

Fractionation of Quail Ovomuroid and Characteristics of the Respective Fractionated Components

Katsuya KOGA, Takao FUKUNAGA and Ryuji YAMAMURA*

(Laboratory of Animal Biochemistry)

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Introduction

In taxonomy, the Japanese quail belongs to the same family, *Phasianidae*, together with the chicken. Although many investigations on the heterogeneity and the fractionation of chicken ovomucoid have been published, there are quite few noticeable investigations on those of quail ovomucoid.

Lineweaver and Murray²¹⁾ firstly isolated ovomucoid from hen egg white and established that it was the component responsible for the trypsin inhibitory activity. Fredericq *et al.*¹³⁾ and Bier *et al.*⁴⁾ found the separation of hen ovomucoid into five components *i.e.* the heterogeneity of ovomucoid by the moving boundary electrophoresis. Subsequently, the ovomucoid preparation obtained by using trichloroacetic acid-acetone method of Lineweaver *et al.* could be further purified and fractionated or it could be isolated directly from egg white by many other researchers^{8, 9, 11, 12, 22, 26, 27)}. Kanamori *et al.*^{15, 16)} reported that hen ovomucoid was fractionated into four components by CM-cellulose chromatography following ammonium sulfate and DEAE-cellulose fractionations and that two of these components were trypsin inhibitors and the other two were apoproteins of flavomuroid. Subsequently, they showed that the different sialic acid concentrations in the four components were responsible for the heterogeneity of ovomucoid. Melamed mentioned also the similar cause as to the heterogeneity²²⁾. Feeney *et al.*¹²⁾ observed the electrophoretic heterogeneity of the Japanese quail ovomucoid prepared by CM-cellulose chromatography following TCA-acetone treatment of egg white, but did not achieve the fractionation of ovomucoid.

Although Asao *et al.*^{2, 3)} repeatedly studied the direct separation of trypsin inhibitor from the quail egg white by CM-cellulose chromatography, they did not succeed in achieving the reproducible separation of ovomucoid fraction. They³⁰⁾, therefore, separated the trypsin inhibitor by TCA-treatment followed by the concentration at about 60°C. The conformation of the inhibitor, however, appeared to be slightly changed.

In the previous report, Koga¹⁷⁾ showed that water-extractable glycoprotein from the heat-coagulated egg white was more in the Japanese quail egg than in the chicken egg, and the electrophoretic behaviors and sugar-protein ratios of both glycoproteins were mutually different. In this report, we describe the fractionation of the Japanese quail ovomucoid and the chemical characteristics of the respective fractionated components.

Experimental Materials and Methods

Materials — The eggs of the Japanese quail (*Coturnix coturnix japonica*) were secured from the

* Present address: Shōkei Women College, Kumamoto 862

Laboratory of Animal Breeding, Faculty of Agriculture, Kagoshima University within 20 hours after being laid. The egg whites were carefully separated from yolks and the whole egg white was blended with a homogenizer at a slow speed to be used as the experimental sample.

Separation of ovomucoid from egg white — An equal volume of saturated ammonium sulfate solution was added to the homogenized egg white, whilst being stirred gently. The globulin precipitate was removed by centrifuging and the supernatant was adjusted to pH 4.6; to be 0.65 saturation of ammonium sulfate. After allowed to stand overnight, the precipitated ovalbumin and conalbumin mixture was discarded, leaving ovomucoid in solution. To the solution was added the crystalline ammonium sulfate, to get full saturation. The precipitate obtained was crude ovomucoid and it was dissolved in water and dialyzed against water at 5°C, until ammonium ion disappeared.

Removal of contaminant from crude ovomucoid — Crude ovomucoid in 0.05 M acetate buffer solution, pH 4.40 was slowly passed through the DEAE-cellulose column (1.8 × 15 cm) equilibrated with the same buffer solution for the removal of a small amount of contaminant such as ovalbumin and conalbumin, referring to the procedure of Kanamori *et al.*¹⁵⁾

Fractionation of ovomucoid — The purified ovomucoid adsorbed on the DEAE-cellulose column as described above was eluted by a linear salt gradient with the concentrations from zero to 0.2 M NaCl in 0.05 M acetate buffer, pH 4.40.

The effluent was collected in each 5 ml. The electric conductivity meter (from M & S Instrument Inc.) was equipped to the top of the column. The concentration of salt in the flow system was determined by measuring the electric conductivity of the eluting buffer. The protein concentration in each 5 ml fraction was estimated by measuring the absorbance at 280 nm with Hitachi-101 Spectrophotometer. Further fractionation of each component separated by DEAE-cellulose column chromatography was achieved by the chromatography with a pH gradient elution on CM-cellulose column (1.8 × 20 cm). The pH values of acetate buffer employed were from 4.0 to 5.5 and ionic strength, 0.025. Additionally, the apparatus for gradient and stepwise elution with salt shown in Fig. 1, was employed to obtain a good separation of flavoprotein. Each aqueous solution of salt-free component, after dialyzed against water, was concentrated on Carbowax 6000 (polyethylene glycol), and lyophilized to be used as sample for the estimation of chemical composition and molecular weight.

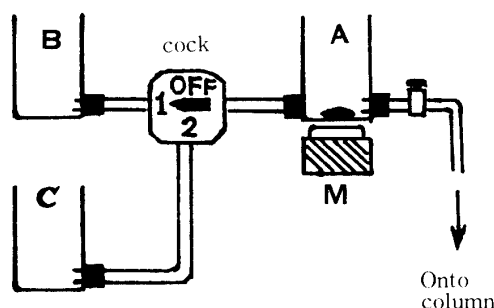


Fig. 1. The apparatus for gradient and stepwise elution.

A: 0.05 M acetate buffer (pH 4.40)

B: 0.2 M NaCl in the same buffer

C: 0.3 M NaCl in the same buffer

Elution method

1) Gradient elution (NaCl, 0→0.038 M) cock 1: open

2) Stepwise elution (NaCl, 0.038 M) cock: off

3) Gradient elution (NaCl, 0.038 M→0.25 M) cock

2: open, M: Magnetic stirrer

Disc gel electrophoresis — Each component from ovomucoid was subjected to the polyacrylamide gel electrophoresis to inspect the purity, according to the procedure described by Nagai²³).

Estimation of molecular weight — Estimation of molecular weight of each component was done by the Sephadex gel-filtration method proposed by Whitaker²³) and Andrews¹). The column from Shōei Works Co. and Sephadex G-100 from Pharmacia, Uppsala, Sweden were employed. The gel chromatography was performed by using the up-flow system of 0.05 M carbonate buffer (pH 10.0, $\mu=0.10$) with flow rate of 1 ml per min.

Examination of combining of each component with riboflavin — Each component from ovomucoid and a slight excess of authentic riboflavin were dissolved altogether to be associated in 0.05 M phosphate buffer, pH 7.0, which is the best binding pH¹⁵), and transferred into the respective cellulose membrane tube. Only riboflavin solution was poured into another tube. These solutions were thoroughly dialyzed against the same buffer until the yellowish color of riboflavin in the control tube disappeared. The spectra of these inner solutions were subsequently measured within the range from 250 nm to 500 nm. As will be described later in this paper, flavoprotein dissociates to the apoprotein and riboflavin at pH 4.0. Therefore, the binding ratio between the two was determined by measuring the concentrations of the apoprotein and the riboflavin solutions. The concentration of the former was calculated from the equation of calibration curve for apoprotein, and that of the latter, calculated from the molecular coefficient of riboflavin ($\epsilon=12.2 \times 10^3 \text{ mol}^{-1}, \text{ cm}^{-1}$ at 450 nm).

Analysis of N-terminal amino acids — N-terminal groups of flavoprotein and its apoprotein were determined by using Sanger's reagent, 2, 4-dinitrofluorobenzene^{28, 29}). Separation and identification of DNP-amino acid were performed by paper chromatography with the use of two different buffer systems: *tert*-amyl alcohol saturated with water and *n*-butanol saturated with water, referring to the method of Blackburn *et al.*⁵)

Amino acid analysis — Duplicate samples were hydrolyzed for 24 and 40 hr with the introduced nitrogen gas after removal of air by the suction, at 110°C, in a sealed tube. The hydrolysates were analyzed on a Yanagimoto LC-5S type amino acid analyzer.

Carbohydrate analysis — The neutral sugar determination was carried out by the phenol-sulfuric acid method¹⁰) and subsequently by gas-liquid chromatography, using Shimazu GC gas chromatograph, according to the procedures of Sweeley *et al.*³¹) and Bolton *et al.*⁷), which were described in our previous report¹⁸) in detail. Glucose was used as an internal standard. Because the majority of the neutral sugar had been identified with mannose in the preliminary experiment, the neutral sugar content was calculated from the following equation as mannose: $y=0.02x$, where y is the absorbance at 490 nm; x is the concentration (γ/ml) of mannose. This equation was employed in the case when y was below 0.7. The identification and determination of hexosamine were carried out according to the Pearson's procedure²⁵) followed by the Elson-Morgan method modified by Boas⁶). The detail was described in our previous report¹⁹). Amount of the identified glucosamine was computed from the equation: $y=15.1 \times 10^{-3}x$, where y is the absorbance at 530 nm; x , γ/ml . Additionally, hexosamine and N-acetylneuraminic acid were also analyzed by using the gas-liquid chromatograph, referring to the report of Bolton *et al.*⁷)

Assay of antitryptic activity with casein substrate — The assay of antitryptic activity of each component with the casein substrate was performed, essentially according to the description of Laskowski (Kunitz method)²⁰).

Bovine trypsin (crystallized 2 times) from Miles Co., Hammarsten casein and 0.1 M phosphate buffer, pH 7.6 were employed. A mixture of 1 ml of trypsin solution containing 1×10^{-3} M CaCl_2

and 0.5 ml of inhibitor solution was preincubated at 37°C for 10 min. And 0.5 ml of 2% casein was added; the assay mixture was incubated at 37°C for 10 min. The reaction was stopped by the addition of 3 ml of 5% trichloroacetic acid. The absorbance ($A_{280 \text{ nm}}$) of the supernatant after the centrifugation was measured.

Blank assay was simultaneously performed. Inhibitory activity was expressed as the decreasing of tryptic activity ($A_{280 \text{ nm}}$) per one mole of inhibitor. Specific inhibitory activity = $(I_0 - I_{0.5}) / \mu\text{M}$. inhibitor, where I_0 is the tryptic activity in the case when no inhibitor was added to the assay medium; $I_{0.5}$ is the activity when 0.5 ml of inhibitor was added to it.

Results and Discussion

Fractionation of ovomucoid by CM-cellulose chromatography following DEAE-cellulose chromatography — Crude ovomucoid separated from the Japanese quail egg white by the ammonium sulfate fractionation was purified by being adsorbed on DEAE-cellulose column. The ovomucoid was then separated into four components (A, B, C and D) by a linear salt gradient elution as shown in Fig. 2, though mutually each component was not separated distinctly.

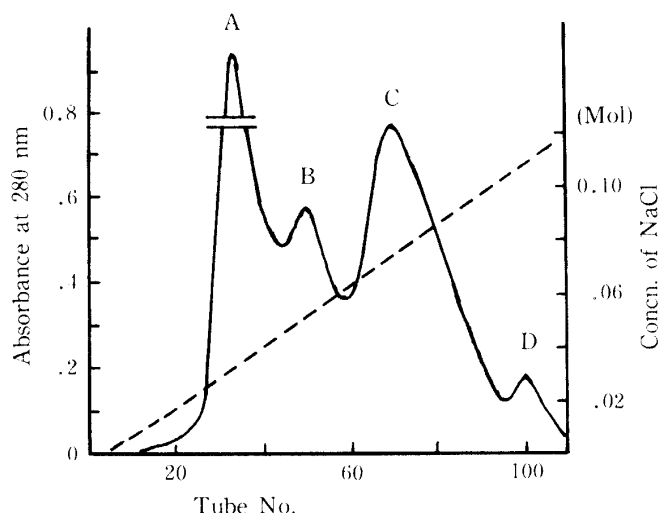


Fig. 2. DEAE-cellulose column chromatography of quail ovomucoid.

Column: 1.8×15 cm, Fraction size: 5 ml. Buffer: 0.05 M acetate, pH 4.40.

—: Protein, ----: Concentration of NaCl.

Relatively large amount of component C showed visible yellowish color, seeming to be flavo-protein. Component D appeared in small amount. When more protein was adsorbed on DEAE-cellulose column, a poor separation was observed: components A and B were not separately eluted. Each component separated incompletely as shown in Fig. 2 was desalted by dialyzing against water and then applied on CM-cellulose column to be eluted with a linear pH gradient. The elution profiles of each component were represented in Fig. 3. Component A from DEAE-cellulose column was distinctly separated into two components (I and II) having the eluting pH values of 4.5 and 5.1; component B appeared as the only one component (corresponding to comp. II), which was eluted at pH 5.1. From these results, component A was ascertained to be involving relatively large amount of component II. Component C was separated into three components and the first component passed through the CM-cellulose column at pH 4.0 was identified with riboflavin on the

basis of an absorption spectrum curve. Since the other two components were respectively eluted at pH 4.8 and 5.1, the former one was fixed to be an apoprotein of flavoprotein. This apoprotein is capable of recombining with riboflavin as will be described later. In the report on the fractionation of hen egg white proteins, Rhodes *et al.*²⁶⁾ described that the flavin moiety dissociated from the apoprotein at a pH more acid than 4.2 and recombined with that by increasing the pH above 4.2. To obtain a clear separation between components II and III, the device for bringing a clear separation of component C from component B by the DEAE-cellulose chromatography is necessary. Therefore, the apparatus shown in Fig. 1 was employed on the fractionation of the purified ovomucoid by the DEAE-cellulose chromatography. The procedure employed consisted of the gradient and the stepwise elutions: component C was eluted stepwise immediately after the mixture of components A and B was sufficiently eluted.

As shown in Fig. 4, component C was distinctly fractionated as a single peak. This com-

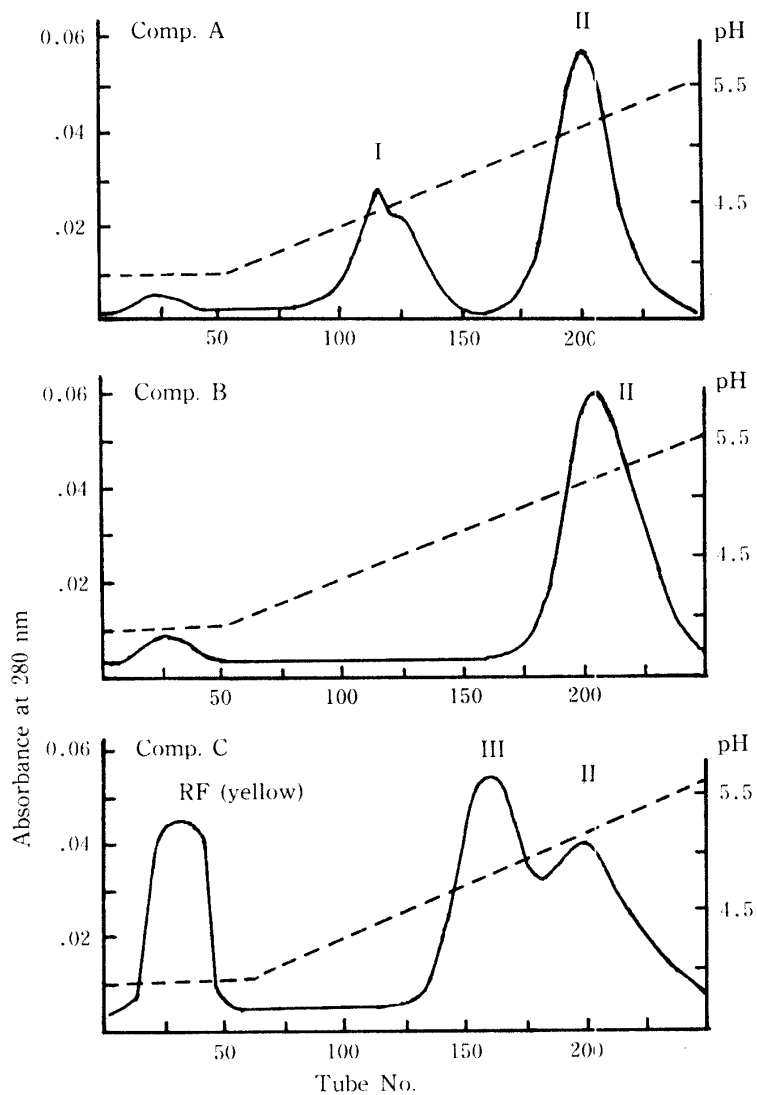


Fig. 3. CM-cellulose column chromatography of ovomucoid fractions separated by DEAE-cellulose chromatography.

Column: 1.8 × 20 cm, Fraction size: 5 ml. Buffer: 0.025 M acetate, pH 4.00 → 5.50 with a linear gradient
 —: Protein, ----: pH

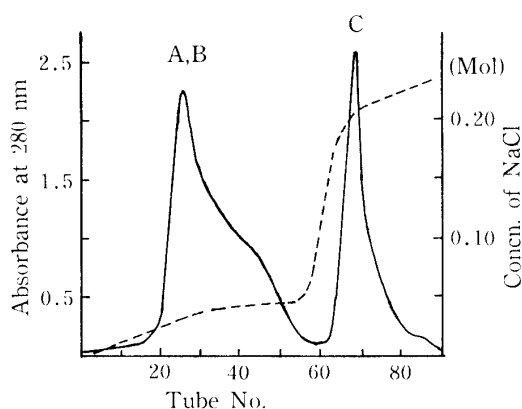


Fig. 4. DEAE-cellulose chromatography of quail ovomucoid to separate comp. A, B mixture and comp. C. Chromatographic condition was the same as that in Fig. 2. —: Protein, ----: Concentration of NaCl

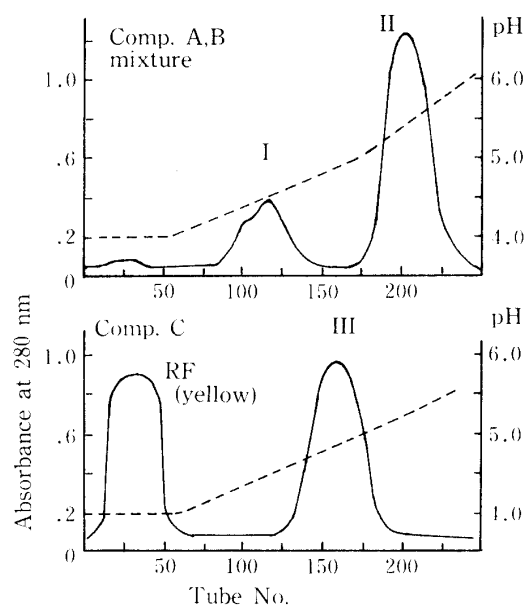


Fig. 5. CM-cellulose chromatography of comp. A, B mixture and C separated by DEAE-cellulose chromatography (Fig. 4). Column: 1.8×20 cm, Fraction size: 5 ml. Buffer: 0.025 M, pH 4.0 \rightarrow 6.0 with a linear gradient, —: Protein, ----: pH

ponent, flavoprotein was subsequently dissociated to riboflavin and apoprotein by CM-cellulose chromatography (Fig. 5). Since the amount of component D was quite small and not easy to be separated, the absorption spectra of the parts located ahead and behind the peak and the peak-part were respectively measured. Consequently, the part ahead the peak of component D gave the absorption maximum at 280 nm; the part behind peak and the peak-part showed respectively the maximum at 265 nm, which was the absorption of riboflavin (Fig. 6). Further studies on component D should be carried out. As the dissociation of riboflavin from the apoprotein was observed by the CM-cellulose chromatography at pH 4.0, but not observed by the DEAE-cellulose chromatography, authors presumed that riboflavin combined with the amino side-chain of the apoprotein.

Disc electrophoresis of each component — Components II, III and C showed respectively single narrow band which proved the high purity, on the disc electrophoresis with polyacrylamide gel. However, component I showed closely migrated two bands (Fig. 7).

Equations of calibration curves for each component refined — The respective calibration curves for components I, II and III were made by using their different concentrations of aqueous solutions. Precise concentrations were calculated from the dry weight. The equations obtained were $y = 4.78 \times 10^{-3}x$ for component I, $y = 13.5 \times 10^{-3}x$ for component II, $y = 14.0 \times 10^{-3}x$ for component III, where y is the absorbance at 280 nm, x is the concentration (mg %) (Fig. 8).

Molecular weights of each component — Molecular weights of the components I and III estimated by the gel-filtration on Sephadex G-100 were respectively 40000, and that of component II was 36500. Those values were considerably larger than the molecular weight of hen ovomucoid previously reported^{9, 32}.

On the flavoprotein — The result of the examination for combination of each component with riboflavin (RF) by the dialyzing method was given in Table 1. Absorption spectra of the inner solutions after the dialysis showed that the component I-RF mixture had an absorption maximum

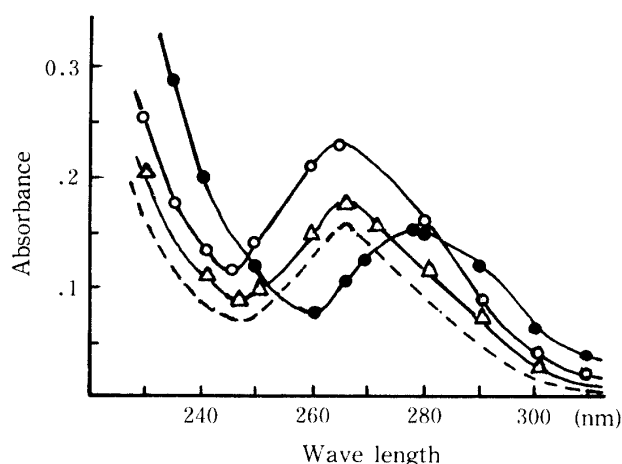


Fig. 6. Absorption spectra of comp. D (Fig. 2) and riboflavin.

—●—●—: The part ahead comp. D peak (in Fig. 2),
 —○—○—: The peak of comp. D,
 —△—△—: The part behind comp. D peak,
 - - - - : Authentic riboflavin.

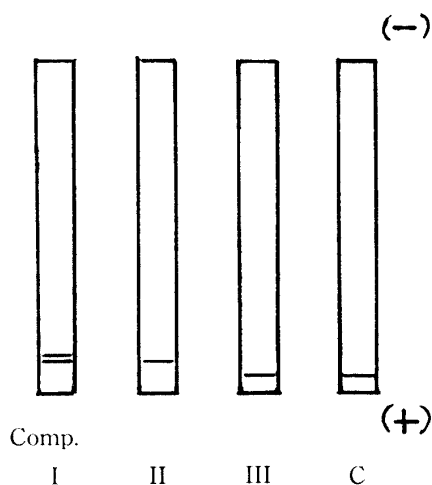


Fig. 7. Disc electrophoretic patterns of components I, II, III and flavo-protein (comp. C in Fig. 2) in polyacrylamide gel.

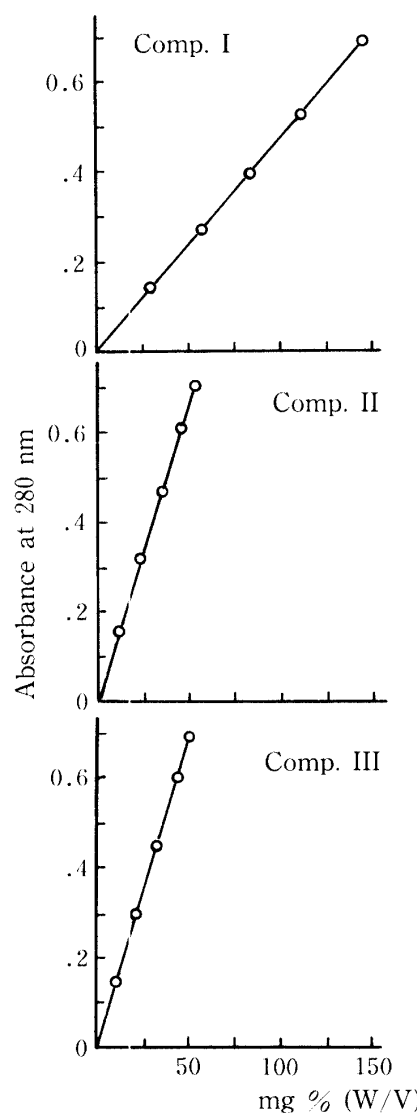


Fig. 8. Relationships between absorbances (A_{280nm}) and concentrations of comp. I, II and III aqueous solutions.

at 280 nm, while component II-RF mixture and component III-RF mixture had respectively the absorption maxima at 280 nm, near 445 nm (Fig. 9). Components II and III were thus found to have the capacity of combining with riboflavin. However, the flavoprotein from the egg white was the associate of component III and riboflavin.

To elucidate this discrepancy, after riboflavin was added to the egg white homogenate, the separation of ovomucoid by DEAE-cellulose chromatography was performed, according to the foregoing description. The separated component B (corresponding to component II) showed yellowish color as component C did (Fig. 10). These results suggest that component III has a

Table 1. Examination for the combining of each component from ovomucoid with riboflavin

	Comp. I +RF	Comp. II +RF	Comp. III +RF	RF
Color of inner soln. after dialysis	—	+	+	—

RF: Riboflavin. The dialysis was thoroughly performed until the yellowish color of RF in the control tube disappeared.

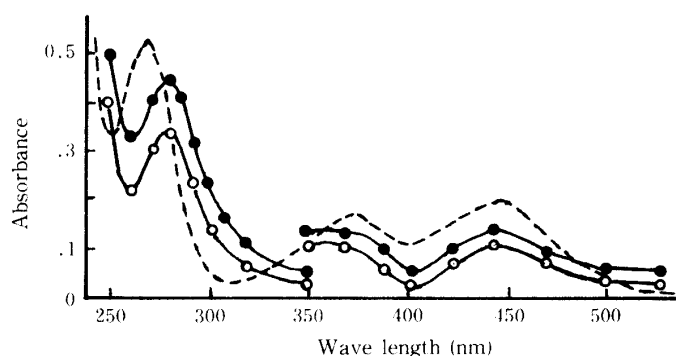


Fig. 9. Absorption spectra of comp. II-RF, comp. III-RF associates and free RF.

—●—●—: Comp. II-RF associate, —○—○—: Comp. III-RF associate,
 ———: RF (riboflavin).

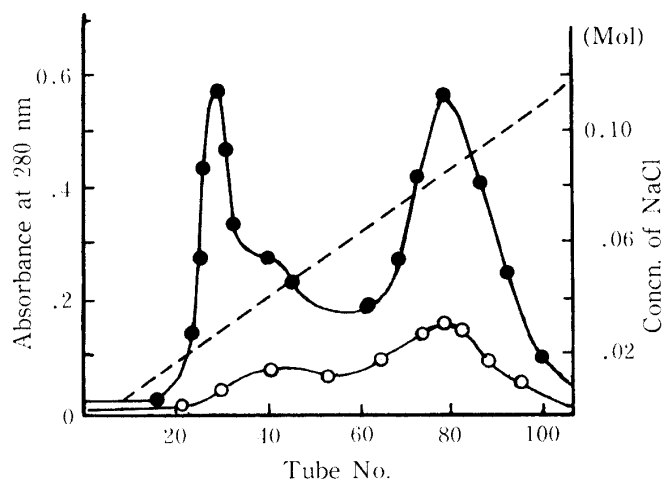


Fig. 10. DEAE-cellulose chromatography of the egg white-RF mixture after ammonium sulfate fractionation. Chromatographic condition was same as that in Fig. 2.

—●—●—: Absorbance at 280 nm, —○—○—: Absorbance at 450 nm.

predominant affinity for RF or RF-derivative than component II does. Further studies should be carried out to elucidate this problem. The binding molar ratio between the apoprotein (comp. III) and the prosthetic group, RF was ascertained to be 1:1, being coincident with that in flavoprotein from hen ovomucoid obtained by Kanamori *et al.*¹⁵⁾ DNP-apoprotein and DNP-flavoprotein preparations were obtained by the method of Sanger^{28,29)}. The intensity of yellowish color of the

former was thicker than that of the latter, despite the flavoprotein being originally yellowish. From this fact and the chromatographic behavior of flavoprotein on the CM-cellulose column, authors presumed that RF is combining with the amino side-chain of the apoprotein, which is to be dinitrophenylated. The acid hydrolysate of DNP-protein was separated into the ether- and acid-soluble fractions. No DNP-amino acid was observed on paper chromatograms of ether soluble part, while only ϵ -DNP-lysine was observed on those of acid soluble part. Namely, the absence of N-terminals in the both of flavoprotein and apoprotein was ascertained. N-terminals in them were considered to be substituted by the acyl group. Subsequently, non dinitrophenylated amino acids in DNP-flavoprotein and DNP-apoprotein were analyzed. The results compared with the amino acids in apoprotein were shown in Table 2. These analytical values demonstrated the following facts: A few equal lysine residues remain both in DNP-apoprotein and in DNP-flavoprotein, and arginine residues are not dinitrophenylated in bicarbonate solution; both the histidine and tyrosine residues are completely dinitrophenylated.

Table 2. Relative amounts of non dinitrophenylated amino acids from DNP-flavoprotein and DNP-apoprotein (Values are molar numbers in 1 mole protein)

Amino acid	DNP-flavoprotein	DNP-apoprotein	Apoprotein*
Lysine	3	3	16
Histidine	0	0	12
Ammonia	23	20	24
Arginine	7	7	7
Aspartic A.	28	26	26
Threonine	10	10	10
Serine	29	29	29
Glutamic A.	40	38	39
Proline	10	10	10
Glycine	11	11	11
Alanine	14	14	17
Cystine/2	19	19	22
Valine	5	5	7
Methionine	7	7	9
Isoleucine	4	4	6
Leucine	12	12	15
Tyrosine	0	0	18
Phenylalanine	10	10	10

* Amino acid composition was calculated from that of component III in Table 3.

It is well known that *O*-DNP-tyrosine is colorless. *Im*-DNP-histidine might be decomposed as shown in Sanger's report²⁸⁾. From these results, the reactive site of the apoprotein combined with RF was presumed to be lysine or histidine residues.

Antitryptic activity of each component — Component I showed the strong antitryptic activity, while components II and III having the capacity of combining with RF showed no antitryptic activity, respectively. As the component I appeared as two bands migrated closely on the disc electrophoresis, the antitryptic activities of the parts located ahead and behind the component I peak on the CM-cellulose chromatogram were estimated. Specific inhibitory activities of the former and the latter were 48 and 403, respectively. The former may be essentially non inhibitory

protein and the weak inhibition is probably due to the contamination with the latter.

Chemical compositions of each component — Concerning the amino acid compositions, component I was different from the components II and III, as shown in Table 3. Component I, the antitryptic component contained more lysine, aspartic acid, threonine, glycine, valine, than the components II and III did, while histidine, serine, glutamic acid, alanine, methionine, tyrosine and tryptophan amounts in the former were respectively smaller than those in the latter. Small amounts of histidine, methionine and tryptophan in component I were noticeable. The amino acid composition of component I was similar to that of the chicken ovomucoid purified by Osuga and Feeney²⁴⁾, as represented in Table 3. Davis *et al.*⁹⁾ described that the composition of hen ovomucoid purified by the authors was in good agreement with that reported by Osuga *et al.*,²⁴⁾ and the presence of tryptophan in the trypsin inhibitory component from hen ovomucoid reported by Kanamori *et al.*¹⁶⁾ was due to the contamination with other proteins. Waheed *et al.*³²⁾ also indicated the absence of tryptophan in the trypsin inhibitor from the hen ovomucoid. A small amount of tryptophan in that from the quail ovomucoid was presumed to be due to the contamination with a little amount of non-inhibitory protein. The amino acid compositions of components II and III were similar one another. Carbohydrate compositions were represented in Table 4, together with those of the chicken ovomucoid⁸⁾ for the comparison between them. The glucosamine contents obtained by the Pearson's procedure and by the gas chromatography were similar one another. The contents in Table 4 are, therefore, the values obtained by the latter. The carbohydrates of each component from the quail ovomucoid consisted of mannose (major constituent), fucose, glucosamine and N-acetylneuraminic acid, being considerably different from those of the hen ovomucoid. The

Table 3. Amino acid compositions of each component from the quail ovomucoid (Values are grams of amino acid residues in 100 g protein)

Amino acid	Comp. I	Comp. II	Comp. III	Purified chicken ovomucoid
Lysine	7.60	5.56	5.85	6.23
Histidine	trace	5.30	5.03	2.11
Arginine	3.78	3.56	3.21	3.51
Aspartic A.	14.38	9.21	8.83	13.11
Threonine	6.59	3.22	3.11	5.27
Serine	3.84	7.34	7.37	3.89
Glutamic A.	9.36	14.56	15.05	6.87
Proline	3.25	3.07	2.71	2.67
Glycine	3.92	1.75	1.84	3.28
Alanine	1.68	3.32	3.58	2.97
Cystine/2	6.99	5.29	6.53	6.38
Valine	6.48	2.11	2.01	5.67
Methionine	1.55	3.63	3.57	0.89
Isoleucine	1.57	2.54	2.05	1.29
Leucine	3.86	5.02	5.14	4.93
Tyrosine	4.19	9.36	8.62	3.90
Phenylalanine	5.04	4.37	4.14	2.79
Tryptophan	0.80	3.10	2.84	0

Tryptophan value was calculated from the ultraviolet absorption determination. Values in the purified chicken ovomucoid were calculated from the data of Osuga and Feeney (1968).

Table 4. Carbohydrate compositions of each component from the quail ovomucoid (Values are grams of carbohydrate in 100 g protein)

	Total neutral sugar	Fucose	Mannose	Galactose	Glucosamine	NANA
Comp. I	5.66	0.99	4.01		2.41	1.32
Comp. II	3.84	1.50	2.31		1.37	0.16
Comp. III	4.24	1.60	2.88		1.66	1.23
Chicken ovomucoid	9.66*		4.7**	1.0**	14.6**	0.5**

Carbohydrates except total neutral sugar were analyzed by the gas-Liquid chromatography. The determination of neutral sugar was performed by the phenol-sulfuric acid and the content was expressed as mannose.

* Calculated from the data of Osuga and Feeney (1968).

** Analytical values of Chatterjee and Montgomery (1962).

NANA: N-acetylneuraminic acid.

mannose and glucosamine contents of component I were higher than those of the components II and III. N-acetylneuraminic acid content of component II was remarkably smaller than that of component III, although the total chemical compositions of the two were mutually similar. This fact may cause the incomplete separation of the two components on the ion exchange cellulose chromatography as in the foregoing description. In the textbook edited by Gottschalk¹⁴⁾, Lin *et al.* have described that the average ratio of mannose to galactose in hen ovomucoid is about 5 to 1. The presence of galactose in each component of the quail ovomucoid was not observed in this investigation. Authors, moreover, have found that the direct separation and purification of a trypsin inhibitor from the quail ovomucoid can be performed by the trypsin-Sepharose affinity chromatography. This is going to be published later in another paper. These experimental results support that only one antitryptic component exists in the quail ovomucoid.

Summary

1. Crude ovomucoid separated from the egg white of the Japanese quail by the fractionation with ammonium sulfate was separated into three protein components (Components I, II and III) by CM-cellulose chromatography following DEAE-cellulose chromatography.

2. Component I eluted from the CM-cellulose column at pH 4.5 was ascertained to be the trypsin inhibitory component, being contaminated with a small amount of non-inhibitory protein. The components II and III eluted from the CM-cellulose column at pH 5.1 and 4.8, respectively, were found to have the capacity of combining with riboflavin by the dialyzing method and the subsequent absorption spectrum estimation. However, only the component III existed in the native egg white as the apoprotein of flavoprotein, which might be distinctly separated.

The binding molar ratio of apoprotein to riboflavin was confirmed to be 1: 1.

3. Component I showed two bands closely migrated on the disc electrophoresis with polyacrylamide, though the components II and III respectively showed single narrow band, which proved to be of high purity.

4. The absence of N-terminal in both of flavoprotein and of apoprotein was ascertained. From the chromatographic behavior and dinitrophenylation of the flavoprotein, the reactive site of

the apoprotein (component III) combining with riboflavin was presumed to be lysine or histidine residue.

5. Molecular weights of the components I and III estimated by the gel-filtration on Sephadex were respectively 40000, and that of component II, 36500.

6. Component I contained smaller histidine, serine, glutamic acid, alanine, methionine, tyrosine and tryptophan amounts, than the components II and III did. Quite small amount of histidine, methionine and tryptophan in the former was noticeable. The amino acid composition of component I was similar to that of chicken ovomucoid purified by others. The compositions of the components II and III were mutually similar.

7. Carbohydrate of each component was found to be consisting of mannose as the major constituent, fucose, glucosamine and N-acetylneuraminic acid, being considerably different from that of hen ovomucoid previously reported. The mannose and glucosamine contents of component I were higher than those of the components II and III.

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