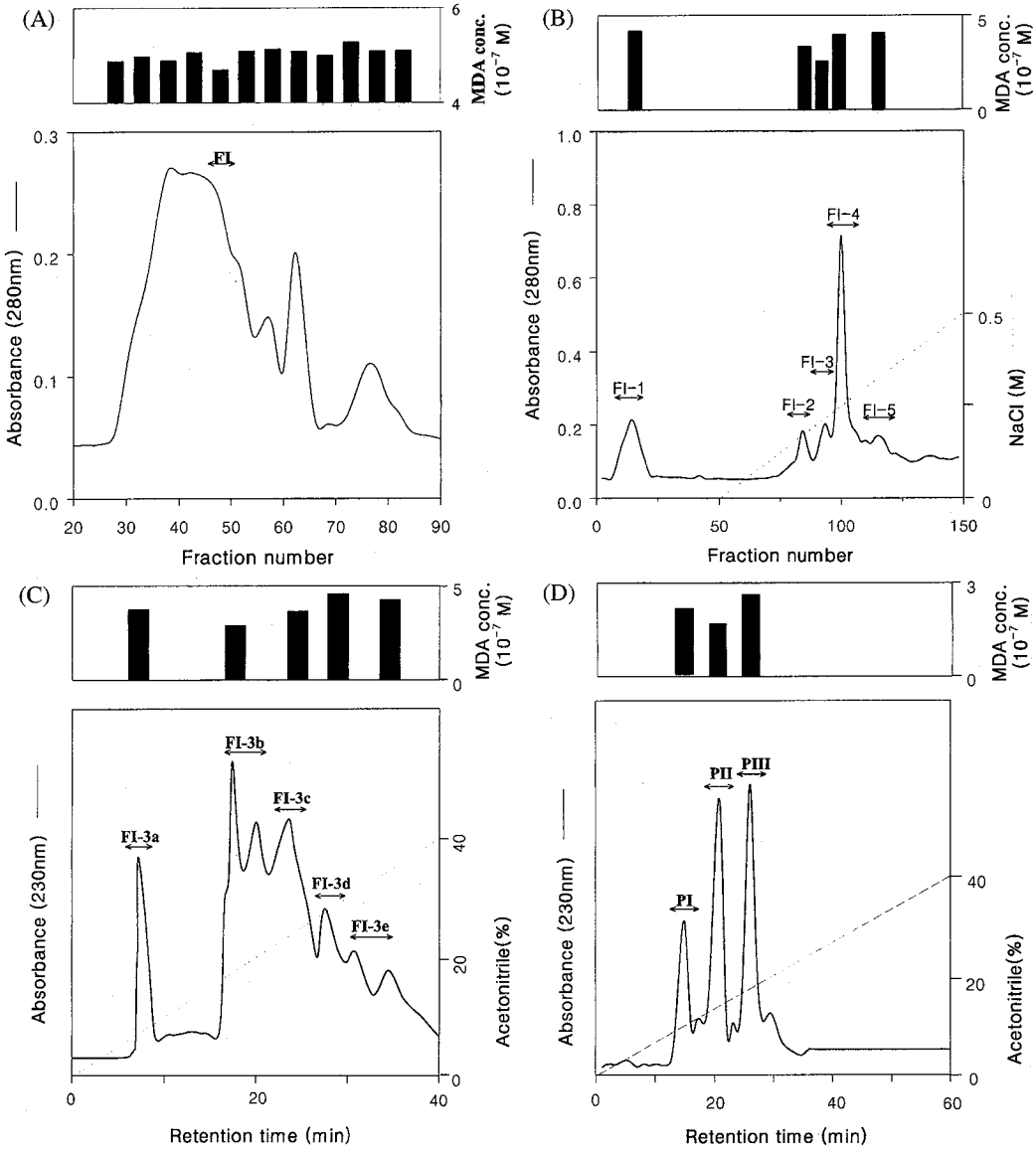

Fig. 2. Purification of antioxidative peptides from second hydrolysate of bovine skin gelatin. (A) Sephadex G-25 chromatography. The second hydrolysate of bovine skin gelatin was applied to a column (2.5 × 90 cm) of Sephadex G-25. The column was eluted with 50 mM sodium phosphate buffer (pH 7.0) at a 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. Fraction indicated solid line were pooled. (C) 1st HPLC chromatography. Active fractions from SP-Sephadex C-25 were applied to a C_{18}-HPLC column (1 × 25 cm) equrilibrated 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) 2nd HPLC chromatography. Pooled fractions from the 1st C_{18}-HPLC column were reapplied to a C_{18}-HPLC column (1 × 25 cm) and eluted with a linear gradient of acetonitrile cantaining 10 mM ammonium acetate at a flow rate of 1 ml/min. Each peak was assayed for antioxidative activity and designated as PI, PII, and PIII according to the order of elution time of peaks. —, peptide (lower panel); ■, antioxidative activity (upper panel).

Measuring the cell viability with a MTT assay assessed the cell injury. As shown in Fig. 4, *t*-BHT (1 mM) caused a decrease of cell viability by 48%. On the other hand, preincubation of the cell with PII (up to 0.5 mg/ml) before *t*-BHP exposure significantly increased approximately 30% of cell viability. When the peptide was compared with α-tocopherol, the protection of cell injury of PII was similar to that of α-tocopherol. These results indicate that PII can protect Donryu rat liver cells from lipid peroxidation induced by *t*-BHT.

Two mechanisms have been reported to explain the killing of hepatocytes by *t*BHP. One mechanism is related to the

Table 1. Amino acid sequences and molecular masses of identified antioxidative peptides

Peptide	Amino acid sequence	FAB-MS (*m/z*)
PI	Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Ala-Hyp	826(M+H)$^+$
PII	Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly	877(M+H)$^+$
PIII	Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp	820(M+H)$^+$

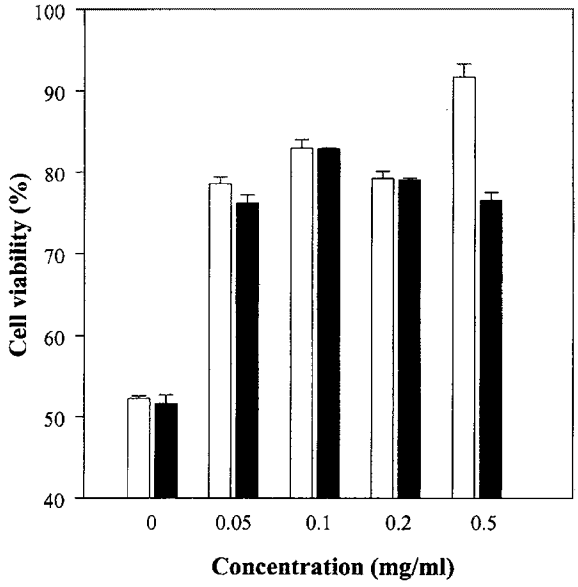

Fig. 3. Effect of the purified peptide (PII) from hydrolysate of bovine skin gelatin on cell viability. Donryu rat liver cells were prepared and incubated with the purified peptide (PII) or α-tocopherol, respectively, at various concentrations. After 17 h, cells were placed in a DMED medium without serum, then incubated with 1 mM *t*-BHP for 2.5 h. A MTT assay was used to measure cell viability. Values are given as the mean± standard error. ❑, purified peptide (PII) from hydrolysate of bovine skin gelatin; ■, α-tocopherol.

peroxidation of cellular membranes (Masaki *et al.*, 1989a), and the second mechanism of cell killing occurs in the absence of lipid peroxidation, and is associated with the loss of the mitochondrial function (Masaki *et al.*, 1989b). Glascott *et al.* (1992) reported that cells treated with an antioxidant, N,N'-diphenyl-p-phenylenediamine (DPPD), exhibited lower cell killing than cells not treated with DPPD.

In conclusion, we purified antioxidative peptides from the gelatin hydrolysates of bovine skin by using consecutive chromatographic methods. The sequences of the isolated antioxidative peptides were as follow: PI, Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Ala-Hyp (MDA concentration, 2.26×10^{-7} M); PII, Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-hyp-Gly (MDA concentration, 1.63×10^{-7} M), and PIII, Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp (MDA concentration, 2.78×10^{-7} M). The peptides isolated in this study had three hydroxyproline and three proline residues. The amino acid residues should play important roles in the antioxidative activity. These results of the antioxidative activities of the purified peptides on the TBA method and cell viability with MTT assay suggest that the antioxidative activity of PII is superior to that of PI and PIII, and the protection of cell injury of PII is similar to that of α-tocoperol. Thus, it is concluded that the purified peptide, PII, from the hydrolysate of bovine skin is a natural antioxidant that has potent antioxidative activity.

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