A Comparative Study of Aggregated and Disaggregated Ovomucin during Egg White Thinning

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A definite amount of aggregated ovomucin, 0.05 g/100 mL of egg white, remained insoluble during storage of egg white from 20 to 60 days. The sialic acid content in disaggregated ovomucin obtained by gel filtration on Sepharose 4B of the supernatant of stored egg white increased during storage. The ovomucin elution pattern on Sepharose 4B of thinned egg white obtained after a 20-day storage was similar to that of fresh thick white homogenized with barbital buffer, pH 8.6, rather than that of fresh thick white in the presence of 0.01 M mercaptoethanol. These results suggest that noncovalent disaggregation of ovomucin occurs during thinning without disulfide cleavage.

Many investigators have been concerened with elucidating the mechanism of egg white thinning. Since the swollen gel structure of thick white is mainly composed of ovomucin, many arguments have been focusing on the degradation of ovomucin. It has been proposed during thinning that either (1) ovomucin is depolymerized by the reduction or alkaline hydrolysis of disulfide bonds (MacDonnell et al., 1951; Donovan et al., 1972; Tomimatsu and Donovan, 1972; Beveridge and Nakai, 1975) or (2) ovomucin–lysozyme interaction is responsible for the rigidity of the gel structure of the thick white and the interaction decreases gradually (Cotterill and Winter, 1955; Brooks and Hale, 1961; Robinson and Monsey, 1972a). However, little is known about the chemical and physicochemical changes that ovomucin actually undergoes in egg white during natural thinning.

In the previous papers (Kato et al., 1971; Kato and Sato, 1971, 1972), we showed that ovomucin consisted of the carbohydrate-rich component, named F-component, and the carbohydrate-poor component, named S-component. During thinning, the former component was gradually solubilized from ovomucin gel in thick white, while the latter was not (Kato et al., 1971; Kato and Sato, 1972). Robinson and Monsey (1971, 1972b) also obtained similar results on the behavior of ovomucin during thinning, and they designated the carbohydrate-poor component as α-ovomucin and the carbohydrate-rich component as β-ovomucin.

To lend further support to our hypothesis, we studied the characterization of aggregated ovomucin obtained from gel-like precipitates by ultracentrifugation of egg white at 59000g and disaggregated ovomucin obtained from gel filtration on Sepharose 4B of the supernatant after ultracentrifugation of egg white. Changes in the aggregated and disaggregated ovomucin thus obtained were followed during thinning. In addition, the elution pattern on Sepharose 4B column of thinned egg white was compared with that of reduced egg white.

MATERIALS AND METHODS

Separation of Thick White. All eggs were collected from hens within 24 h after laying, and the shells were sterilized by 70% ethanol. Thick white was separated from thin white by using a sieve and was stored in flasks at 30 °C for a given amount of days. All procedures to separate the thick white were aseptically carried out in a germ-free box.

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Isolation of Aggregated and Disaggregated Ovomucin. The homogenized thick white was separated into a gel-like precipitate and supernatant by sedimentation at 59000g for 60 min. The gel-like precipitates were washed with 2% KCl solution 5 times and finally washed with water until the washing contained no chloride. The precipitate was designated as aggregated ovomucin. On the other hand, 2 mL of supernant was diluted with 8 mL of 0.05 M barbital buffer, pH 8.6, and then 10 mL of diluted supernatant was applied to a Sepharose 4B column (3.2 × 28 cm). The samples were eluted with 0.05 M barbital buffer, pH 8.6. The flow rate was adjusted to 20 mL/h. The effluent was collected in each fraction of 3 mL. Each fraction was followed by measuring the extinction at 280 nm for protein. In addition, each fraction was followed by measuring the sialic acid according to the thiobarbituric acid method (Warren, 1959) and the interaction with lysozyme according to the turbidimetric method (Kato et al., 1975) in order to locate ovomucin. The first fraction that emerged in the void volume was collected and was designated as disaggregated ovomucin.

Reduction of Fresh Thick White. Fresh thick white was reduced with 0.01 M mercaptoethanol for 10 min at 20 °C, and then the reduced egg white was applied to Sepharose 4B column, as described above.

Carbohydrate Analysis. Total hexose content was determined by orcinol method in which an equimolar mixture of galactose and mannose was used as the standard. Galactose and mannose were determined by gas–liquid chromatography (Sweeley et al., 1963). Samples (0.5–5 mg) were hydrolyzed with 1 N H_2SO_4 at 100 °C in sealed ampules for 3 h. After removal of SO_4^{2-} by passage down a Dowex 1 column, the hydrolysates were evaporated to dryness. The hydrolysates were reacted for 5–10 min at 20 °C with 0.1 mL of a pyridine–hexamethyldisilazane–trimethylsilyl chloride (10:2:1 by volume) mixture. Samples (0.5–1.0 μL) of the trimethylsilylation reaction mixture were injected on to a glass column (2 m) packed with 5% SE-30 on Chromosorb W (60–80 mesh). The column was maintained at 180 °C until all of the hexose peaks had emerged. Total hexosamine content was determined by the modified Elson–Morgan's method (Neuhaus et al., 1957), in which a equimolar mixture of galactosamine and glucosamine was used as a standard. Samples (5 mg) were hydrolyzed with 3 N HCl at 100 °C for 4 h in sealed ampules. After hydrolysis the hydrolysates were evaporated to dryness. Glucosamine and galactosamine in the dried residues were determined by using a Hitachi 034 amino acid analyzer. Sialic acid was determined by the thiobarbituric acid method after hydrolysis of samples (5 mg) with 0.05 N H_2SO_4 at 75 °C for

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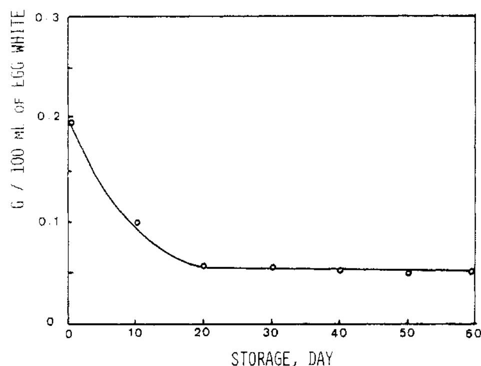

Figure 1. Changes in the content of aggregated ovomucin during storage of egg white.

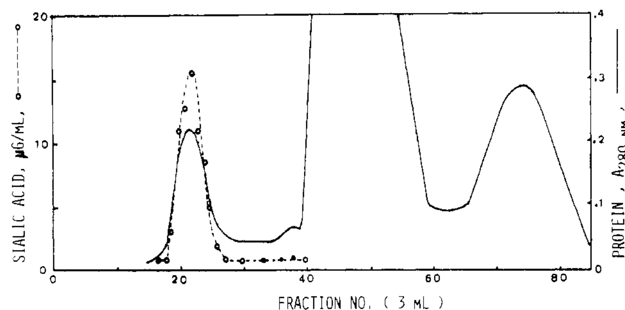

Figure 2. Elution pattern on a Sepharose 4B column of fresh thick white homogenized with barbital buffer.

Table I. Carbohydrate Composition of Aggregated Ovomucin in Stored Egg White

	carbohydrate composition, g/100 g		
	fresh	20 days	60 days
hexose	12.0	5.5	5.0
mannose	3.0	3.8	3.6
galactose	9.0	1.7	1.4
hexosamine	12.2	6.8	5.8
glucosamine	8.2	6.8	5.8
galactosamine	4.0	0	0
sialic acid	7.2	0.8	0.7

1 h in sealed ampules (Warren, 1959).

RESULTS

The fresh and stored thick white were throughly separated into a gel-like precipitate and clear supernatant by sedimentation at 59000*g* for 60 min. The content of aggregated ovomucin decreased during storage, as shown in Figure 1. However, an appreciable amount of aggregated ovomucin, 0.05 g/100 mL of stored egg white, remained insoluble even after the thick white had been stored aseptically for 60 days at 30 °C. The carbohydrate compositions and contents of aggregated ovomucin obtained after 60-day storage were almost similar to those after 20-day storage (Table I). The insoluble aggregated ovomucin obtained after 20-day storage was previously identified as carbohydrate-poor glycoprotein (α-ovomucin) which contained mainly mannose and glucosamine as carbohydrates (Kato and Sato, 1972). Therefore, these results show that α-ovomucin remains insoluble even after 2-month storage of egg white at 30 °C. The aggregated ovomucin in egg white after 60-day storage was readily solubilized by adding mercaptoethanol, suggesting that disulfide cleavage of ovomucin did not occur in egg thinning. The remarkable decreases of galactose, galastosamine, and sialic acid in ovomucin during storage suggest the release of β-ovomucin which contains a large amount of these carbohydrates. This will herein after be described.

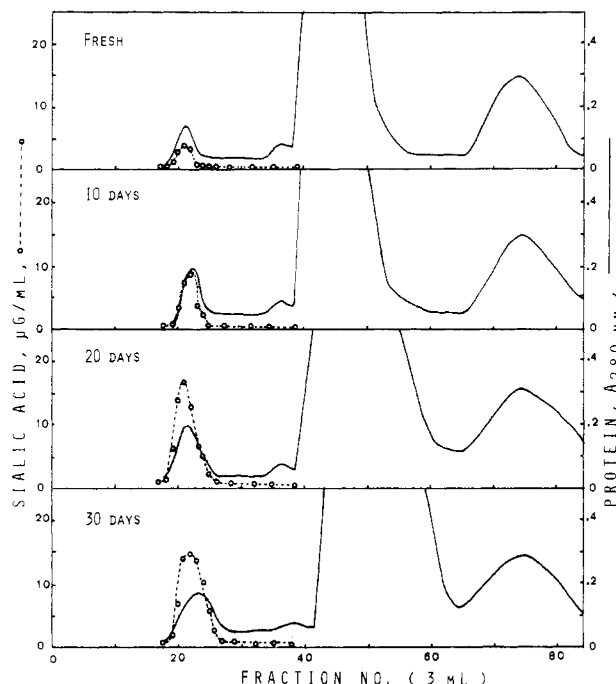

Figure 3. Elution patterns on a Sepharose 4B column of the supernatant of stored egg white.

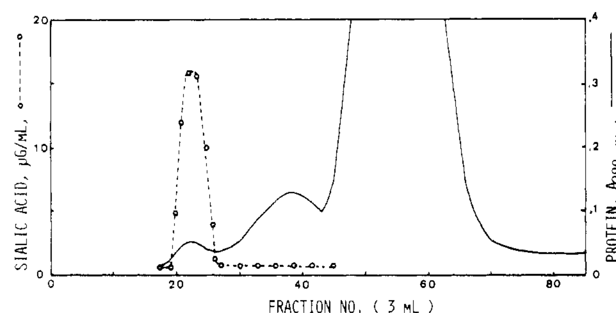

Figure 4. Elution pattern on a Sepharose 4B column of fresh thick white in the presence of 0.01 M mercaptoethanol.

Figure 2 shows the elution pattern on Sepharose 4B column of fresh thick white homogenized with 4 volumes of 0.05 M barbital buffer, pH 8.6, for 1 min at moderate speed. The protein peak that emerged in the void volume overlapped the sialic acid one and showed the interaction with lysozyme. This suggests that the void volume fraction is ovomucin, as reported previously (Young and Gardner, 1972). For investigation of the behavior of disaggregated ovomucin, changes in the elution patterns on Sepharose 4B of supernatants obtained after removal of aggregated ovomucin by sedimentation of stored egg white were followed during thinning, as shown in Figure 3. Since most sialic acids in egg white are contained in ovomucin, changes in the elution patterns of disaggregated ovomucin were followed by the measurement of sialic acid and protein. The supernatant of fresh thick white was not viscous, but that of stored egg white become viscous during storage, due to the disaggregation of ovomucin. A small amount of disaggregated ovomucin was present in the supernatant of fresh thick white, as shown in Figure 3. The ovomucin fraction increased as the egg white was stored. The sialic acid content of ovomucin fraction also increased during storage of egg white, suggesting the release of β-ovomucin which contained mainly galactose, galactosamine, and sialic acid in thinning, as reported previously (Kato and Sato, 1972). It is most interesting that the elution pattern of the ovomucin fraction obtained after 20-day storage of egg

white was appreciably similar to that of fresh thick white homogenized with barbital buffer (shown in Figure 2).

Figure 4 shows the elution pattern on Sepharose 4B column of fresh thick white reduced with 0.01 M mercaptoethanol. The protein profile of ovomucin fraction shows another broader peak of fraction no. 30–42, due to the disulfide cleavage of α-ovomucin, although this broader peak did not emerge in the elution patterns of stored egg white. On the other hand, the sialic acid rich ovomucin, reduced β-ovmucin, was still present in the void volume fraction, suggesting that it was of high molecular weight, although the peak slightly shifted to the lower molecular side.

DISCUSSION

Our hypothesis on a mechanism of thinning was confirmed by following the behavior of aggregated and disaggregated ovomucin during storage of egg white. The carbohydrate-rich ovomucin (β-ovomucin) disaggregatd from ovomucin gel and the carbohydrate-poor ovomucin (α-ovomucin) remained insoluble during egg white thinning. It is an important finding that α-ovomucin polymerized by disulfide bonds remained insoluble for a long-time storage. This is apparently contrary to the hypothesis that ovomucin is depolymerized by the reduction or alkaline hydrolysis of disulfide bonds during thinning.

On the other hand, the ovomucin elution pattern on Sepharose 4B of thinned egg white obtained after 20-day storage was similar to that of fresh thick white homogenized with barbital buffer, pH 8.6, rather than that of fresh thick white in the presence of a reducing reagent.

These results suggest that noncovalent disaggregation of ovomucin occurs during natural thinning, without disulfide cleavage of ovomucin. The ovomucin gel structure of the thick white may be held by noncovalent bonds that are unstable to mild homogenization in mild alkaline solution rather than by the disulfide bond in aggregated α-ovomucin that is not disaggregated during thinning.

Further studies are now being carried out to determine the molecular weight of disaggregated ovomucin during thinning.

LITERATURE CITED

Beveridge, T.; Nakai, S. *J. Food Sci.* **1975**, *40*, 864.
Brooks, J.; Hale, H. P. *Biochim. Biophys. Acta* **1961**, *46*, 289.
Cotterill, O. J.; Winter, A. R. *Poult. Sci.* **1955**, *34*, 679.
Donovan, J. W.; Davis, J. G.; Wiele, M. B. *J. Agric. Food Chem.* **1972**, *20*, 223.
Kato, A.; Imoto, T.; Yagishita, K. *Agric. Biol. Chem.* **1975**, *39*, 541.
Kato, A.; Nakamura, R.; Sato, Y. *Agric. Biol. Chem.* **1971**, *35*, 351.
Kato, A.; Sato, Y. *Agric. Biol. Chem.* **1971**, *35*, 439.
Kato, A.; Sato, Y. *Agric. Biol. Chem.* **1972**, *36*, 831.
MacDonnell, L. R.; Lineweaver, H.; Feeney, R. E. *Poult. Sci.* **1951**, *30*, 856.
Neuhaus, O. W.; Letzring, M. *Anal. Chem.* **1957**, *29*, 1230.
Robinson, D. S.; Monsey, J. B. *Biochem. J.* **1971**, *121*, 537.
Robinson, D. S.; Monsey, J. B. *J. Sci. Food Agric.* **1972a**, *23*, 893.
Robinson, D. S.; Monsey, J. B. *J. Sci. Food Agric.* **1972b**, *23*, 29.
Sweeley, C. C.; Bentley, R.; Makita, M.; Wells, W. W. *J. Am. Chem. Soc.* **1963**, *85*, 2497.
Tomimatsu, Y.; Donovan, J. W. *J. Agric. Food Chem.* **1972**, *20*, 1067.
Warren, L. *J. Biol. Chem.* **1959**, *234*, 1971.
Young, L. L.; Gardner, F. A. *J. Food Sci.* **1972**, *37*, 8.

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Hemoglobin as a Binding Substrate in the Quantitative Analysis of Plant Tannins

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The use of hemoglobin derived from fresh bovine blood as a binding substrate in protein precipitation of tannins is described. The use of a chromoprotein obviates the need for visualizing either precipitated protein or substances remaining in the supernatant. When the appropriate initial hemoglobin concentration is chosen, the relationship between absorbance of hemoglobin remaining in the supernatant and tannin concentration is linear over a wide range of tannin concentrations and for several tannin types. The binding process is relatively unaffected by solution pH. Methods combining the precipitation of hemoglobin with other techniques, such as the Folin–Denis assay, may help elucidate the details of the binding process.

Tannins are polyphenolic compounds which form stable complexes with proteins (Swain, 1979). They are nearly ubiquitous in woody plants (Bate-Smith and Metcalf, 1957) and are widespread in various tissues of herbaceous plants as well, including economically important species (Swain and Hillis, 1959; Chan et al., 1978; Hagerman and Cutler, 1978). The proposed biological actions of tannins include complexing with plant proteins, reducing the digestibility of tissues to insect or vertebrate herbivores, complexing with digestive enzymes (Feeny, 1968, 1969; Williams, 1963; Goldstein and Swain, 1965), or acting as behavioral antifeedants (Bate-Smith, 1973; Goldstein and Swain, 1963). Thus, several techniques which estimate tannin content of plant material by determining the affinity of plant extracts for proteins have appeared in the literature (Bate-Smith, 1973; Chan et al., 1978; Hagerman and Butler, 1978; Swain, 1979; Walter and Purcell, 1979; Boudini et al., 1980).

A useful tannin assay should have the characteristics of simplicity and adaptability to small-sized samples and should not require specialized equipment (Bate-Smith, 1973). The use of the chromoprotein hemoglobin as a binding substrate (Bate-Smith, 1973) meets these requirements but has only been employed for qualitative taxonomic work [e.g., Bate-Smith (1977)]. Hemoglobin concentration can be measured directly by visual spectrophotometry and can be used in the analysis of single leaves (Bate-Smith, 1973; Swain, 1979). In this communication we report on the use of hemoglobin in a quantitative assay for plant tannins. We report here on the

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