Beneficial Effects of Ascorbic Acid on Heat-Induced Fish Gel (Kamaboko) from the Superoxide Anion Radical

Yuka MIYAMOTO* and Kimio NISHIMURA†

Department of Food Science and Nutrition, Doshisha Women’s College of Liberal Arts, Kamigyo-ku, Kyoto 602-0893, Japan

Received August 31, 2005; Accepted October 22, 2005

We examined the participation of the superoxide anion radical \( (\text{O}_2^{-}) \) in the beneficial effects of L-ascorbic acid on heat-induced fish gel (Kamaboko). The generation of a thiol radical \( (\text{S}^{-}) \) in glutathione, ovalbumin, and actomyosin was examined by electron spin resonance spectroscopy coupled with spin trapping. \( \text{O}_2^{-} \) was provided by the photoactivation of riboflavin. The typical line shape for \( \text{S}^{-} \) was observed with the glutathione and ovalbumin samples. A signal different from that for \( \text{S}^{-} \) was detected with the actomyosin sample, and its intensity markedly decreased when the SH groups of actomyosin had been modified. The signal was eliminated when superoxide dismutase was added, but unaffected when catalase or an equivalent amount of heat-inactivated superoxide dismutase or catalase were added. These results suggest that \( \text{S}^{-} \) in actomyosin was produced by the reaction with \( \text{O}_2^{-} \) and that the beneficial effects of L-ascorbic acid are due to a different mechanism in Kamaboko from that in bread.

Key words: vitamin C; improvement mechanism; actomyosin; superoxide anion radical; electron spin resonance (ESR)

Since Jørgensen discovered in 1939 that L-ascorbic acid (AsA) promoted the oxidation of bread dough,1 a variety of studies have examined how it improves bread quality.2–19 Two main hypotheses have been developed, the first based on studies by Every14,15 and the second by Kuninori and Nishiyama16–18 and Grosch et al.16–18 Both hypotheses accept that only the L-threo isomer of AsA is rheologically active and that added AsA is quickly converted to dehydro-L-ascorbic acid (DHA) by ascorbic acid oxidase which is present in wheat flour. Every has proposed that direct oxidative cross-linking of protein thiols to interprotein disulfides by DHA is catalyzed by thiol disulfide oxidoreductase, although this enzyme has not previously been identified in flour. On the other hand, the findings of Kuninori and Nishiyama as well as those of Grosch have indicated a rapid conversion of endogenous reduced glutathione (GSH) to its oxidized form in conjunction with simultaneous reduction of DHA to AsA by glutathione dehydrogenase in the early stages of dough mixing.2,16–18 Thereafter, thiol (SH)-disulfide (SS) interchange occurs between oxidized glutathione and protein thiols, increasing the amount of interprotein disulfides, which improves dough quality. This second hypothesis is also supported by the findings of Koehler19 who used \(^{35}\)S-labeled GSH to confirm that the addition of AsA to dough promoted thiol-disulfide interchange between the cysteine residues of proteins and glutathione.

AsA is also used as an agent for improving the quality of heat-induced fish gel (Kamaboko).20–22 The mechanism for this improvement has been thought to be the same as that proposed by Kuninori and Nishiyama19 and Grosch et al.16–18 because the addition of DHA to raw fish paste (surimi) increased the strength of Kamaboko.20 However, whether this occurs and the details of the mechanism for improving Kamaboko have not yet been determined.

To clarify the effects of AsA on protein, we have previously carried out studies using ovalbumin (OVA).23,24 We found that AsA and DHA enhanced OVA polymer formation by promoting SS bond formation even in the absence of oxidase, reductase, or GSH. In addition, we observed that active oxygen, especially the superoxide anion radical \( (\text{O}_2^{-}) \) generated during the oxidation of AsA, played a role in accelerating polymer formation. These results suggested a mechanism of action distinct from that in bread. Further studies on the effect of AsA on surimi showed that \( \text{O}_2^{-} \) promoted the formation of SS bonds among the myosin heavy chain (MHC) in the absence of oxidase, reductase, or GSH.25–28 Moreover, by using electron spin resonance (ESR) spectroscopy, we detected the thyl radical (\( \text{S}^{-} \)) of Cys due to its reaction with \( \text{O}_2^{-} \).29 Based on these studies, we have proposed the mechanism shown in

---

* To whom correspondence should be addressed. Tel: +81-75-251-4214; Fax: +81-75-251-4289 or 4214; E-mail: knishimu@dwc.doshisha.ac.jp
† Present address: Department of Home Economics, Shukugawa Gakuen College, 6-58 Koshikiiwa-cho, Nishinomiya 662-8555, Japan

Abbreviations: AsA, L-ascorbic acid (vitamin C); DHA, dehydro-L-ascorbic acid; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DTPA, diethylene-triaminepentaacetic acid; ESR, electron spin resonance; GS, glutathione thyl radical; NEM, N-ethylmaleimide; OVA, ovalbumin; PIPES, piperazine-N,N’-bis(2-ethanesulfonic acid); S, thyl radical; SOD, superoxide dismutase
However, this mechanism was deduced from studies on Cys and not on MHC, so it was not clear whether S- had been generated in MHC by O$_2^-$.

Accordingly, in the current study, the formation of S- was examined in actomyosin; we used actomyosin that consisted of approximately 70% myosin. The resonant motion of S- generated in a high-molecular-weight substance such as a protein is easily restricted, so ESR spectra reveal anisotropic line shapes.

Therefore, in initial experiments, we first examined the reaction of O$_2^-$ with GSH, a low-molecular-weight compound, to determine whether the typical trace for S- could be observed by spin trapping ESR spectroscopy. In addition, we used photoactivated riboflavin as the source of O$_2^-$, instead of AsA, because the persistence of the monodehydro-L-ascorbic acid (AsA radical) precluded the ability to detect S-. This preliminary analysis suggested that S- was indeed produced in GSH. ESR also confirmed that O$_2^-$ generated S- in OVA, a small protein. Finally, to determine whether the production of S- accounted for the ability of AsA to improve Kamaboko, we examined whether O$_2^-$ resulted in the production of radical species in actomyosin, a very large protein, and whether it gave an ESR trace consistent with that for S-.

Materials and Methods

Special-grade frozen surimi of walleye pollack, Theragra chalcogramma, containing 4% sucrose, 4% sorbitol, and 0.2% polyphosphoric acid was purchased in a market and stored at -80°C. Reagent-grade OVA was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Reagent grade GSH and 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 of reagent grade from Nacalai Tesque (Kyoto, Japan). Distilled water was pretreated with Chelex 100 resin (100–200 mesh; Bio-Rad, Hercules, CA, USA) to remove metal ions.

Preparation of the GSH and OVA solutions. To prepare SH-blocked GSH, a solution of 24 mM GSH and 80 mM N-ethylmaleimide (NEM) in 0.2 M sodium phosphate buffer at pH 7.0 was mixed for 1 h at 4°C. To prepare SH-blocked OVA, a solution of 8.1% OVA and 80 mM NEM in 0.2 M sodium phosphate buffer at pH 6.0 was mixed for 1 h at 4°C. Control solutions lacking NEM were also prepared for both GSH and OVA.

Preparation of the actomyosin and modified actomyosin concentrates. All steps were performed at 4°C. Actomyosin was prepared as described by Takashi et al. To remove the sarcoplasmic proteins, frozen surimi was homogenized with five volumes of 15 mM sodium phosphate buffer at pH 7.0 containing 0.16 M NaCl and then centrifuged at 3,000 × g for 10 min. This step was repeated two more times. The resulting precipitate was homogenized with 10 volumes of 15 mM sodium phosphate buffer at pH 7.0 containing 0.45 M NaCl and the solution stirred slowly for 2 h. The mixture was then centrifuged for 30 min at 23,000 × g, after which a crude actomyosin solution was obtained as the supernatant. Ten volumes of Chelex-treated water were added, and the solution was centrifuged for 25 min at 11,000 × g. To the recovered precipitate, NaCl was added to a final concentration of 0.45 M to liquefy it. Since this was a semi-solid preparation, we refer to it hereafter as the “actomyosin concentrate.” The modified actomyosin concentrate was prepared in a similar manner except that the precipitate following the removal of sarcoplasmic proteins was homogenized in a buffer containing 40 mM NEM. The protein contents of the actomyosin and modified actomyosin concentrates were determined by the method of Kjeldahl.

Preparation of samples for ESR spectroscopy. ESR spectroscopy of GSH was performed on solutions containing GSH (0, 1, 5, 10, or 20 mM) or modified...
GSH (20 mM). To examine the effects of SOD and catalase on GSH, solutions were prepared containing GSH (20 mM) or modified GSH (20 mM) in the presence of SOD (200 units/ml), catalase (200 units/ml), or an equivalent amount of heat-inactivated SOD or catalase. Studies on OVA were performed on solutions containing 6.75% OVA or modified OVA. To examine the effects of SOD and catalase on OVA, solutions were prepared containing OVA (6.75%) or modified OVA (6.75%) in the presence of SOD (2000 units/ml), catalase (200 units/ml), or an equivalent amount of heat-inactivated SOD or catalase. All of these solutions also contained 1 mM diethylentriaminepentaacetic acid (DTPA), 0.2 μM riboflavin, 75 mM DMPO (as a spin trap reagent), and 0.2 M sodium phosphate buffer at pH 7.0 and 6.0 for GSH and OVA, respectively. Heat-inactivated SOD and catalase were produced by heating 70,000 or 220,800 units/ml of the respective enzyme for 24 h at 110°C under vacuum.

ESR spectroscopy of actomyosin was performed on solutions containing 0.83 g/ml of the actomyosin concentrate, with sodium phosphate buffer replaced by 4.17 mM PIPES buffer at pH 7.0. To examine the effect of SOD and catalase on actomyosin, solutions of actomyosin were prepared containing SOD (200 units/ml), catalase (200 units/ml), or an equivalent amount of heat-inactivated SOD or catalase.

Measurement of the SH content. GSH and OVA solutions were respectively diluted 800- and 250-fold with 0.1 M Tris–HCl buffer at pH 8.0 containing 6 M guanidine hydrochloride and 0.01 M ethylenediamine tetraacetic acid. In respect of actomyosin, 0.6 g of the actomyosin concentrate or modified actomyosin concentrate was dissolved in 14.4 ml of 0.1 M Tris–HCl buffer at pH 8.0 containing 6 M guanidine hydrochloride and 0.01 M ethylenediamine tetraacetic acid. The SH content of each solution was determined according to the method of Ellman. 33)

ESR spectroscopy. Electron spin resonance spectroscopy was carried out with an X-band ESR spectrometer (JES-FA100), using 100-kHz field modulation at room temperature in a 0.4-mm flat cell (ES-LC12; jeol, Tokyo, Japan). White actinic light (520,000 lx) for the GSH experiments, 650,000 lx for the OVA experiments, and 455,000 lx for the actomyosin experiments) from a halogen lamp (Mega Light 100; Hoya-Schott, Tokyo, Japan) was used for flavin excitation. In the case of OVA, the ESR spectra were recorded after 4 min of illumination; otherwise, the ESR measurements were begun just after turning on the white actinic light. The ESR spectra were recorded under the following conditions: center field, 335.8 mT; microwave power, 16 mW; modulation width, 0.1 mT; amplitude, 2000; sweep time, 4 min; and time constant, 0.3 s. The magnetic field strength and apparent signal intensity of the S– adduct were calibrated by using the ESR signals of Mn(II)-doped MgO powder.

### Table 1. SH Content of the ESR Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>SH content (μmoles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (20 mM)</td>
<td>20.2 ± 0.8</td>
</tr>
<tr>
<td>Modified GSH</td>
<td>0**</td>
</tr>
<tr>
<td>OVA</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>Modified OVA</td>
<td>0**</td>
</tr>
<tr>
<td>Actomyosin</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Modified actomyosin</td>
<td>0.05 ± 0.1**</td>
</tr>
</tbody>
</table>

*Each value is presented as the mean ± SD (n = 3).**Significantly different from each kind of unmodified sample (p < 0.01)

Statistical analyses. Unless otherwise stated, each result is presented as the mean ± standard deviation (SD) of at least triplicate samples. The significance of differences was evaluated by Student’s t-test.

Results and Discussion

Measurement of the SH content of the GSH samples for ESR spectroscopy

The SH contents in 1 ml of the GSH and modified GSH solutions (24 mM) were respectively 24.2 ± 0.9 μmoles/ml (n = 3) and nil (n = 3). Using these solutions, we prepared ESR samples for GSH and modified GSH. Based on the measured SH content, the SH contents in 1 ml of the GSH and modified GSH samples for ESR were respectively calculated to be 20.2 ± 0.8 μmoles/ml and nil (Table 1). This shows that all of the SH groups in the modified GSH sample had been blocked by the treatment with NEM.

Detection of the glutathione thyl radical (GS•) by DMPO spin trapping ESR spectroscopy

In these experiments, we first examined the reaction of O2 with GSH, a low-molecular-weight compound, to determine whether the typical trace for S• could be observed by spin trapping ESR spectroscopy. In addition, we used photoactivated riboflavin as the source of O2 instead of AsA, because the persistence of the monodehydro-L-ascorbic acid (AsA radical) precluded the ability to detect S•. ESR measurements were performed on these GSH samples at room temperature and in the presence of the spin trapping reagent, DMPO. The samples, which contained 20 mM GSH or modified GSH, were illuminated by white light (520,000 lx). Before this illumination, no free radical species could be detected in the GSH sample (Fig. 2A). Following illumination, a quartet split with a 1:2:2:1 signal intensity (g = 2.0055) was observed (Fig. 2B). The hyperfine splitting constants of the radical were determined to be ΔN = 1.525 mT and ΛH = 1.611 mT. The obtained values for g and the hyperfine splitting constant corresponded with a DMPO-S• adduct in Cys34–36 suggesting that the shape of the ESR trace was due to the formation of a DMPO-S• adduct. When ESR spectroscopy was carried out on the modified GSH sample, a different hyperfine structure was obtained after
The presence of a DMPO-O₂⁻ adduct in the ESR spectra observed in the absence of GSH (Fig. 2D) is consistent with the observed illumination (Fig. 2C). A similar ESR spectrum was observed for 20 mM GSH in the presence of heat-inactivated SOD was the same as that of the control, strongly supporting the idea that O₂⁻ caused the production of S⁻.

Since O₂⁻ spontaneously disproportionates to H₂O₂ and O₂, it is also possible that H₂O₂ divalent oxidized SH groups to SS via sulfenic acid or univalently to S⁻, as in the case of the horseradish peroxidase-catalyzed oxidation of GSH. We therefore assessed the effect of adding catalase (200 units/ml), a H₂O₂ scavenger, on the ESR spectra for 20 mM GSH. The same line shape and signal intensity as those of the control were obtained in the presence of both catalase and heat-inactivated catalase (data not shown), implying that H₂O₂ did not participate in the generation of GS⁻.

We further calculated the signal intensity of each second main peak in the S⁻ trace relative to the control (Table 2). The mean ± SD for the control was 100.0 ± 10.3% (n = 3), and the relative signal intensities obtained from the samples containing 20 mM GSH and SOD (200 units/ml), heat-inactivated SOD, catalase (200 units/ml), and heat-inactivated catalase were 63.2 ± 14.3% (n = 3), 103.5 ± 4.8% (n = 3), 104.9 ± 18.0% (n = 3), and 103.5 ± 4.7% (n = 3), respectively. Although there was a significant difference between the relative signal intensities of the control and the 20 mM GSH sample containing SOD (p < 0.01), there were not significant differences between the control and other samples. However, the addition of SOD did not completely eliminate S⁻, possibly because some of the O₂⁻ reacted with SH groups faster than it could be scavenged. Since it is known that there is a little difference between the rate constants for O₂⁻ with thiol compounds (10⁻⁵⁻¹0⁶ M⁻¹ s⁻¹) and O₂⁻ with SOD (4.8 × 10⁶ M⁻¹ s⁻¹), these results confirmed that S⁻ in GSH shown in Fig. 2B was due to O₂⁻ but not H₂O₂. Although S⁻ in GSH has been previously proposed, this is the first time that this species has been experimentally confirmed.

### Dependence of GS⁻ generation on the GSH concentration

We next examined the dependence of the GS⁻ signal on the concentration of GSH. Figure 3A shows the ESR trace for the 20 mM GSH control and is the same as that of GS⁻ in the absence of GSH (Fig. 2D). The observed line shapes in both Figs. 2C and D are consistent with the presence of a DMPO-O₂⁻ adduct. These results indicate the possibility that O₂⁻ participated in the production of S⁻ in this system.

To understand the reaction mechanism for the formation of GS⁻, we examined the effect of adding SOD as a O₂⁻ scavenger. In the presence of SOD (200 units/ml), the 20 mM GSH sample (Fig. 2E) showed the same quartet splitting as that of the control (Fig. 2B), although its signal intensity was significantly weaker than the control. This suggests that the generation of GS⁻ was due to the action of O₂⁻. However, all proteins are known to have some radical-scavenging activity. Therefore, to exclude this effect we also performed the ESR experiment in the presence of heat-inactivated SOD. As shown in Fig. 2F, the ESR spectrum observed for 20 mM GSH in the presence of heat-inactivated SOD was the same as that of the control, strongly supporting the idea that O₂⁻ caused the production of S⁻.

**Table 2.** DMPO-GSH Radical Adduct in the 20 mM GSH Samples

<table>
<thead>
<tr>
<th>Additive</th>
<th>Signal intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>100.0 ± 10.3</td>
</tr>
<tr>
<td>SOD</td>
<td>63.2 ± 14.3**</td>
</tr>
<tr>
<td>Heat-inactivated SOD</td>
<td>103.5 ± 4.8</td>
</tr>
<tr>
<td>Catalase</td>
<td>104.9 ± 18.0</td>
</tr>
<tr>
<td>Heat-inactivated catalase</td>
<td>103.5 ± 4.7</td>
</tr>
</tbody>
</table>

Each value is presented as the mean ± SD (n = 3). The relative second peak intensity is shown compared to the control. ESR measurements were carried out under illumination by white actinic light at 650,000 lx.

**Significantly different from the control (p < 0.01)**
SH content in the OVA samples for ESR spectroscopy

We investigated whether this could be detected in a small protein by using OVA. The SH contents in 1 ml of the OVA and modified OVA solutions were 5.1 ± 0.1 μmoles/ml (n = 3) and nil (n = 3), respectively. Using these solutions, we prepared OVA and modified OVA samples for ESR spectroscopy. The SH contents in 1 ml of the ESR samples of OVA and modified OVA were calculated to be 4.3 ± 0.1 μmoles/ml and nil, respectively (Table 1). This shows that all of the SH groups in the modified OVA sample had been blocked by NEM.

Detection of S− in OVA by DMPO spin trapping ESR spectroscopy

In this and following experiments, the line shape shown in Figs. 2B and 3A was considered to indicate the presence of S−. ESR spectra for the OVA and modified OVA samples were recorded before and after illumination with white light (650,000 lx). Prior to this illumination, no ESR signal derived from DMPO could be detected for the OVA sample (Fig. 4A). However, after 4 min of illumination, quartet splitting with a 1:2:2:1 signal intensity was detected (Fig. 4B). The obtained signal showed characteristic line shapes for S− in GSH presented in Fig. 2B. Samples containing 10 and 5 mM GSH gave the same clear quartet splitting with a 1:2:2:1 signal intensity, although the signal intensity was weaker as the concentration of GSH was reduced (Figs. 3B and C). Moreover, as the GSH concentration was further decreased, the quartet signal weakened and new peaks, presumably due to O2− (arrow), emerged and became stronger (Figs. 3D and E). These results indicate that a concentration above 5 mM GSH was needed to obtain the typical line shape for GS−/C1.

The resonant motion of S−/C1 generated in high-molecular-weight substances such as protein is easily restricted, causing the ESR signal to be anisotropic. However, GSH has a low-molecular-weight, and we confirmed a signal characteristic of S−/C1 in the GSH experiments.

SH content in the OVA samples for ESR spectroscopy

We investigated whether this could be detected in a small protein by using OVA. The SH contents in 1 ml of the OVA and modified OVA solutions were 5.1 ± 0.1 μmoles/ml (n = 3) and nil (n = 3), respectively. Using these solutions, we prepared OVA and modified OVA samples for ESR spectroscopy. The SH contents in 1 ml of the ESR samples of OVA and modified OVA were calculated to be 4.3 ± 0.1 μmoles/ml and nil, respectively (Table 1). This shows that all of the SH groups in the modified OVA sample had been blocked by NEM.

Detection of S− in OVA by DMPO spin trapping ESR spectroscopy

In this and following experiments, the line shape shown in Figs. 2B and 3A was considered to indicate the presence of S−. ESR spectra for the OVA and modified OVA samples were recorded before and after illumination with white light (650,000 lx). Prior to this illumination, no ESR signal derived from DMPO could be detected for the OVA sample (Fig. 4A). However, after 4 min of illumination, quartet splitting with a 1:2:2:1 signal intensity was detected (Fig. 4B). The obtained signal showed characteristic line shapes for S− in GSH presented in Fig. 2B. Samples containing 10 and 5 mM GSH gave the same clear quartet splitting with a 1:2:2:1 signal intensity, although the signal intensity was weaker as the concentration of GSH was reduced (Figs. 3B and C). Moreover, as the GSH concentration was further decreased, the quartet signal weakened and new peaks, presumably due to O2− (arrow), emerged and became stronger (Figs. 3D and E). These results indicate that a concentration above 5 mM GSH was needed to obtain the typical line shape for GS−/C1.

The resonant motion of S−/C1 generated in high-molecular-weight substances such as protein is easily restricted, causing the ESR signal to be anisotropic. However, GSH has a low-molecular-weight, and we confirmed a signal characteristic of S−/C1 in the GSH experiments.

SH content in the OVA samples for ESR spectroscopy

We investigated whether this could be detected in a small protein by using OVA. The SH contents in 1 ml of the OVA and modified OVA solutions were 5.1 ± 0.1 μmoles/ml (n = 3) and nil (n = 3), respectively. Using these solutions, we prepared OVA and modified OVA samples for ESR spectroscopy. The SH contents in 1 ml of the ESR samples of OVA and modified OVA were calculated to be 4.3 ± 0.1 μmoles/ml and nil, respectively (Table 1). This shows that all of the SH groups in the modified OVA sample had been blocked by NEM.

Detection of S− in OVA by DMPO spin trapping ESR spectroscopy

In this and following experiments, the line shape shown in Figs. 2B and 3A was considered to indicate the presence of S−. ESR spectra for the OVA and modified OVA samples were recorded before and after illumination with white light (650,000 lx). Prior to this illumination, no ESR signal derived from DMPO could be detected for the OVA sample (Fig. 4A). However, after 4 min of illumination, quartet splitting with a 1:2:2:1 signal intensity was detected (Fig. 4B). The obtained signal showed characteristic line shapes for S− in GSH presented in Fig. 2B. Samples containing 10 and 5 mM GSH gave the same clear quartet splitting with a 1:2:2:1 signal intensity, although the signal intensity was weaker as the concentration of GSH was reduced (Figs. 3B and C). Moreover, as the GSH concentration was further decreased, the quartet signal weakened and new peaks, presumably due to O2− (arrow), emerged and became stronger (Figs. 3D and E). These results indicate that a concentration above 5 mM GSH was needed to obtain the typical line shape for GS−/C1.

The resonant motion of S−/C1 generated in high-molecular-weight substances such as protein is easily restricted, causing the ESR signal to be anisotropic. However, GSH has a low-molecular-weight, and we confirmed a signal characteristic of S−/C1 in the GSH experiments.
and Cys. When the sample contained modified OVA, we obtained a completely different ESR spectrum after illumination (Fig. 4C) that was consistent with the presence of a DMPO-O$_2^-$ adduct and similar to the signal obtained for a sample lacking GSH (Fig. 2D). These findings strongly suggest that the signal in Fig. 4B was due to S\(^-\).

To determine the type of radical species responsible for the generation of S\(^-\) in OVA, we examined the effect of SOD (2,000 units/ml) as an O$_2^-$ scavenger. We also examined the effect of an equivalent amount of heat-inactivated SOD to that in the control for the effect of protein. As shown in Fig. 4D, the ESR signal obtained from the OVA sample that included SOD was very weak. In contrast, the addition of heat-inactivated SOD to OVA (Fig. 4E) resulted in a similar line shape and intensity to those in the control lacking an added enzyme (Fig. 4B). The addition of catalase (200 units/ml), to examine the role of H$_2$O$_2$, or an equivalent amount of heat-inactivated catalase also resulted in a line shape and intensity similar to those of the control lacking an added enzyme (data not shown).

We further calculated the signal intensity of each second main peak relative to that of the control (Table 3). The mean ± SD of the relative signal intensity for the control was 100.0 ± 19.9% (n = 3), and the values for the OVA sample including SOD (2,000 units/ml), heat-inactivated SOD, catalase (200 units/ml), and heat-inactivated catalase were 120.8 ± 26.0% (n = 3), 120.8 ± 26.0% (n = 3), 105.2 ± 18.9% (n = 3), and 118.7 ± 27.9% (n = 3), respectively. There was a significant difference between the intensities of the signal for the control and for the OVA sample including SOD (p < 0.01), but no significant differences were apparent between the control and the other samples. These results suggest that the decrease in intensity shown in Fig. 4D was due to O$_2^-$, but not to H$_2$O$_2$, and that O$_2^-$ causes the generation of S\(^-\) in OVA.

OVA has a low molecular weight, which allowed us to detect S\(^-\) in the protein even though ESR signals for proteins tend to be anisotropic.\(^{30}\) We suspect that our ability to detect S\(^-\) in OVA depended on the concentration of SH groups, because an SH concentration at or above approximately 5 μmoles/ml was sufficient to allow the observation of the typical line shape for S\(^-\).

We therefore suspect that we would also be able to detect this radical in larger proteins as long as there were enough SH groups present.

**Table 3.** DMPO-OVA Radical Adduct in the OVA Samples

<table>
<thead>
<tr>
<th>Additive</th>
<th>Signal intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>100.0 ± 19.9</td>
</tr>
<tr>
<td>SOD</td>
<td>42.7 ± 9.2**</td>
</tr>
<tr>
<td>Heat-inactivated SOD</td>
<td>120.8 ± 26.0</td>
</tr>
<tr>
<td>Catalase</td>
<td>105.2 ± 18.9</td>
</tr>
<tr>
<td>Heat-inactivated catalase</td>
<td>118.7 ± 27.9</td>
</tr>
</tbody>
</table>

Each value is presented as the mean ± SD (n = 3). The relative second peak intensity is shown compared to the control. ESR measurements were carried out under illumination by white actinic light at 455,000 lx. **Significantly different from the control (p < 0.01).

**Determination of protein content in the actomyosin samples for ESR spectroscopy**

We next examined whether we could detect the generation of S\(^-\) by O$_2^-$ in actomyosin which has a very high-molecular-weight. The amounts of protein per 1 g of the actomyosin and modified actomyosin concentrates were 15.4 ± 1.0 (n = 3) and 17.8 ± 2.0 (n = 3) mg/g, respectively. Thus, there was no significant difference between the actomyosin and modified actomyosin concentrates. This also shows that the method used for preparing actomyosin was reproducible.

**Estimation of the SH content in the actomyosin samples for ESR spectroscopy**

The SH contents per 1 g of the actomyosin and modified actomyosin concentrates were 1.08 ± 0.1 (n = 3) and 0.06 ± 0.1 (n = 3) μmoles, respectively. The samples for ESR spectroscopy were made from these concentrates, the SH contents per 1 ml of the actomyosin and modified actomyosin samples being 0.9 ± 0.1 and 0.05 ± 0.1 μmoles, respectively (Table 1). Thus, almost all of the SH groups in actomyosin had been blocked by the treatment with NEM.

**Detection of the radicals in actomyosin by DMPO spin trapping ESR spectroscopy**

Prior to illuminating with white light (455,000 lx), no ESR signal could be detected in the actomyosin sample (Fig. 5A), but a signal with four main peaks was detected after illumination (Fig. 5B). The line shape of this signal was not similar to that of S\(^-\) (Fig. 2B), but rather was characteristic of O$_2^-$ when actomyosin was replaced with modified actomyosin, the intensity of the signal was markedly less, although the line shape did not change (Fig. 5C). These results indicate that the signal intensity of the radical generated in actomyosin depended on the SH content of the actomyosin sample and that the four peaks shown in Fig. 5B were partly derived from S\(^-\). The SH content of the actomyosin sample was essentially the same (0.9 ± 0.1 μmoles/ml) as that in 1 mM GSH, and in that case, a trace similar to that of O$_2^-$ was also observed, despite the presence of S\(^-\). The line shape obtained for the actomyosin sample was also similar to that of the 1 mM GSH sample. These facts also support the idea that the signal obtained for the actomyosin sample was at least partly due to S\(^-\).

To determine the type of radical species that generated the radicals in actomyosin, we examined the effect of adding SOD (200 units/ml). This resulted in a nearly undetectable ESR signal (Fig. 5D). When SOD was replaced by heat-inactivated SOD (Fig. 5E), the line shape and intensity of the signal were similar to those of the control (Fig. 5B). The addition of catalase (200 units/ml) or an equivalent amount of heat-inacti-
For the actomyosin sample was partly due to S/C1 modified actomyosin sample, indicating that the trace signal intensities of the actomyosin sample and respectively. There was a significant difference between 99% (n = 3). The relative second peak intensity of signal as those of the control (data not shown). The intensities of each second main peak in the ESR results show that the radicals which were partly derived for the actomyosin sample and the other signals. These significant differences were apparent between the signal for the actomyosin sample and for the sample was also a significant difference in intensities between the signal for the actomyosin sample and for the actomyosin sample containing SOD (p < 0.05). No significant differences were apparent between the signal for the actomyosin sample and the other signals. These results show that the radicals which were partly derived from S had been generated in actomyosin by O2− and not by H2O2.

It has been thought that the mechanism by which AsA improved heat-induced fish gel (Kamaboko) was the same as that proposed by Kuninori and Nishiyama2) and Grosh et al.16–18) for bread. However, the results presented here suggest that the beneficial effect of AsA was due to the mechanism proposed by Nishimura et al. (Fig. 1), which involves O2− generated during the oxidation of AsA. Active oxygen commonly seems to be a trigger for aging and life-style related or other diseases,45) so the search for food ingredients, which can extinguish active oxygen, such as anthocyanin, has been extensive out.46–50) However, in the case of food processing (especially Kamaboko), we can suggest from the results of this study the positive side of active oxygen that has a beneficial effect on food quality.

In contrast to Kamaboko, yeast rapidly consumes O2 in leavened dough, making it difficult for the oxidation of AsA to occur without oxidase. On the other hand, microorganisms are not present in surimi, and AsA can be easily oxidized because a sufficient level of O2 is present. Indeed, the generation of O2− from AsA has been reported in surimi.27) Accordingly, our results suggest that, in unleavened bread, the beneficial effect of AsA occurs by the same mechanism as that in Kamaboko. Studies in our laboratory are now examining the validity of this hypothesis.

**Acknowledgments**

We thank Professor Kunihiro Tajima at Kyoto Institute of Technology for his critical reading of this manuscript. This work was supported in part by grant-aid for scientific research (C) (2) (14580150) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.
References


