Antioxidative Activity of Soluble Elastin Peptides

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Insoluble elastin was rendered soluble by pepsin digestion and HCl treatment. The antioxidative activity of pepsin-solubilized elastin (PSE) and acid-solubilized elastin (ASE) was investigated. The peroxide value and carbonyl value of oleic acid in the presence and absence of PSE and ASE after oxidation at high temperature (75−90 °C) were evaluated. Both PSE and ASE were effective inhibitors of the oxidation of oleic acid. The antioxidative activity of PSE and ASE was enhanced in the presence of citric acid as a synergist. Both PSE and ASE are thought to be valuable as antioxidants. Since the preparation of ASE was easier than the preparation of PSE, ASE could therefore be more useful as an antioxidative agent. The antioxidative activity of low molecular weight peptides obtained from ASE could be found by the ferric thiocyanate method.

Keywords: *Elastin; antioxidant; antioxidative peptide*

INTRODUCTION

Elastin has been found as a major protein component of elastic tissues such as the arterial wall, ligament, and skin (Serafini-Fracassini et al., 1975; Spina et al., 1975). Elastin is unique in that its amino acid composition is rich in such nonpolar amino acids (>80%) as Gly, Ala, Val, and Pro (Field et al., 1978). Elastin also has specific cross-linkages of desmosine, isodesmosine (Partridge et al., 1963; Thomas et al., 1963), lysinonorleucine, and merodesmosine (Starcher et al., 1967; Franzblau et al., 1965). Consequently, elastin is highly insoluble in diluted acids, diluted alkalis, hot water, and various denaturants, leading to difficulty in its structural analysis and utilization. Some chemical solubilization processes have already been reported (Lowry et al., 1941; Partridge et al., 1955; Lansing et al., 1952; Moschetto et al., 1974). However, the utilization of elastin has not progressed so far. To develop a new method for utilizing elastin, we have been studying the nature of solubilized elastin peptides. In a previous paper (Hattori and Takahashi, 1993), we clarified that pepsin-solubilized elastin (PSE) had good affinity for oil and good emulsifying ability. PSE stabilized both O/W type and W/O type emulsions. PSE was unique in that it was especially effective for stabilizing the W/O type emulsion.

Since PSE had strong affinity for oil and good emulsifying ability, we postulated that elastin peptides would have some protective effect such as antioxidative activity on the degradation of oil. Many studies on antioxidative agents have been carried out, and many synthetic antioxidants have already been developed. Antioxidants for food additives should satisfy the following requirements: (1) effectiveness at low concentration; (2) safety; (3) no color, odor, or strange taste; (4) effectiveness for processed food as well as for the raw material; (5) analyzability in food; and (6) low cost. Since most synthetic antioxidants cannot satisfy these requirements, many searches for antioxidants from natural materials including food have been and are being carried out. For example, tocopherols from plants (Liebler, 1990), peptides from spices (Srinivas et al., 1992), gingerol-related compounds and diarylheptanoids from herbs (Kikuzaki and Nakatani, 1993), bromophenols from marine algae (Fujimoto et al., 1985, 1986), metabolic products from microorganisms (Ishikawa et al., 1985), Maillard reaction products (Bedinghaus and Ockerman, 1995), hydrophobic proteins (Wang et al., 1991), and peptides from protein hydrolysate (Chen et al., 1995) have been proved to be antioxidative.

Our purpose in the present study was to confirm the antioxidative activity of elastin peptides on lipid oxidation. We thought that an understanding of the potential function of elastin peptides might lead to new ideas to utilize this abundant protein as a food additive. We prepared soluble elastin peptides by pepsin digestion and HCl treatment. The antioxidative effect of both PSE and acid-solubilized elastin (ASE) was investigated.

MATERIALS AND METHODS

Preparation of Soluble Elastin Peptides. Insoluble elastin (IE) was prepared from minced bovine ligamentum nuchae as described previously (Hattori and Takahashi, 1993) by referring to the method of Partridge et al. (1955). In brief, minced fresh bovine ligamentum nuchae was suspended in a 10% sodium chloride solution to remove globular proteins. After washing with distilled water, the sample was defatted with acetone/ether, before autoclaving at 2 kg/cm^2 for 1 h. The autoclaving procedure was repeated seven times. IE was recovered by lyophilization and then pulverized by cooling with dry ice/acetone.

PSE was prepared as described previously (Hattori and Takahashi, 1993). Twenty grams of IE was suspended in 1000 mL of 0.5 M acetic acid, 200 mg of pepsin (EC 3.4.23.1, Sigma, 3200 units/mg) was added to the suspension, and the reaction mixture was incubated at 39 °C for 18 h with gentle stirring. The reaction was stopped by adjusting the pH to >12.0 with a 30% NaOH solution. The reaction mixture was neutralized with 1 M HCl and centrifuged at 12 000 rpm for 30 min at 25 °C. The resulting supernatant was dialyzed against distilled

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water and lyophilized, the lyophilized material being designated PSE.

ASE was prepared by dissolving IE (25 g) in 0.5 M HCl (1000 mL) at 80 °C for 7 h. The reaction was stopped by neutralization with 1 M NaOH, before the reaction mixture was centrifuged at 3000 rpm for 5 min at 4 °C. The resulting supernatant was dialyzed against distilled water and lyophilized, the lyophilized material being designated ASE. To obtain low molecular weight peptides from ASE, ASE (1 g) was hydrolyzed in 1 M HCl (40 mL) at 80 °C for 48 h. Hydrolysis was stopped by neutralization with 1 M NaOH. The hydrolysate was deionized by electrical dialysis with a Micro Acilyzer (Asahi Chemical Industry, Tokyo, Japan) and lyophilized, the low molecular peptides from ASE being designated ASE-L.

Determination of the Amino Acid Composition. PSE and ASE were hydrolyzed in 6 M HCl at 110 °C for 20 h in vacuo. An amino acid analysis of the hydrolysate was carried out on an automatic amino acid analyzer (model 835, Hitachi, Tokyo, Japan).

To determine the desmosine and isodesmosine contents, PSE and ASE were hydrolyzed in 6 M HCl at 110 °C for 48 h in vacuo, and the hydrolysate was analyzed by ion-pair chromatography. A TSK gel ODS-80$_{TM}$ column (4.6 i.d. × 150 mm, Tosoh, Tokyo, Japan) was equilibrated with 0.09 M methanesulfonic acid (pH 2.0) containing 5.4 mM sodium heptanesulfonate and 10% (v/v) acetonitrile. The hydrolysate was applied to the column and eluted at a flow rate of 1.0 mL/min at 25 °C, the absorbance being monitored at 275 nm.

Measurement of the Molecular Weight of Elastin Peptides. The molecular weights of PSE and ASE were measured by size exclusion chromatography (SEC). A TSK gel G3000SW$_{XL}$ column (7.8 i.d. × 300 mm, Tosoh, Tokyo, Japan) was equilibrated with a 0.067 M phosphate buffer containing 0.3 M NaCl at pH 7.0. The sample (100 μg/50 μL) was applied to the column and eluted at a flow rate of 1.0 mL/min. The absorbance was monitored at 280 nm.

The molecular weight of ASE-L was also measured by SEC. A Superdex peptide HR 10/30 column (10.0 i.d. × 300 mm, Pharmacia LKB) was equilibrated with a 0.02 M phosphate buffer containing 0.25 M NaCl at pH 7.2. The sample (200 μg/100 μL) was applied to the column and eluted at a flow rate of 1.0 mL/min. The absorbance was monitored at 230 nm.

Evaluation of the Antioxidative Activity of Elastin Peptides. Elastin peptides (PSE and ASE) were rendered soluble in 0.1 M ammonium hydroxide solution containing 90% ethanol and then added to oleic acid. After the solvent was removed by evaporation at 30 °C, oleic acid containing elastin peptides was obtained. To clarify the effect of synergist on the antioxidative activity of elastin peptides, oleic acid containing the elastin peptides and 0.02% citric acid was also prepared. Citric acid was first dissolved in propylene glycol and added to oleic acid together with elastin peptides. Control sample was prepared according to the same procedure without adding elastin peptides and citric acid. The antioxidative activity of the elastin peptides was evaluated after oxidation at high temperature (75, 80, 85, and 90 °C) by measuring the peroxide value (POV) and carbonyl value (COV).

POV was measured by putting a sample (1 g) into an Erlenmeyer flask (100 mL) and adding an acetic acid/chloroform (3:2) solution (25 mL). After nitrogen gas was flushed into the Erlenmeyer flask, a saturated KI solution (1 mL) was next added. The flask was immediately sealed, shaken gently, and allowed to stand in the dark for 10 min. After distilled water (30 mL) was added with vigorous stirring, the solution was titrated with 0.01 N $Na_2S_2O_3$, using a 1% starch solution (1 mL) as an indicator.

COV was evaluated for a measured sample (10−100 mg), which was washed with benzene (10 mL) into a measuring flask (50 mL). After a 4% trichloroacetic acid/benzene solution (4 mL) and a 0.05% 2, 4-DNPH/benzene solution (5 mL) had been added, the mixture was heated at 60 °C for 30 min. After cooling to room temperature, the mixture was colored by adding 4% NaOH in ethanol solution (10 mL). After dilution to 50 mL with ethanol and standing for 30 min, the absorbance at 440 nm was measured.

Table 1. Amino Acid Composition of PSE and ASE[a]

AA	IE	PSE	ASE	ASE-L	bovine ligamentum nuchae elastin[b]
4-Hyp	10	12	5	40	8.1
Asp	7	11	7	6	5.8
Thr	9	11	9	14	9.3
Ser	9	12	11	12	8.7
Glu	18	20	23	13	15.4
Pro	143	134	124	106	115.5
Gly	298	292	247	249	328.1
Ala	202	223	326	188	227.0
Cys	10	6	10	2	0
Val	147	149	111	177	131.6
Met	2	1	1	1	0
Ile	26	25	17	34	23.9
Leu	64	51	44	74	59.4
Tyr	7	11	12	16	5.9
Phe	34	31	38	31	29.3
Hyl	3	2	3	2	0.5
Lys	4	5	5	4	3.3
His	1	1	1	1	0.5
Arg	7	3	6	29	5.8
Des	2.4[c]	3.2[c]	3.1[c]	1.7[c]	5.4
Ide	2.1[c]	2.2[c]	5.4[c]	23.0[c]	10.1

[a] Residues per 1000 amino acid residues. [b] Field et al. (1978). [c] mol/10^5 g.

The antioxidative activity of ASE-L was evaluated according to the ferric thiocyanate method (Mitsuda et al., 1966; Osawa and Namiki, 1981; Chen et al., 1995). ASE-L (100 μg) was dissolved in 10 mL of ethanol (99.5%), 10 mL of a 50 mM phosphate buffer (pH 7.0), and 0.13 mL of linoleic acid (final concentration of 2×10^{-2} M). The solution was filled with distilled water to 25 mL and stored at 60 °C. At intervals, aliquots of the reaction mixture were taken for measuring the oxidation by the ferric thiocyanate method. In brief, 2.35 mL of 75% ethanol, 50 μL of 30% ammonium thiocyanate, and 50 μL of a 20 mM ferrous chloride solution in 3.5% HCl were added to 50 μL of the reaction mixture with stirring. After 5 min, the absorbance at 500 nm was measured. Ascorbic acid (100 μg, 200 μg) and δ-tocopherol (1 mg) were used as controls.

RESULTS AND DISCUSSION

Structural Features of PSE and ASE. IE obtained from bovine ligamentum nuchae was rendered completely soluble by both pepsin digestion at 39 °C for 18 h and HCl treatment at 80 °C for 7 h. The yields of PSE and ASE from IE were about 27 and 23%. Although there was little difference in the yields, the preparation procedure for ASE was easier than that for PSE.

The amino acid compositions of PSE and ASE shown in Table 1 are similar to those of the IE preparation and native elastin from bovine ligamentum nuchae reported by Field et al. (1978), suggesting that PSE and ASE have peculiar amino acid sequences rich in hydrophobic residues. PSE and ASE contained amounts of desmosine and isodesmosine similar to those in IE.

The SEC patterns of PSE and ASE indicate a relatively homogeneous molecular weight with a major peak of about 25 000 as shown in Figure 1.

Antioxidative Activity of Elastin Peptides. The elastin peptides were insoluble in oleic acid when directly mixed. Hence, the elastin peptides were first rendered soluble in a 0.1 M ammonium hydroxide solution containing 90% ethanol and then added to oleic acid.

The POV of the oleic acid increased with increasing oxidation time (0−5 h) and temperature (75−90 °C) (data not shown). The antioxidative activity of elastin peptides was evaluated after oxidation at 80 °C for 3 h,

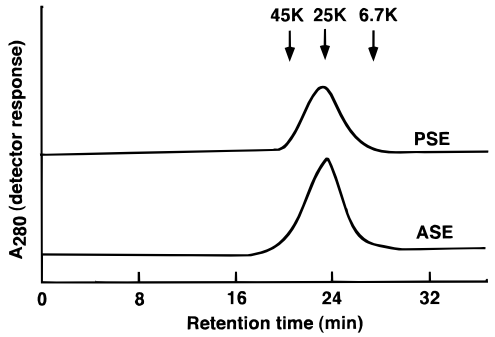

Figure 1. Size-exclusion chromatograms for PSE and ASE. SEC conditions: column, TSK gel G3000SW$_{XL}$ (7.8 i.d. × 300 mm, Tosoh); sample, 100 μg/50 μL; mobile phase, 0.07 M phosphate buffer containing 0.3 M NaCl (pH 7.0); flow rate, 1.0 mL/min; detection, absorbance at 280 nm.

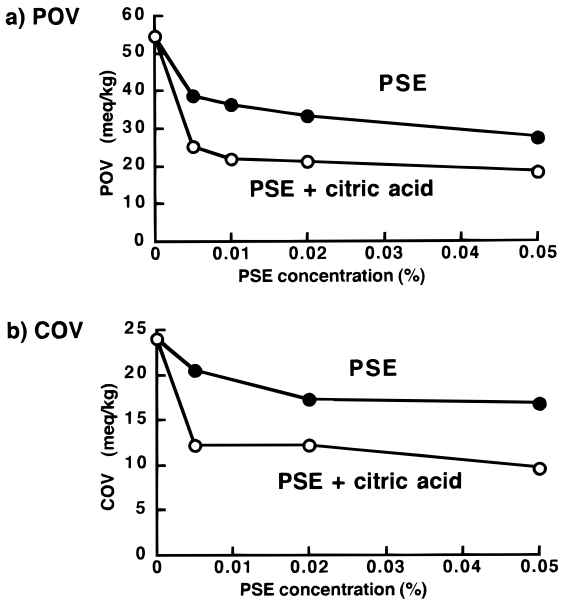

Figure 2. POV and COV data for oleic acid in the presence of PSE after oxidation at 80 °C for 3 h: (●) in the presence of PSE; (○) in the presence of PSE and 0.02% citric acid.

by which time POV of oleic acid had sufficiently increased. POV and COV data for oleic acid in the presence of PSE at various concentrations (0−0.5%) are shown in Figure 2. Each oxidation has been repeated three times, and each titration was done in quadruplicate or triplicate. Each titration contained a standard deviation of 1−4% of the average value. In Figure 2, results from one oxidation experiment are presented. PSE showed antioxidative activity >0.005%, and this antioxidative activity was markedly enhanced in the presence of a synergist (0.02% citric acid). In general, commercially available antioxidants are used at the concentration of 0.02%. PSE was also effective at 0.02% and is thought to be a valuable antioxidant from protein.

The antioxidative activity of both ASE and PSE was evaluated at the concentration of 0.02% in the presence or absence of citric acid (Figure 3). In the presence of ASE, the POV and COV of oleic acid were both markedly suppressed. ASE as well as PSE showed antioxidative activity in this condition. The antioxidative activity of ASE was similar to that of PSE. Since both ASE and PSE were colorless, tasteless, and odorless, they could each be used as a food additive. Because the preparation of ASE was easier than that of PSE, ASE is thought to be more useful as an antioxidative agent.

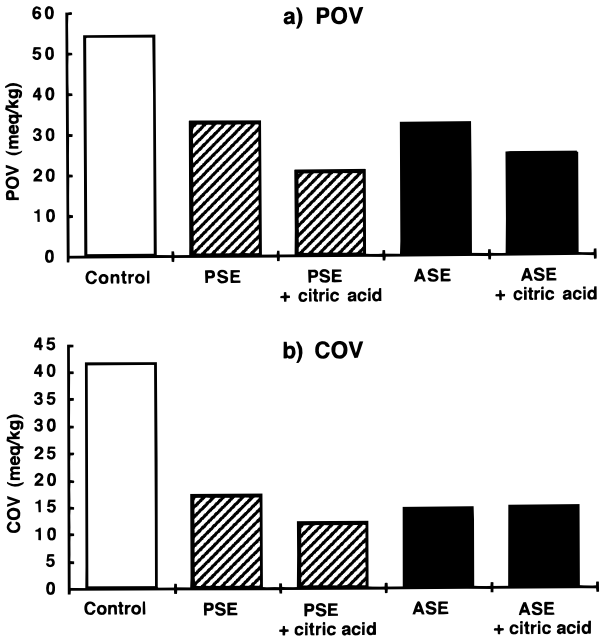

Figure 3. POV and COV data for oleic acid in the presence of elastin peptides after oxidation at 80 °C for 3 h. Concentration: PSE, ASE, and citric acid, 0.02%.

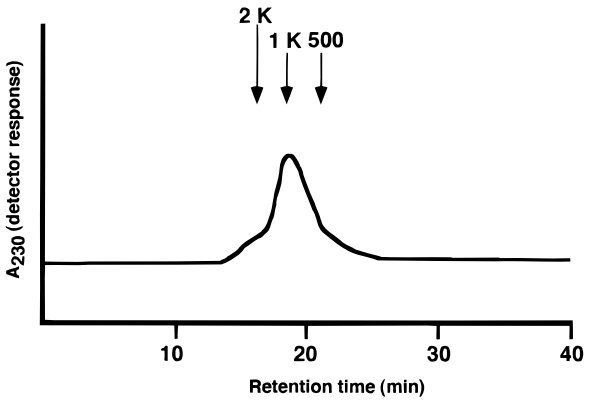

Figure 4. Size-exclusion chromatogram for ASE-L. SEC conditions: column, Superdex peptide HR 10/30 column (10.0 i.d. × 300 mm, Pharmacia); sample, 200 μg/100 μL; mobile phase, 0.02 M phosphate buffer containing 0.25 M NaCl (pH 7.2); flow rate, 1.0 mL/min; detection, absorbance at 230 nm.

To investigate whether elastin peptides with low molecular weight had antioxidative activity, ASE-L was subjected to further investigation. The amino acid composition of ASE-L was rich in hydrophobic residues (Table 1). The molecular weight of ASE-L was measured by SEC with a Superdex peptide HR 10/30 column (10.0 i.d. × 300 mm, Pharmacia LKB) as 350−2700 Da, with a major peak of 1000 Da (Figure 4). ASE-L was also tasteless and odorless and is thought to be applicable as a food additive. The antioxidative activity of ASE-L was evaluated according to the ferric thiocyanate method (Figure 5), ascorbic acid and δ-tocopherol being used as controls. Although ascorbic acid had poor antioxidative activity, the activity of δ-tocopherol was strong. ASE-L (100 μg) showed a very strong antioxidative activity comparable to that of 1 mg of δ-tocopherol. The antioxidative activity of low molecular weight peptides obtained from ASE could thus be found. These results indicate in particular that elastin peptides are effective as antioxidant not only in an oil system but also in a water/alcohol system and are expected to become valuable antioxidants from natural sources.

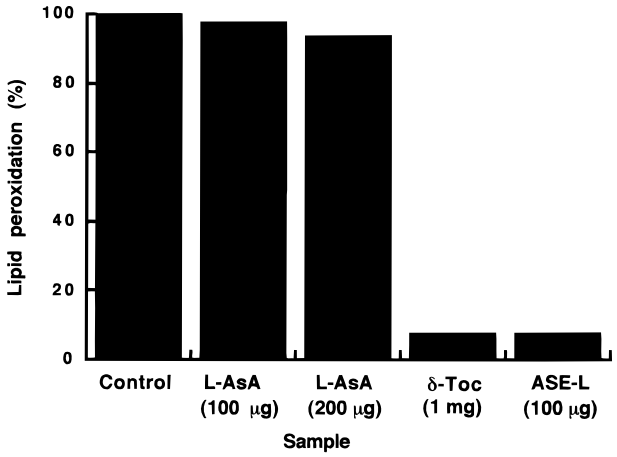

Figure 5. Antioxidative activity of small elastin peptides. The antioxidative activity of peptides with low molecular weight from ASE (ASE-L) was evaluated according to the ferric thiocyanate method. Amounts: ASE-L, 100 μgl L-ascorbic acid (L-AsA), 100, 200 μg; δ-tocopherol (δ-Toc), 1 mg.

The ability of elastin peptides as radical scavengers was evaluated by their reactivity to the phenol reagent (Wako Pure Chemical Industries, Osaka, Japan). Phenol diluted with an equal volume of Milli Q water (100 μL) was added to a 0.1% elastin peptide solution (1 mL). When a radical scavenger exists, the color of the reagent solution changes from yellow to blue-purple. δ-Tocopherol reacted to the phenol reagent, whereas the elastin peptides did not (data not shown); therefore, elastin peptides are thought not to have ability as radical scavengers. The expected mechanism for the antioxidative activity of the elastin peptides includes the following possibilities: (1) chelating ability for metal ions and (2) good affinity for oil, which would prevent the release of hydrogen or the binding of oxygen. The precise antioxidative action of the elastin peptides needs to be elucidated.

Concluding Remarks. We were able to prepare two kinds of soluble elastin peptides, PSE and ASE, with antioxidative activity. The antioxidative activity of both PSE and ASE was enhanced in the presence of citric acid as a synergist. Since ASE was easier to prepare than PSE, ASE is thought to be more useful as an antioxidative agent. Low molecular weight peptides obtained from ASE also showed antioxidative activity. Isolation of the antioxidative peptide(s) from elastin peptides should be carried out, and the mechanism for the antioxidative activity of the peptide(s) needs to be elucidated.

ABBREVIATIONS USED

IE, insoluble elastin; PSE, pepsin-solubilized elastin; ASE, acid-solubilized elastin; ASE-L, low molecular peptides from ASE; L-AsA, L-ascorbic acid; δ-Toc, δ-tocopherol; POV, peroxide value; COV, carbonyl value; SEC, size exclusion chromatography.

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