J. Home Econ. Jpn. Vol. 43 No. 3 185–191 (1992)

Effects of Several Kinds of Buffers and Ionic Strength on the Polymerization of Ovalbumin by Ascorbic Acid

Kimio NISHIMURA* and Masaru OHTSURU

Department of Food and Nutrition, Yamaguchi Women's University, Sakurabatake, Yamaguchi 753, Japan

Examinations were carried out with ovalbumin (OVA) as the protein to clarify the beneficial effects of ascorbic acid on food.

First, in order to elucidate the difference in turbidity of ovalbumin (OVA) dissolved in several kinds of 0.2 M buffer at pH 6, resulting from ascorbic acid (AsA) during incubation at 50°C, the effects of several chelating agents and of two metal ions were investigated. Second, phosphoric acid buffers with an ionic strength of 0.26 to 1.0 were used to examine the influence of increasing ionic strength on OVA turbidity to clarify the mechanism for polymerization of OVA by AsA.

The results obtained from an earlier investigation suggested that one of the causes of the difference in turbidity with various buffers may have been due to metal ion contaminants.

An increase in ionic strength in the phosphoric acid buffer inhibited polymer formation derived from OVA, indicating that both electrostatic and polar bonding occurred in the formation of the polymer.

(Received March 4, 1991)

Keywords: ascorbic acid, ovalbumin, polymerization, beneficial effect, electrostatic bond.

INTRODUCTION

L-AsA is often used for improving food, in particular, during baking and "kamaboko" (a heat-induced gel made from raw fish paste) manufacture. AsA improve the baking process for dough and the quality of surimi (raw fish paste). The beneficial effects of AsA on bread dough have been widely investigated. The popularly accepted explanation suggests that DHA, which is the oxidizing form of AsA, oxidizes sulfhydryl compounds to disulfides and also forms intermolecular bonds.[1)2)] However, some other hypotheses on the effect of AsA on food protein have been proposed,[3)–5)] so that the definitive answer is still not known.

In previous papers,[6)7)] in order to clarify the action of AsA and DHA upon protein, we investigated the effect of AsA and DHA on protein, using OVA as a food protein source. The dough or surimi system is so complex that there are a number of unclarified points regarding the action of AsA and DHA on protein. We observed that 0.05% of AsA or DHA generated turbidity in a 1% OVA solution of pH 6 at 50°C. During incubation, both large polymers (high MW products) and low MW products were formed from OVA. Furthermore, the structural modifications incurred by oxygen radicals (especially the superoxide anion radical) increased the surface hydrophobicity of OVA, the oxygen radicals being generated by the autoxidation of AsA[8)] during incubation. The polymers were formed by the resulting hydrophobic interaction, and this was followed by the formation of disulfide bonds. Disulfide bonds have been suggested to participate

*Present address: Department of Food and Nutrition, Kochi Women's University, Eikokuji-cho, Kochi 780, Japan

Abbreviations: AsA, ascorbic acid; DHA, dehydroascorbic acid; DKG, 2,3-diketo-gulonic acid; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(2-aminoethylether)-*N*, *N*, *N'*, *N'*-tetraacetic acid; MW, molecular weight; NBT, nitro blue tetrazolium; NTA, nitrilotriacetic acid; OVA, ovalbumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

in the polymer formation in gluten[1)2)] and in actomyosin.[9)] During the investigation, we noticed that polymers were produced in a 0.2 M phosphoric acid buffer, but did not in a 0.2 M maleic acid buffer, in spite of the same conditions of pH 6 and 50°C.

In this paper, we report the effects of several chelating agents and iron and copper ions on the turbidity in various buffers, in order to elucidate the cause of the difference in turbidity of an OVA solution between the two buffers. Next, we attempt to clarify the mechanism for polymerization by AsA with a phosphoric acid buffer (described in previous papers by Nishimura *et al.*[6)7)]). Thus, we examine the influence of ionic strength in the phosphoric acid buffer on the turbidity of OVA, the degradation of AsA and the generation of the superoxide anion radical.

MATERIALS AND METHOD

Materials. OVA (A 5503) was obtained from Sigma Chemical Co., and the other chemicals used were of reagent grade from Nacalai Tesque Inc. or from Wako Pure Chemical Industries Ltd. When OVA was applied to SDS-PAGE, sub-bands above the main one were observed, as described in a previous paper.[6)] Because these sub-bands almost disappeared with the addition of 2-mercaptoethanol, the OVA might have contained a dimer or trimer and may have been slightly contaminated.

Measurement of the turbidity of OVA. The 0.2 M phosphoric acid, malonic acid, citric acid, malic acid, succinic acid, phosphorous acid and maleic acid buffers were prepared by adding aqueous NaOH to solutions of these respective acids. A mixture of 1% OVA and 0.05% AsA in each 0.2 M buffer at pH 6 was incubated at 50°C for 44 hr. The absorbance at 600 nm after a 44-hr incubation is defined as 100% of turbidity in the phosphoric acid buffer. As a blank test, each buffer containing only OVA or AsA was incubated. The effect of ionic strengths of 0.26 to 1.0 in the phosphoric acid buffer were also investigated.

SDS-PAGE analysis. After incubating at 50°C, 1 ml of an OVA solution was mixed with an equal volume of 0.125 M Tris-HCl buffer at pH 6.8 containing 4% SDS and 20% glycerol. After heating at 100°C for 5 min, the mixture was applied to SDS-PAGE, electrophoresis being carried out on a slab gel at 20 mA for 3–4 hr according to Laemmli's method.[10)]

Effects of chelating agents and metal ions on turbidity. In order to examine the participation of divalent cations in the turbidity of OVA, four chelating agents EDTA, EGTA, NTA or DTPA were added to the reaction mixture. Ferrous chloride and cuprous chloride were also added, excepting the phosphoric acid buffer in which both salts were insoluble. A 1% OVA solution in each buffer at pH 6 containing 0.05% AsA and the chelating agent or metal ion at a 10^{-3} M level was incubated at 50°C for 44 hr. Its absorbance at 600 nm was measured and compared with that of a reaction mixture without the chelating agent or metal ion.

Measurement of AsA and DHA plus DKG. The AsA and DHA plus DKG concentrations were determined according to the method of Roe *et al.*[11)]

Measurement of the superoxide anion radical. The generation rate of the superoxide anion radical was measured according to the method described by Beauchamp and Fridovich.[12)] Each buffer containing 0.005% AsA and 5×10^{-5} M NBT was incubated at 50°C, and the increase in absorbance at 560 nm was then measured.

Unless otherwise noted, all experiments were duplicated, and the average ± standard deviation was calculated for each. Statistical differences were evaluated by Student's *t*-test.

RESULTS AND DISCUSSION

Turbidity of OVA in several kinds of buffer.

During the previous investigation,[7)] we noticed that polymers were produced in the 0.2 M phosphoric acid buffer, but did not in the 0.2 M maleic acid buffer, in spite of the same condition of pH 6 and 50°C. In order to examine the cause of such difference with each buffer, an investigation using OVAs dissolved in several kinds of 0.2 M buffer at pH 6 was carried out.

The relative turbidity of OVA in each 0.2 M buffer depended on the kind of buffer as shown in Table 1, although the molar concentration of each buffer was the same. Based on the degree of turbidity, the buffers could be roughly divided

Table 1. Comparison of turbidity in various buffers at pH 6

Buffer (0.2 M)	Turbidity (%)
Phosphoric acid	100
Malonic acid	86.7±5.3
Citric acid	24.8±4.7
Malic acid	19.6±3.2
Succinic acid	19.5±0.5
Phosphorous acid	0
Maleic acid	0

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

into three groups. The first group (strong turbidity) involved the phosphoric acid and malonic acid buffers; the second group (mild turbidity), the citric acid, malic acid and succinic acid buffers; and the third group (no turbidity), the phosphorous acid and maleic acid buffers.

Figure 1 shows the change in MW of OVA in the phosphoric acid, malic acid and succinic acid buffers during incubation at 50°C and pH 6. As described in the previous paper,[6] sub-bands above the main one, which might have been di- or trimers, were observed in native OVA (line A) and incubated OVA without AsA (lines B–D). High MW products (band a), which could not enter the spacer gel, and low MW products (band b), which migrated to the top of the run, were produced by AsA (line E). In spite of adding AsA, the amounts of both MW products in the malic acid and succinic acid buffers (lines F and G) were small, and they were not apparent at all in the phosphorous acid and maleic acid buffers (data not shown), in relation to the turbidity in each buffer as shown in Table 1.

Effects of chelating agents and metal ions on OVA turbidity in various buffers

AsA oxidation was accelerated by metal ions, which could have been due to the participation of metal contaminants in the reagents. Consequently, the effects of four chelating agents and two metal ions on the turbidity of OVA were investigated by incubating at 50°C for 44 hr, the results being shown in Table 2. In the phosphoric acid buffer, ferrous chloride and cuprous chloride were insoluble; therefore, the influence of these two metal ions could not be examined. The four chelating agents acted differently with respect to the occurrence of OVA turbidity. EDTA depressed the turbidity in the malic acid and succinic acid buffers to about 62 and 25%, respectively, although no marked depression occurred in the citric acid buffer. However, the turbidity increased in the phosphoric acid and malonic acid buffers. Although EGTA mildly suppressed the development of turbidity in the phosphoric acid and succinic acid buffers, no significant effects were observed in the malonic acid, citric acid or malic acid buffers. We observed no inhibiting effect of NTA in the five buffers, the turbidity accelerating overall. DTPA inhibited turbidity in the same five buffers, almost completely in the phosphoric acid buffer.

Copper ions (1 mM) rapidly accelerated the

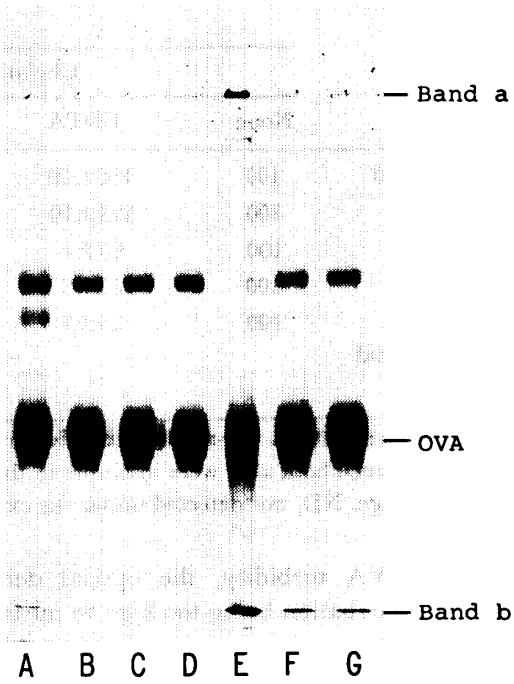

Fig. 1. Different SDS-PAGE patterns for OVA incubated in various buffers

One % OVAs dissolved in a 0.2 M phosphoric acid, malic acid or succinic acid buffer with and without 0.05% AsA were incubated at 50°C and pH 6 for 44 hr, and applied to SDS-PAGE. A, native OVA; B, OVA without AsA in the phosphoric acid buffer; C, OVA without AsA in the malic acid buffer; D, OVA without AsA in the succinic acid buffer; E, OVA with AsA in the phosphoric acid buffer; F, OVA with AsA in the malic acid buffer; G, OVA with AsA in the succinic acid buffer.

Table 2. Effects of chelating agents and divalent cations in various buffers at pH 6.0

Buffer (0.2 M)	Turbidity (%)						
	Chelating agent (1 mM)					Cation (1 mM)	
	None	EDTA	EGTA	NTA	DTPA	Fe^{2+}	Cu^{2+}
Phosphoric acid	100	136±19	68±8	111±13	4±1	−	−
Malonic acid	100	123±10	98±10	116±9	29±2	193±10	+
Citric acid	100	90±4	100±6	131±11	27±3	270±31	+
Malic acid	100	62±3	96±4	154±16	40±1	164±7	+
Succinic acid	100	25±7	73±9	121±7	33±6	127±6	+
Phosphorous acid						ND	+
Maleic acid						ND	+

Duplicate determinations were independently carried out to obtain the means and standard deviations shown. −, not measured since both salts were insoluble in the phosphoric acid buffer; +, not measured due to high turbidity outside the range. ND, not detected under the experimental conditions.

progress of OVA turbidity, the optical density after a 44-hr incubation being too high to measure. The addition of iron ions promoted the turbidity of OVA in the malonic, citric, malic and succinic acid buffers; but it did not affect the phosphorous or maleic acid buffers, no turbidity of OVA being produced by AsA in either buffer.

In the phosphoric acid buffer, the chelating agents, except for DTPA, did not depress the turbidity of OVA. One chelating agent, EDTA, actually promoted the turbidity significantly. It is known that DTPA is used as an iron chelator,[13] and that phosphoric acid buffers may contain some iron.[14,15] Moreover, it has been reported that EDTA inhibited not iron-[13] but copper-catalyzed oxidation reactions of AsA,[16] and that it prompted the oxidation of AsA in the presence of iron ions and oxygen or hydrogen peroxide.[17] These results show that, in the phosphoric acid buffer, contaminating iron ions catalyzed the oxidation of AsA, so that the promotion of the oxygen radical was enhanced. In fact, the relative amount of AsA in the phosphoric acid buffer containing 1 mM DTPA, after an 8-hr incubation, was greater than that in the buffer without DTPA (data not shown).

The occurrence of OVA turbidity in the malonic acid and citric acid buffers was strongly promoted by metal ions and inhibited by DTPA, indicating contamination by iron of both buffers. Depression of the turbidity of OVA in the malic acid and succinic acid buffers by both EDTA and DTPA, and its enhancement by iron and copper ions, indicate the possibility that both buffers contained not only iron but also copper, and that two metal ions took part in the oxidation of AsA.

The addition of iron ions to the phosphorous acid and maleic acid buffers did not influence the turbidity of OVA; however, the addition of copper ions did promote turbidity. Since AsA was easily oxidized in the presence of copper ions,[18] the large increase in the generation of the oxygen radical by the more rapid oxidation of AsA must have caused the turbidity. Although it is known that iron ions also catalyze the oxidation of AsA,[19] in this case, the increase of the generating oxide radical by adding iron ions might not have been enough alone to generate OVA turbidity. We observed that the degradation of AsA by copper ions was greater than that by iron ions (data not shown). Thus, OVA was not damaged, and no OVA turbidity occurred. In the other buffers, the difference in turbidity between the iron and copper ions might have been for the same reason. Judging from the foregoing results, we can assume that one of the causes for the difference in turbidity with various buffers can be ascribed to metal ion contaminants in each buffer.

To discover other causes for the difference, we examined the relationships between changes in AsA, the generation rate of the superoxide anion radical, ionic strength and the turbidity of OVA in several kinds of buffer. Although we did not identify a distinct relationship, the degradation of AsA and generation of the superoxide anion radical occurred even in the phosphorous acid buffer,

being as much as those occurring in the phosphoric acid buffer (data not shown). However, Fig. 1 indicates that OVA was not attacked by oxygen radicals. These results imply the possibility that another cause for the difference in turbidity was due to the nature of each buffer, although we have not yet learned the actual cause. Accordingly, further investigation on this point is necessary.

Relationship between the turbidity of OVA, changes in AsA, generation rate of the superoxide anion radical, and the ionic strength in a phosphoric acid buffer

In order to clarify the polymerization process in OVA by AsA, the effect of ionic strength was examined in the phosphoric acid buffer, for which an investigation on the turbidity of OVA by AsA has been carried out,[6)7)] using an ionic strength ranging from 0.26 to 1.0. The results are shown in Figs. 2 and 3, indicating that the turbidity of OVA decreased with increasing ionic strength (Fig. 2). According to densitometric measurements taken from the SDS-PAGE patterns in Fig. 3, the residual amount of OVA with AsA after incubation was still 34%, indicating that OVA cleavage had not been depressed.

These results suggest that increasing ionic strength inhibits the polymer formation of OVA in spite of the increase in surface hydrophobicity promoted by OVA damage, which was suggested in a previous paper.[7)] Thus, in addition to hydrophobic inter-

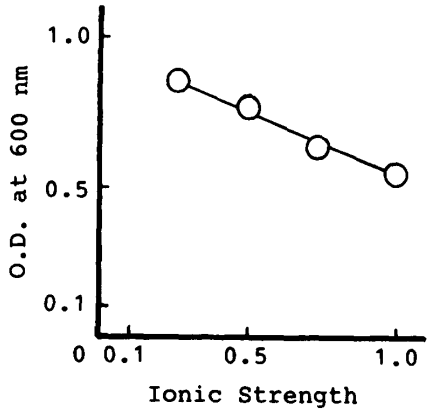

Fig. 2. Effect on turbidity of increasing ionic strength of the phosphoric acid buffer

Phosphoric acid buffers with an ionic strength of 0.26 to 1.0 were prepared, 1% OVA in each buffer with 0.05% AsA was incubated at 50°C and pH 6 for 44 hr, and the optical density at 600 nm was measured.

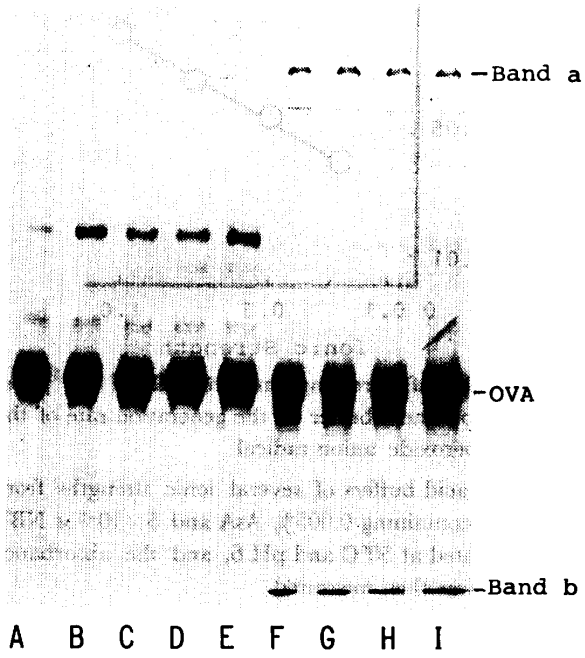

Fig. 3. Different SDS-PAGE patterns for OVA incubated in phosphoric acid buffers of various ionic strength

One % OVAs in several phosphoric acid buffers of ionic strength from 0.26 to 1.0 were incubated at 50°C and pH 6 for 44 hr in the presence or absence of 0.05 % AsA, and then applied to SDS-PAGE. A, native OVA; B, ionic strength of 0.26 without AsA; C, ionic strength of 0.5 without AsA; D, ionic strength of 0.75 without AsA; E, ionic strength of 1.0 without AsA; F, ionic strength of 0.26 with AsA; G, ionic strength of 0.5 with AsA; H, ionic strength of 0.75 with AsA; I, ionic strength of 1.0 with AsA.

Table 3. Changes of AsA in phosphoric acid buffers having various ionic strengths

Ionic strength	Amount of AsA (%)			
	Incubation time (hr)			
	0	4	8	20
0.26	100	56±5	31±1	5±2
0.5	100	60±4	41±2	9±2
0.75	100	58±2	39±2	9±6
1.0	100	60±4	41±1	10±3

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

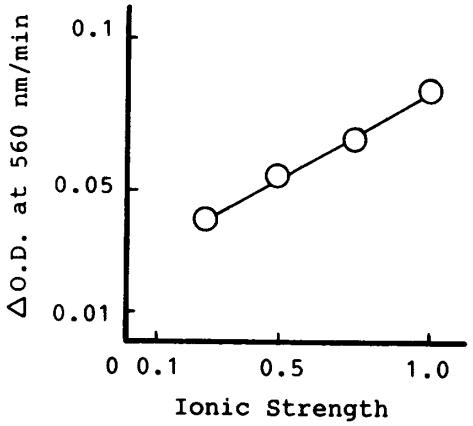

Fig. 4. Effect of increasing ionic strength of the phosphoric acid buffer on the generation rate of the superoxide anion radical

Phosphoric acid buffers of several ionic strengths from 0.26 to 1.0 containing 0.005% AsA and 5×10^{-5} M NBT were incubated at 50°C and pH 6, and the absorbance at 560 nm was then measured.

action between OVA molecules, other interactions such as electrostatic and polar bonding (which tend to be reduced by ions in solvents), must also be considered in the role of polymer formation.

The changes of AsA in these buffers during incubation were followed (Table 3). Although the amount of AsA decreased with time, no marked influence from different ionic strength on the degradation of AsA was apparent, except for a slightly lower value detected at 0.26 after an 8-hr incubation. The generation rate of the superoxide anion radical was also measured (Fig. 4), the rates increasing with increasing ionic strength.

Because the oxidation of AsA was not promoted, this generation could not have been due to a catalytic effect stimulated by an increase of contaminating iron in the phosphoric acid buffer.[14)15)] However, the reason for the generation of the superoxide anion radical with increasing ionic strength is not known at present, and further examination is therefore necessary.

Based on the results from this study, and those from the previous papers,[6)7)] we can form certain conclusions about the polymer formation of OVA by AsA in a phosphoric acid buffer at pH 6. Oxygen radicals produced during the oxidation process of AsA catalyzed by iron ions, which contaminated the phosphoric acid buffer. In particular, the superoxide anion radical seemed to specifically cleave peptide bonds, generating low MW products. As a result, the OVA conformation changed, increasing the surface hydrophobicity of OVA and then freeing sulfhydryl groups on the surface. High MW polymers were then formed by hydrophobic interaction and such other interactions as electrostatic and polar bonding, which appeared to participate from this study, resulting in the formation of disulfide bridges between adjacent free sulfhydryl groups.

However, our results obtained for OVA differ from the findings for dough and surimi.[1)2)9)] Therefore, it is necessary to investigate whether polymerization occurs in accordance with this process in a dough or surimi system. An examination of surimi is now in progress.[20)-21)]

This work was supported in part by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1) Elkassabany, M. and Hoseney, R.C.: *Cereal Chem.*, **57** 85 (1980)
2) Nicolas, J., Gustafsson, S. and Drapron, R.: *Lebensm. Wiss. Technol.*, **13**, 308 (1980)
3) Johnston, W.R. and Mauseth, R.E.: *Bakers Dig.*, **46**, 20 (1972)
4) Ewart, J.A.D.: *J. Sci. Food Agric.*, **36**, 101 (1985)
5) Pfeilsticker, K. and Marx, F.: *Z. Lebensm. Uuters. Forsh.*, **182**, 191 (1986)
6) Nishimura, K., Ohtsuru, M. and Nigota, K.: *J. Agric. Food Chem.*, **37**, 1539 (1989)
7) Nishimura, K., Ohtsuru, M. and Nigota, K.: *J. Agric. Food Chem.*, **37**, 1544 (1989)
8) Murata, A. and Kitagawa, K.: *Biosci. Biotech. Biochem.* **37**, 1145 (1973)
9) Yoshinaka, R., Shiraishi, M. and Ikeda, S.: *Nippon Suisan Gakkaishi*, **38**, 511 (1972)
10) Laemmli, U.K.: *Nature*, **227**, 680 (1970)
11) Roe, J.H., Mills, M.B., Oesterling, M.J. and Damron, C.M.: *J. Biol. Chem.*, **174**, 201 (1948)
12) Beauchamp, C. and Fridovich, I.: *Anal. Biochem.*, **44**, 276 (1971)
13) Halliwell, B.: *FEBS Lett.*, **92**, 321 (1978)
14) Wong, S.F., Halliwell, B., Richmond, R. and Skowroneck, W.R.: *J. Inorg. Biochem.*, **14**, 127 (1981)
15) Wayner, D.D.M., Burton, G.W. and Ingold, K.U.: *Biochim. Biophys. Acta*, **884**, 119 (1986)
16) Järger, H.: *Parmazie*, **3**, 536 (1948)
17) Timberlake, C.F.: *J. Sci. Food Agric.*, **11**, 258 (1960)

18) Ogata, Y., Kosugi, Y. and Morimoto, T.: *Tetrahedron*, **24**, 4057 (1968)
19) Murata, A.: *Protein, Nucleic Acid Enzyme*, **20**, 33 (1975)
20) Nishimura, K., Ohtsuru, M. and Nigota, K.: *Nippon Suisan Gakkaishi*, **56**, 959 (1990)
21) Nishimura, K., Ohishi, N., Tanaka, Y. and Sasakura, C.: *Biosci. Biotech. Biochem.*, **56**, 24 (1992)

アスコルビン酸による卵白アルブミンの重合化に対する緩衝液種およびイオン強度の効果について

西村公雄*,**，大鶴　勝*

(* 山口女子大学，** 現 高知女子大学)

平成3年3月4日受理

アスコルビン酸（AsA）の食品品質改良効果を明らかにするために，タンパク質として卵白アルブミン（OVA）を用いて以下の検討を行った．

各種 0.2 M 緩衝液（pH 6）に溶かした OVA 溶液は，AsA によって 50℃ でインキュベーションすると白濁（重合化）するが緩衝液の種類によってその濁度が著しく異なった．そこで，まず，各種緩衝液に対するキレート剤と金属イオンの効果を調べた．次に，0.26 から 1.0 のイオン強度に調整したリン酸緩衝液を用いて AsA によって生じる OVA の濁度に対するイオン強度の影響を検討した．

得られた結果は，緩衝液中に存在する金属イオンが AsA の酸化を促すことで白濁を生じさせることが，各種緩衝液による OVA の白濁の違いの原因の一つであることを示していた．

また，リン酸緩衝液のイオン強度増加により AsA による OVA の重合体形成が抑えられたのは，重合体形成に静電的および極性結合が関与しているためであると推定した．

キーワード：アスコルビン酸，卵白アルブミン，重合化，改良効果，静電結合，極性結合．