Effect of Ascorbic Acid and Dehydroascorbic Acid on Ovalbumin

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The occurrence of turbidity in OVA solution (pH 6) by dehydroascorbic acid (DHA) at 50 °C was further studied to investigate the reaction mechanism of low molecular weight (MW) products formed from OVA. The addition of radical generator to the reaction mixture enhanced the acceleration of occurrence of turbidity whereas removal of air from the reaction mixture and addition of free-radical scavengers or reductants into the reaction mixture prevented the turbidity formation. Hydrogen peroxide had little effect on the occurrence of turbidity. The occurrence of the OVA turbidity by ascorbic acid (AsA) under the same condition was also studied. The addition of hydrogen peroxide to the reaction mixture accelerated the occurrence of turbidity while the reductants prevented it. These results support that oxygen radicals were responsible for the occurrence of turbidity and that free radicals were produced during the oxidation process of AsA. Even when DHA was used instead of AsA, free radicals were still produced from AsA, which was formed from the reduction of DHA during incubation. The experiments using specific radical scavengers indicate that $^{\bullet}O_2^-$, $^{\bullet}OH$, and $^1O_2$ were involved in the occurrence of turbidity and that the occurrence of turbidity would be mainly derived from the function of $^{\bullet}O_2^-$. The amino acid composition of low-MW products from OVA by DHA was comparable to that of native OVA except for aspartic acid and tyrosine, being 3 and 6 times as much as that of OVA, respectively, indicating that the peptide bond cleavage by oxygen radicals might be somewhat specific.

In the previous paper (Nishimura et al., 1989), we investigated the effect of dehydroascorbic acid (DHA) on ovalbumin (OVA) and observed that 0.05% DHA generated turbidity in 1% OVA solution under the condition of pH 6 and 50 °C and that during the incubation both giant polymers (high molecular weight, (MW) products) and low-MW products were derived from OVA. Furthermore, it was elucidated that formation of polymers was not dependent on disulfide bond, which had been strongly suggested to be participated in polymer formation in gluten and actomyosin (Yoshinaka et al., 1972; Elkassabany et al., 1980; Nicolas et al., 1980) but dependent on hydrophobic interaction.

Yano et al. (1976) reported that the reaction of DHA with an amino acid generated two types of free-radical species, and it was also reported that certain free radicals damaged DNA, RNA, polysaccharide, and protein, etc. (Murata et al., 1975; Murata and Uike, 1976; Uchida and Kawakishi, 1986; Gutteridge and Wilkins, 1983). The occurrence of low-MW products from OVA by DHA with the rise of turbidity suggested that OVA was cleaved by DHA during the incubation at pH 6, 50 °C. Thus, the occurrence of turbidity might depend on free radicals. We investigated the effects of free-radical scavengers and generator on the OVA turbidity. Moreover, in order to confirm the participation of oxygen radical, which was a kind of free radical, in the occurrence of turbidity, specific scavengers of each oxygen radical specie were examined.

In addition, the amino acid composition of low-MW products from OVA by DHA was investigated and compared with that of native OVA.

MATERIALS AND METHODS

Materials. DHA, AsA, and OVA described in the previous paper (Nishimura et al., 1989) were used. The other reagents (oxidants, reductants, and radical scavengers, etc.) were special reagent grades.

Measurement of Turbidity. A 1% OVA solution in 0.2 M phosphate buffer, pH 6, containing 0.05% DHA or AsA was incubated at 50 °C for 20 h. The absorbance at 600 nm after 20-h incubation was assigned as 100% of turbidity. As the blank test, the same buffer containing only OVA or DHA or AsA was incubated. As the turbidity of commercial-grade OVA differed depending on lot numbers, the extent of turbidity of OVA by DHA or AsA was newly measured whenever the commercial grade of OVA having a different lot number was used.

Effect of Air on Turbidity. In order to examine the influence of air on the occurrence of turbidity, the test tube containing 1% OVA solution, pH 6, with 0.05% DHA was evacuated first, then closed, and incubated at 50 °C for 20 h.

Examination of Radical Scavengers, Generator, Oxidant, and Reductants on Turbidity of OVA. In order to examine the participation of free radicals on turbidity of OVA, (2-aminoethyl)isothiouronium bromide hydrobromide (AET) and 2-mercaptoethylamine hydrochloride (MEA) were used as free-radical scavengers and 2,2′-azobis(2-amidinopropane) (ADC), which produces free radicals, was used as a free-radical generator. Further, in order to investigate the participation of the oxygen radical, the following chemicals were used: 1,2-dihydroxybenzene-3,5-disulfonic acid (Tiron) and L-adrenalin as $^{\bullet}O_2^-$ scavenger; 1,4-diazabicyclo[2.2.2]octane (DABCO), NaN_3, and hydroquinone as $^1O_2$ scavenger; KBr, KI, and KCN as $^{\bullet}OH$ scavenger. In addition, hydrogen peroxide as oxidant and L-cysteine, 2-mercaptoethanol (2-ME), glutathione, and dithiothreitol as reductant were used in order to confirm the condition of occurrence of oxygen radicals. Each reagent described above was added to 1% OVA solution (pH 6) containing 0.05% DHA or AsA at the 10^{-3} or 10^{-2} M level, and the reaction mixture was incubated at 50 °C for 20 h. Its absorbance at 600 nm was measured and compared with that of reaction mixture without it.

Measurement of AsA and DHA plus 2,3-Diketogulonic Acid (DKG). AsA and DHA plus DKG concentrations were determined by the method of Roe et al. (1948).

SDS-PAGE Analysis. The procedure was the same as described previously (Nishimura et al., 1989) according to the method of Laemmli (1970).

Extraction of Low Molecular Weight Products from Gel after SDS-PAGE. After electrophoresis, the preparative SDS-

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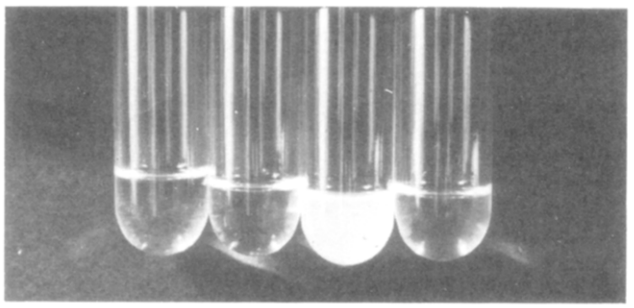
Table I. Effect of Free-Radical Scavengers and Generator on Turbidity of OVA[a]

type	name[b]	concn, M	rel turbidity, %
	none	0	100
scavenger	AET	1×10^{-3}	61.1 ± 9.7
		1×10^{-2}	13.2 ± 3.3
	MEA	1×10^{-3}	78.5 ± 6.1
		1×10^{-2}	6.8 ± 0.6
generator	ADC	1×10^{-3}	106.4 ± 2.5
		1×10^{-2}	212.0 ± 7.3

[a] Duplicate determinations were independently carried out to obtain the means and standard deviations shown. [b] Chemical abbreviations: AET, (2-aminoethyl)isothiouronium bromide hydrobromide; MEA, 2-mercaptoethylamine hydrochloride; ADC, 2,2′-azobis(2-amidinopropane).



A B C D

Figure 1. Photos of OVA solution incubated under various conditions: (A) native OVA solution without incubation; (B) in air without DHA; (C) in air with DHA; (D) in vacuo with DHA.

PAGE gel was soaked in methanol–water–acetic acid solution (10:83:7, v/v) with continuous shaking until the protein became visible as turbid white bands on removal of SDS from the gel. The visible band of low-MW products was cut out with a razor blade and homogenized with distilled water. After centrifugation at 3000*g* for 10 min, the supernatant was used for amino acid analysis.

Amino Acid Analysis. The supernatant of protein extract was hydrolyzed with 6 N HCl in a sealed and evacuated tube at 110 °C for 24 h. Amino acid analysis was performed with an analyzer (Hitachi KLA-5). The amino acid composition is the mean of two independent determinations. Unless otherwise noted, in all experiments duplicate determinations were independently carried out and the averages ± standard deviation were calculated.

RESULTS AND DISCUSSION

The results from examination of radical scavengers and generator on turbidity of OVA are shown in Table I. In the reaction mixture containing 10^{-2} M AET or MEA, turbidity was drastically depressed, while it was accelerated by the addition of ADC to about 210% at the 10^{-2} M level, suggesting that free radicals were responsible for the occurrence of turbidity.

The influence of air on turbidity of OVA is shown in Figure 1. A 1% OVA solution, pH 6, without 0.05% DHA did not produce turbidity after incubation (B) whereas a reaction mixture with 0.05% DHA produced turbidity during incubation (C) but not in vacuo (D). These results suggest that both DHA and air (Figure 1), especially oxygen radical, which is a kind of free radical (Table I), contributed to the occurrence of turbidity.

Superoxide anion radical ($^{\bullet}O_2^-$), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$) are known as oxygen radicals (Asada, 1976). The participation of these oxygen radicals was examined (Table II). Tiron and L-adrenaline, which catch $^{\bullet}O_2^-$ particularly (Miller and Rapp, 1973; Misra and Fridovich, 1972), significantly suppressed the occurrence of turbidity even at the 10^{-3} M level. However, hydroquinone, NaN_3, and DABCO known as specific $^1O_2$ scavenger (Foote et al., 1970; Hasty et al., 1972; Ouannes and Wilson, 1968) and KI, KBr, and KCN known as specific scavengers for $^{\bullet}OH$ (Asada, 1976) fairly depressed the occurrence of turbidity at the concentration of 10^{-2} M and slightly at the 10^{-3} M level. These results show that the occurrence of turbidity depends on three oxygen radical species, especially, $^{\bullet}O_2^-$.

Table II. Effect of Specific Scavengers for $^{\bullet}O_2$, $^{\bullet}OH$, or $^1O_2$ on Turbidity of OVA by DHA[a]

type	name[b]	concn, M	rel turbidity, %
	none	0	100
$^{\bullet}O_2^-$ scavenger	Tiron	1×10^{-3}	21.7 ± 10.8
		1×10^{-2}	0.5 ± 0.6
	L-adrenaline	1×10^{-3}	28.2 ± 6.4
		1×10^{-2}	0
$^1O_2$ scavenger	hydroquinone	1×10^{-3}	68.1 ± 1.3
		1×10^{-2}	14.0 ± 7.8
	NaN_3	1×10^{-3}	47.7 ± 6.8
		1×10^{-2}	6.0 ● 3.5
	DABCO	1×10^{-3}	82.0 ● 17.5
		1×10^{-2}	48.9 ± 16.9
$^{\bullet}OH$ scavenger	KI	1×10^{-3}	72.4 ± 20.5
		1×10^{-2}	23.6 ± 1.3
	KBr	1×10^{-3}	94.3 ± 1.6
		1×10^{-2}	18.6 ● 2.1
	KCN	1×10^{-3}	103.6 ± 10.5
		1×10^{-2}	18.7 ± 6.2

[a] Duplicate determinations were independently carried out to obtain the means and standard deviations shown. [b] Chemical abbreviations: Tiron, 1,2-dihydroxybenzene-3,5-disulfonic acid; DABCO, 1,4-diazabicyclo[2.2.2]octane.

It was reported that DNA, RNA (Murata et al., 1975; Murata and Uike, 1976), polysaccharide (Uchida and Kawakishi, 1986), and protein (Gutteridge and Wilkins, 1983) were damaged by oxygen radicals and that among $^{\bullet}O_2^-$, $^{\bullet}OH$, and $^1O_2$ $^{\bullet}OH$ was mainly responsible for damage. However, the occurrence of turbidity by DHA depended mainly on $^{\bullet}O_2$ among the oxygen radicals (Table II), strongly suggesting that $^{\bullet}O_2$ damaged OVA easier than $^{\bullet}OH$ under the condition as described in Materials and Methods. The cause of the preponderance of $^{\bullet}O_2$ is not known at this time and is being investigated.

Relationship between the change of turbidity and of relative amounts of AsA and DHA was investigated, shown in Figure 2. DHA plus DKG decreased rapidly to about 10% of the added amount for the first 2 h. Longer incubation time decreased them gradually. The amount of AsA, which was 0% at 0 h, increased with incubation time up to about 15% for the first 2 h and then dropped to 0% at the 20-h incubation. The turbidity generated gradually as the amounts of AsA and DHA plus DKG decreased, suggesting that oxygen radicals might have been formed during oxidation of AsA, DHA and DKG.

The effects of oxidant or reductant on turbidity of 1% OVA solution with 0.05% DHA are shown in Table III. An oxidant (H_2O_2) depressed the occurrence of turbidity slightly and reductants (L-cysteine, 2-ME, glutathione, dithiothreitol) suppressed it almost completely at the concentration level of 10^{-2} M. These results do not support that oxygen radicals were produced during the oxidation process of DHA and DKG.

In general, oxygen radicals are known to be formed during autoxidation of AsA to DHA (Asada, 1976). Therefore, we carried out the same experiments by using AsA instead of DHA. AsA generated turbidity of OVA and produced both high- and low-MW products as DHA did in the previous paper (data not shown) (Nishimura et

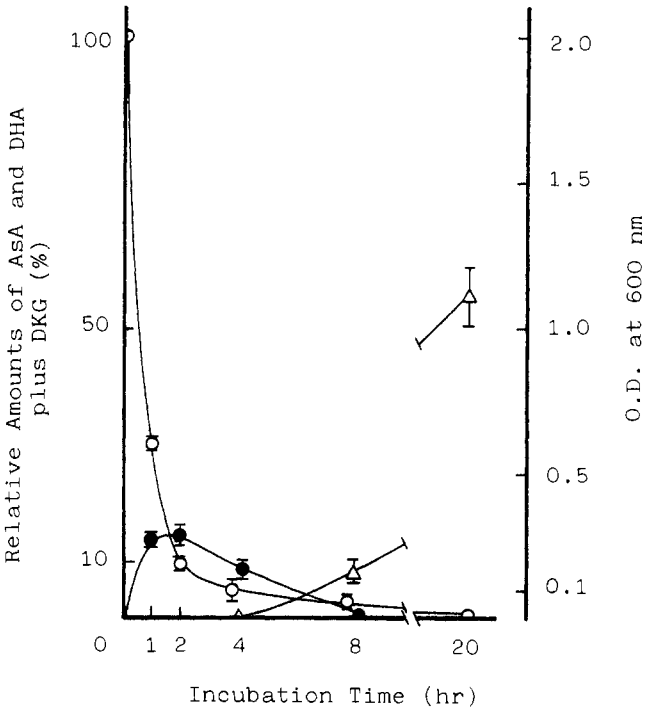


Figure 2. Changes of relative amounts of AsA and DHA plus DKG during occurrence of turbidity in OVA solution with DHA: (●) AsA; (○) DHA plus DKG; (△) turbidity of OVA. The bars show the standard deviations of duplicate determinations.

Table III. Effect of Oxidant or Reductant on Turbidity of OVA by DHA or AsA[a]

type	name	concn, M	rel turbidity,% DHA	rel turbidity,% AsA
	none	0	100	100
oxidant	H_2O_2	1×10^{-3}	84.6 ± 3.9	130.2 ± 17.2
		1×10^{-2}	60.7 ± 12.7	214.2 ± 20.0
reductant	L-cysteine	1×10^{-3}	33.7 ± 16.0	38.3 ± 12.3
		1×10^{-2}	0.4 ± 0.6	0.8 ± 0.5
	2-mercapto-ethanol	1×10^{-3}	52.9 ± 10.5	34.7 ± 27.8
		1×10^{-2}	1.9 ± 0.5	1.1 ± 0.5
	glutathione	1×10^{-3}	65.5 ± 9.9	13.0 ± 6.3
		1×10^{-2}	4.3 ± 1.6	0.4 ± 0.3
	dithiothreitol	1×10^{-3}	47.1 ± 12.6	5.2 ± 1.5
		1×10^{-2}	2.0 ± 3.5	4.1 ± 2.2

[a] Duplicate determinations were independently carried out to obtain the means and standard deviations shown.

al., 1989). Figure 3 shows that the amount of AsA did not decrease as quickly as DHA plus DKG did (Figure 2) during the incubation at 50 °C and remained about 30% of the original added amount even after 8-h incubation. Turbidity generated in the similar rate in Figure 2 with a decrease of AsA (Figure 3). In 1% OVA solution with 0.05% AsA, the oxidant (H_2O_2) accelerated the occurrence of turbidity to over 200% at 10^{-2} M (Table III). On the other hand, reductants depressed the occurrence of turbidity almost completely at 10^{-2} M and decreased turbidity to 5.2–38.3% at 10^{-3} M concentration of each reductant.

Even though the decreasing rate of AsA (Figure 3) was slower than that of DHA (Figure 2) during incubation at 50 °C, the decrease in AsA was accompanied by a rise of turbidity (Figure 3). Furthermore, the occurrence of turbidity by AsA was accelerated by oxidant and depressed by reductants (Table III). These results suggest that oxygen radicals must have been generated during the oxidation process of AsA, as is generally known (Asada, 1976). Therefore, the turbidity of OVA by DHA may be due to the oxygen radicals produced in the oxidation process of

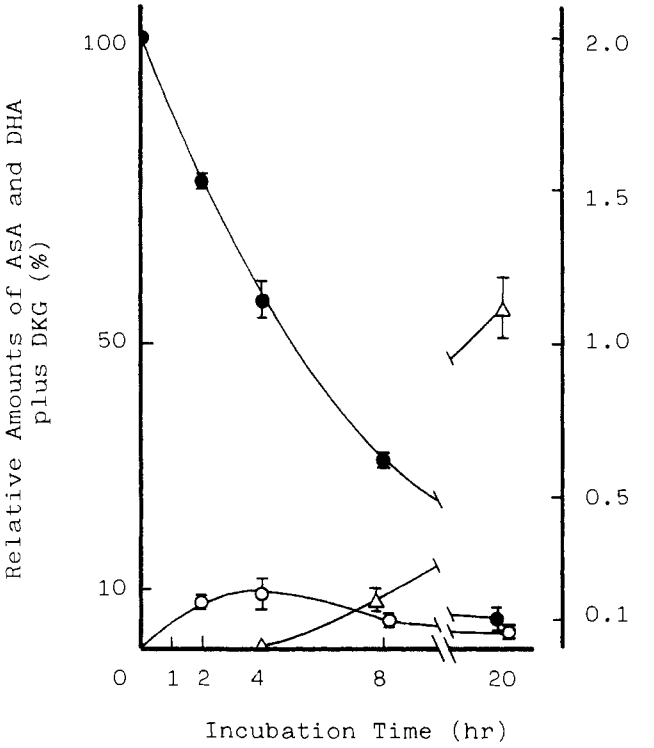


Figure 3. Changes of relative amounts of AsA and DHA plus DKG during occurrence of turbidity in OVA solution with AsA: (●) AsA; (○) DHA plus DKG; (△) turbidity of OVA. The bars show the standard deviations of duplicate determinations.

Table IV. Amino Acid Compositions[a] of OVA and Low Molecular Weight Products Formed from OVA[b]

amino acid	OVA[c]	low-MW products
Cys	0.8	1.5
Asp	3.9	12.2
Thr	1.9	2.3
Ser	4.5	5.1
Glu	6.3	6.5
Gly	2.3	(374.0)
Ala	4.3	4.6
Val	3.8	5.3
Met	1.9	
Ile	3.0	3.4
Leu	4.0	5.0
Tyr	1.1	6.2
Phe	2.5	3.6
Lys	2.5	2.5
His	1	1
Arg	2.4	2.5

[a] Arbitrary relative values to the histidine value being 1. [b] The amino composition is the mean of two independent determinations. [c] From Forthergill and Forthergill (1979).

AsA, which was formed from the reduction of DHA during the incubation (Figure 2). Oxygen radicals were presumably generated from the reoxidation of AsA. Therefore, the suppression of turbidity of OVA solution in the presence of DHA by reductants (Table III) must have been due to the inhibition of reoxidation of AsA by reductants.

Amino acid composition of low-MW products from OVA is compared with that of OVA reported by Fothergill and Fothergill (1970) in Table IV. Amino acid compositions had relative values, compared to that of histidine being 1. The amino acid composition of low-MW products was comparable to that of native OVA except for aspartic acid and tyrosine being 3 and 6 times as much as that of native OVA, respectively. Unusually high amounts of glycine from the low-MW products might have resulted from contamination of the buffer used for the electrophoresis.

This result suggests that the peptide bond cleavage by oxygen radicals might be somewhat specific. The reason for cleavage on the aspartic acid and tyrosine residues is not clear, and a further study is needed.

On the basis of results obtained from this study and the previous paper (Nishimura et al., 1989), the turbidity of OVA resulted from the formation of both high- and low-MW products. Oxygen radicals, especially, $^{\bullet}O_2^-$ produced in the oxidation process of AsA, cleaved peptide bonds somewhat specifically to generate small molecules in such a way the conformation of OVA changed somewhat to increase surface hydrophobicity as reported by Kato et al. (1984). Then, the high-MW polymers were formed by hydrophobic interactions.

However, our result obtained by using OVA differs from the findings of dough and surimi (Yoshinaka et al., 1972; Elkassabany et al., 1980; Nicolas et al., 1980). The cause may depend on the different conditions of temperature, DHA and AsA concentrations, flour or muscle components, protein structure, and ancillary ingredients such as lipid that are found on dough or surimi. Therefore, it is necessary that we investigate whether polymerization occurs in accordance with this process in the dough or surimi system. So, examination on surimi are being carried out now.

Registry No. DHA, 490-83-5; AsA, 50-81-7; $^{\bullet}O_2^-$, 11062-77-4; $^{\bullet}OH$, 3352-57-6; O_2, 7782-44-7.

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