

Effect of L-Ascorbic Acid and Superoxide Anion Radical on the Rheological Properties of Wheat Flour-Water Dough

M. NAKAMURA¹ and T. KURATA²

ABSTRACT

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L-Ascorbic acid (AsA) plays an important role in the rheological properties of bread dough and as an improver. Addition of AsA and iron, as heavy metal ions, affected the rheological properties, especially hardness of flour-water dough prepared under aerobic conditions. Addition of both AsA and iron increased the hardness of regular flour-water dough as compared with control dough; using oxygen-saturated water further increased the dough hardness. Dough mixed with AsA under nitrogen gas showed significantly decreased dough hardness when compared with dough mixed with AsA under air. The improved effect of AsA on dough

hardness may be due to oxygen radicals generated during AsA oxidation. The effect of the superoxide anion radical (O_2^-) on dough hardness was examined by using both O_2^- scavenging and O_2^- generating systems. In an O_2^- generating system (xanthine and xanthine oxidase), dough hardness increased. In an O_2^- scavenging system (superoxide dismutase and catalase), dough hardness was similar to that of the control. It was strongly indicated that hardness of dough containing AsA was much influenced by the O_2^- generated in the oxidation process of AsA.

L-ascorbic acid (AsA) and its related compounds play an important role in the modification of rheological properties of foods, especially as an improver for dough preparation in bread production (Jorgensen 1939). However, the improvement mechanism of AsA in dough formation is not clearly understood.

It has been suggested that rheological properties of bread dough were improved in the presence of reducing substances, such as AsA, that directly or indirectly affect the structure and intermolecular interaction of protein molecules in dough (Johnston and Mauseth 1972, Bekes et al 1994). Also, it is generally accepted that the rheological properties of dough and its three-dimensional network are dependent on the interaction of sulfhydryl group (SH) and disulfide (SS) bonds of protein molecules (Tsen and Bushuk 1963, Johnston and Mauseth 1972).

Previously (Nakamura and Kurata 1997), we described the effects of AsA and its related compounds on the dough hardness during mixing. The addition of either reduced form or oxidized form of glutathione (GSH) decreased dough hardness. The SH group in the reduced form of GSH is believed to cleave a portion of the cross-linking SS bonds among protein molecules and create new free SH groups. The addition of an oxidized form of GSH might partly increase the SH-SS interchange reaction between protein molecules in the dough. On the other hand, addition of either AsA or dehydro-L-AsA (DHA), especially AsA, to dough increases hardness when compared with the control. However, DHA had less effect on dough hardness than AsA did. Therefore, the improvement mechanism of AsA in flour-water dough might be not only due to DHA as an oxidant, as commonly believed, but also due to the oxidation process of AsA to DHA (Nakamura and Kurata 1997).

AsA has been known for many years to be easily oxidized to DHA, which is the first chemically stable oxidation product by one- or two-electron oxidation reactions. The oxidation of AsA, in water at neutral pH, is accelerated in the presence of transition metals (Khan and Martell 1967a,b and 1968; Ogata et al 1968). Grant and Sood (1980) and Pfeilsticker and Roeung (1980, 1982) showed that a nonenzymic oxidation of AsA, mediated by copper

or iron ions, plays an important role in its improver action. In this oxidative reaction, some free radical species were formed. These free radical species such as superoxide anion radical (O_2^-) or hydroxyl radical ($\bullet OH$) promote the oxidative degradation of proteins (Samuni et al 1983; Shinar et al 1983; Levine 1984; Marx and Chevion 1985; Uchida and Kawakishi 1986, 1987, and 1989). Kurata et al (1996a,b) proposed that the autoxidation mechanism of AsA included the formation mechanism of O_2^- , even in the absence of heavy metal ions. Moreover, Sonntag et al (1993) had obtained evidence that O_2^- oxidized the thiolate ion of dithiothreitol (DTT) to SS. Nishimura et al (1989, 1992) reported that the oxygen radicals generated from AsA might have participated in the turbidity of ovalbumin and polymerization of crude actomyosin.

The experimental results described previously (Nakamura and Kurata 1997) and the reports mentioned above suggest the possible involvement of O_2^- in SH-SS interchange reaction. The improvement effect of AsA in dough might be due mainly to O_2^- generated during the AsA oxidation process. The O_2^- would affect intra- or intermolecular SH-SS interchange reaction of proteins in dough and would form a three-dimensional network. However, the behavior of AsA in dough preparation is so complex that there are a number of questions regarding the action AsA against protein.

The objectives of this study were to clarify the improving effect of O_2^- generated in the AsA oxidation process on the rheological properties of flour-water dough during mixing. Then we studied the hardness of dough containing AsA and metals that promote O_2^- generation. Furthermore, we examined the dough hardness in sample containing an O_2^- generating system or an O_2^- scavenging system.

MATERIALS AND METHODS

Reagents

The chemical substances were commercially obtained. Superoxide dismutase (SOD) from bovine erythrocyte (EC 1.15.1.1) (3,000-4,000 units/mg, MW 32,000) and catalase from bovine liver (EC 1.11.1.6) (5,900 units/mg, MW 240,000) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Xanthine oxidase (XOD) from buttermilk (EC 1.2.3.2) (1.16 units/mg, MW 270,000) was obtained from Oriental Yeast Co., Ltd. (Osaka, Japan). Xanthine monosodium salt was obtained from ICN Bio-medicals Inc. (Aurora, OH). The other chemicals used were reagent grade. Wheat flour was supplied by Nissin Flour Milling Co., Ltd. (Tokyo). It contained 12.3% protein ($N \times 5.7$) and 0.42% ash.

¹Yamazaki Baking CO, LTD. 3-15-6 Chitose, Sumida-ku, Tokyo 130, Japan. Corresponding author.

²Institute of Environmental Science for Human Life, Ochanomizu University. 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112, Japan.

Hardness Measurement

The dough was mixed with a mixer (CS-10, Kanto kongoki kogyo Co., Ltd., Tokyo, Japan). After mixing, a 55-g dough sample was measured for hardness using a Rheoner (RE-33005, Yamaden Co. Ltd., Tokyo, Japan) (Nakamura and Kurata 1997). In dough mixed with a mortar and a pestle, the hardness was measured as described above, except for the sample height (10 mm), clearance (5 mm), and plunger (3 mm i.d.).

Effect of Iron on Flour-Water Dough Containing AsA

Dough consisting of 400 g of flour and 260 mL of deionized and distilled water or an aqueous solution of AsA and iron was mixed at room temperature under aerobic and anaerobic conditions in a CS-10 Kanto mixer specially designed for bread dough making. Under aerobic conditions, we used either regular water or oxygen-saturated water for dough mixing. Concentrations of AsA added to dough were 10 and 100 ppm. Concentration of ferric chloride, hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) added to dough was 0.936 $\mu\text{mol/g}$ of flour. Oxygen-saturated water was prepared by aeration of O_2^- gas into deionized distilled water, under stirring, for 30 min. In dough with added FeCl_3 , 130 mL of FeCl_3 solution was first added to flour and mixed for 1 min, followed by the addition of 130 mL of AsA solution and mixed for 11 min. Mixing was done at medium speed (250 rpm) and held for 12 min.

Under anaerobic conditions, degassed water was used for dough mixing. Degassed water was prepared by the filtration of deionized and distilled water with a multilayered composite hollow-fiber membrane module (Mitsubishi Rayon Co., Ltd. Tokyo, Japan) under nitrogen gas to remove dissolved oxygen in water. The mixing bowl was filled with nitrogen gas to prevent access of air, and flour was stirred in lightly under nitrogen in the mixer for 30 min to remove oxygen. Then, mixing started on the medium speed (250 rpm) and was held for 12 min.

Effect of Superoxide Generators on Flour-Water Dough

Dough consisting of 3 g of flour and 2 mL of water or aqueous solution of additives was mixed at room temperature with a mortar and a pestle. Xanthine oxidase (XOD) and xanthine as an O_2^- generating system were added to dough. As the optimum pH for XOD was pH 10.2, xanthine and XOD were dissolved in 0.1M sodium

phosphate buffer (pH 10.2). Ethylenediaminetetraacetic acid (EDTA) was used to chelate the contaminated trace metal ions in the sodium phosphate buffer. Buffer (1 mL) containing $5 \times 10^{-3} M$ xanthine and $1 \times 10^{-3} M$ EDTA was added to flour and mixed for 30 sec. After mixing, 1 mL of buffer containing XOD ($1.1 \times 10^{-6} M$ or $4.4 \times 10^{-6} M$) was added to the dough and mixed for 2.5 min.

Effect of Superoxide Scavengers on Flour-Water Dough

Dough consisting of 3 g of flour and 2 mL of water or aqueous solution of additives was mixed at room temperature with a mortar and a pestle. Superoxide dismutase (O_2^- scavenger) or catalase (H_2O_2 scavenger) were added to dough together with AsA. AsA solution (1 mL, 10 ppm) was added to flour and mixed for 30 sec. After mixing, 1 mL of water containing $1 \times 10^{-6} M$ SOD or $2 \times 10^{-7} M$ catalase was added to the dough and mixed for 2.5 min.

Determination of AsA and DHA

AsA was extracted from wheat flour dough as described previously (Nakamura and Kurata 1997). Dough sample (10 g) was ground with sea sand in 5% metaphosphate solution using a mortar. The extracts were centrifuged at $3,000 \times g$ for 5 min and then filtered successively with 0.8- and 0.45- μm membrane filters (Toyo Roshi Kaisha Ltd., Tokyo, Japan). The clear supernatants obtained were used for the analysis of AsA.

The AsA and DHA contents in the dough extraction were measured by using HPLC. Analysis was conducted using an HPLC system (Shimadzu Co. Ltd., Kyoto, Japan), model LC-9A pump, equipped with an electrochemical detector (model LC-4B BAS Co. Ltd., Tokyo, Japan), with a glassy carbon working electrode, a rheodyne loop injector (20 μL , Rheodyne, Cotati, CA), and a recorder (model C-R6A) attached to the HPLC system. Separation was made with Inertsil ODS-2 column, 4.6 mm i.d. \times 15 cm (GL Science Co. Ltd., Tokyo, Japan). The mobile phase was 0.05M sodium phosphate buffer (pH 2.30) containing 1 μM EDTA. The mobile phase buffer was filtered through 0.2- μm membranes (Toyo Roshi) and degassed by ultrasonic generator (model 50a, MRK Inc., Tokyo, Japan) under reduced pressure before use. Flow rate was 0.7 mL/min. The column temperature was maintained at room temperature (25–28°C) and the potential of the detector was

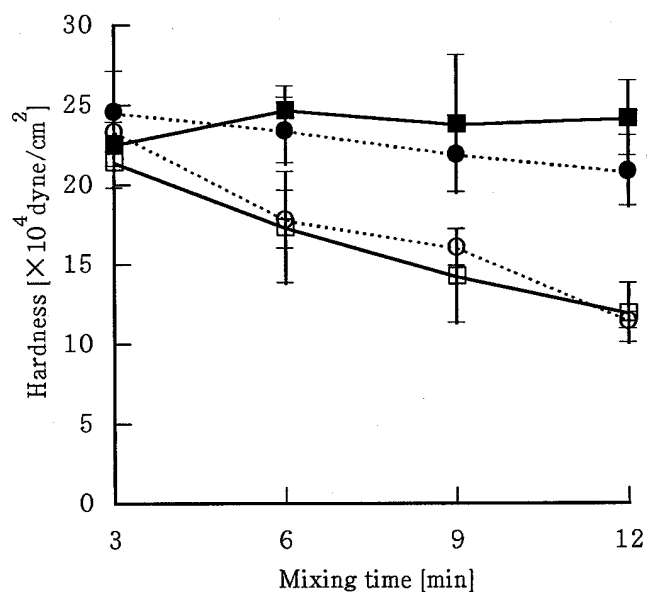


Fig. 1. Effect of L-ascorbic acid (AsA) with or without FeCl_3 on dough hardness. Flour-water dough control (○); 100 ppm of FeCl_3 (□); 10 ppm of AsA (●); 10 ppm of AsA and 100 ppm of FeCl_3 (■). Average \pm standard errors of four or more replicates. ** = Significantly different at $P < 0.01$.

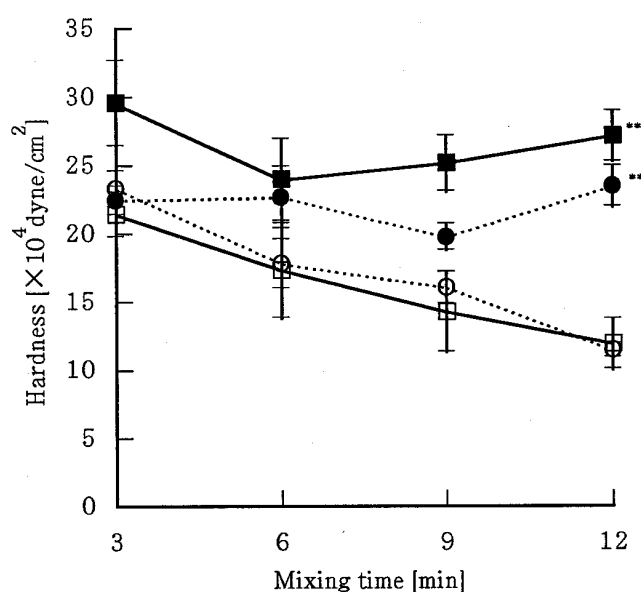


Fig. 2. Effect of L-ascorbic acid (AsA) with or without FeCl_3 on dough hardness. Flour-water dough control (○); 100 ppm of FeCl_3 (□); 100 ppm of AsA (●); 100 ppm of AsA and 100 ppm of FeCl_3 (■). Average \pm standard errors of four or more replicates. ** = Significantly different at $P < 0.01$.

set at +0.6 V vs. Ag/AgCl reference electrode. AsA concentrations in the sample were 0.005–0.01 mg%.

Quantitative determinations of AsA were made by using a calibration curve. The amount of DHA in the sample was estimated by the reduction of DHA to AsA with DTT. The reduction procedure was done as follows. The pH values of the sample solution were adjusted to pH 7.4 with 0.05M phosphate buffer; 1% DTT solution was added to a final concentration of 0.1% and incubated at 35°C for 30 min. After incubation, the sample was diluted with water suitable for HPLC analysis.

RESULTS AND DISCUSSION

Superoxide Generated by AsA Oxidation During Dough Mixing

We have investigated the effect of AsA and its related compounds on the rheological properties of flour-water dough during mixing. Previous results indicated that the improving effect of AsA was probably not by DHA produced from AsA in dough but by the intermediate oxidation products such as O_2^- produced in the oxidation process of AsA (Nakamura and Kurata 1997). Hardness was adopted as the parameter for the evaluation of the rheological properties of water-flour dough.

When a control flour-water dough was mixed, hardness decreased from an initial 23×10^4 dyne/cm² to 11×10^4 dyne/cm² as the mixing time of the dough increased from 3 to 12 min (Figs. 1 and 2). The hardness of the dough containing FeCl₃ was almost the same as that of the control dough. The dough containing 10 ppm of AsA using regular water maintained hardness at $\approx 23 \times 10^4$ dyne/cm² during dough mixing (Fig. 1). The dough containing 10 ppm of AsA and 100 ppm of FeCl₃ using regular water maintained hardness at $\approx 25 \times 10^4$ dyne/cm² during dough mixing (Fig. 1). There were significant differences ($P < 0.01$) in the dough hardness of the dough containing additives and control dough mixed for 12 min. After mixing for 12 min, hardness of dough containing AsA was increased in FeCl₃ (Fig. 1). Similarly, the dough containing 100 ppm of AsA and 100 ppm of FeCl₃ using regular water maintained hardness at $\approx 25 \times 10^4$ dyne/cm² during dough mixing (Fig. 2). O_2^- generation during AsA oxidation is accelerated by the heavy metal ion (Khan and Martell 1967a,b, and 1968; Ogata et al 1968). These results suggest that the effect of AsA on dough

hardness might depend on the concentration of O_2^- generated from oxygen molecule by the one electron migration from AsA.

The hardness of the dough prepared with oxygen-saturated water that promotes the generation of O_2^- during dough mixing was measured. The hardness of the control flour-water dough with oxygen-saturated water decreased from $\approx 18 \times 10^4$ dyne/cm² to 11×10^4 dyne/cm² during mixing (Figs. 3 and 4). This result with oxygen-saturated water was comparatively similar to the result with regular water shown in Figs. 1 and 2. The dough containing 10 ppm of AsA and 100 ppm of FeCl₃ maintained hardness at $\approx 25 \times 10^4$ dyne/cm² during mixing (Fig. 3). The dough containing 100 ppm of AsA and 100 ppm of FeCl₃ maintained hardness at $\approx 27 \times 10^4$ dyne/cm². These data showed that the hardness of the dough with

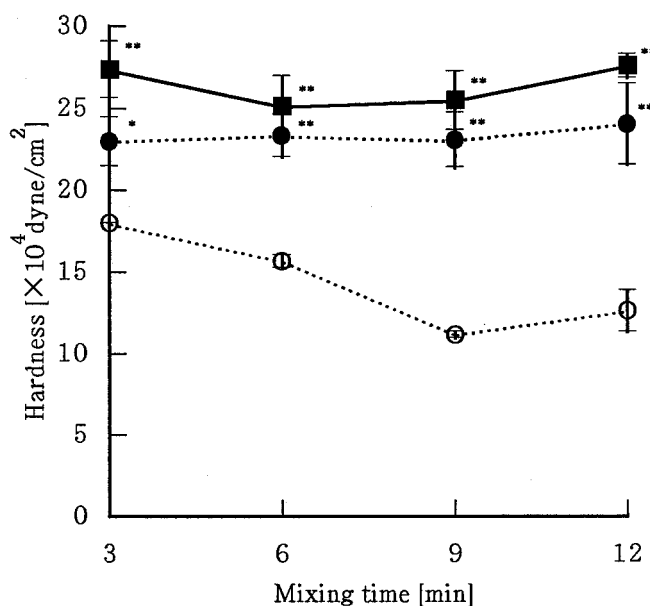


Fig. 4. Effect of L-ascorbic acid (AsA) with or without FeCl₃ on dough hardness. Dough was prepared with oxygen-saturated water. Flour-water dough control (○); 100 ppm of AsA (●); 100 ppm of AsA and 100 ppm of FeCl₃ (■). Average \pm standard errors of four or more replicates. ** = Significantly different at $P < 0.01$.

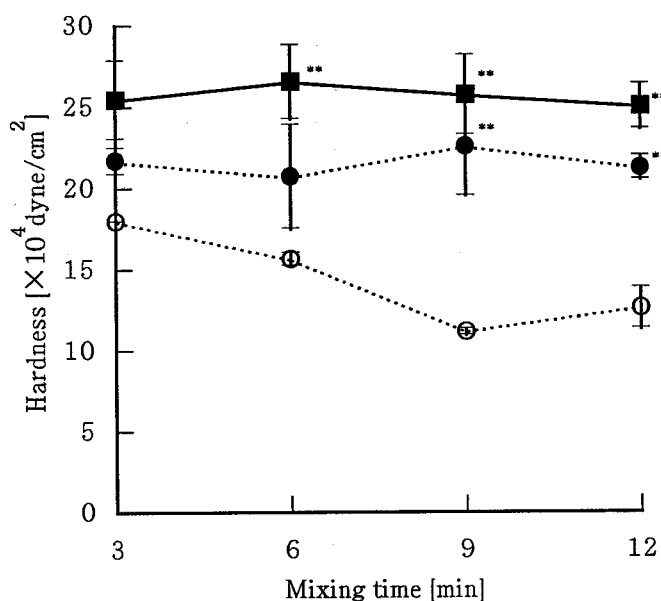


Fig. 3. Effect of L-ascorbic acid (AsA) with or without FeCl₃ on dough hardness. Dough was prepared with oxygen-saturated water. Flour-water dough control (○); 10 ppm of AsA (●); 10 ppm of AsA and 100 ppm of FeCl₃ (■). Bars show the standard errors. ** = Significantly different at $P < 0.01$.

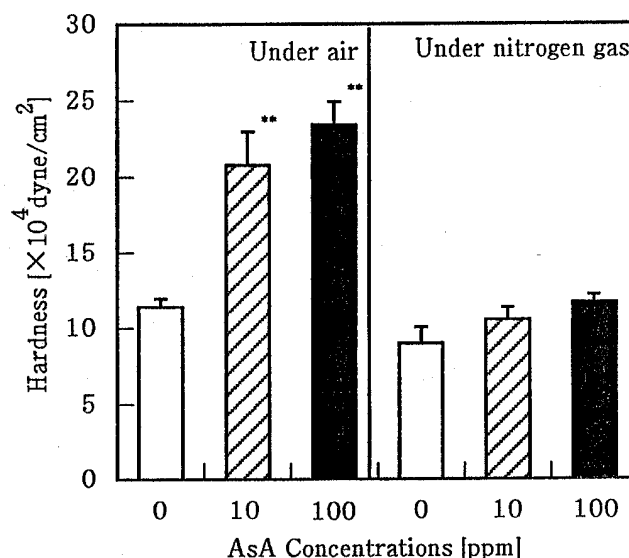


Fig. 5. Effect of L-ascorbic acid (AsA) on dough prepared under air or nitrogen gas for control, 10 ppm of AsA, and 100 ppm of AsA, respectively. Average \pm standard errors of four or more replicates. ** = Significantly different at $P < 0.01$.

AsA and FeCl₃ was increased after mixing for 12 min (Fig. 4). However, there is no statistical significant difference between the hardness of the dough prepared with regular water and oxygen-saturated water.

The hardness of the dough prepared with or without AsA under air or nitrogen gas is given in Fig. 5. The hardness of the dough sample prepared under nitrogen gas with 10 or 100 ppm of AsA were significantly decreased compared to those prepared under air.

Oxygen radicals are produced during AsA oxidation process in the presence of metals (Khan and Martell 1967a,b, and 1968; Ogata et al 1968) or in the absence of metals (Kurata et al 1996a,b). Generated oxygen radicals promote the oxidative degradation of various food and biological materials (Chiou 1983; Levine 1983; Samuni et al 1983; Shinar et al 1983; Marx and Chevion 1985; Uchida and Kawakishi 1986, 1987, and 1989; Uchida et al 1989). In particular, Sonntag et al (1993) had obtained evidence that O₂⁻ acts as a chain carrier in certain chain oxidation reactions that are induced as well as propagated by the O₂⁻ radical, and the thiolate ion of DTT is reported to be oxidized to a SS bond. Food proteins such as gluten in dough usually include SH groups and intra- or intermolecular SS bonds. Therefore, it could be possible that thiolate ion from SH groups might react with O₂⁻ and consequently produce intermolecular SS bonds.

Determination of Ascorbic Acid in Dough

Parallel with the measurement of dough hardness, determination of AsA and its oxidation products were also undertaken to

TABLE I
Degradation of L-Ascorbic Acid (AsA) During Dough Mixing

		Remaining AsA (ppm) After 12 min of Mixing			
		Regular Water ^a		O ₂ Saturated Water	
		No Iron	With Iron	No Iron	With Iron
Total C	10 ppm	7.3 ± 0.34	7.2 ± 0.01	7.3 ± 2.0	7.3 ± 0.30
AsA		1.6 ± 0.07	0.9 ± 0.05	1.3 ± 0.42	1.1 ± 0.30
DHA ^b		5.8 ± 0.26	6.3 ± 0.03	6.0 ± 2.4	6.2 ± 0.00
Total C	100 ppm	96.6 ± 3.02	100.5 ± 6.3	105.6 ± 2.2	95.4 ± 0.88
AsA		65.4 ± 1.41	49.0 ± 6.8	70.5 ± 9.0	48.1 ± 2.3
DHA		31.2 ± 2.52	51.3 ± 13	35.1 ± 6.6	47.4 ± 1.4

^a Deionized and distilled water.

^b Dehydro-L-AsA.

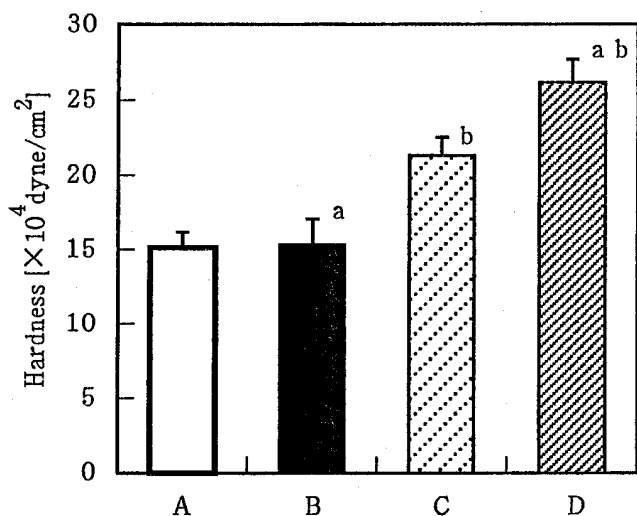


Fig. 6. Effect of superoxide anion radical generators on dough hardness. Columns A–D: Flour-water dough control, 5 × 10⁻³M xanthine, 5 × 10⁻³M xanthine and 1.1 × 10⁻⁶M xanthine oxidase (XOD), and 5 × 10⁻³M xanthine and 4.4 × 10⁻⁶M XOD, respectively. Average ± standard errors of four or more replicates. Values with the same letters are significantly different at P < 0.01 and P < 0.05, respectively.

estimate the corresponding chemical changes that occurred during mixing. We then considered the possible correlation between the chemical changes and the changes in rheological properties of these doughs. Remaining AsA in flour-water dough mixed for 12 min is listed in Table I. The AsA remaining in the dough containing AsA with FeCl₃ was less than that in the dough containing only AsA. However, there seemed to be no significant difference in the rate of AsA oxidation in the dough prepared with oxygen-saturated water or with regular water. As shown in Figs. 1–4, the trends in hardness with or without FeCl₃ or oxygen-saturated water were similar to the trends in the remaining amount of AsA in dough shown in Table I. As the rate of AsA oxidation was increased during dough mixing, the hardness of dough increased.

Effect of O₂⁻ Generating System or O₂⁻ Scavenging System on Dough Hardness

The effect of O₂⁻ on dough hardness was examined by using both an O₂⁻ scavenging system and an O₂⁻ generating system.

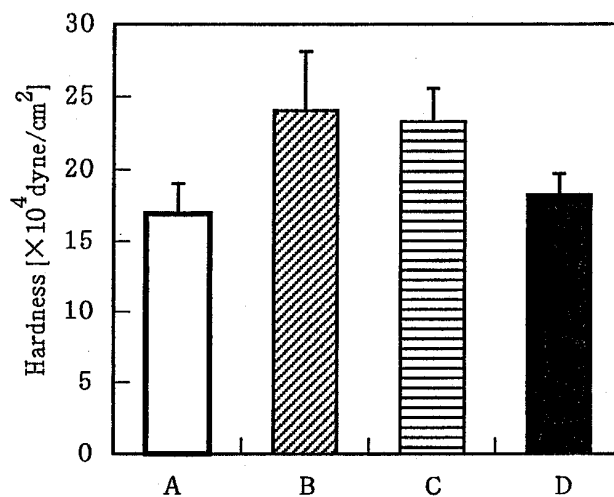


Fig. 7. Effect of superoxide dismutase (SOD) and catalase on dough hardness. Columns A–D, Flour-water dough control, 10 ppm of AsA, 10 ppm of AsA and 2 × 10⁻⁷M catalase, and 10 ppm of AsA and 1 × 10⁻⁶M SOD, respectively. Bars show the standard errors.

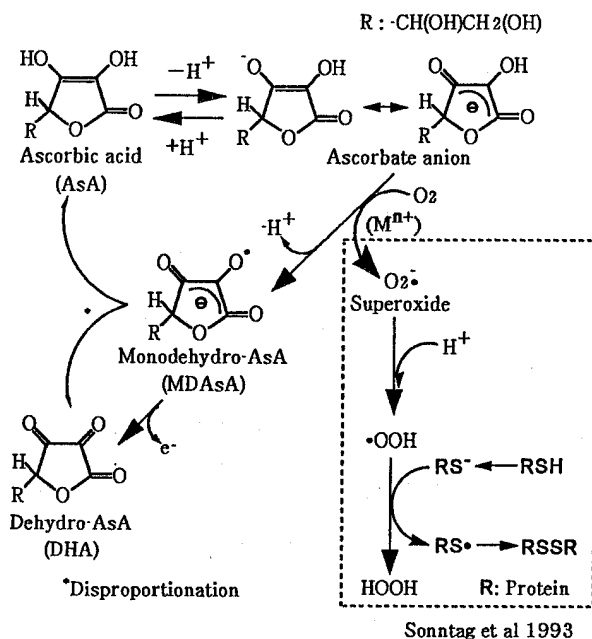


Fig. 8. Reaction scheme of L-ascorbic acid (AsA) and superoxide in dough.

Effect of O_2^- radical generators, XOD and xanthine system, on dough hardness is shown in Fig. 6. When control flour-water dough was mixed for 3 min with mortar and pestle, the dough hardness was $\approx 15 \times 10^4$ dyne/cm². The hardness of dough mixed with xanthine and EDTA in sodium phosphate buffer (pH 10.2) was 15×10^4 dyne/cm², almost the same as that of the control dough. Addition of XOD ($1.1 \times 10^{-6}M$) increased the dough hardness to 21×10^4 dyne/cm². At higher XOD concentrations ($4.4 \times 10^{-6}M$), dough hardness increased to $25-26 \times 10^4$ dyne/cm². Thus, O_2^- generated from XOD and xanthine systems was contributed to the formation of dough hardness when compared to control dough.

The effects of SOD and catalase on dough hardness are given in Fig. 7. When control flour-water dough was mixed for 3 min, the hardness of dough was $\approx 16 \times 10^4$ dyne/cm². The hardness of the dough containing only 10 ppm of AsA was 24×10^4 dyne/cm². The hardness of the dough containing 10 ppm of AsA, catalase, and SOD was similar to that of the control. O_2^- is generated in the autoxidation of AsA and SOD catalyzes O_2^- dismutation very efficiently. Catalase eliminated H_2O_2 , the reaction products of O_2^- dismutation by the catalytic reaction. However, as shown in Fig. 7, the hardness of the dough containing both 10 ppm of AsA and catalase was 23×10^4 dyne/cm², which was similar to the hardness of dough containing only 10 ppm of AsA. H_2O_2 had no significant effect on the dough hardness, while O_2^- and its related products such as the hydroperoxyl radical ($\bullet OOH$) did have some effect.

The data described above suggests that improvement function of AsA was mainly attributable to the formation of the O_2^- radical, the first reduction product of oxygen in the autoxidation process of AsA. Thus, the O_2^- produced supposedly affected the intra- or intermolecular SH-SS interchange reaction between protein molecules in dough, resulting in the formation of three-dimensional network. The formation of O_2^- in control dough was detected with nitro blue tetrazolium (NBT), which showed color change from initial yellow to purple or violet during the O_2^- generation suggested that the superoxide anion radical was formed in the dough containing AsA (data not shown). A possible reaction scheme of AsA in dough is illustrated in Fig. 8. DHA, the major stable oxidation product of AsA, is produced by the two successive one-electron oxidation process of AsA or by the disproportionation of reactive monodehydroascorbic acid (MDAsA) as the first one-electron oxidation product of AsA. In these processes, O_2^- is generated by the transfer of an electron from AsA to dioxygen with the formation of MDAsA. O_2^- formed will be immediately reduced to H_2O_2 by the dismutation reaction, accompanied by the production of hydroperoxyl radical ($\bullet OOH$), which might oxidize thiolate ion to thiyl radical, forming an SS bond (Sonntag et al 1993). These reaction pathways might be operative in the production process of bread dough.

Hardness of dough containing AsA may be related to the O_2^- generating process of AsA oxidation, and generated O_2^- was believed to affect the structure of protein. In this reaction process, O_2^- might bring about reactive hydroperoxyl radicals that would yield thiyl radicals or protein thiyl radicals to give intermolecular protein SS bonds (Fig. 8). Therefore, both the oxidation effect of DHA and the O_2^- formation during AsA oxidation seemed to be involved in the SH-SS interchange reaction and thus contribute to the improvement of the rheological properties of flour-water dough.

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