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Studies on Changes in Stored Shell Eggs

Part VII. Changes in the Physicochemical Properties of Ovomucin Solubilized by Treatment with Mercaptoethanol during Storage

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Being solubilized by treatment with 0.01 M mercaptoethanol, the ovomucin gel(B) was found in free boundary electrophoresis to contain subunits which were consisted of two components. Changes in the physicochemical properties of all the insoluble ovomucin gel(B) and sol(B) obtained from stored egg white were studied after this treatment.

The fast moving component of the ovomucin gel(B) in free boundary electrophoresis decreased during storage and disappeared completely after 30 days. On the other hand, the fast moving component of the ovomucin sol(B) increased during storage.

The acid mucoprotein concentration of the ovomucin gel(B) in acrylamide gel electrophoresis decreased and that of the ovomucin sol(B) increased during storage, although the protein pattern did not show significant changes.

The interaction of the ovomucin gel(B) with lysozyme decreased whereas that of the ovomucin sol(B) increased during storage.

By summarizing these results, a model of ovomucin gel structure and a mechanism of egg white thinning were proposed.

It was reported in the previous work[1] that the carbohydrate content of the ovomucin gel(B) obtained from stored egg white decreased remarkably as the storage proceeded. For further consideration on this change in ovomucin during storage, it is important to study the physicochemical properties of ovomucin obtained from stored egg white. It is necessary to solubilize ovomucin, because most part of ovomucin are insoluble in dilute alkaline solution. In the previous work,[2] we reported that ovomucin was solubilized dissociating into subunits by treatment with thioglycolate or hydrogen peroxide.

This paper describes a study on the physicochemical properties of ovomucin obtained from stored egg white, which was solubilized by treatment with 0.01 M mercaptoethanol.

MATERIALS AND METHODS

Separation and storage of egg white. All eggs were collected from a strain of White Leghorn layers within 24 hr after laying, and the shell was sterilized with 70% ethanol. Thick white and thin white were separated and the former was stored in sterilized flasks with cotton plug at 30°C for given days. All procedures were aseptically carried out: all materials such as a separater, flasks, *etc.* were sterilized prior to use and all procedures were carried out in a bacteriological inoculating room. Bacteriological tests

1) A. Kato, R. Nakamura and Y. Sato, *Agr. Biol. Chem.*, **34**, 1009 (1970).
2) R. Nakamura, A. Kato and Y. Sato, *Nippon Nogeikagaku Kaishi*, **43**, 105 (1969).

were made by standard plate counts at 30°C. Absence of growth in all tests after 4 days' incubation was termed sterile.

Preparation of the ovomucin gel(B) and sol(B). The stored thick white was separated into gel and sol parts by ultracentrifugation for 60 min at $59,000 \times g$. The gel part was washed with 2% KCl solution directly without diluting with water until the washing was free from protein. The gel-like precipitate was finally washed with water until the washing was free from chloride. The gel-like precipitate was designated as ovomucin gel(B). One volume of the sol part was diluted with three volumes of water, its pH was adjusted to 6.0 with 1 N H_2SO_4 and then it was centrifuged for 15 min at $1000 \times g$. The precipitate was washed with 2% KCl solution until the washing was free from protein and was finally washed with water until the washing was free from chloride. The precipitate was designated as ovomucin sol(B).

Solubilization of ovomucin gel(B) and sol(B). Ovomucin gel(B) and sol(B) were dialyzated against Menzel buffer (pH 9.6, ionic strength 0.25) and added to it 0.01 M mercaptoethanol. The mixture was stored at 20°C for 30 min. After solubilization of ovomucin gel(B) and sol(B) each solution was dialyzated against Menzel buffer until the solution was free from mercaptoethanol. This solution was used in all experiments.

Chemical analysis. Total nitrogen was determined by Kjeldahl method. Hexose content was determined by orcinol method in which an equimolar mixture of galactose and mannose was used as the standard. Sialic acid was determined by thiobarbituric acid method.[4] Each content was indicated as percentage of dry matter.

Free boundary electrophoresis. Free boundary electrophoresis was performed with a Hitachi Model D-I Tiselius apparatus. All runs were performed with Menzel buffer (pH 9.6, ionic strength 0.25) at 4°C for 60 min at 60 volts, 6 milliamps.

Acrylamide gel electrophoresis. Horizontal acrylamide gel electrophoresis was carried out with the discontinuous buffer system. The gel buffer contained 0.08 M Tris, 0.004 M EDTA and 0.013 M boric acid; the electrode buffer contained 0.3 M boric acid and 0.05 M sodium hydroxide. Electrophoresis was performed at 4°C for 6 hr at 200 volts, 15 milliamps. Solution of 1% amide black in 5% acetic acid was used to stain protein portion after electrophoresis and solution of 5% alcian blue in 30% acetic was used to stain acid mucoprotein portion. Excess dye was removed by washing with 2% acetic acid.

Ovomucin-lysozyme interaction. Solution of ovomucin(B), 0.5% in concentration, solubilized by treatment with 0.01 M mercaptoethanol and 1.0% solution of lysozyme were used to measure the extent of ovomucin-lysozyme interaction. One tenth ml of ovomucin(B) solution was added to 3 ml of lysozyme solution. The final pH of the mixture was adjusted to 6.0. After shaking the mixture, turbidity was determined by measuring absorbances at 600 mμ. The extent of ovomucin-lysozyme interaction was shown in terms of turbidity.

RESULTS

Ovomucin gel(B) was solubilized by treatment with 0.01 M mercaptoethanol. By this treatment, 2% of ovomucin gel(B) was disintegrated into low molecular materials which were dialyzed out through cellophane membrane (Table I). No significant change was observed in chemical composition of ovomucin

TABLE I. DISINTEGRATION RATE OF OVOMUCIN GEL(B) TREATED WITH MERCAPTOETHANOL

Disintegration rate of ovomucin gel(B) treated with 0.01 M mercaptoethanol was obtained by the following calculation;

$$\text{Disintegration rate} = \frac{a-b}{a}$$

a: Dry weight of inner dialyzate of ovomucin gel(B)

b: Dry weight of inner dialyzate of the mercaptoethanol-treated ovomucin gel(B)

No. of Exp.	Disintegration rate			
	I	II	III	Mean
Ovomucin gel(B)	0	0	0	0
Mercaptoethanol-treated ovomucin gel(B)	0.01	0.02	0.02	0.02

3) N. L. Allport, "Colorimetric Analysis," Vol. I, Chapman and Hall Ltd., 1957, p. 82.

4) L. Warren, *J. Biol. Chem.*, **234**, 1971 (1959).

gel(B) after treatment with mercaptoethanol (Table II). Electrophoretic patterns of ovomucin gel(B) treated with mercaptoethanol showed two components, with relative mobility of about 100 and 150, same as those of the untreated ovomucin gel(B), although relative area ratio was different from that of the untreated ovomucin gel(B) (Fig. 1, Table III).

TABLE II. CHANGES IN CHEMICAL COMPOSITION OF OVOMUCIN GEL(B) TREATED WITH MERCAPTOETHANOL

Values were shown in terms of percentage of dry matter.

	Total	Hexose	Sialic acid
Ovomucin gel (B)	9.5	12.2	9.7
Mercaptoethanol-treated ovomucin gel(B)	9.3	12.6	9.8

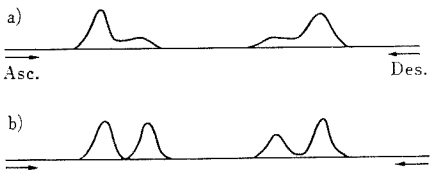

FIG. 1. Electrophoretic Pattern of Ovomucin Gel(B) Treated with Mercaptoethanol.

a) Ovomucin gel(B)
b) Ovomucin gel(B) treated with mercaptoethanol.
All runs were performed in Menzel buffer, pH 9.6, ionic strength 0.25, at 4°C for 60 min.

TABLE III. RELATIVE MOBILITY OF OVOMUCIN GEL(B) TREATED WITH MERCAPTOETHANOL

Relative mobility was shown taking the values of the slow moving component of ovomucin gel(B) as 100.

	Relative Mobility M_1	M_2
Ovomucin gel(B)	100 (7)	150 (3)
Ovomucin gel(B) treated with mercaptoethanol	100 (5)	150 (5)

M_1: Slow moving component in Fig. 1
M_2: Fast moving component in Fig. 2
( ): Relative area ratio

Electrophoretic patterns of ovomucin gel(B) and sol(B) treated with mercaptoethanol changed remarkably during storage. The fast moving component of ovomucin gel(B) in free boundary electrophoretic patterns decreased progressively as the storage proceeded and disappeared completely after 30 days. On the other hand, the fast moving component of ovomucin sol(B) increased during storage (Fig. 2, Table IV).

The protein patterns of ovomucin gel(B) in horizontal acrylamide gel electrophoresis showed a broad band at anionic side which

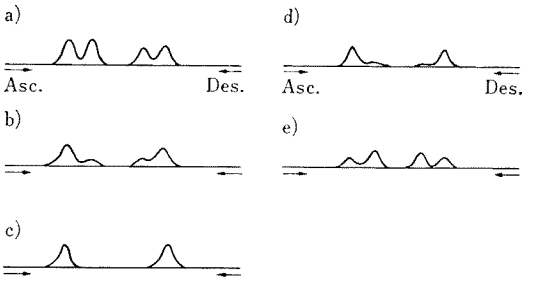

FIG. 2. Changes in Electrophoretic Patterns of Ovomucin Gel(B) and Sol(B) during Storage.

a) Ovomucin gel(B) in newly laid egg white. b) Ovomucin gel(B) in egg white stored for 10 days. c) Ovomucin gel(B) in egg white stored for 30 days. d) Ovomucin sol(B) in newly laid egg white. e) Ovomucin sol(B) in egg white stored for 15 days. All runs were performed in Menzel buffer at 4°C for 60 min.

TABLE IV. RELATIVE MOBILITY AND RELATIVE AREA RATIO IN ELECTROPHORETIC PATTERNS OF OVOMUCIN GEL(B) AND SOL(B) IN STORED EGG WHITE

	Relative mobility M_1	M_2	Relative area ratio M_1	M_2
a)	100	150	5	5
b)	110	150	7	3
c)	110	—	10	0
d)	100	150	9	1
e)	110	150	4	6

a)~e): Same as that of Fig. 2
M_1: Slow moving component in Fig. 2
M_2: Fast moving component in Fig. 2

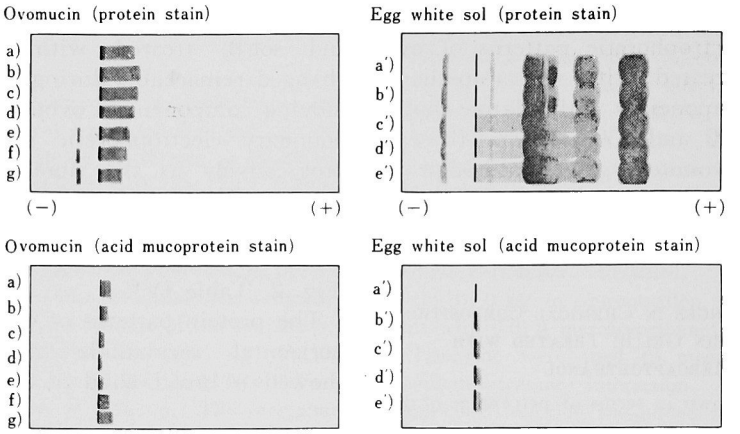

FIG. 3. Changes in Acrylamide Gel Electrophoretic Patterns of the Ovomucin Gel(B) and Sol(B) during Storage.

a) Ovomucin gel(B) in newly laid egg white. b) Ovomucin gel(B) in egg white stored for 10 days. c) Ovomucin gel(B) in egg white stored for 20 days. d) Ovomucin gel(B) in egg white stored for 30 days. e) Ovomucin sol(B) in newly laid egg white. f) Ovomucin sol(B) in egg white stored for 10 days. g) Ovomucin sol(B) in egg white stored for 20 days.

a′) Newly laid egg white sol. b′) Egg white sol stored for 10 days. c′) Egg white sol stored for 20 days. d′) Egg white sol stored for 30 days. e′) Egg white sol stored for 40 days.

was as yet unidentified, and those of ovomucin sol(B) had, beside this broad band at anionic side, another sharp band at cationic side which appeared to be lysozyme. No significant change in the protein stain patterns was observed during storage.

The acid mucoprotein patterns of ovomucin gel(B) in horizontal acrylamide gel electrophoresis showed a blue broad band near slot origin. As the storage proceeded, the following observations were made. The blue broad band of ovomucin gel(B) diminished progressively, whereas that of ovomucin sol(B) increased.

The protein stain patterns of egg white sol, separated from egg white gel by ultracentrifugation, treated with mercaptoethanol showed a slightly broad band at anionic side in acrylamide gel electrophoresis. On the other hand, the acid mucoprotein stain patterns of egg white sol treated with mercaptoethanol showed only a blue broad band near slot origin (Fig. 3).

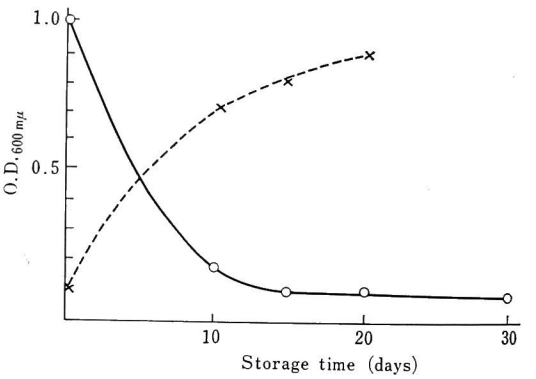

FIG. 4. Changes in the Interaction with Lysozyme of the Ovomucin Gel(B) and Sol(B) during Storage.

○—○ Ovomucin gel(B), ×---× Ovomucin sol(B)

While the interaction of ovomucin gel(B), treated with mercaptoethanol, with lysozyme decreased, that of ovomucin sol(B) increased progressively as the storage proceeded (Fig. 4).

DISCUSSION

According to our observation ovomucin gel(B) obtained from stored egg white insolubilized itself progressively as the storage proceeded, although ovomucin gel(B) obtained from a newly laid egg white was slightly soluble in dilute alkaline solution. Therefore, to study the physicochemical properties of ovomucin gel(B) obtained from stored egg white, it was necessary to solubilize it. We reported previously[2] that ovomucin(B) was solubilized by treatment with thioglycolate. But it seems that thioglycolate tends to lose the reducing force during storage. To avoid ambiguity in the results due to the loss of the reducing force of thioglycolate, ovomucin gel(B) was solubilized by treatment with mercaptoethanol.

By treatment with 0.01 M mercaptoethanol, the degree of disintegration into low molecular weight materials was very low and chemical composition did not show large changes.

Free boundary electrophoretic patterns of the mercaptoethanol-treated ovomucin gel(B) showed two components as in the case of the untreated ovomucin gel(B) which was solubilized slightly with dilute alkaline solution. The difference in relative area ratio between the mercaptoethanol-treated and the untreated seems to show that the fast moving component of the untreated ovomucin gel(B) is hard to be solubilized with dilute alkaline solution. It is suggested from these results that ovomucin gel(B) is dissociated into subunits which are consisted of two components by treatment with mercaptoethanol as well as thioglycolate.

It is suggested from changes in free boundary electrophoretic patterns of ovomucin gel(B) obtained from stored egg white that the fast moving component of ovomucin gel is released into egg white sol progressively as the storage proceeds. This is also supported by the result that the fast moving component of ovomucin sol(B) obtained from stored egg white increased during storage.

Horizontal acrylamide gel electrophoretic patterns of ovomucin gel(B) and sol(B) obtained from stored egg white showed more interesting results. While the protein stain patterns of ovomucin gel(B) and sol(B) did not reveal large changes during storage, the acid mucoprotein stain patterns changed remarkably. Whereas the blue broad band near slot origin of ovomucin (B) dimished progressively, the blue broad band of ovomucin sol(B) increased as the storage proceeded. By these results it is supported that the acid mucoprotein is released from ovomucin gel during storage.

This is supported further by the result that the blue band in horizontal acrylamide gel electrophoretic patterns of egg white sol increases during storage.

It was suggested in the previous report[1] that the carbohydrate rich component in ovomucin gel(B) was released into the sol part of stored white and the carbohydrate poor component remained insoluble during storage. Therefore, corresponding to changes in the electrophoreric patterns of ovomucin, it is considered that the carbohydrate rich component, perhaps acid mucoprotein, is the fast moving component and the carbohydrate poor component is the slow moving component in free boundary electrophoresis.

O. J. Cotterill *et al.*[5] reported that precipitate resulted from the addition of lysozyme to ovomucin and that quantity of the precipitate indicated the degree of interaction of ovomucin with lysozyme. In this paper, the turbidity resulting from the addition of lysozyme to ovomucin is taken as indication of the degree of ovomucin-lysozyme interaction. Although ovomucin was solubilized by treatment with 0.01 M mercaptoethanol, the turbidity resulting from the addition of lysozyme to ovomucin or the interaction of ovomucin obtained from stored egg white with lysozyme

5) O. J. Cotterill and A. R. Winter, *Poultry Sci.*, **34**, 679 (1955).

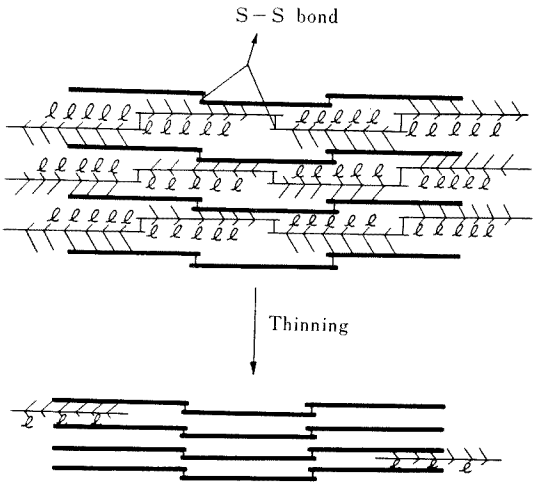

FIG. 5. A Model of Ovomucin Gel Structure and Mechanism of Egg White Thinning.

Carbohydrate poor component
Carbohydrate rich component
Lysozyme

could be measured. It is very interesting that changes in the properties of ovomucin during storage affect on the interaction with lysozyme. The interaction with lysozyme of ovomucin gel(B) decreased and that of ovomucin sol(B) increased as the storage proceeded. It was reported in the previous work[6] that the interaction of ovomucin with lysozyme in the thick white was stronger than that in the thin white.

Therefore, it is suggested that the fast moving component in free boundary electrophoresis interacts with lysozyme more strongly than the slow moving component, and the newly laid thick white gel may owe its swollen rigid gelatinous structure partly to ovomucin-lysozyme interaction.

Summarizing these results, we can demonstrate a model of ovomucin gel structure and a mechanism of egg white thinning. The model is shown in Fig. 5. According to the model, the swollen rigid gelatinous structure of ovomucin gel is supported both by the carbohydrate poor, slow moving component, which is the core portion of ovomucin gel remaining insoluble during storage; and by the carbohydrate rich, fast moving component, which is the prosthetic portion of ovomucin gel giving the swollen gelatinous structure by interaction with lysozyme. It is natural that either the carbohydrate poor or rich component must be kept by disulfide-bond, because ovomucin gel(B) is solubilized completely by treatment with 0.01 M mercaptoethanol and is found to dissociate itself into subunits. Furthermore, it is concluded that during storage the prosthetic portion of ovomucin gel is released from ovomucin gel and the swollen rigid gelatinous structure of ovomucin gel changes into contracted gelatinous structure.

6) A. Kato, R. Nakamura and Y. Sato, *Agr. Biol. Chem.*, **34**, 854 (1970).