Purification and Characterization of Antioxidative Peptides from Protein Hydrolysate of Lecithin-Free Egg Yolk

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ABSTRACT: The protein extracted from lecithin-free egg yolk, normally discarded by lecithin processing plants, was hydrolyzed with the aid of Alcalase, a commercial enzyme. The hydrolysate was separated through a series of ultrafiltration membranes with molecular weight cutoffs of 10, 5, and 1 kDa; and three types of permeates including 10 K (permeate from 10 kDa), 5 K (permeate from 5 kDa), and 1 K (permeate from 1 kDa) were obtained. The antioxidative efficacy of hydrolysates so obtained was investigated and compared with α-tocopherol. Furthermore, two different peptides showing strong antioxidative activity were isolated from the hydrolysates by using consecutive chromatographic methods including ion exchange chromatography on a SP-Sephadex C-25 column, gel filtration on a Sephadex G-25 column, and high-performance liquid chromatography on an octadecylsilane column. The purity of the peptides was identified using capillary electrophoresis. The isolated peptides were composed of 10 and 15 amino acid residues, and both contained a leucine residue at their N-terminal positions.

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The term antioxidant is defined as any substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate. Antioxidants can act at different levels in an oxidative sequence. This may be illustrated by considering one of the many mechanisms by which oxidative stress can cause damage by stimulating the free radical chain reaction of lipid peroxidation. Free radical chain reactions within a material may be inhibited either by adding chemicals that retard the formation of free radicals (preventive antioxidants) or by introducing substances that compete for the existing radicals and remove them from the reaction medium (chain-breaking antioxidants).

Lipid oxidation is of great concern to the food industry and consumers because it leads to the development of undesirable off-flavors and potentially toxic reaction products (1). Synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, *tertiary*-butylhydroquinone, and propyl gallate may be added to food products to retard lipid oxidation (2). However, use of synthetic antioxidants in food products is under strict regulation due to the potential health hazards caused by such compounds (3). Therefore, search for natural antioxidants as alternatives to synthetic ones is of great interest among researchers. Several studies have described the antioxidative activity of proteins such as milk casein (4), soy protein (5), bovine serum albumin (6), oil seed protein (7), wheat gliadin (8), beach pea (9), evening primrose (10) and maize zein (11). Amino acids have also been reported to exhibit antioxidant activity against linoleic acid oxidation in a freeze-dried emulsion (12). Recently, six antioxidative peptides were isolated from the hydrolysate of a soybean protein, β-conglycinin (13). These peptides were composed of 5 to 16 amino acid residues and included hydrophobic amino acids, Val and Leu, at the N-terminus and Pro, His, or Tyr in their sequences. However, little is known about the structure of antioxidative peptides from various food proteins.

In this study, we examined the antioxidative effect of enzymatic hydrolysate of a lecithin-free egg yolk protein (EYP), a by-product of lecithin extraction from egg yolk. Two antioxidative peptides were isolated from the hydrolysate so obtained, and their amino acid sequences were determined.

MATERIALS AND METHODS

Materials. Lecithin-free EYP (protein content, 76%) was donated by Doosan Co. (Yongin, Korea). Alcalase (0.6 AU/g) was acquired from Novo Co. (Novo Nordisk, Bagsvaerd, Denmark); and 2-thiobarbituric acid (TBA), linoleic acid, α-tocopherol, ammonium thiocyanate, SP-Sephadex C-25, and Sephadex G-25 were purchased from Sigma Chemical Co. (St. Louis, MO). The ultrafiltration system (Minitan^{TM} System) and membranes for the fractionations of each hydrolysate were purchased from Millipore Co. (Bedford, MA). Fetal bovine serum (FBS), antibiotic/antimycotics were obtained from Gibco Co. (Gibco BRL, Grand Island, NY), and malondialdehyde (MDA) was purchased from Fluka Co. (Fluka Chimie AG, Buchs, Switzerland). Dulbecco's modified Eagle's medium (DMEM), *tert*-butyl hydroperoxide (*t*BHP) and 1,1,3,3-tetraethoxypropane (TEP) were also acquired from Sigma Chemical Co.

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Preparation of lecithin-free EYP hydrolysates (EYPH). The hydrolysis of lecithin-free EYP (100 g) was performed according to a method described previously by Park *et al.* (14). EYP (1%, wt/vol) was prepared in 10 L of 0.1 M disodium hydrogen phosphate-sodium dihydrogen phosphate buffer (pH 7.0) and digested with Alcalase for 18 h at 50°C. The resulting solution was first passed through a 10 kDa molecular weight cutoff (10 kDa MWCO) membrane. A portion of the solution was removed immediately, and the solution was then pumped sequentially through 5 and 1 kDa membranes. The respective permeates passed through each membrane, 10 K hydrolysate (permeate from 10 kDa MWCO membrane), 5 K hydrolysate (permeate from 5 kDa MWCO membrane) and 1 K hydrolysate (permeate from 1 kDa MWCO membrane), were lyophilized and then stored at −20°C until use.

Molecular weight distribution profile. Molecular weight distributions of the hydrolysates were determined by gel permeation chromatography (GPC) using a high-performance liquid chromatography (HPLC) system (Hewlett-Packard, Palo Alto, CA). Two GPC columns, Zorbax PSM 300 and 60 (Hewlett-Packard), with exclusion limits of 3×10^3–3×10^5 Da (6.2 µm, 25 × 4.6 cm) and 110–10^3 Da (6.2 µm, 25 × 4.6 cm), were connected in series, and the hydrolysates were chromatographed and monitored at 230 nm at room temperature.

Measurement of antioxidative activity. Antioxidative activity of EYPH was measured in a linoleic acid model system according to the methods of Osawa *et al.* (15). The sample (1.3 mg) was dissolved of 10 mL of 50 mM phosphate buffer (pH 7.0) and added to a mixed solution that consisted of 0.13 mL of linoleic acid and 10 mL of 99.5% ethanol. Then the total volume was adjusted to 25 mL with distilled water. The mixed solution in a conical flask with screw cap was incubated at 40 ± 1°C in a dark room, and the degree of oxidation was evaluated by measuring the TBA value and peroxide value (PV). The TBA value was measured using a modified version of the method of Ohkawa *et al.* (16). 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 adjusted to pH 3.5 using 10 N NaOH, and 1.5 mL of 0.8% TBA solution. The mixture was incubated at 5°C for 1 h, and then heated at 95°C for 1 h in the dark. The TBA value was measured by reading the absorbance at 532 nm. The PV was measured according to the method of Mitsuda *et al.* (17). The reaction solution (100 µL) incubated in a linoleic acid model system described above (15) was mixed with 4.7 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 2×10^{-2} M ferrous chloride solution in 3.5% HCl. After 3 min, the PV was measured by reading the absorbance at 500 nm after color development with $FeCl_2$ and thiocyanate at different intervals at 40 ± 1°C during the incubation period. All analyses were run in triplicate and averaged.

Isolation of the antioxidative peptides and determination of amino acid sequences. The lyophilized 5 K hydrolysate was dissolved in 20 mM sodium acetate buffer (pH 4.0) and fractionated by ion exchange chromatography on a SP-Sephadex C-25 column (4 × 40 cm) equilibrated and eluted with a linear gradient of NaCl concentrations from 0 to 1.0 M. Fractions of 3 mL were collected at a flow rate of 120 mL/h. The fractions showing antioxidative activity were pooled and lyophilized. The lyophilized fraction was dissolved in 50 mM sodium phosphate buffer (pH 7.0) and loaded onto a Sephadex G-25 gel filtration column (2.5 × 90 cm) which had previously been equilibrated with the same buffer. The column was then eluted with the same buffer, and the fractions exhibiting antioxidative activity were pooled and lyophilized. The antioxidative fraction was dissolved in distilled water and separated using reversed-phase HPLC on Primesphere 10 C_{18}-HC 120 (10 µm, 1.0 × 25 cm; Phenomenex, Macclesfield, United Kingdom) column using a linear gradient of acetonitrile (0–50% in 60 min) in 0.1% trifluoroacetic acid (TFA) at 2.0 mL/min. The elution peaks were monitored at 215 nm, and their antioxidative activities were measured using the same method. The active peaks were concentrated using a centrifugal evaporator. The peaks representing antioxidatively active compounds were rechromatographed on the same column using a linear gradient of acetonitrile (0–30% in 40 min) in 0.1% TFA at a flow rate of 2.0 mL/min. The purity was established using capillary electrophoresis (Bio-Rad Co., Cambridge, MA), and the sequences of peptides were determined by automated Edman degradation with PerkinElmer 491 protein sequencer (Branchburg, NJ).

Lipid peroxidation in cultured human liver cells. Normal human hepatocytes (Chang cell line) were obtained from the Japan Health Sciences Foundation (JHSF, Tokyo, Japan). The hepatocytes were maintained on 75-cm^2 plastic flasks (Falcon Co., Paigton, Devon, United Kingdom), at a range of 10^6 to 10^7 cells/flask, in 15 mL of DMEM containing 4.4 µL/mL antibiotic-antimycotic (10,000 units/mL penicillin G sodium, 10,000 µg/mL streptomycin sulfate, and 25 µg/mL amphotericin B as Fungizone R in 0.85% saline), 150 µg/mL $NaHCO_3$, 58.5 µg/mL glutamine, and 10% heat-inactivated (55°C for 15 min) FBS (Gibco BRL) (complete DMEM) at 37°C in an atmosphere of 5% CO_2/95% room air, and subcultured every 2 d.

Lipid peroxidation, as determined by the accumulation of TBA-reactive substances (TBARS) released into the medium, was measured using a modified version of the method of Glascott *et al.* (18). Briefly, the hepatocytes were planted in 75-cm^2 plastic flasks at a density of 5×10^4 cells/flask in 15 mL complete DMEM medium at 37°C under an atmosphere of 5% CO_2/95% room air. After incubation for 2 h at 37°C in an atmosphere of 5% CO_2/95% room air, the cultures were rinsed twice with prewarmed phosphate-buffered saline (PBS) solution to remove unattached dead cells. Fresh complete DMEM (7 mL), with or without a final concentration of 10 µg/mL of the peptide with the highest antioxidative activity, was added and the hepatocytes were incubated overnight (18–20 h). After the cells were washed twice with prewarmed PBS solution, 5 mL of DMEM without FBS (incomplete DMEM) was added to the flasks, and the cells were treated with 225 µM *t*BHP. After *t*BHP treatment for 2 h, the medium was removed to an-

other flask, and cells were gathered by scraping. In addition, trichloroacetic acid (TCA) was added to the mixture of scraped cells and removed medium (4.5% final concentration). The scraped cells were sonicated for 20 s and centrifuged (1,300 × *g*, 10 min) to pellet the protein. One milliliter of TCA supernatant was added to 2 mL of TBA solution prepared with 0.45% TBA and 7.5% acetic acid adjusted to pH 4.15 with 10 N NaOH. The reaction solution was placed in a boiling water bath for 15 min, cooled to room temperature, and the absorbance was read at 532 nm using a spectrophotometer (Hitachi U-3210, Tokyo, Japan). TBARS were quantified using TEP as the standard. TEP was dissolved in 0.01 N HCl to produce MDA, and this was used to generate a standard curve for TBARS.

Protein in each sample was determined by the method of Smith *et al.* (19), using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Molecular weight profiles. EYPH was separated using three ultrafiltration membranes (10, 5, and 1 kDa MWCO membranes) according to molecular size, and thus three types of hydrolysates (10 K, 5 K, and 1 K hydrolysate) were obtained. The molecular weight distributions varied according to the MWCO size of the membrane used (Fig. 1). The 10 K hydrolysate had a size distribution from 12 to 6.5 kDa, except for the presence of peaks of 5 kDa and 2 kDa. The pattern of the molecular weight distribution of the 5 K hydrolysate was located from 5.5 to 1 kDa, except for the appearance of peaks of 7 kDa and 700 Da, and major peaks were 5, 2, and 1 kDa. The 1 K hydrolysate showed three major peaks of 900, 700, and 350 Da. The result of molecular weight profiles of each hydrolysate showed a distinct decrease of molecular weights according to pore size of membrane.

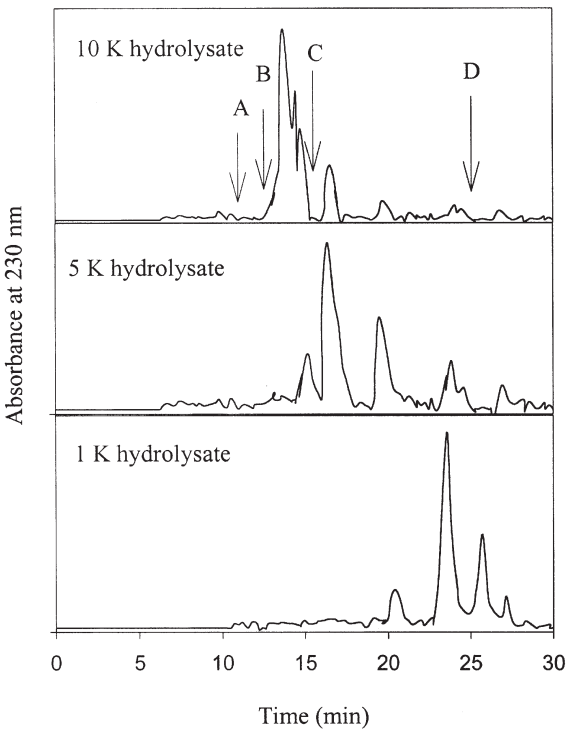

FIG. 1. Molecular weight distribution profiles of 10, 5, and 1 K hydrolysate on high-performance liquid chromatography (HPLC) with gel permeation chromatography column. HPLC operation was carried out with deionized water as mobile phase at flow rate of 1.0 mL/min. (A) Carbonic anhydrase (MW 29,000 Da); (B) cytochrome C (MW 12,327 Da); (C) aprotinin (MW 6,000 Da); (D) pentaphenylalanine (MW 753.9 Da).

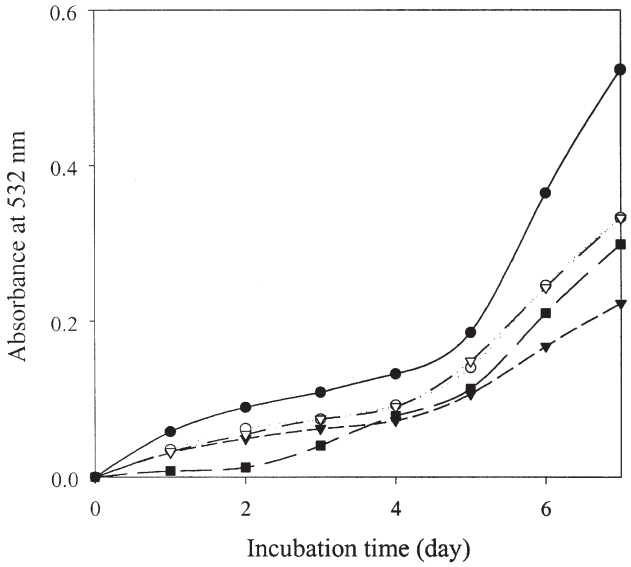

FIG. 2. Antioxidative activities of Alcalase hydrolysates from lecithin-free egg yolk protein in linoleic acid autoxidation system measured by the thiobarbituric acid (TBA) method. ●, Control; ○, 10 K hydrolysate; ▼, 5 K hydrolysate; ▽, 1 K hydrolysate; ■, α-tocopherol.

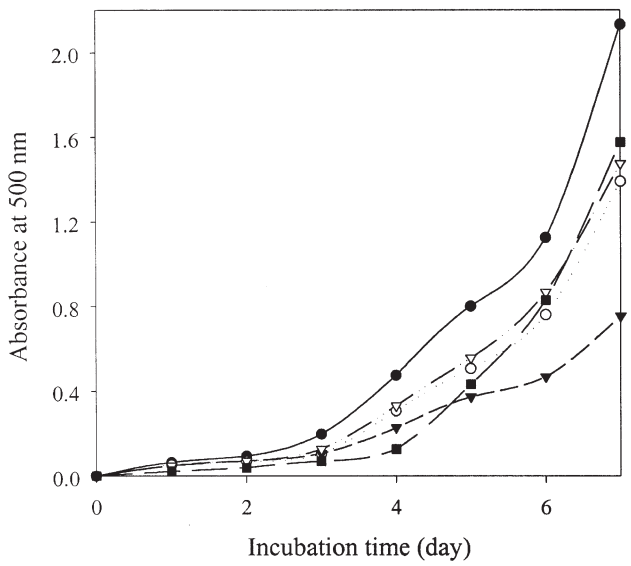

FIG. 3. Antioxidative activities of Alcalase hydrolysates from lecithin-free egg yolk protein in linoleic acid autoxidation system measured by the ferric thiocyanate method. ●, Control; ○, 10 K hydrolysate; ▼, 5 K hydrolysate; ▽, 1 K hydrolysate; ■, α-tocopherol.

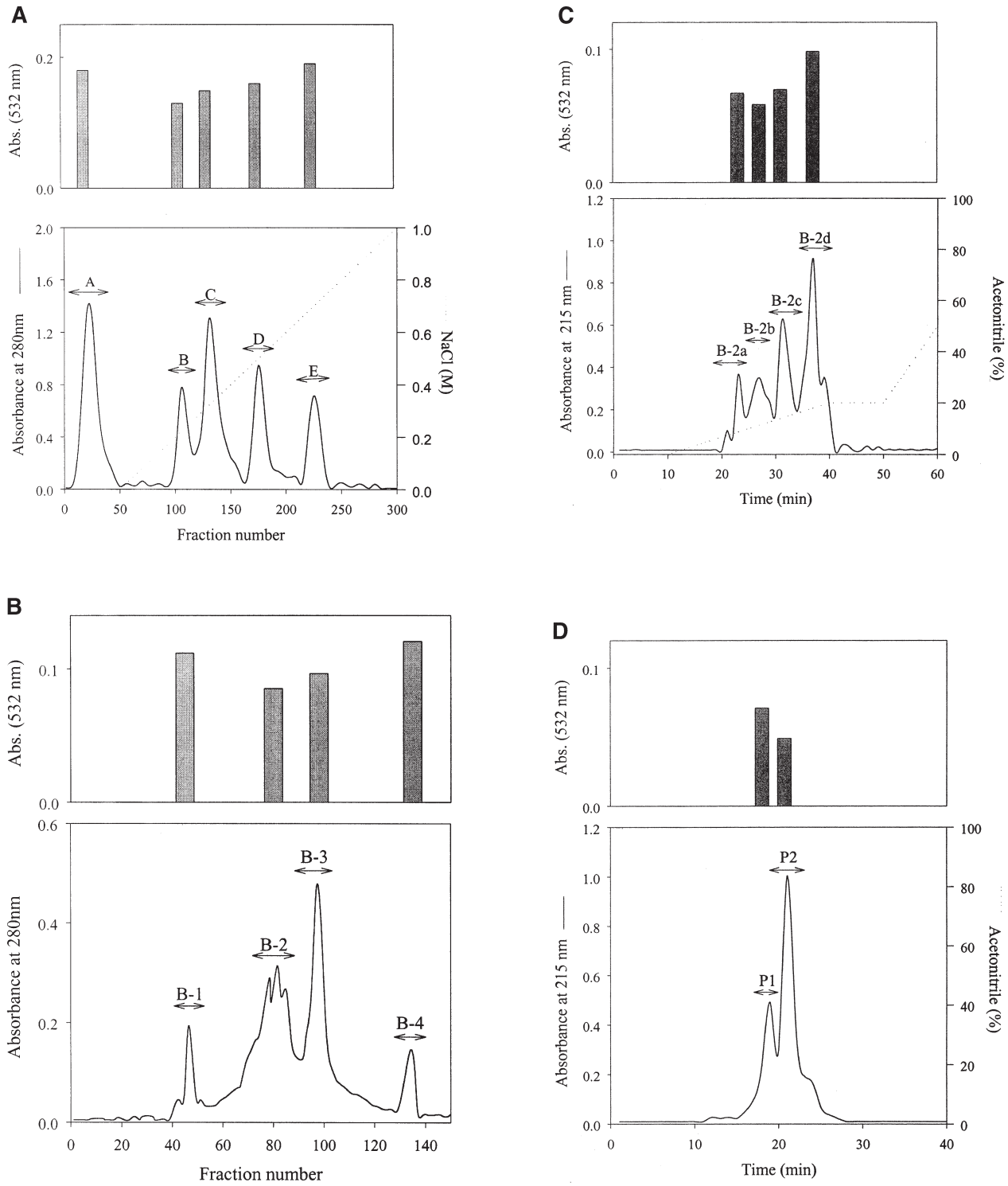

FIG. 4. Isolation of antioxidative peptides. (A) Separation of 5 K hydrolysate by SP-Sephadex C-25 column chromatography (lower panel) and antioxidative activities of the fractions (upper panel) measured by TBA method after 6 d. Elution was performed at a flow rate of 2 mL/min with a linear NaCl gradient (0–1 M) in 20 mM sodium acetate buffer, pH 4.0. (B) Rechromatography of fraction B from Figure 4A on Sephadex G-25 gel chromatography (lower panel) and antioxidative activities of the fractions (upper panel) measured by the TBA method after 6 d. Elution was done at a flow rate of 1 mL/min in 0.1 M sodium phosphate buffer, pH 7.0. (C) Reversed-phase HPLC pattern on a Primesphere 10 C-18 column of fraction B-2 from Figure 4B eluted on the gel chromatography (lower panel) and antioxidative activities of the fractions (upper panel) measured by the TBA method after 6 d. HPLC operation was carried out with 50% acetonitrile as mobile phase at a flow rate of 2 mL/min using an ultraviolet (UV) detector at 215 nm. (D) Further separation of subfraction B-2b renewed-phase HPLC. Elution profiles (lower panel) and antioxidative activities of the fractions separated by reversed-phase HPLC (upper panel) measured by the TBA method after 6 d. The HPLC operation was carried out with 30% acetonitrile as mobile phase at flow rate of 2 mL/min using a UV detector at 215 nm. For abbreviations see Figures 1 and 2.

Antioxidative activity of EYPH. There are only a few reports on the antioxidative efficacy of amino acids. Tryptophan and histidine showed high antioxidant activity whereas glycine and alanine showed only weak activity, and methionine and cysteine had an antioxidative effect in soybean oil (20). However, all amino acids have been shown to have antioxidant activity in some systems, which probably reflects the antioxidant nature of the NH_3R group (21). The use of a protein or a hydrolysate for the improvement of the antioxidative activity in functional foods might be more practical than the use of amino acids, because proteins and hydrolysates have other desired functional properties. The antioxidative activity of soybean protein hydrolysates has been documented (22). In a recent paper, the antioxidative effect of peptides derived from the enzymatic hydrolysates of fish skin gelatin was described (23).

In this study, the antioxidative activity of three hydrolysates fractionated from EYP was investigated and compared with that of α-tocopherol, a widely used natural antioxidant. The 5 K hydrolysate was the most effective and exhibited activities—as reflected in TBA (Fig. 2) and PV (Fig. 3)—that were 30 and 43% better than that for α-tocopherol, respectively.

These results indicate that the antioxidative activity of proteins or peptides depends on their molecular weight.

Isolation of antioxidative peptides and determination of amino acid sequences. To identify the antioxidative peptides derived from lecithin-free EYP, the protein was hydrolyzed with Alcalase, and the three kinds of permeates (10, 5, and 1 K hydrolysate) were obtained using ultrafiltration membranes with 10, 5, and 1 kDa MWCO. The 5 K hydrolysate (41.7 g) was then separated using ion exchange chromatography on an SP-Sephadex C-25 column and fractionated into five portions. When these fractions were tested for antioxidative activity, fraction B was found to possess a strong activity and was then lyophilized (Fig. 4A). The lyophilized fraction B (4.63 g) was subjected to size exclusion chromatography on Sephadex G-25 and fractionated into four portions. Fraction B-2 (3.57 g) exhibited the strongest antioxidative activity, although antioxidative activity was widely observed for all fractions (Fig. 4B). This fraction was further separated by reversed-phase HPLC using a 0.1% TFA-acetonitrile system and fractionated to B-2a, B-2b, B-2c and B-2d; the subfraction B-2b possessed the highest antioxidative activity (Fig. 4C). The subfraction B-2b (0.94 g) was further separated by reversed-phase HPLC using the same solvent system. Two antioxidative peptides (P1 and P2) were finally obtained from the Alcalase hydrolysate of lecithin-free EYP, and the yields estimated by lyophilization of P1 and P2 were 183.5 and 573.1 mg, respectively (Fig. 4D). In addition, the purity of P1 and P2 was identified using capillary electrophoresis (data not shown).

The amino acid sequences of these peptides were as follows. Antioxidative peptide P1 was composed of 10 amino acid residues, Leu-Met-Ser-Tyr-Met-Trp-Ser-Thr-Ser-Met, and antioxidative peptide P2 had 15 residues; Leu-Glu-Leu-His-Lys-Leu-Arg-Ser-Ser-His-Trp-Phe-Ser-Arg-Arg. Both had leucine at their N-terminal positions. In addition, antioxidative peptide P2 exhibited activity, as reflected in TBA, that was 61% higher than for α-tocopherol (data not shown). The amino acid residues at the N-termini of dipeptides have been demonstrated to be antioxidative in an oil system (24). It is probable that the amino acid residues play a role in increasing the interaction between peptides and fatty acids.

The antioxidative activity of histidine-containing peptides has been reported (25,26). This activity may be attributed to the chelating and lipid radical-trapping ability of the imidazole ring. The antioxidative activities of histidine-containing peptides were higher than that of histidine itself. This was partly explained by the increase of hydrophobicity of peptides and fatty acids. As shown in this study, one peptide of the two peptides contained histidine residue in its sequence. The other peptide contained tyrosine residue, which is a potent hydrogen donor. Previously, many proteins have been reported to have strong antioxidative activity against the peroxidation of lipid or fatty acids systems (27,28). Three antioxidative peptides previously isolated from egg white albumin contained histidine residues at the second residue in their sequences (29), and all of the six antioxidative peptides isolated from β-conglycinin contained a proline residue in their sequences. As a result, antioxidative activity of peptides is thought to be related to their molecular weight and amino acid sequence.

The effect of antioxidative peptide P2 on lipid peroxidation of hydrolysate of EYP, induced by *t*BHP, was also investigated using cultured normal human liver cells. In general, the accumulation of MDA, as measured by TBARS in the medium, is a sensitive index of the peroxidation of cellular lipids in cultured cells intoxicated with *t*BHP. Lipid peroxidation in hepatocytes cultured overnight with the antioxidant peptide P2 was significantly lower than in hepatocytes cultured without P2 (2.6 ± 0.1 vs. 1.9 ± 0.1 μM MDA, mean ± standard error of the mean, $P < 0.05$).

Two mechanisms have been reported to explain the killing of hepatocytes by *t*BHP. One is related to the peroxidation of cellular membranes (30), and the other occurs in the absence of lipid peroxidation and is associated with the loss of mitochondrial function (31). Glascott *et al.* (18) reported that cells treated with the antioxidant *N*,*N*′-diphenyl-*p*-phenylenediamine (DPPD) exhibited lower cell killing than cells not treated with DPPD.

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