Note

Effects of L-Ascorbic Acid and Superoxide Anion Radical on the Polymerization of Ovalbumin

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L-Ascorbic acid (AsA) plays an important role in food, especially in the rheological properties of bread dough. So in this study, we estimated the effect of AsA on the polymerization of proteins using ovalbumin (OVA). OVA was separated into 4 fractions by size-exclusion high-performance liquid chromatography (SE-HPLC) on the basis of molecular weight. When the OVA was incubated at 55°C, the relative areas of fraction 1 (molecular weight of more than 350,000) increased. The relative area of fraction 1 of OVA added with AsA, and of the OVA with superoxide anion radical (O_2^-) generating system was increased. Furthermore, the remaining sulfhydryl group content in OVA with the O_2^- generating systems showed a significant decrease from control. Thus, it was suggested that the O_2^- produced during the autoxidation of AsA might react with the sulfhydryl groups, and consequently affect the polymerization of OVA.

L-Ascorbic acid (AsA) has been widely used as an antioxidant in food processing and preservation to improve food quality. AsA and its related compounds are also suggested to play an important role in the modification of the rheological properties of foods and foodstuffs, and AsA has been used to improve the bread industry (Jorgensen, 1939).

AsA is known to be easily oxidized to dehydro-L-ascorbic acid (DHA), which is the first chemically stable oxidation product by one- or two-electron oxidation reaction. In this oxidative reaction, some free radical species, such as superoxide anion radical (O_2^-) or hydroxyl radical (·OH), were formed. Reactive oxygen radicals such as O_2^- are known to be produced during the AsA oxidation process in the presence of metals (Khan & Martell, 1967a, b, 1968; Ogata *et al*., 1968), or there absence (Kurata *et al*., 1996a, b). In previous papers (Nakamura & Kurata, 1997a, b), we strongly suggested that the hardness of the dough added with AsA was significantly influenced by O_2^- generated during the AsA oxidation process, and reported that O_2^- was believed to be somehow involved in the formation of the characteristic rheological properties of flour-water dough (Nakamura & Kurata, 1997b). It is generally accepted that the rheological properties of dough and its three-dimensional network are dependent on the interaction of the sulfhydryl groups (SH) and disulfide bonds (SS) of protein molecules (Tanaka & Bushuk, 1973a, b, c; Jones *et al*., 1974, Bloksma, 1972). Moreover, we suggested that the amount of relatively large molecular weight protein, which was extracted from dough mixed with or without AsA, significantly increased due to the production of intermolecular SS bonds (Nakamura & Kurata, 1998). AsA is also known to be involved in the polymerization of protein by SS bonds formed between protein molecules (Nishimura *et al*., 1989a, b, 1990, 1992). Thus, O_2^- generated during the oxidation process of AsA was

suggested to be involved in the polymerization of wheat protein molecules. However, the dough system is very complex, and the polymerization mechanism of protein by O_2^- generated during the oxidation process of AsA has not yet been clarified.

In this study, ovalbumin (OVA) was selected as the protein sample because it is a typical water-soluble protein included in food, and OVA was easily investigated from the polymerized by intermolecular disulfide bonds among the OVA molecules (Shimada & Matsushita, 1980a, b; Buttkus, 1974) in comparison with wheat protein. The effect of AsA and O_2^- generated during the oxidation process of AsA on the polymerization mechanism of OVA was examined using SE-HPLC, because the number of SH groups and SS bonds of OVA had been determined (Fothergill & Fothergill, 1969) and the experimental results of SE-HPLC were expected to be easily analyzed.

Materials and Methods

Reagents All the chemical reagents used were reagent grade and purchased from commercial sources. Superoxide dismutase (SOD) from bovine erythrocyte ((EC 1.15.1.1) 3000–4000 units/mg, MW 32,000) and catalase from bovine liver ((EC 1.11.1.6) 5900 units/mg, MW 240,000) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka). Xanthine oxidase (XOD) from buttermilk (EC 1.2.3.2, 1.16 unit/mg, MW 270,000) was obtained from the Oriental Yeast Co., LTD. (Osaka). Xanthine monosodium salt was obtained from ICN Biochemicals Inc. (Aurora, Ohio). The other reagents were purchased from commercial sources. Wheat flour was supplied by the Nissin Flour Milling Co., Ltd. (Tokyo), and contained 12.3% protein (N×5.7) and 0.42% ash.

Size-exclusion high performance liquid chromatographic analysis The SE-HPLC was carried out using a Shimadzu (Kyoto) HPLC system, that consisted of a model LC-6A pump, a model SPD-6A ultraviolet wavelength detector, a rheodyne loop

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injector (20 μl, Rheodyne, Cotani, CA), and a recorder (Model L-R6A). A TSK G4000 SW (Tosoh, Tokyo) SE-HPLC analytical column (7.5×300 mm) was used with a TSK guard column (Tosoh). The mobile phase was 0.05 M sodium phosphate buffer which was filtered through a 0.2 μm membrane filter (Toyo Roshi Kaisha, Ltd, Tokyo) and degassed by an ultrasonic generator under reduced pressure before use. The flow rate was 0.7 ml/min. The column temperature was maintained at the room temperature of 20–25°C. Absorbance was measured at 214 nm. The concentrations of the samples were about 0.2 mg protein/ml. The apparent molecular weights of the major peaks were estimated by calibrating the column with a protein or polysaccharide standard of blue dextran (2,000,000), thyroglobulin (669,000), β-galactosidase (116,000), OVA (45,000), trypsin inhibitor (20,100) and cytochrome C (12,400).

Polymerization of OVA OVA was incubated with AsA, DHA, the O$_2^-$ generating system, or the O$_2^-$ scavenging system to study the effect of the additives on its polymerization. OVA and the additives were dissolved in 0.1 M sodium phosphate buffer (pH 7.4), and the final concentration of OVA was 1%. The concentrations of AsA and DHA incubated with 1% OVA were 0.1, 1 and 10 mg/100 ml. As the O$_2^-$ generating system, the xanthine-XOD system was used. XOD and xanthine were incubated with 1% OVA. Ethylenediaminetetraacetic acid (EDTA) was used to chelate the contaminated trace metal ions in the sodium phosphate buffer. The concentrations of xanthine, XOD and EDTA were 5 mM, 4.4 μM and 1 mM, respectively. The SOD (O$_2^-$ scavenger) or catalase (H$_2$O$_2$ scavenger) was incubated with 1% OVA. The concentrations of the SOD and catalase were 1 μM and 0.2 μM, respectively. The various OVA mixtures were incubated at 55°C in a water bath for 300 min. After incubation, the polymerization of OVA was estimated by SE-HPLC.

Sulfhydryl groups determination The SH contents in 1% OVA were determined according to the methods of Obata *et al.* (1989) using 2,2′-dithiobis-(5-nitropyridine) (DTNP). The reaction mixture containing 1% OVA was diluted with 0.1 M sodium phosphate buffer (pH 6.80) in a centrifugal precipitation tube (16 mm×6.9 mm,11PA, Hitachi Co., Ltd, Tokyo) to the final concentration of 0.1% OVA. Then, 0.5 mM of DTNP-ethanol was added to this diluted solution, and allowed to stand for 20 min at room temperature. After 20 min, 10% hydrogen perchloride was added to the solution, and centrifuged for 20 min at 12,000 rpm. After centrifugation, absorbance of the solution was read at 386 nm. Cysteine was used as the standard for the determination of sulfhydryl groups.

Results and Discussion

SE-HPLC patterns OVA was separated by SE-HPLC into 4 fractions on the basis of molecular weight. Figure 1A shows the typical elution pattern of 1% OVA dissolved in 0.05 M sodium phosphate buffer (pH 7.4) and the elution pattern of OVA incubated at 55°C is given in Fig. 1B. The percentages of the relative peak areas under these fractions were calculated from the area of the size-exclusion high-performance liquid chromatogram (SE-HPLCm). The averaged molecular weights of these fractions were as follows: fraction 1 (F1) >350,000; fraction 2 (F2) 60,000–350,000; fraction 3 (F3) 20,000–60,000; and fraction 4 (F4) <20,000. F3 seems to correspond to the monomer, and F2 to the dimer, trimer, tetramer and oligomer of OVA, and

F1 to the polymer of OVA. As shown in Fig.1B, the relative area of F2 and F3 decreased during incubation for 30 min, while the relative area of F1 increased during incubation for the same period. The relative area of F1 of OVA reduced with dithiothreitol significantly decreased (data not shown); the protein molecules of F1 were thought to contain a number of intermolecular disulfide bonds. Kitabatake *et al.* (1987) suggested that gel filtration of the OVA gel, obtained at neutral pH and low ionic strength, showed that the amount of OVA monomer decreased and another peak appeared. This peak was a soluble aggregate that was composed of monomers and oligomers connected by an SS bridge or bridges (Kitabatake *et al.*, 1987). Hatta *et al.* (1986) described that the SS bridge among the OVA molecules was not essential for forming the gel, however, the formation of SS bonds was probably involved in the first step of coagulation (Shimada & Matsushita, 1980a, b), and heating caused OVA to polymerize by an intermolecular sulfhydryl-disulfide exchange to form a network (Shimada & Matsushita, 1980a, b; Buttkus, 1974). Although the OVA mixture did not seem to be a typical gel, polymerization of OVA might be promoted under this condition. Therefore, the F1 of OVA seemed to be composed of polymers connected by the SS bonds.

Effect of AsA and the O$_2^-$ scavenging system on the poly-
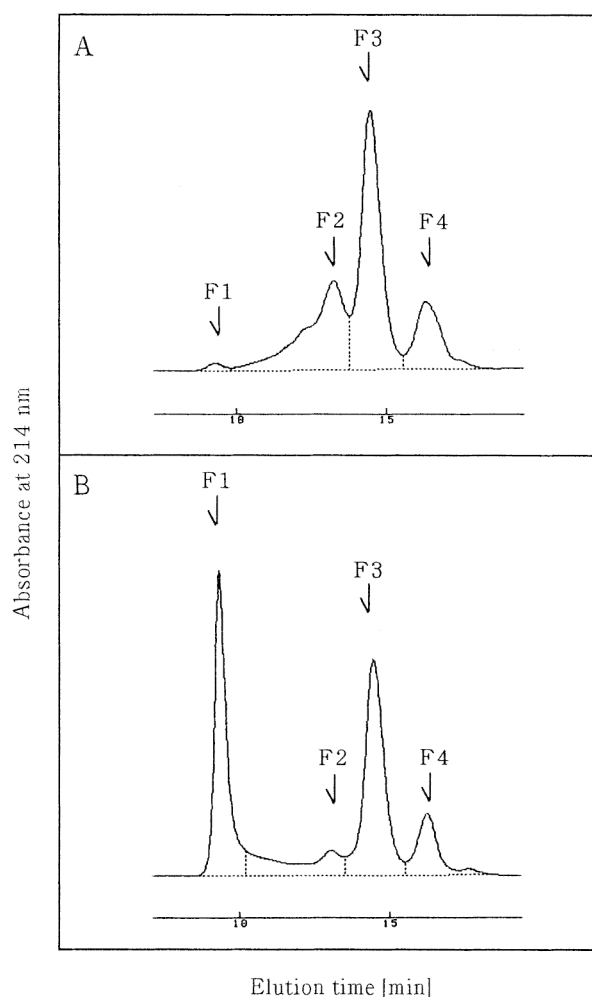
Fig. 1. Size-exclusion high performance liquid chromatograms of ovalbumin (OVA) dissolved in phosphate buffer (pH 7.4). Concentration of OVA was 1%. A: before incubation; B: after incubation for 300 min at 55°C.
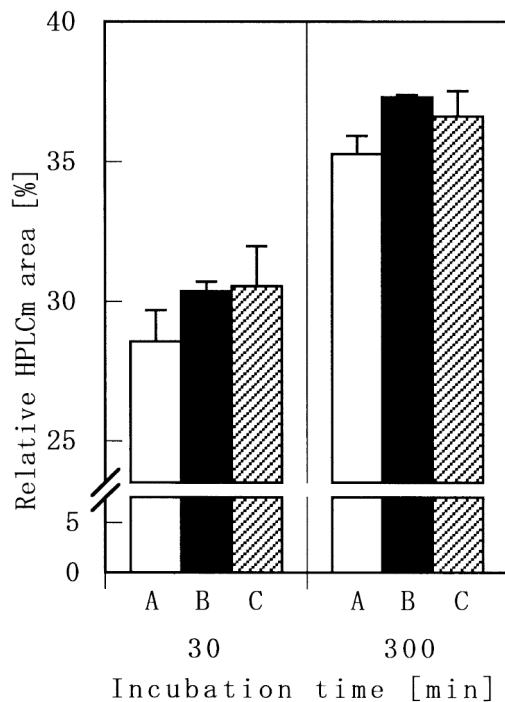
Fig. 2. Effects of superoxide scavenging systems on relative area of the F1 of SE-HPLCm of ovalbumin (OVA). The concentration of OVA was 1% and it was incubated for 30 and 300 min at 55°C with 1 mg/100 ml AsA (B), 1 mg/100 ml AsA, 1 μM superoxide dismutase (SOD, superoxide scavenger) and 0.2 μM catalase (hydrogen peroxide scavenger) (C) and the control (A). Bars show standard errors.

merization of OVA   The effect of AsA or O_2^- on the relative peak area separated by SE-HPLC of 1% OVA in 0.05 M sodium phosphate buffer (pH 7.4) was examined using O_2^- scavenging systems. The effects of SOD and catalase and/or AsA on the relative area of F1 separated by SE-HPLC of 1% OVA are given in Fig. 2. When the control OVA was incubated for 30 min, the relative area of F1 was about 28%. The relative area of F1 with 1 mg/100 ml AsA added and incubated for 30 min increased to 37%. The remaining ratio of the SH group content of the OVA is shown in Table 1. A decrease in the SH group contents in control was observed during incubation. The remaining SH group in 1% OVA after a 30 min-incubation without AsA was about 94% at 55°C. When 1% OVA was incubated with 1 mg/100 ml AsA for 30 min, the remaining SH group content was about 89%. Moreover, the remaining SH group content of OVA added with AsA,
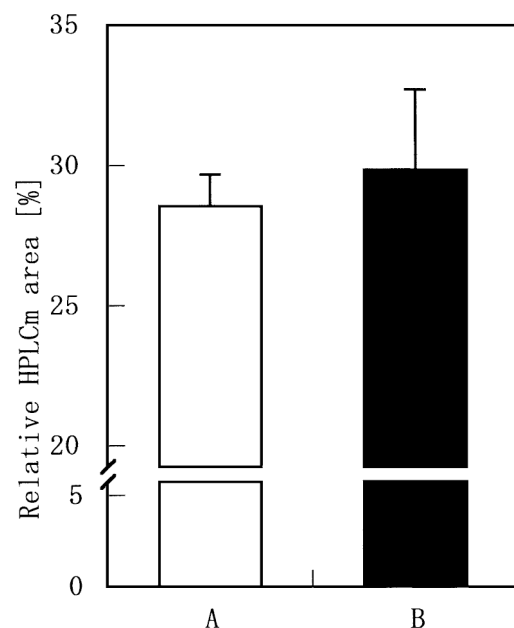
SOD and catalase were about 92%, similar to that of the control. After a 300 min-incubation, the remaining SH group content of OVA was significantly decreased compared with the content when only AsA was added. These results suggested that O_2^- was generated by the oxidation process of the AsA oxidized SH groups of OVA, consequently, OVA polymerized during the incubation. Moreover, the remaining SH group content of OVA incubated with DHA (1 mg/100 ml) decreased to 94% after the 300 min-incubation (data not shown). These results suggested that the formation of an SS bond tended to be more accelerated by AsA than DHA. As shown in Fig. 2, the relative area of F1 increased in the presence of AsA which might suggest that the SH groups contained in OVA were responsible for the formation of the SS bond and thus protein polymerization. AsA accelerated the decrease in SH groups, thereby suggesting that the formation of the polymer of OVA occurred due to the O_2^- produced during the AsA oxidation process.

Effect of the O_2^- generating system on the polymerization of OVA   The effect of the O_2^- generating system, XOD and the xanthine system, on the relative area of F1 separated by SE-HPLC of 1% OVA incubated at 55°C for 30 min is given in Fig 3. When the 1% OVA incubated with O_2^- generating systems for 30 min, the relative area of F1 of the OVA had an increased tendency compared with control. The relative area of F3 of the O_2^- generating systems added OVA, which was composed of the OVA monomer, was slightly decreased (data not shown). The remaining SH group content of the OVA added O_2^- generating systems, and the XOD and xanthine system is given in Table 2. The SH group content of OVA with the O_2^- generating system was about 80%, which was observed as a significant decrease from control after a 30 min-incubation. Thus, the O_2^- generated from XOD and the xanthine system were believed to contribute to the polymerization of OVA as compared to the control OVA. The results described above suggested that the polymerization of OVA

Table 1. Effect of superoxide scavenging systems on the content of sulfhydryl group in ovalbumin.

	Incubation time [min]		
	0	30	300
Control	100	93.7±2.7	62.1±2.2
AsA	100	88.6±5.2	53.9±5.2*,a
AsA with SOD and catalase	100	91.6±3.4	67.0±5.7a

Each value is presented as the mean±SE (*n*=5) and is expressed as a percent with the initial amount of sulfhydryl group of ovalbumin (OVA) regarded as 100%. OVA was dissolved to 1% in 0.1 M phosphate buffer (pH 7.4) and incubated for 0, 30 and 300 min at 55°C without AsA; with 1 mg/ 100 ml AsA; 1 mg/100 ml AsA, 1 μM superoxide dismtase (SOD, superoxide scavenger) and 0.2 μM catalase (hydrogen peroxide scavenger). *, Significantly different at *p*<0.05 from control. Values with the same letters are significantly different (*p*<0.05).

Fig. 3. Effects of superoxide generating systems on the relative area of the F1 of SE-HPLCm of ovalbumin (OVA). The concentration of OVA was 1% and it was incubated for 30 min at 55°C with 5 mM xanthine and 4.4 μM xanthine oxidase (XOD) (B) and the control (A). Bars show standard errors.

Table 2. Effect of superoxide generating systems on the content of sulfhydryl group in ovalbumin.

	Incubation time [min]	
	0	30
Control	100	93.7±2.7
Xanthine and XOD	100	79.6±4.2**

Each value is presented as the mean±SE ($n=5$) and is expressed as percent with the initial amount of sulfhydryl group of ovalbumin (OVA) regarded as 100%. OVA was dissolved to 1% in 0.1 M phosphate buffer (pH 7.40) and incubated for 30 min at 55°C with 5 mM xanthine and 4.4 μM xanthine oxidase (XOD). **, Significantly different at **$p<0.01$ from control.

was related to O_2^- generating system, and generated O_2^- was considered to affect the structure of the OVA. The OVA soluble aggregate is known to be composed of monomers and oligomers connected by an SS bridge or bridges (Kitabatake *et al.*, 1987), Sonntag *et al.* (1993) obtained evidence that O_2^- acts as a chain carrier in certain chain oxidation reactions, and the thiolate ion of dithiothreitol was reported to be easily oxidized to form SS bonds. From these results described above, both the oxidative effect of DHA and O_2^- formation during AsA oxidation seemed to be involved in the sulfhydryl-disulfide interchange reaction and seemed to promote the polymerization of OVA by enhanced SS bond formation.

In a previous paper (Nakamura & Kurata,1997b), we suggested that the hardness of the dough added with AsA was significantly influenced by O_2^- generated during the oxidation process of AsA, and O_2^- was believed to be somehow involved in the formation of the characteristic rheological properties of dough. Moreover, we investigated the effect of AsA or O_2^- on the polymerization of protein in dough (Nakamura & Kurata,1998). The relative area of fractions 1 and 2, separated by SE-HPLC (molecular weights of more than 130,000), extracted from dough with AsA and from the dough with the O_2^- generating system increased (Nakamura & Kurata,1998). That the O_2^- produced during the autoxidation of AsA thus appears to affect the polymerization of flour protein in dough. Therefore, it was suggested that O_2^- affects the intra- or intermolecular SH-SS interchange reaction among protein molecules in dough, resulting in the formation of a three-dimensional network.

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