Effect of β -Ovomucin on the Solubility of α -Ovomucin and Further Inspection of the Structure of Ovomucin Complex in Thick Egg White

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The effect of β -ovomucin on the solubility of α -ovomucin was studied in the presence or absence of lysozyme. The α -ovomucin content in a mixed solution of α -ovomucin was calculated with a simultaneous equation devised on the basis of the differences in the protein and carbohydrate contents of the two ovomucin.

β -Ovomucin inhibited the formation of α -ovomucin aggregates in a salt-free solution or in a slightly acid one. α -Ovomucin formed an insoluble complex with lysozyme below pH 10 at the ionic strength of 0.01, and β -ovomucin formed an insoluble complex with lysozyme below pH 6. β -Ovomucin inhibited the formation of an insoluble α -ovomucin complex with lysozyme in neutral or slightly alkaline pH regions.

It was presumed that bound β -ovomucin inhibits the aggregation of the α -ovomucin-lysozyme complex in thick egg white and results in the formation of a gel structure.

Ovomucin is a structural glycoprotein that is widely distributed in egg white,¹⁾ chalazae²⁾ and the outer layer of the vitelline membrane,³⁾ and is characterized by a high molecular weight and its ability to form a gel structure. There are two different types of ovomucin complex in hen egg white; one is an insoluble ovomucin, which accounts for the high viscosity of the egg white, and the other is a soluble ovomucin.⁴⁾ Both ovomucins are composed of subunit structures of α -ovomucin and β -ovomucin^{5~7)} with molecular weights of 1.8×10^5 and 4.0×10^5 , respectively.⁷⁾ The difference in β -ovomucin content between the two types seems to be the main reason for the difference in viscometric behaviours.⁸⁾

It has been shown that ovomucin can interact strongly with egg white lysozyme.^{9~16)} The interaction between ovomucin and egg white proteins has also been investigated.¹⁷⁾ However, interaction of ovomucin with lysozyme or other egg white proteins has been studied using an ovomucin mixture composed of α -ovomucin and β -ovomucin. Little work

has been done on the interaction between α -ovomucin and β -ovomucin.

The purpose of the present study was to determine the interaction between α -ovomucin and β -ovomucin in the presence and absence of lysozyme and to discuss the role of α -ovomucin, β -ovomucin and lysozyme in the gel structure of the ovomucin complex in thick egg white.

MATERIALS AND METHODS

Materials. α -Ovomucin and β -ovomucin were prepared from the gel fraction of newly laid thick egg white by the method described previously.⁸⁾ Lysozyme, sialic acid and chondroitin sulfate were purchased from Seikagaku Kogyo Ltd., dextran and dextran sulfate from Pharmacia Co., bovine serum albumin (BSA) from Sigma Co. and glutamic acid from Katayama Chemical Co.

Measurement of α -ovomucin and β -ovomucin in the mixed solution. The concentration of protein in a solution containing α -ovomucin or β -ovomucin alone was determined by using a calibration curve for the dry matter based on the absorbance at 280 nm or that of the solution treated with phenol sulfuric acid reagent at 490 nm.¹⁸⁾ α -

Ovomucin content in the solution containing β -ovomucin was calculated with the following simultaneous equation based on the different protein and carbohydrate contents of α -ovomucin and β -ovomucin;

$$A_{280} = C(\alpha A_1 + \beta A_2)$$

$$A_{490} = C(\alpha B_1 + \beta B_2)$$

$$\alpha + \beta = 1$$

A_{280} and A_{490} are the absorbances at 280 nm and 490 nm for the mixed protein solution. A_1 and A_2 , which are 0.963 and 0.349, respectively, correspond to the absorbance at 280 nm for α -ovomucin and β -ovomucin at a concentration of 1 mg/ml (Fig. 1). B_1 and B_2 , which are 0.704 and 1.766, respectively, are the values corresponding to the absorbances at 490 nm for α -ovomucin and β -ovomucin at a concentration of 1 mg/ml (Fig. 1). Alpha and beta are the ratio of α -ovomucin and β -ovomucin in total ovomucin. C is the concentration of total ovomucin. The concentration of α -ovomucin in a mixed protein solution was estimated by multiplying α by C .

Determination of effect of various materials on the solubility of α -ovomucin and β -ovomucin. A known amount of α -ovomucin solution (4 or 6 mg/ml) was mixed with an equal volume of a solution containing 4 mg/ml glutamic acid, various carbohydrates, BSA, β -ovomucin and/or lysozyme at pH 7 or 11. The mixed solution was adjusted to an appropriate pH and an ionic strength of 0.01 with a small volume of 1 N HCl and 1 M NaCl. After standing for 30 min, the mixed solution was centrifuged at $1500 \times g$ for 15 min and then the absorbance at 280 nm was measured. The same solution was treated with phenol sulfuric acid reagent and the developed color measured at 490 nm. α -

Ovomucin content in the solution containing the carbohydrate was calculated from the absorbance at 280 nm. α -Ovomucin content in the solution containing BSA or lysozyme was calculated from the absorbance at 490 nm by the phenol sulfuric acid method.¹⁸⁾ β -Ovomucin content in the solution containing lysozyme was also calculated from the absorbance at 490 nm. α -Ovomucin content in the solution containing β -ovomucin and lysozyme was calculated with the simultaneous equation after the removal of lysozyme with a carboxymethyl cellulose column equilibrated with 0.1 M Tris-HCl buffer (pH 8) containing 0.15 M NaCl.

Solubility is shown as a percentage of the α -ovomucin content in the supernatant to that in the initial mixed solution.

RESULTS AND DISCUSSION

It is fairly troublesome to estimate quantitatively the content of an individual protein in a solution containing more than two kinds of protein with no specific activity. The relative amounts of α -ovomucin and β -ovomucin in the solubilized ovomucin complex have been estimated by free boundary electrophoresis,⁵⁾ density gradient electrophoresis,^{15,19)} analytical ultracentrifugation²⁰⁾ and DEAE cellulose column chromatography.⁸⁾ There are some disadvantages to these procedures. The major one is time; it takes several hours to determine one sample. Hillier²¹⁾ developed a method which can easily estimate the amount of an individual protein in a mixture of whey proteins by spectrophotometric scanning of the stained protein bands following polyacrylamide gel electrophoresis. Although this method can estimate multiple samples in one run, it is not useful for ovomucin because α -ovomucin and β -ovomucin cannot be separated well on polyacrylamide gel due to their high molecular weight.⁷⁾ It has been shown that α -ovomucin is different in protein and carbohydrate contents from β -ovomucin.⁶⁾ Therefore, we attempted to estimate the amounts of α -ovomucin and β -ovomucin in the mixed solution based upon the difference in the protein and carbohydrate contents of the two ovomucins.

A linear relationship was observed between the absorbance at 280 nm and 490 nm and the amount of protein for α -ovomucin and β -

FIG. 1. Correlation between the Absorbance at 280 nm and 490 nm and the Concentrations of α -Ovomucin and β -Ovomucin.

○, α -ovomucin at 280 nm; ●, α -ovomucin at 490 nm; △, β -ovomucin at 280 nm; ▲, β -ovomucin at 490 nm.

FIG. 2. Correlation between Ratio of α -Ovomucin Calculated with the Simultaneous Equation Devised and the Mixing Ratio of α -Ovomucin and β -Ovomucin.

Total protein concentration was 1 mg/ml. Open circles represent the ratio of α -ovomucin calculated and the solid line represents theoretical values.

FIG. 3. Effect of β -Ovomucin on the Solubility of α -Ovomucin in the Solution with No Salt.

An equal volume of various concentrations of β -ovomucin solution (0~1.7 mg/ml) was mixed with 4 mg/ml α -ovomucin solution at pH 7 followed by thorough dialysis against distilled water.

ovomucin (see Fig. 1). The method of Lowry *et al.*²²⁾ was not useful for determination of protein because of the poor linearity between the absorbance and amount of β -ovomucin due to its extremely high carbohydrate content. To confirm the validity of the simultaneous equation devised, the ratio of α -ovomucin in an ovomucin mixture containing known amounts

FIG. 4. Effect of β -Ovomucin on the Solubility of α -Ovomucin at Various pHs.

○, none; ●, 0.2 mg/ml; △, 0.4 mg/ml; ▲, 0.8 mg/ml; □, 4.0 mg/ml of β -ovomucin solution was mixed with an equal volume of 4 mg/ml α -ovomucin solution and then each mixed solution was adjusted to an appropriate pH with 1 N HCl.

of α -ovomucin and β -ovomucin was determined with the simultaneous equation. The results are presented in Fig. 2. The ratio of α -ovomucin calculated from the equation showed extremely good correlation with the theoretical value. Therefore, it is considered reasonable to determine the solubility of α -ovomucin by using the equation in the present study.

Figure 3 shows the solubility of α -ovomucin when an α -ovomucin solution containing various concentrations of β -ovomucin was dialyzed against distilled water thoroughly. α -Ovomucin showed low solubility in salt-free water, whereas it remained soluble in the presence of small amount of β -ovomucin. It is considered that the isolated soluble ovomucin which is composed of 87% α -ovomucin and 13% β -ovomucin⁸⁾ remains soluble when dialyzed against distilled water because of the protective effect of β -ovomucin on the solubility of α -ovomucin.

Figure 4 shows the effect of β -ovomucin on the solubility of α -ovomucin at various pHs. Although about half of the α -ovomucin in the solution containing no β -ovomucin precipitated at pH 5, half of the α -ovomucin in the

TABLE I. EFFECT OF AMINO ACID, PROTEINS AND CARBOHYDRATES ON THE pH OF THE FORMATION OF α -OVOMUCIN AGGREGATES

	pH*		pH*
Without	5.0	Glucose	4.7
Glutamic acid	4.9	Sialic acid	5.3
Serum albumin	4.9	Dextran	4.9
Lysozyme	10.0	Dextran sulfate	<1.5
β -Ovomucin	3.3	Chondroitin sulfate	<1.5

* pH is that of half-precipitation of α -ovomucin when an equal volume of an additive (4 mg/ml) was added to the α -ovomucin solution (4 mg/ml) and the pH of the mixed solution was lowered with 0.1 N HCl.

solution containing β -ovomucin precipitated at a more acid pH. α -Ovomucin precipitated below pH 4 in the solution containing equal amounts of α -ovomucin and β -ovomucin. Almost an equal amount of β -ovomucin precipitated with α -ovomucin in spite of the fact that β -ovomucin in the solution containing no α -ovomucin did not precipitate at any pH (data not shown). It is, therefore, considered that β -ovomucin inhibits the formation of α -ovomucin aggregates in the slightly acid pH region due to formation of a complex of α -ovomucin and β -ovomucin and this complex may precipitate in a more acid pH region. Kato *et al.*¹⁷⁾ showed that ovomucin can interact with some egg white proteins and that sialic acid in ovomucin is essential for the ovomucin-protein interaction. Acid polysaccharides such as dextran sulfate and chondroitin sulfate showed a pronounced protective effect on the solubility of α -ovomucin (Table I). Therefore, the protective effect of β -ovomucin was presumably due to the large amount of carbohydrate side chains and their negative charges.

Figure 5 shows the solubility of α -ovomucin or β -ovomucin in either an α -ovomucin or a β -ovomucin solution containing an equal amount of lysozyme at various pHs and an ionic strength of 0.01. The solubility of α -ovomucin rapidly decreased at pHs below 10, whereas a major part of β -ovomucin remained soluble at pHs above 6. It is suggested that α -ovomucin is insoluble and β -ovomucin is sol-

FIG. 5. Solubility of α -Ovomucin and β -Ovomucin in the Solution Containing Lysozyme at Various pHs.

○, α -ovomucin; ●, β -ovomucin. Each solution of 4 mg/ml α -ovomucin or β -ovomucin was mixed with an equal volume of 4 mg/ml lysozyme solution at pH 11, and then the pH of the mixed solution was adjusted with 1 N HCl followed by adjusting of the ionic strength to 0.01^{*} with 1 M NaCl.

FIG. 6. Effect of NaCl on the Solubility of α -Ovomucin and β -Ovomucin in the Solution Containing Lysozyme.

○, α -ovomucin; ●, β -ovomucin. One volume of various concentrations (0~1 M) of NaCl solution was added to nine volumes of α -ovomucin or β -ovomucin solution (2 mg/ml) containing an equal amount of lysozyme at pH 7 and an ionic strength of 0.01.

uble in the presence of an equal amount of lysozyme in neutral or slightly alkaline pH regions which naturally occur in egg white. α -Ovomucin became soluble as the ionic strength was increased, as shown in Fig. 6. Although

FIG. 7. Effect of β -Ovomucin on the Solubility of α -Ovomucin in the Solution Containing Lysozyme.

●, pH 7; ○, pH 8. Equal volumes of 6 mg/ml α -ovomucin, 6 mg/ml lysozyme and various concentrations (0.6~6 mg/ml) of β -ovomucin solutions were mixed at pH 11, and then each mixed solution was adjusted to pH 7 or 8 with 1 N HCl followed by adjusting of the ionic strength to 0.01 with 1 M NaCl.

the solubility of β -ovomucin apparently increased above the concentration of 60 mM NaCl, this increase was due to the presence of turbid aggregated protein molecules which were not sedimented by centrifugation. Since Kato *et al.*¹⁶⁾ showed that β -ovomucin interaction with lysozyme was much more extensive than that of α -ovomucin in a higher ionic strength, these turbid aggregated protein molecules might be the result of interaction between β -ovomucin and lysozyme.

Figure 7 shows the solubility of α -ovomucin in the solution containing 2 mg/ml α -ovomucin, 2 mg/ml lysozyme and various concentrations of β -ovomucin adjusted to pH 7 or 8 at the ionic strength of 0.01. The solubility of α -ovomucin showed no decrease in the solution which contained more than 1 mg/ml β -ovomucin at pH 7 and 8. It is generally accepted that the pH of newly laid egg white is 7.5~8.0 and that it increases to about 9.5 on storage. Although the ionic strength of egg white was estimated to be about 0.1 based on the amount of inorganic salts in it,²³⁾ the ionic strength may be considerably reduced by a large amount of egg white proteins. Sato *et al.*¹⁹⁾ assumed that a high concentration of

ovalbumin may reduce ionic activity in their simulated experiments on egg white thinning. Therefore, it is considered that the actual ionic strength in egg white is much lower than 0.1. Thus, it is possible that β -ovomucin's inhibiting action on α -ovomucin aggregate formation in the presence of lysozyme occurs in conditions like those for egg white.

There is no direct evidence for the interaction of ovomucin with lysozyme in thick egg white. However, the lysozyme content in the gel fraction of thick egg white was higher than that in the liquid fraction,²⁴⁾ and the ovomucin-lysozyme interaction is considered important for the formation of the rigid gelatinous structure in the thick white, in the light of the mechanical properties of the thick white.¹¹⁾ Therefore, it is quite possible that the α -ovomucin-lysozyme complex does not form the aggregates with the protective effect of bound β -ovomucin, so the α -ovomucin-lysozyme complex covered with β -ovomucin in thick white can form a gel structure.

β -Ovomucin is reported to dissociate gradually from the ovomucin complex and to solubilize into the liquid fraction of thick white, whereas α -ovomucin remains insoluble during the natural thinning.^{14,20)} It has been proposed that the ovomucin complex is depolymerized by alkaline hydrolysis of disulfide bonds^{25,26)} or by alkaline β -elimination of the O-glycosidically linked carbohydrate unit,²⁷⁾ or that a gradual dissociation of the ovomucin-lysozyme complex takes place when eggs are stored.^{10,11,13)} Kato *et al.*²⁸⁾ suggested that α -ovomucin in ovomucin gel remains insoluble during long term storage because it is polymerized by disulfide bonds. However, Sato *et al.*¹⁹⁾ showed that about equal amounts of α -ovomucin and β -ovomucin were solubilized when the lysozyme-free insoluble ovomucin complex was aseptically stored at pH 9.6 and an ionic strength at 0.01 for 15 days; moreover, a major part of the α -ovomucin was solubilized on storage at an ionic strength of 0.1. Therefore, it is rather reasonable to consider that α -ovomucin remains insoluble during storage due to the formation of an in-

soluble complex with lysozyme.

The present findings suggest that the gel structure of the ovomucin complex in thick egg white consists of an α -ovomucin-lysozyme complex covered with β -ovomucin and that disaggregation of β -ovomucin from the ovomucin complex in thick white by an alkaline reaction during storage, as proposed by Kato *et al.*²⁷⁾ or Donovan *et al.*²⁵⁾ leads to insolubilization of the α -ovomucin-lysozyme complex.

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