

Production of Thiyl Radical on a Peptide Derived from Wheat Protein by Superoxide Anion Radical

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ABSTRACT

Cereal Chem. 83(5):472–477

In the current study, we examined the role of superoxide anion radical (O_2^-) in the improvement of bread dough by L-ascorbic acid. Because of difficulties in detecting thiyl radicals in the presence of L-ascorbic acid, we replaced the latter with riboflavin, which produces O_2^- upon photoactivation. Nitro blue tetrazolium dye confirmed that O_2^- was produced in dough upon photoactivation of riboflavin. Electron spin resonance spectroscopy coupled with spin trapping showed that, in a solution

containing riboflavin and the hydrolyzed gluten peptide (GP-1), thiyl radicals are produced upon photoactivation. Addition of superoxide dismutase but not catalase suppressed the production of thiyl radicals on GP-1. These results suggest that the O_2^- produced during the oxidation of L-ascorbic acid in dough generates thiyl radicals on gluten proteins. This, in turn, would increase the production of interprotein disulfide bonds and result in an improvement in bread structure.

Since Jorgensen (1939) discovered that L-ascorbic acid (AsA) promotes oxidation of bread dough, a variety of studies have examined how it improves bread quality (Nicolas et al 1980; Ewart 1985; Pfeilsticker and Marx 1986; Kuninori and Nishiyama 1993; Sarwin et al 1993; Nakamura and Kurata 1997a,b, 1998; Hahn and Grosch 1998; Every et al 1999, 2000; Grosch and Wieser 1999; Koehler 2003). The effects of AsA are now thought to be due to its oxidation to dehydro-L-ascorbic acid (DHA) by ascorbic acid oxidase or transition metals (Fig. 1) (Kuninori and Nishiyama 1993). DHA is reduced back to AsA by glutathione dehydrogenase in conjunction with the oxidation of reduced glutathione. Oxidized glutathione, in turn, increases the formation of disulfide (SS) bridges in wheat proteins through a SS-sulfhydryl (SH) exchange reaction. Consequently, a network structure develops in the dough, resulting in an improvement in loaf structure (Sarwin et al 1993; Hahn and Grosch 1998; Grosch and Wieser 1999; Koehler 2003). An alternative hypothesis is that thiol disulfide oxidoreductase contributes to disulfide bridge formation after AsA is converted to DHA, thereby promoting the formation of a protein network in bread dough (Every et al 1999, 2000).

In a series of previous studies on the beneficial effects of AsA in heat-induced fish gel (Kamaboko) (Nishimura et al 1992a,b, 1994, 1996; Miyamoto and Nishimura 2006), it was found that superoxide anion radical (O_2^-) generated during the oxidation of AsA in the presence of transition metals such as iron removes hydrogen radicals from SH groups on myosin, resulting in the production of thiyl radicals ($S\cdot$). Due to the reaction of pairs of $S\cdot$, SS bonds are formed between myosin heavy chains, creating a network structure and therefore increasing the strength of the gel (Fig. 2) (Nishimura et al 1996). As described above, this mechanism is distinct in two important ways from the mechanism by which AsA improves bread.

Based on results from size-exclusion high-performance liquid chromatography, Nakamura and Kurata (1997a,b, 1998) suggested that, as in the fish gel, the promotion of protein polymerization in flours by AsA was due to O_2^- . In the present study, using soluble wheat peptide (trade name GP-1), we examined

whether this hypothesis, depicted in Fig. 2, explains the ability of AsA to improve bread quality, because the detection of $S\cdot$ in dough was very difficult.

MATERIALS AND METHODS

Materials

Wheat peptide (GP-1) was supplied by the Fine Chemical Laboratory of Nisshin Flour Milling (Tokyo, Japan). Biochemical-grade superoxide dismutase (SOD) from bovine erythrocytes and catalase from bovine liver were obtained from Wako Pure Chemicals (Osaka, Japan). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO, 99.5% pure) was from Labotec (Tokyo, Japan). The other chemicals used were reagent-grade and obtained from Nacalai Tesque (Kyoto, Japan). Distilled water was pretreated with Chelex 100 Resin (100–200 mesh, Bio-Rad Laboratories, Hercules, CA) to remove metal ions.

Preparing Flour-Water Dough Containing AsA

Flour-water dough was prepared according to the method of Nakamura and Kurata (1997a,b, 1998). To prepare flour-water dough, 200 g of flour and 40 mL of 15 mmol/L sodium phosphate buffer, pH 7.0, containing 8 mmol/L of nitro-blue tetrazolium (NBT) were mixed for 30 sec using a National SD-BT3 mixer (Matsushita Electric Industrial, Osaka, Japan). The dough was then mixed for 30 sec with 40 mL of 15 mmol/L sodium phosphate buffer, pH 7.0, with or without 80 mmol/L of 1,2-dihydroxybenzene-3,5-disulfonic acid disodium salt (Tiron), that was used as an O_2^- scavenger. Finally, the dough was mixed for 11 min with 50 mL of 15 mmol/L sodium phosphate buffer, pH 7.0, with or without 0.06% AsA. Water-flour doughs containing only NBT (control for AsA); NBT and AsA (AsA dough); or NBT, AsA, and Tiron (Tiron dough for AsA) were made. Final concentrations of NBT, AsA, and Tiron were 1 mmol/L, 0.01%, and 9.7 mmol/L, respectively. After mixing, 40 g of each dough was vacuum-packed and incubated at 30°C for 30 min. The coloration was observed as an indication of the reduction of NBT by O_2^- .

Preparing Flour-Water Dough Containing Riboflavin (Rf)

Flour-water dough containing Rf was prepared as described for AsA-containing flour-water dough except using Rf in place of AsA. Flour-water dough including NBT (control dough for Rf); NBT and Rf (Rf dough); or NBT, Rf, and Tiron (Tiron dough for Rf) contained final concentrations of 0.5 mmol/L NBT, 30 μ mol/L of Rf, and 9.7 mmol/L of Tiron. After mixing, 40 g of each dough was vacuum-packed and illuminated with

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sunlight for 30 min. The coloration was observed as an indication of the reduction of NBT by O_2^- .

Tricine-SDS-PAGE Analysis

Mixtures of 20% GP-1 and 0, 0.001, 0.01, 0.1, 1, or 10% 2-mercaptoethanol (2-ME) in 25 mmol/L of piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer, pH 7.0, were incubated overnight at room temperature. A portion of each GP-1 mixture (1 mL) was combined with an equal volume of 0.125 mol/L of Tris-HCl buffer, pH 6.8, containing 4% SDS, 20% glycerol, and 1% bromophenol blue. Each mixture was applied to a Tricine SDS-PAGE gel, and electrophoresis was conducted at 30V for 2–3 hr, followed by 100V for 5–6 hr as described by Schägger and Jagow (1987). After electrophoresis, the gels were soaked for 30 min in stationary solution containing 10% acetic acid and 40% methanol, stained for 60 min with Bio-Safe Coomassie dye (Bio-Rad, Hercules, CA) and then destained in distilled water.

Preparing GP-1 Solution

All steps were performed at 4°C. A solution of 25 mmol/L of PIPES buffer, pH 7.0, containing 6% GP-1 was centrifuged at $5,000 \times g$ for 10 min. A 40-mL portion of the supernatant was dialyzed against 25 mmol/L of PIPES buffer, pH 7.0, for two days. Next, the dialysis tube was submerged in polyethylene glycol (#20,000) to reduce the volume fourfold. This concentrated solution contained 24% GP-1 and is the GP-1 solution referred to in subsequent experiments. The amount of protein content in the solution was determined according to the method of Lowry (1951).

Preparing SS-Reduced and Nonreduced GP-1

All procedures were performed at 4°C. A mixture of the GP-1 solution containing 1% 2-ME was stirred overnight to reduce SS bonds and then dialyzed against 25 mmol/L of PIPES buffer, pH 7.0, to remove the 2-ME. The contents of the dialysis tube were collected and centrifuged at $3,000 \times g$ for 5 min, and the supernatant was used as the SS-reduced GP-1. Nonreduced GP-1 was prepared in the same manner except that 2-ME was omitted.

Preparing Samples Containing GP-1 for ESR Spectroscopy

A solution of 20% (w/v) nonreduced GP-1, 1 mmol/L of diethylenetriaminepentaacetic acid (DTPA), 0.2 μ mol/L of Rf, 75 mmol/L of DMPO (a spin-trap reagent), and 25 mmol/L of PIPES buffer, pH 7.0, was prepared for ESR spectroscopy. The same solution was also produced using SS-reduced GP-1 in place of the nonreduced GP-1. In addition, solutions of GP-1 or SS-reduced GP-1 containing SOD (2,000 units/mL), catalase (200 units/mL), or the same amounts of heat-inactivated SOD or catalase were prepared. Heat-inactivated enzymes were generated by heating solutions of 70,000 units/mL of SOD or 220,800 units/mL of catalase for 24 hr at 110°C under vacuum.

Measuring SH Content

Nonreduced and SS-reduced GP-1 were diluted 250-fold with 0.1 mol/L of Tris-HCl buffer, pH 8.0, containing 6 mol/L of guanidine hydrochloride and 0.01 mol/L of ethylenediamine tetraacetic acid. The amount of SH content in each solution was determined according to the method of Ellman (1959) and was used to calculate the quantities of SH content in each ESR sample.

ESR

The ESR spectra were obtained with an X-band ESR spectrometer (JES-FA100, JEOL, Tokyo, Japan) using a 100-kHz field modulation and a 0.4-mm thick flat cell (ES-LC12, JEOL, Tokyo, Japan). White actinic light (650,000 lx) from a halogen lamp (Mega Light100, Hoya-Schott, Tokyo, Japan) was used to excite the flavin molecules. The measurement was started just

after turning on the white actinic light. The conditions for recording ESR spectra were 335.8 mT center field; 16 mW microwave power; 0.1 mT modulation width; 2000 amplitude; 4 min sweep time; and 0.3 sec time constant. Magnetic field strength and apparent signal intensity of the $S\cdot$ adducts were calibrated using the ESR signal from Mn(II)-doped MgO powder.

Statistical Analyses

Unless otherwise stated, results represent the mean \pm standard deviation (SD) of at least triplicate independently measured samples. The significance of differences was evaluated using Student's *t*-test.

RESULTS AND DISCUSSION

Detecting Superoxide Anion Radical Generated During Oxidation of AsA in Flour-Water Dough

To examine the role of O_2^- in the polymerization of protein by AsA, we first examined whether O_2^- is generated in dough during the oxidation of AsA. AsA dough, Tiron dough, and control dough for AsA were prepared and then incubated for 30 min at 30°C. O_2^- production was measured with NBT, which is converted into a colored product in the presence of O_2^- . Differences in the coloration of the three kinds of dough were not noticeable immediately after preparation (Fig. 3A–C). Incubation did not change the color of the control dough (Fig. 3D), but the AsA dough became purple (Fig. 3E). This coloration was not as intense in the Tiron-containing dough (Fig. 3F)

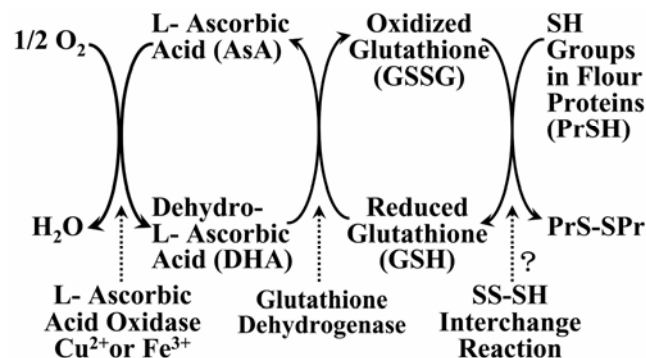


Fig. 1. Scheme for improving dough by AsA (Kuninori and Nishiyama 1993). AsA in dough is oxidized to DHA by ascorbic acid oxidase or transition metals. Reduction of DHA to AsA by glutathione dehydrogenase also results in the oxidation of reduced glutathione. This oxidation increases the formation of disulfide bridges among flour proteins through disulfide-sulfhydryl exchange.

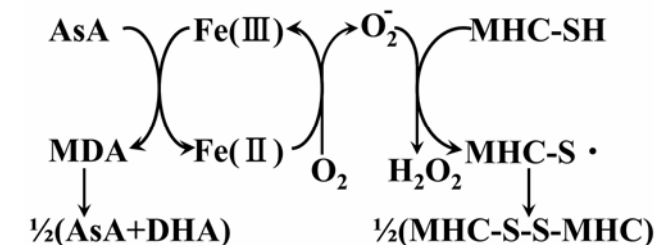


Fig. 2. Scheme for superoxide anion radical-dependent polymerization of myosin heavy chain (MHC) (Nishimura et al 1996). Fe(II) is shown as an example of a trace metal ion that catalyzes the auto-oxidation of AsA. Monodehydro-L-ascorbic acid (AsA radical) (MDA), the univalently oxidized product of AsA, is spontaneously disproportionated to AsA and DHA. AsA reduces molecular oxygen, forming O_2^- . The resulting O_2^- removes hydrogen radicals from sulfhydryl groups on myosin heavy chain, generating $S\cdot$. Two $S\cdot$ can then combine to form an SS bond.

compared with the AsA dough (Fig. 3E). These results suggest that O_2^- is generated in flour-water dough during the oxidation of AsA, and this supports earlier proposals (Nishimura et al 1996; Miyamoto and Nishimura 2006) that AsA improves the quality of dough by generating O_2^- .

Detecting Superoxide Anion Radical Generated by Photoactivated Rf in Flour-Water Dough

The ESR spectroscopy of AsA was unable to detect $S\cdot$, and only the monodehydro-L-ascorbic acid (AsA radical) was detected because of its persistence. Therefore, we used photoactivated

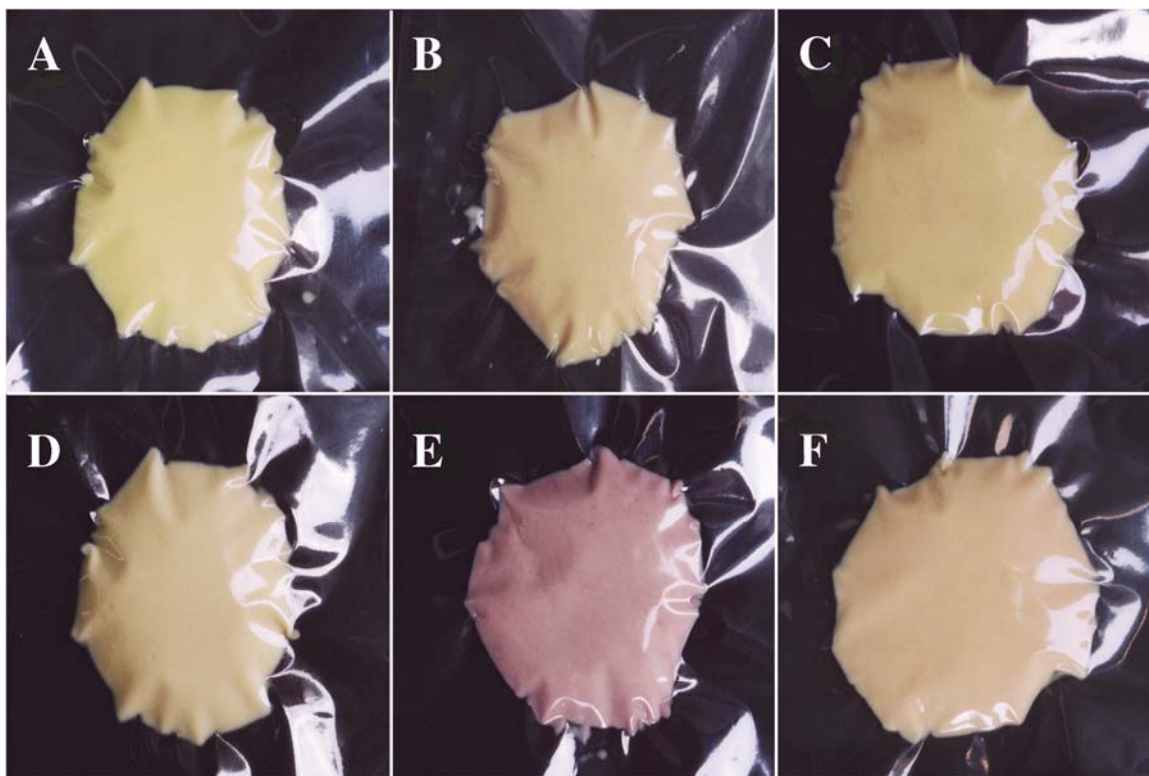


Fig. 3. Occurrence of O_2^- in AsA-containing dough before (A–C) and after (D–F) incubation. Dough containing 1 mmol/L of NBT (control dough for AsA A and D); 0.01% AsA and 1 mmol/L of NBT (AsA dough B and E); or 0.01% AsA, 1 mmol/L of NBT and 9.7 mmol/L of Tiron (Tiron dough for AsA C and F) was incubated at 30°C for 30 min.

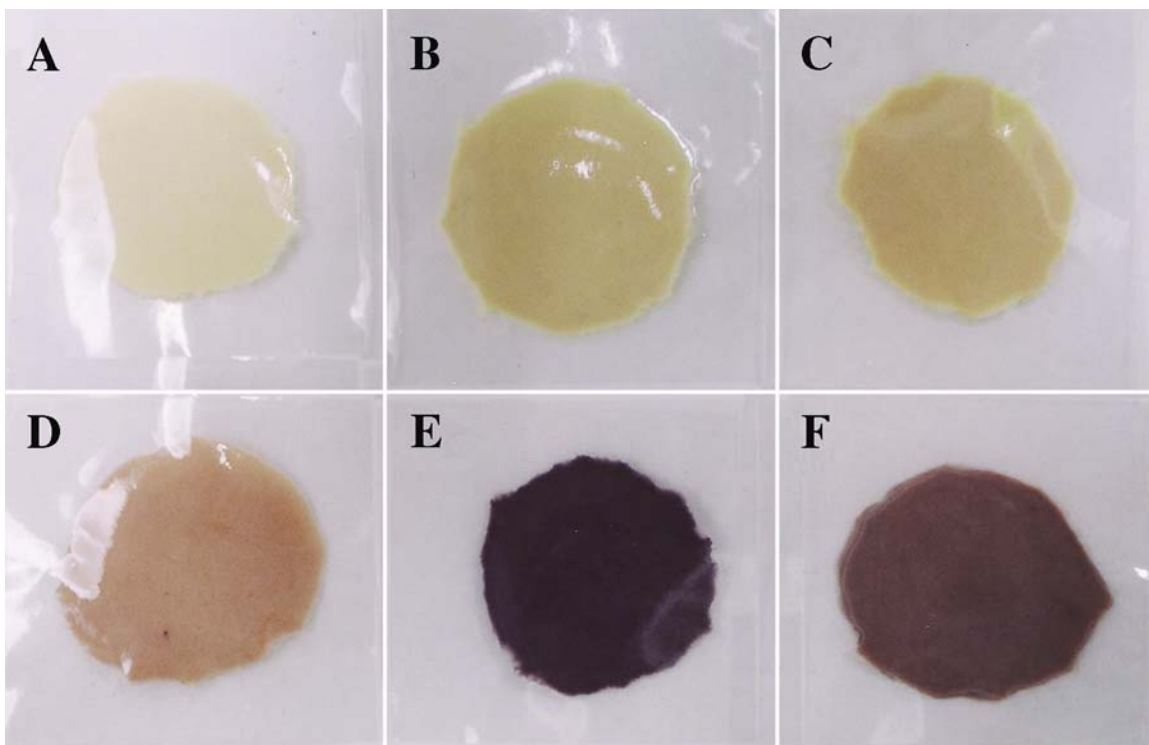


Fig. 4. Occurrence of O_2^- in Rf-containing dough before (A–C) and after (D–F) exposure to sunlight. Dough containing 0.5 mmol/L of NBT (control dough for Rf [A and D]); 30 μ mol/L of Rf and 0.5 mmol/L of NBT (Rf dough [B and E]); or 30 μ mol/L of Rf, 0.5 mmol/L of NBT, and 9.7 mmol/L of Tiron (Tiron dough for Rf [C and F]) was exposed to sunlight for 30 min.

Rf as a source of O_2^- (Nishimura et al 1996; Miyamoto and Nishimura 2006) to investigate whether O_2^- could be generated in flour-water dough. Rf dough, control dough for Rf, and Rf and Tiron dough were exposed to sunlight for 30 min. Before exposure to sunlight, a yellow color was observed in all three doughs due to the presence of Rf (Fig. 4A–C). Following exposure to sunlight, the control dough for Rf changed to pale red (Fig. 4D), whereas the Rf dough became dark purple (Fig. 4E). This dark red change in color was not as intense in the Rf and Tiron dough (Fig. 4F). Based on these results, it is clear that O_2^- is generated upon photoactivation of Rf in the flour-water dough. For this reason, Rf was used as a source of O_2^- in further experiments instead of AsA.

Effect of 2-ME concentration on GP-1

For studies of GP-1 where the SS bond is reduced, it was necessary to remove the reducing agent by dialysis. Higher concentrations of reducing agent, which can result in a greater degree of protein denaturation, require longer dialysis times. Therefore, to reduce the chance of protein denaturation, we determined the lowest concentration of 2-ME necessary to reduce the SS bond on GP-1. Tricine SDS-PAGE showed that the untreated GP-1 (as obtained from the manufacturer) migrated as a mixture of bands (MW \approx 1,000 to 26,600) (Fig. 5, lane 1), suggesting that GP-1 was not single peptide but rather a mixture of various peptides obtained by the hydrolysis of gluten. Although the addition of $<0.1\%$ 2-ME hardly affected the electrophoretic pattern of GP-1 (Fig. 5, lanes 2–4), the presence of $>1\%$ 2-ME caused the appearance of major bands with MW \approx 26,000, 14,000, and 6,500 (Fig. 5, lanes 5 and 6). This suggests that nearly all of the SS bonds in GP-1 are cleaved in the presence of 1% 2-ME. Therefore, the cleavage of SS bonds in further experiments was accomplished with 1% 2-ME.

Measuring SH Content in GP-1 Samples Used for ESR

Conversion of 5,5'-dithiobis (2-nitrobenzoic acid) to a yellow product was used to measure the SH content of GP-1 (Ellman 1959). The SH content in 1 mL of nonreduced and SS-reduced

GP-1 solution was $0.07 \pm 0.08 \mu\text{mol/mL}$ ($n = 9$) and $7.1 \pm 2.6 \mu\text{mol/mL}$ ($n = 9$), respectively. The SH content was therefore calculated to be $0.05 \pm 0.07 \mu\text{mol/mL}$ for the GP-1 ESR sample and $5.9 \pm 2.2 \mu\text{mol/mL}$ for the SS-reduced GP-1 ESR sample. These results show that 1% 2-ME effectively reduced SS bonds in GP-1.

Detecting GP-1 Thiyl Radicals by DMPO Spin-Trapping ESR

The ESR measurements were made with these samples at room temperature and in the presence of DMPO as a spin-trapping reagent. We examined the effect of illumination with white light (650,000 lx) on a 20% (w/v) solution of nonreduced GP-1 containing 1 mmol/L of DTPA, 0.2 $\mu\text{mol/L}$ of Rf, 75 mmol/L of DMPO, and 25 mmol/L of PIPES buffer, pH 7.0 (GP-1 sample). We also examined the same solution containing SS-reduced GP-1 in place of the nonreduced GP-1 (SS-reduced GP-1 sample). Before illumination, there was a weak signal for free radical species in the SS-reduced GP-1 sample (Fig. 6A). After illumination of the SS-reduced GP-1 sample, a quartet splitting with a 1:2:2:1 signal intensity ($g = 2.0052$) was obtained (Fig. 6B). The hyperfine splitting constants for the radical were determined to be $A_N = 1.522 \text{ mT}$ and $A_H = 1.670 \text{ mT}$, respectively. The obtained g and hyperfine splitting constant values corresponded with one DMPO-glutathione thiyl radical adduct (Ross et al 1984; Buettner 1987; Rota et al 1999). This suggests that the observed ESR spectrum was due to the presence of DMPO-S• adducts. Finally, when the GP-1 sample was used for the same ESR measurements, a different hyperfine structure (Fig. 6C) was obtained after illumination. A similar ESR spectrum was observed in the absence of GP-1 or SS-reduced GP-1 (data not shown). The observed ESR line was consistent with of the presence of a DMPO- O_2^- adduct. These results indicated that O_2^- participates in the production of S• in this system.

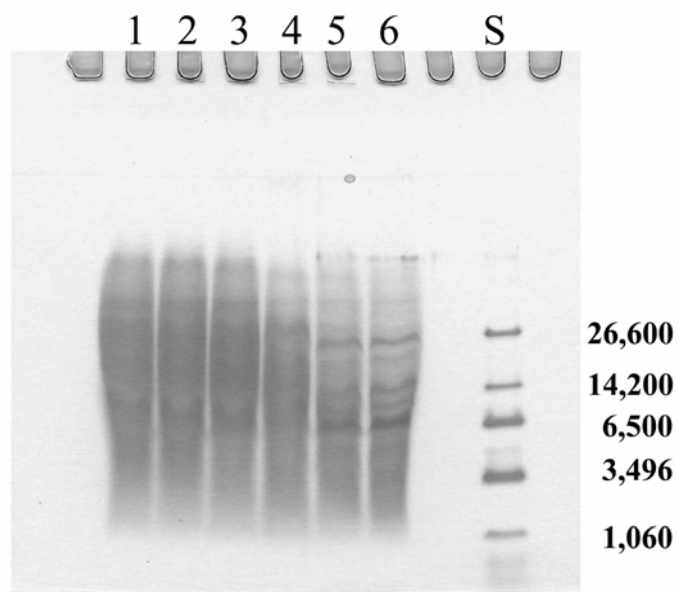


Fig. 5. Effect of 2-ME on GP-1. Solution of 20% GP-1 containing 0 (lane 1), 0.001 (lane 2), 0.01 (lane 3), 0.1 (lane 4), 1 (lane 5), or 10% 2-ME (lane 6) was incubated overnight at room temperature and analyzed by Tricine SDS-PAGE, molecular mass standards (lane S).



Fig. 6. Detection of S• on SS-reduced GP-1 by DMPO spin-trapping ESR spectroscopy. Solution of 20% SS-reduced GP-1, 1 mmol/L of DTPA, 2 $\mu\text{mol/L}$ of Rf, and 75 mmol/L of DMPO in 25 mmol/L of PIPES buffer, pH 7.0 (SS-reduced GP-1 sample) at room temperature in darkness (A) or in white light at 650,000 lx (B). Trace (C) was recorded under identical conditions as for (B) except using a solution of nonreduced GP-1.

To understand the mechanism of S• formation on GP-1, we examined the effect of using SOD as an O₂⁻ scavenger. In the presence of SOD (2,000 units/mL), the same quartet splittings were observed with the SS-reduced GP-1 reaction mixture (Fig. 7B) as without SOD (Fig. 7A), but the signal intensity was much weaker when SOD was present. This result suggested that the generation of S• on GP-1 was due to the action of O₂⁻. However, all proteins have some radical scavenging activity (Davies 1987; Soriani 1994; Halliwell and Gutteridge 1999). Then we examined the ESR spectrum of SS-reduced GP-1 containing heat-inactivated SOD. The ESR spectrum for the heat-inactivated SOD sample (Fig. 7C) had the same line shape and similar intensity as the sample lacking SOD (Fig. 7A). These results strongly suggest that the generation of S• on GP-1 was due to the action of O₂⁻.

O₂⁻ spontaneously disproportionates to H₂O₂ and O₂. The resulting H₂O₂ can divalently oxidize SH groups to SS through sulfenic acid (Armstrong and Buchanan 1978) or it can univalently oxidize them to S• as in the case of the horseradish peroxidase-catalyzed oxidation of glutathione (Harman et al 1986). Therefore, the decrease of intensity in Fig. 7B may be

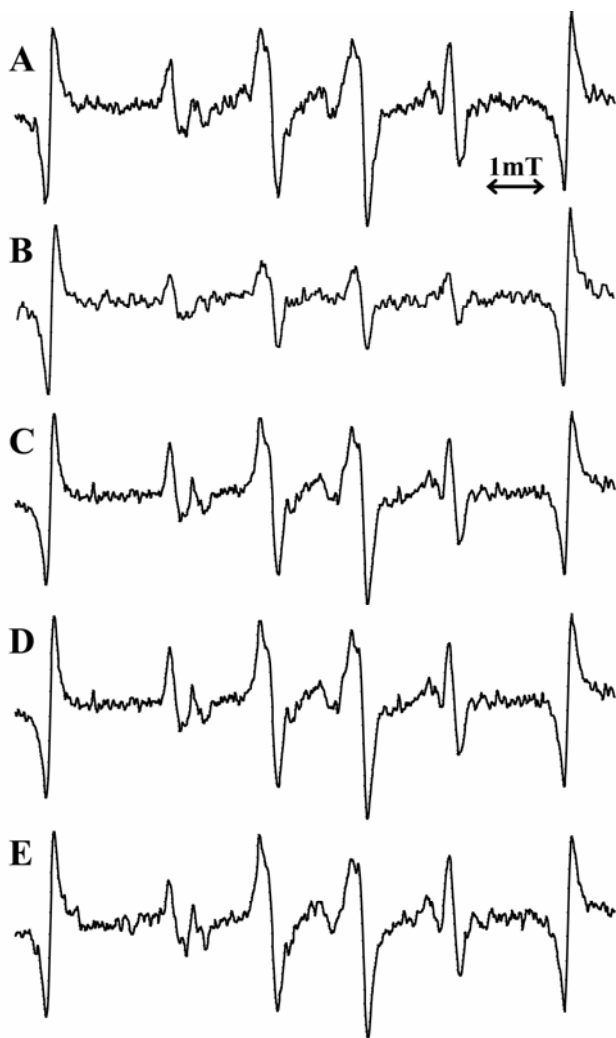


Fig. 7. Effect of SOD and catalase on occurrence of S• in SS-reduced GP-1. Solution of 20% SS-reduced GP-1, 1 mmol/L of DTPA, 2 μmol/L of Rf, and 75 mmol/L of DMPO in 25 mmol/L of PIPES buffer, pH 7.0 (SS-reduced GP-1 sample) was placed at room temperature illuminated by white light at 650,000 lx (A). Traces were recorded under identical conditions as trace (A) except in the presence of 2,000 units/mL of SOD (B), an equivalent amount of heat-inactivated SOD (C), 200 units/mL of catalase (D), or equivalent amount of heat-inactivated catalase (E).

derived not only from O₂⁻ but also from H₂O₂. To determine whether this occurred, we examined the effects of catalase, which scavenges H₂O₂. In the presence of catalase (200 units/mL) (Fig. 7D) or heat-inactivated catalase (Fig. 7E), the ESR spectrum after illumination of SS-reduced GP-1 had the same shape and intensity as the sample containing SS-reduced GP-1 alone (Fig. 7A). These results indicated the generation of S• on GP-1 was due to the action of O₂⁻.

The relative signal intensity of each second main peak in the S• trace was calculated as a percent of the mean signal intensity for SS-reduced GP-1 alone (Fig. 6B) as shown in Table I. The mean ± SD of the relative signal intensity for SS-reduced GP-1 alone was 100 ± 23.9% (*n* = 9). For SS-reduced GP-1 containing SOD, heat-inactivated SOD, catalase, and heat-inactivated catalase, the values were 58.3 ± 6.5% (*n* = 10), 109.1 ± 14.9% (*n* = 10), 81.4 ± 14.2% (*n* = 6), and 117.1 ± 12.6% (*n* = 10), respectively. Although there was a significant difference between the signal intensities of the SS-reduced GP-1 control and the SOD-containing sample (*P* < 0.05), significant differences were not found between the control and any of the other samples. These results suggest that the decrease in the intensity shown in Fig. 7B is due to O₂⁻ but not H₂O₂ and, furthermore, that the O₂⁻ causes the generation of S• on GP-1. It was already reported that the occurrence of S• became the trigger of the chain reaction that follows. Once the RS• has been formed, the radical chain reaction propagates to form RSSO•, RSR•, O₂⁻ and RS• radicals. The chain reaction is terminated finally to form disulfides (RSSR) (Saez et al 1982), suggesting strongly that disulfides were formed in this system, though we did not prove it experimentally. Of course, a real dough is a very complex system and contains a lot of LMW thiols like GSH. We can not deny the possibility that such thiols would provide the same products by recombination of glutathionyl radicals to GSSG and would inhibit protein depolymerization as postulated by Grosch. However, detecting the occurrence of S• on GP-1 indicates strongly that the S• on wheat protein occurs by O₂⁻ in a real dough, resulting in the formation of SS bridges between wheat proteins, although some parts of O₂⁻ were reacted with thiols such as GSH. Then we think that our advocated mechanism coexists with other systems as postulated by Grosch and Wieser (1999).

CONCLUSIONS

Collectively, our results confirmed that O₂⁻ is generated in dough and that it produces S• on GP-1 peptides, and therefore on wheat protein. This supports the findings of Nakamura and Kurata (1997a,b, 1998) that AsA promotes the polymerization of protein in flours by producing O₂⁻. This also supports the mechanism proposed by Nishimura et al (1996) (Fig. 2). However, this does not eliminate the mechanism proposed by Kuninori and Nishiyama (1993) (Fig. 1), and advocated by Grosh et al (Sarwin et al 1993; Hahn and Grosch 1998; Grosch and Wieser 1999), and Koehler (2003) for the improvement of

TABLE I
Relative Signal Intensity of SS-Reduced GP-1 Radical in ESR^a

Additives	<i>n</i>	Signal Intensity (%)
None (Control)	9	100.0 ± 23.9
SOD	10	58.3 ± 6.5*
Heat-inactivated SOD	10	109.1 ± 14.9
Catalase	6	81.4 ± 14.2
Heat-inactivated catalase	10	117.1 ± 12.6

^a Values represent the mean values ± SD. ESR of each sample was measured under illumination. Relative signal intensity of the second peak was compared with the control. *, Significantly different from the control (*P* < 0.05).

bread because the process of fermentation in breadmaking would consume most of the O₂, making the spontaneous oxidation of AsA without oxidase difficult. Thus, the improvement of bread by AsA through O₂⁻ production (Fig. 2) can apply only to breads made without yeast. In fact, our results suggest that enough O₂ is present in unleavened bread dough for AsA to produce sufficient amounts of O₂⁻ to improve bread quality. Further studies on the production of S• in wheat protein using O₂⁻ are currently being conducted in our laboratory.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (2) (14580150) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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[Received September 2, 2005. Accepted April 10, 2006.]