Studies on egg protein
VI The trypsin inhibitor and flavomucoid fraction of ovomucoid*

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Summary The ovomucoid was purified by the DEAE-C treatment after the ammonium sulfate fractionation of egg white. It is demonstrated by the CM-C column chromatography that the ovomucoid consisted of four protein components (I-IV). The antitryptic activity was present only in components I and II, and both components inhibit trypsin by forming the equimolar complexes. The antitryptic activity was inactivated completely by the heating at 100°C for 20 minutes, at 90°C for 30 minutes and 80°C for 170 minutes at pH 8.0. The components III and IV are the apoprotein of falvomucoid K_1 and K_2; both components bind flavin to form the equimolar complex. The optimum pH for FR-binding was 7.0 and the flavin-binding capacity was inactivated by heating for 55 minutes at 80°C and also 2 hours of heating at 70°C.

In the previous papers[1], the characteristics of the constituent proteins of ovomucoid which was fractionated by the carboxymethyl-cellulose (CM-C) column chromatography, were reported. The ovomucoid was purified by heat treatment after the ammonium sulfate fractionation of the egg white in those studies. The ovomucoid is not coagulated by heating, and is known as the relatively heat stable protein, but it seemed that the denaturation of the ovomucoid could not be avoided to some extent, since the inactivation of the trypsin inhibitor was observed during those purification procedures. Therefore, we have been looking for a milder method of preparing ovomucoid to obtain the biochemically active protein, and the following procedure proved to be satisfactory, namely, the ovomucoid was obtained by ammonium sulfate fractionation followed by the further purification with the diethyl-aminoethyl-cellulose (DEAE-C) column treatment under mild conditions. The fractionation of purified ovomucoid was carried out with CM-C column chromatography.

By the above procedure, the ovomucoid was fractionated into four components and each component was isolated and purified, and it was found that two of these components were shown to be the trypsin inhibitor and the other two were show to be the apoprotein of the flavomucoid K_1 and flavomucoid K_2, which had been reported in previous papers.[1]

In the present paper deals with the fractionation and isolation methods for four component proteins of ovomucoid and the results of investigations on the

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antitryptic activity and flavin-binding capacity of each component.

Experimental

Preparation and Fractionation of Ovomucoid

a) *The Ammonium Sulfate Fractionation*--The egg white (6 litres) was separated from domestic hen's (white leghorn sp.) egg (10 kg.), and an equal volume of saturated ammonium sulfate solution was added to it. The precipitate was discarded after filtration. The filtrate was adjusted to pH 4.6, by N-sulfuric acid and was left to stand overnight, then the ovalbumin was crystallized. The crystallines were filtered off, and the filtrate was adjusted to pH 7.0 with N-ammonium hydroxide solution, and solid ammonium sulfate was added to 0.63 saturation. After filtration, the filtrate was saturated with ammonium sulfate to precipitate crude ovomucoid. The precipitates were dissolved in a minimum volume of water and dialyzed against running tap water for 24 hours, and the dialyzate was lyophilized. The yield of crude ovomucoid was 35 g.

b) *DEAE-C column treatment*--The fibrous DEAE-C (F-DEAE-C) was prepared from rayon pulp by the method of Peterson and Sober[2] with several modifications. The F-DEAE-C (0.8 meq. N./g., 10 g.) was suspended in a 0.05 M sodium acetate buffer, pH 4.4, and was homogenized slightly. The suspension was packed to the column (2.5×35 cm.) and equilibrated to the starting buffer. Crude ovomucoid (5 g.) in a 0.05 M soium acetate buffer, pH 4.4 was applied to the column. The column was washed by the same buffer to remove the contaminating proteins which were present in the crude ovomucoid preparation. When no trace of protein was detected in the washings, the elution of ovomucoid was initiated with the introduction of a 0.05 M sodium acetate buffer, pH 3.6, containing 0.5 M sodium chloride to the column. These procedures were repeated, and protein containing fractions of the eluate were dialyzed and lyophilized. The yield of purified ovomucoid was 10 g.

c) *CM-C Column Chromatography*--The purified ovomucoid was further fractionated by fibrous CM-C (F-CM-C) which was prepared from the rayon pulp. The F-CM-C (D. S.=0.170, 5 g.) was suspended in a 0.05 M sodium acetate buffer, pH 4.0, and homogenized for about one minute, and was then packed to the column (2×20 cm.) and equilibrated with the same buffer. Lyophilized, purified ovomucoid (1 g.) was dissolved in 20 ml. of starting buffer, and applied to the column. The column was washed by the same buffer, and washing was continued until no fluorescence of flavin which dissociated from flavomucoid, was detected in the effluent. The fractionation of ovomucoid was carried out by stepwise elution from the CMC-column with a sodium acetate buffer of 0.05 ionic strength. Four kinds of buffer were used in 600 ml. portion, having the pH 4.4, 4.6, 4.8 and 5.0. The effluent solution was collected in 6 ml. fractions by the fraction collector.

Each fraction was pooled and dialyzed in the Visking tubing against water and after dialysis, the tubes were then immersed in a concentrated solution of Carbowax 6000 in a cold place to concentrate the protein solution. The protein

solution was then lyophilized.

Method of Assay for Antitryptic Activity—The spot plate estimation method of Rhodes et al.[3] was modified. As the substrate of trypsin, *p*-toluene sulfonyl L-arginine methyl ester (*p*-TAME) was used, and it was synthesized essentially based on the method of Bergmann et al.[4]. Trypsin (E. Merck & Co. containing about 50% magnesium sulfate) was dissolved in a 0.004 M acetic acid which contained 0.02 M calcium chloride. The nitrogen content of the trypsin solution was determined by the indophenol method of Lubochinsky et al.[5]. The procedure for the assay of antitryptic activity was as follows. A 0.05 ml. portion of trypsin solution containing 6.25 μg. of trypsin, and 0.1 ml. of ovomucoid solutions containing 0—15 μg. of each component of the ovomucoid, were placed in the test tube (1×5 cm.) and pre-incubated at 37°C for 2 minutes. Then a 0.2 ml. of substrate-buffer-indicator solution [0.02 M *p*-TAME, 0.015 M tris (hydroxymethyl)-aminomethane at pH 8.2, and 0.002% phenol red, respectively] which had been equilibrated to the same temperature, was added to the above solution and shaken at 37°C. The time required for the red color of the indicator to change to the standard yellow was measured. The color change was brought about by the release of free carboxylic residues from the substrate by the esterase action of trypsin. The retardation of the substrate hydrolysing rate from the control experiment was determined.

The Method of Determining the Thermal Resistance of Antitryptic Activity—The thermal resistance of antitryptic activity was tested with the component II. The component II was dissolved in a 0.006 M tris buffer, pH 8.0 and the protein concentration was prepared to the consistency of 125 μg. per ml. This solution was heated in test tubes capped by glass beads at 80°C, 90°C and 100°C. Aliquots were taken out at suitable time intervals, and after cooling, 0.02 ml. of each solution was added to 0.05 ml. of trypsin solution (8.8 μg. of trypsin). It was then pre-incubated for 2 minutes at 37°C and then 0.2 ml. of a mixture of substrate-buffer-indicator was added. As the control experiment, the native component II solution (2.5 μg. in 0.02 ml. of buffer) was added to the enzyme solution. The substrate hydrolysing rate of trypsin in the control experiment was retarded at the rate of 89 seconds to 362 seconds.

Determination of Flavin-binding Capacity

a) *Fluorescence Quenching Titration Method*—Under illumination of the ultra violet lamp, 1 ml. of the standard riboflavin (FR) solution (FR dissolved in phosphate buffer, $\Gamma/2=0.05$, pH 7.0) was titrated by a solution of each ovomucoid component. The titration was carried out until the quenching of the fluorescence of FR was attained. The Flavin concentration was calculated from the molecular extinction coefficient at 450 mμ., $\varepsilon=12.2\times10^3$ mole^{-1} cm^{-1}.[6] The protein concentration of the ovomucoid solution was estimated from the extinction at 280 mμ., using $E_{cm}^{mg/ml}=0.45$.[7]

b) *Dialysis Method*—An excess of the FR solution was added to the protein solution which was dissolved in various buffers, and dialyzed against various buffers

for two days. In the FR was dissolved in various buffers and dialyzed against each buffer at the same condition. The extinction at 280 mμ. for the protein concentration and at 455 mμ.* for bound FR concentration was measured.

The Method of Determining the Thermal Resistance of Flavinbinding Capacity— The proteins of component III and IV were dissolved in phosphate buffer ($I'/2=0.05$, pH 7.0) and the solution was heated at 70°C, 80°C, 90°C and 100°C. One ml. of the standard FR solution was titrated by protein solutions which were taken up at various time intervals in heat treatment. Thus the thermal stability of the flavin binding capacity was investigated.

Results

The Fractionation of Ovomucoid— The components which were eluted from the CMC-column with each buffer, pH 4.4, 4.6, 4.8 and 5.0 were named components I, II, III and IV respectively. The typical elution pattern is shown in Fig. 1. The yields for components I, II, III and IV from one gram of purified ovomucoid were 120 mg., 400 mg., 300 mg. and 100 mg. respectively and the recovery of total protein was 93 per cent of the original ovomucoid which had been applied to the CM-C column.

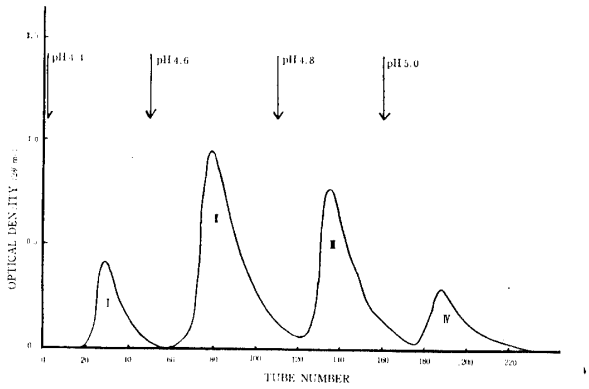

Fig. 1. F-CMC Column Chromatogram of Ovomucoid.

Purified ovomucoid (1 g) was applied to the column (2×20 cm) of F-CMC, elution was carried out stepwise increase of the pH of 0.05 M sodium acetate buffer.

Antitryptic Activity of each Component of Ovomucoid—Fig. 2. show the results of the determination of antitryptic activity with each component of ovomucoid.

As shown in Fig. 2. components I and II had identical activity. If the complete inhibition of the trypsin (6.25 μg.) in these experiment were defined as that it takes more than 500 seconds to hydrolyse 4 μ moles of substrate, the amount of compo-

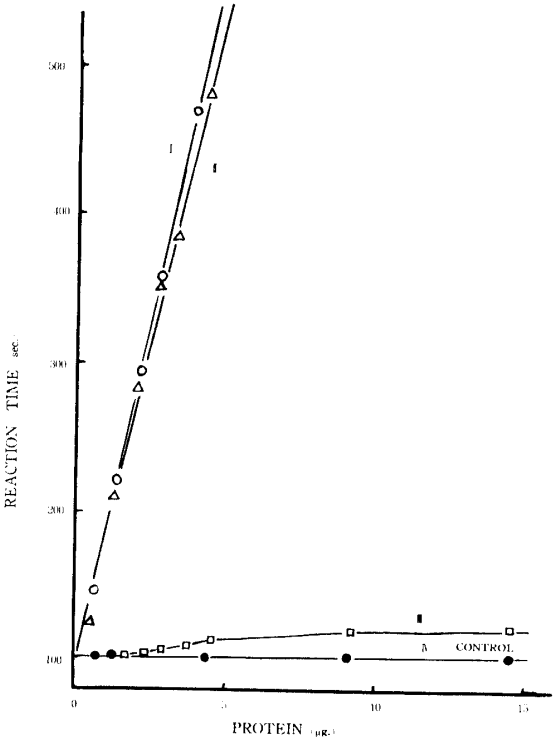

Fig. 2. Antitryptic Activity of each Component of Ovomucoid.

The reaction mixture contained 6.25 μg. of trypsin, 4μ moles of *p*-TAME as the substrate, 0.03 mmoles tris buffer pH 8.2, 4 μg. of phenol red as an indicator and 0-15 μg. of ovomucoid components in a total volume of 0.3 ml.

* The absorption maximum at 450 mμ. of FR shifted to 455 mμ., when it was combined with protein.

nents I and II, needed to complete inhibition were 5 μg. in each case. The weight ratio of trypsin to ovomucoid was one to 0.8. The components III and IV had no antitryptic activity.

Thermal Resistance of Antitryptic Activity—The results of the experiments with heat treated component II (2.5 μg. in 0.02 ml.) are listed in Table 1.

Table 1: Thermal Resistance of Antitryptic Activity

Duration of heating (min.)	Substrate hydrolysing rate (sec.)		
	100°C	90°C	80°C
0	362	362	362
1	323		
2.5	287		
5	262	360	
10	181	352	
15	105	306	
20	90		357
22		141	
25		95	
30		89	
35			330
55			325
75			274
85			254
95			247
110			219
130			179
140			131
150			121
165			99
170			90

The protein of component II, 125 μg. per ml. in 0.006 M tris buffer pH 8.2 was heated at 80°C, 90°C and 100°C, the aliquots were taken out at suitable time intervals and 0.02 ml of heated protein solution (2.5 μg) was added to the trypsin (8.8 μg) solution, and preincubated for 2 minutes at 37°C, and then, a substrate-buffer-indicator mixture solution (0.9 ml) was added.

Assuming that the hydrolysis time of 362 seconds to be 100% antitryptic activity, the activity percentage of heated inhibitor is plotted against the heating time in Fig. 3. As shown in Fig. 3., the inhibitory activity was reduced to about 50 per cent by the heat-treatment at 100°C for only 7 minutes, and was inactivated completely by heating for 18 minutes. At 90°C, the activity was reduced to one half by the heating for 18 minutes, and inactivated by the heating for 30 minutes, but the treatment at 80°C, the antitryptic activity was compara-

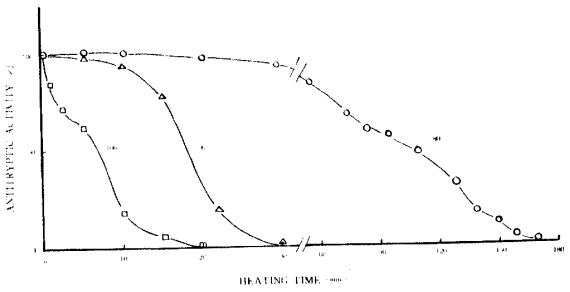

Fig. 3. Antitryptic Activity of heated Ovomucoid.

The test system was the same as table I, the hydrolysis time of 273 seconds (362-99 seconds) is defined as the full activity of the inhibitor.

tively stable, and 105 minutes of heating was needed for the reduction of activity to 50 percent, and for the complete inactivation, continuing the heat-treatment for 170 minutes was necessary.

The Flavin binding Capacity of each Ovomucoid Component and of the Flavomucoid K_1 and K_2—The results of determining the flavin binding capacity by both methods are shown in Table II and Table III.

Table II: Flavin-binding Capacity of each Ovomucoid Components by the Titration Method.

Ovomucoid	Protein conc. mg./ml.	Titration ml.	Protein moles	Flavin moles	Molar ratio FR/protein
I	0.11	>10	3.92×10^{-3}	2.45×10^{-3}	<0.063
II	0.083	>10	2.96 〃	〃	<0.083
III	0.127	0.52	2.35×10^{-3}	〃	1.040
IV	0.067	0.96	2.46 〃	〃	1.000

Under the radiation of UV ray. 1 ml of the standard riboflavin solution, in a phosphate buffer $I'/2=0.05$, pH 7.0, was titrated with the protein solution, in the same buffer, the flavin concentration was estimated from the extinction at 450 mμ, and the molarity of the protein was calculated from the extinction at 280 mμ. and using the value of 28,000 as the molecular weight of ovomucoid.

Table III: Flavin-binding Capacity of each Ovomucoid Component by Dialysis Method.

Ovomucoid	Protein conc. mg./ml.	Protein molarity moles/ml.	Flavin conc. OD 455 mμ	Bound FR moles	Molar ratio FR/protein
I	0.110	3.92×10^{-3}	0.004	3.27×10^{-10}	0.08
II	0.083	2.96 〃	0.005	4.10 〃	0.14
III	0.127	4.53 〃	0.054	4.42×10^{-9}	0.98
IV	0.067	2.39 〃	0.031	2.54 〃	1.06

An excess of riboflavin solution, in a phosphate buffer $I'/2=0.05$, pH 7.0, was added to the protein solution, and dialyzed in the Visking tubing against the same buffer for two days with several changes. The bound flavin was estimated with the dialyzate from the optical density at 455 mμ; the protein molarity was calculated from optical density; and the value of 28,000 for the molecular weight of ovomucoid was used.

The conclusion drawn from these experiments was that the proteins of components III and IV were apoproteins of flavomucoid K_1 and flavomucoid K_2 respectively, and shown the flavin-binding capacity to form the equimolar complexes, and proteins of components I and II had no flavin-binding capacity.

Effect of Buffer, and pH on the Flavin-binding Capacity and its Thremal Lability—To investigate the effects of different buffers besides of phosphate, on the flavin-binding capacity of components III and IV, the following buffers of ($I'/2=0.05$, pH 7.0) borate, barbital and tris were prepared. The protein of components III and IV were dissolved in each buffer and the excess of FR solution was added. The mixture was dialyzed against the same buffer, the bound FR concentration was determined from the extinction at 455 mμ. as described above. As a result, each buffer did not have any effect on the flavin-binding capacity and these proteins formed the equimolar complexes with FR in any medium.

The effect of pH on the flavin-binding capacity was investigated in eight buffers which had the same ionic strength of 0.05: acetate buffers (pH 3.6, 4.0 and 5.0),

phosphate buffers (pH 6.0, 7.0 and 8.0), carbonate buffers (pH 9.0 and 10.0). Proteins of components III and IV were dissolved in above each buffers and to those solution was added the excess of FR dissolved in the same buffer and the mixture was dialyzed against each buffer for two days. The bound flavin was calulated from the extinction at 455 mμ. The results which were obtained with component III are shown in Fig. 4.

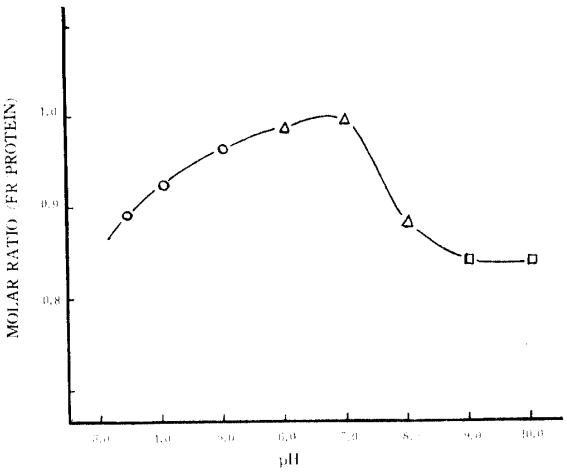

Fig. 4. Effect of pH on the Flavin-binding Capacity.

○—○ acetate; △—△ phosphate; □—□ carbonate buffer. The protein of component III was dissolved in each buffer, and the excess of riboflavin solution was added. The concentration of bound flavin was estimated by the dialysis method.

From the results of this experiment, it was found that the flavin-binding capacity showed at its maximum at pH 7.0 and was stable within the pH ranges of 5.0 to 7.0. The results of the experiment on the thermal stability of the flavin-binding activity are shown in Fig. 5.

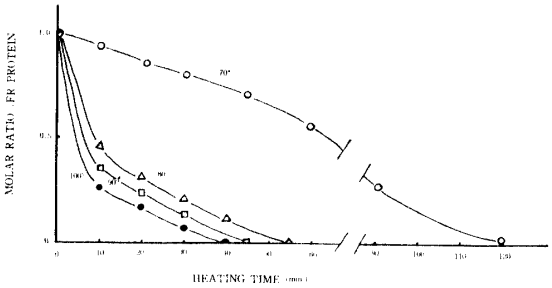

Fig. 5. Thermal Stability of the Flavin-binding Capacity of Ovomucoid.

The protein of component III was dissolved in a phosphate buffer, $I/2=0.115$, pH 7.0 (0.1 mg/ml. of buffer), and was heated at 70°C, 80°C, 90°C and 100°C. The heated solution was taken up at suitable time intervals and titrated into 1 ml. of standard riboflavin solution.

By heat treatment above 80°C, the flavin-binding capacity is inactivated remarkably even in a short time of heating, but by the heating at 70°C for 30 minutes, the activity remained over 80 per cent, and two hours of heating was necessary to inactivate this capacity.

Discussion

The preparative method to obtain a native ovomucoid which was proposed by Lineweaver et al.[8] is questionable, because it involved the treatment of protein at low pH, and in such a condition, the flavin moiety of ovomucoid would be dissociated. The method which was the modification of Warner et al.[9] in the previous papers, also involved the heat-treatment of the sample. The partial denaturation of protein was supposed from the results of the studies on the thermal stability of antitryptic activity and the differences in the pattern of CM-C column chromatography on heat treated ovomucoid and that of the present study. Heat treated ovomucoid was fractionated with CM-C chromatography into five components (I—V), and components I and II in those studies corresponded to component I of the present study. It is suggested that this component is probably resolved into two components by the heat denaturation of the protein. A milder method for preparation of ovomucoid, has been

sought to avoid these disadvantages, and the following procedure has proved to be satisfactory, that is, after ammonium sufate fractionation of egg white, ovomucoid was further purified by the DEAE-C column treatment instead of the heating procedure, and the ovomucoid was fractionated by CM-C column chromatography into four components (I—IV), and each component was available in a highly purified form. The relation between the trypsin inhibitor of egg white and the ovomucoid had been reported by Ball et al.[10] Meyer et al.[11] and Linweaver et al.[8] and on electrophoretic studies on the ovomucoid have also been reported by many workers[12,13,14] Notably, in the report by Bier et al.[15] the ovomucoid was fractionated into five components by means of electrophoresis convection and it showed each component to have identical activity of the trypsin inhibitor. Recently, Rhodes et al.[3] also fractionated the ovomucoid into three components with the CM-C column chromatography. Feeney et al.[16] reported that only one component of ovomucoid, which was fractionated by CM-C column chromatography after treatment with the trichloroacetic acid-acetone system, had antitryptic activity. In the present study, it was found that the ovomucoid consisted of four protein components and only two of these components (I and II) have an antitryptic activity, and these components (I and II) show absolutely identical activity and simillar thermal stability. The weight ratio of trypsin to ovomucoid is 1 to 0.8, and from this results it is assumed that the inhibition occured when ovomucoid and trypsin were combined to form the equimolar complex. Supposing that the molecular weight of ovomucoid is 28,000, the molecular weight of trypsin is calculated as 35,000 this value coincides with the molecular weight of trypsin reported by Bull[17] from the data of the spread monolayer experiment. The flavoprotein in egg white was first reported by Bain et al.[18] and they considered that the flavin in egg white is combined with conalbumin. Forsythe et al.[19] and Mahlar et al.[20] also supported the above concept. In 1956 it was found, by chance in the studies on conalbumin in this laboratory[1] that the riboflavin in egg white is found in combination specifically with ovomucoid but not with conalbumin, and this flavoprotein is present as a natural constituent in the egg white. The ovomucoid was further purified and fractionated with CM-C column chromatography into the four components I, II, III and IV in the present study, and it was demonstrated that the protein of components III and IV have the flavin binding capacity to form the equimolar complex. The flavoproteins which were reconstituted by the combination of FR and components III and IV, were named as falvomucoid K_1 and flavomucoid K_2 respectively. Rhodes et al.[2] also reported the presence of the new flavoprotein-apoprotein system in egg white. They obtained two components of apoprotein which contained phosphorous, and suggested that these components were different from ovomucoid. But the results of the previous papers and the present study demonstrated that the components III and IV of ovomucoid are the apo-protein of flavomucoid K_1 and K_2 and no phosphorous was detected in either protein component (III and IV) when analyzed by the microdetermination method of Chen et al.[22] The phosphorous reported to be present in apoprotein of Rhodes et al.[21] is supposed to be derived from the ovalbumin which is contained from the conatamination in the ovomucoid preparations.

要 約 卵白から硫安分別，DEAE-C 処理によってオボムコイドを純化し，CM-C カラムクロマトグラフィーによりオボムコイドは4成分たんぱく（I～IV）から組成されていることを明らかにした．

オボムコイド4成分たんぱくのうち，I，IIにのみ抗トリプシン活性が認められ，ともにトリプシンと等モル作用して阻害することがわかり，またその活性はpH 8.0 において 100°，20分；90°，30分；80°，170分の加熱処理で完全に失活した．

III および IV 成分たんぱくは，フラボムコイド K_1，K_2 のアポたんぱくであり，いずれも FR と等モルで結合し，その結合割合は pH 7.0 付近が最も高く，また結合能は 80° 以上の加熱では55分以内で，70° 加熱では2時間でなくなった．

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