Stabilization of L-Ascorbic Acid by Superoxide Dismutase and Catalase

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The effects of superoxide dismutase (SOD) and catalase on the autoxidation rate of l-ascorbic acid (ASA) in the absence of metal ion catalysts were examined. The stabilization of ASA by SOD was confirmed, and the enzyme activity of SOD, which scavenges the superoxide anion formed during the autoxidation of ASA, contributed strongly to this stabilization. The stabilization of ASA by catalase was observed for the first time; however, the specific enzyme activity of catalase would not have been involved in the stabilization of ASA. Such proteins as bovine serum albumin (BSA) and ovalbumin also inhibited the autoxidation of ASA, therefore it seems that non-specific interaction between ASA and such proteins as catalase and BSA might stabilize ASA and that the non-enzymatic superoxide anion scavenging ability of proteins might be involved.

Key words: l-ascorbic acid; autoxidation; superoxide dismutase; catalase; stabilization

ASA is an important antioxidant in food and biological systems and, when acting as an antioxidant, the oxidation reaction of ASA necessarily occurs. It is known that ASA is easily oxidized to yield dehydro-L-ascorbic acid (DASA) via monodehydro-L-ascorbic acid (MDASA). This oxidation process is known to be accelerated by heavy metal ions; however, although the reaction rate is very slow, it can also proceed without a metal ion catalyst. On the other hand, it has been reported that such reducing reagents as thiourea, diithiothreitol and uric acid, such chelating agents as EDTA, diethylenetriamine-N,N',N'',N'''-pentaaetic acid (DETPAC), phosphates and citrates, such sugars as sucrose, sugar syrup, glucose and maltose, as well as glycerol and propylene glycol each inhibited the autoxidation of ASA. It was also reported that such amino acids as His and Gln, such peptides as Gly-Gly-His and Gly-His-Lys, and such proteins as serum albumin, ceruloplasmin and protamine protected the degradation of ASA. The chelation of trace metal ions in an ASA solution by proteins, peptides, or amino acids has been indicated to account for the stabilization mechanism of ASA in most of these foregoing reports. However, it was also suggested in some reports that possible direct interaction between protein-related compounds and ASA might contribute to the stabilization of ASA in a solution. Thus, the mechanism for stabilizing ASA has not been fully elucidated.

It has also been reported that the superoxide anion was formed during the oxidation of ASA in an aqueous solution in both the absence and presence of a metal catalyst. In regard to the formation mechanism for the superoxide anion, we have proposed in previous papers that ASA autoxidation proceeded via the C(2) oxygen adduct of ASA, and that the superoxide anion would be directly released from the C(2) oxygen adduct of ASA to form MDASA. It is well known that hydrogen peroxide was formed from the superoxide anion during the oxidation of ASA by a reaction involving the superoxide anion with ASA or MDASA and disproportionation of the superoxide anion. It has also been reported that the reaction of DASA with the superoxide anion in an aqueous solution might result in the formation of ASA. In the presence of a heavy metal ion catalyst, the hydroxy radical is well known to be produced from hydrogen peroxide by the Fenton reaction. Therefore, substances which have some radical scavenging effects might stabilize ASA. Scarpa et al. have reported that SOD protected the degradation of ASA due to the scavenging superoxide anion formed during the autoxidation of ASA, but it has not been clarified whether the enzymatic superoxide scavenging effect of SOD would satisfactorily account for the stabilization of ASA. The effect of catalase, a hydrogen peroxide scavenger, on the oxidation of ASA in the absence of metal ion catalysts has not yet been studied.

In this study, the effects of SOD, catalase, and such other proteins as BSA and γ-globulin on the autoxidation reaction of ASA in the absence of a metal ion catalyst were examined. The mechanism for the stabilization of ASA by SOD and catalase will be discussed.

Materials and Methods

Materials. ASA was obtained from Wako Pure Chemical Industries and further purified by recrystallization. Cu, Zn-SOD of bovine erythrocytes, bovine catalase, egg albumin (ovalbumin), γ-globulin from human serum, and egg-white lysozyme were obtained from Wako Pure Chemical Industries. BSA was from Sigma Chemical Co. Ultra-refined water with an electrical resistance of 18 MΩ·cm was used throughout the experi-

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Abbreviations: ASA, l-ascorbic acid; SOD, superoxide dismutase
Autodioxidation reaction of ASA. Recrystallized ASA was dissolved in a 0.07 M potassium phosphate buffer (pH 7.4) at a concentration of 50 μM, and oxygen gas was bubbled through the solution at a flow rate of 200 ml/min for 30 min at 35°C. The buffer solution was passed through a Chelex-100 column (Bio-Rad Laboratories) to remove any trace heavy metal ions.

a) Effect of proteins. The effect of SOD and catalase on the autodioxidation of ASA was examined by carrying out the autodioxidation reaction in the presence of 10⁻⁶ M SOD and (or) 2 × 10⁻⁷ M catalase as reported by Scarpa et al. The effect of various proteins on the autodioxidation of ASA was examined by running the autodioxidation reaction in the presence of 5 × 10⁻⁷ M BSA, ovalbumin or lysozyme, or of 33 mg/ml of γ-globulin. Inactivated catalase was prepared by heating a catalase solution at 90°C for 5 min. The effect of inhibitors of SOD on the autodioxidation of ASA was examined by adding 1.5 mM KCN or 3 mM sodium azide to the autodioxidation reaction mixture in the presence of 10⁻⁶ M SOD and 2 × 10⁻⁷ M catalase.

b) Effect of hydrogen peroxide. The autodioxidation reaction of ASA was carried out without bubbling any oxygen gas in the presence of 0, 50, 100, 200 and 250 μM hydrogen peroxide.

Determination of the remaining amount of ASA. The amount of remaining ASA in the reaction mixture was determined by using a Shimadzu double-beam UV-180 spectrophotometer. It was estimated by the absorbance of the reaction solution at 265 nm, the wavelength of the absorption maximum of ASA in the neutral pH region.

Statistical analysis. Significant differences between two groups were evaluated by Student's t-test.

Results and Discussion

Effects of SOD and catalase

Figure 1 presents the effects of SOD and catalase on the ratio of remaining ASA during the autodioxidation of ASA in a buffer solution. The ratio of remaining ASA was significantly larger in the presence of SOD and (or) catalase than in the absence of these radical-scavenging enzymes. It has been reported that catalase was added to the reaction mixture to avoid the involvement of hydrogen peroxide in the oxidation reaction of ASA and also to scavenge hydrogen peroxide, the possible inhibitor of SOD. The mechanism for the inactivation of SOD by exposure to hydrogen peroxide has recently been reported. The remaining amount of ASA was significantly larger in the presence of SOD that in its absence, which agreed with the results of the earlier study. It has also been reported that SOD scavenged the superoxide anion which was formed during the autodioxidation of ASA. However, the oxidation of ASA was also suppressed in the presence of catalase, and this phenomenon has never been reported before. It has not previously been clarified whether only the enzymatic superoxide anion scavenging ability of SOD is involved in the stabilization of ASA, nor whether the enzymatic ability of catalase contributes to the stabilization of ASA.

Effects of proteins

Figure 2 presents the effects of various proteins that are considered to have non-specific activity for scavenging active oxygen species on the ratio of remaining ASA during the autodioxidation of ASA in a buffer solution, together with those of SOD and catalase. The ratio of remaining ASA was significantly larger in the presence
of BSA or ovalbumin than in the absence of such a protein. Although the effect was smaller, γ-globulin and lysozyme also tended to stabilize ASA in a buffer solution. Since these proteins stabilized ASA in the buffer solution, it seemed that some non-enzyme effects of SOD and catalase might have been involved in the stabilization of ASA.

**Enzyme effect of catalase**

The reactivity of ASA with hydrogen peroxide formed during the autoxidation of ASA was examined. Figure 3 shows the effect of hydrogen peroxide in the ratio of remaining ASA during the autoxidation of ASA. Although the ratio of remaining ASA was smaller in the presence of 200 μM of hydrogen peroxide (four times larger than the concentration of ASA) than that of the control, an almost negligible effect of hydrogen peroxide on the autoxidation of ASA was apparent. It was thus reconfirmed that the reactivity of ASA with hydrogen peroxide was very low, as has generally been believed. Figure 4 presents the enzymatic effect of catalase on the autoxidation of ASA in a buffer solution. In the presence of inactivated catalase, the ratio of remaining ASA during the autoxidation of ASA was approximately equal to that in the presence of catalase and was significantly larger than that in the absence of catalase (control). These results suggest that the enzymatic ability of catalase, the removal of hydrogen peroxide, does not contribute to the stabilization of ASA. Although the exact mechanism has not been clarified, it is considered that some non-enzyme effects of catalase might have been involved in the stabilization of ASA, just as was observed in the case of the proteins (Fig. 2).

**Enzyme effect of SOD**

Figure 5 shows the effect of inhibitors on SOD during the autoxidation of ASA in a buffer solution. In the presence of KCN or sodium azide, both being inhibitors of SOD, the ratio of remaining ASA was smaller than that in the absence of the inhibitor, being approximately equal to that of the control (in the absence of SOD). It was confirmed that KCN or azide had no effect on the lability of ASA. Therefore, the decrease in the ratio of remaining ASA in the presence of an inhibitor would be attributable to the loss in enzyme activity of SOD. Therefore, the significant contribution of the specific enzyme effect of SOD, its superoxide anion-scavenging ability, to the stabilization of ASA was reconfirmed. It is unclear, however, whether a non-enzyme effect of SOD might have contributed to the stabilization of ASA or

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**Fig. 3.** Effect of Hydrogen Peroxide on the Autoxidation Rate of ASA.

Each value is the mean±SD (n=4) and is expressed as a ratio, with the initial amount of ASA regarded as 100%. The reaction was carried out for 30 min without bubbling oxygen gas.

*p<0.05 significantly different from the result without hydrogen peroxide.

**Fig. 4.** Effect of Catalase on the Autoxidation Rate of ASA.

Each value is the mean±SD (n=4) and is expressed as a ratio, with the initial amount of ASA regarded as 100%. The reaction was carried out for 30 min.

*p<0.01 significantly different from the control (without catalase).

**Fig. 5.** Effect of SOD Activity on the Autoxidation Rate of ASA.

Each value is the mean±SD (n=4) and is expressed as a ratio, with the initial amount of ASA regarded as 100%. The reaction was carried out for 30 min.

*p<0.01 significantly different from the control (with catalase).
not.

As already described, the enzyme effect of SOD on the stabilization of ASA was confirmed in this study. The stabilization of ASA by catalase in the absence of metal ion catalysts was newly found, although the stabilization mechanism is considered to have been different from that of SOD. The enzyme ability of catalase would not have contributed to the stabilization of ASA. Some proteins, like BSA and ovalbumin, stabilized ASA; therefore, a similar type of non-enzyme effect of catalase might be involved in the stabilization of ASA. The stabilization of ASA by some proteins, peptides and amino acids has also been reported, and it was suggested that a possible direct interaction between ASA and protein-related compounds stabilized ASA against autoxidation. It was reported that His, Glu, GSSG, Gly-Gly-His and Gly-His-Lys were especially effective for inhibiting the autoxidation of ASA.13) It has also been reported that prolamine, which contains many basic amino acids such as Arg, stabilized ASA in an aqueous solution, and the possible formation of a complex of ASA with prolamine was suggested by chromatographic analysis.15) Possible hydrogen bonding of ASA with some amino acid residues in peptides has been suggested to provide a stabilizing effect on ASA,13) although the detailed mechanism for this has not been clarified. Meucci et al. have reported a possible interaction between ASA and BSA from the results of UV and fluorescence spectroscopy.20) Therefore, the stabilization of ASA by catalase and some proteins that was found in this study might be accounted for by an interaction between ASA and the proteins. However, the possible non-enzymatic superoxide anion scavenging ability of these proteins might also have affected the stabilization of ASA. It has been reported that such protein-related compounds as ceruloplasmin23) peroxidase,23) catalase23) and metallothionein24,25) GSH25) N-acetyl-Cys20) and Cys25) showed superoxide anion scavenging ability, although the effectiveness was not as strong as that of SOD.

In summary, the stabilization of ASA by SOD was reconfirmed to be mainly due to the specific enzymatic superoxide scavenging ability of SOD. However, it is suggested that the stabilization of ASA by catalase and some other proteins could be accounted for by two mechanisms, one involving non-specific interaction between ASA and protein, and the other, the non-enzymatic superoxide anion-scavenging ability of protein.

References