Fractionation of Pigeon Egg White Proteins and Chemical Compositions of Ovalbumin and Ovomucoid

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Introduction

The studies on the constitutional proteins of the avian egg whites are significant from the standpoint of the comparative biochemistry and phylogenesis. In earlier days, Bain and Deutsch[1] performed electrophoretic comparative studies on the egg whites of different birds belonging to various orders of Galliformes, Anseriformes and Columbiformes, and found the difference among the electrophoretic patterns of various egg whites. Feeney and co-workers carried out comparative biochemical investigations on the constitutional proteins of the egg white from different avian species. After the analyses of the egg whites of 25 different species or varieties, they found large differences in several constituents among the different egg whites.[2]

As to the pigeon egg white, only relatively small amount of lysozyme and large amount of sialic acid could be appreciated in their reports. Rhodes *et al.*[3] isolated the ovomucoids from egg whites of eleven different avian species and divided them into four classes on the basis of their inhibitory activities against proteinases. Feeney *et al.*[4] asserted that the heterogeneity of ovomucoid appeared to be a general phenomenon in avian egg whites.

Clark *et al.*[5] compared the properties of chicken conalbumin with those of the conalbumins from a variety of avian egg whites. Moreover, a number of investigations on the chemical composition of several avian ovalbumins and ovomucoids were introduced and discussed in the book: "Glycoproteins" edited by Gottschalk, recently.[6] The investigations on the pigeon egg white proteins have not been shown in the above-mentioned papers and others. Differing from chickens and quails, the domestic pigeon belongs to the Columbiformes and has a characteristic laying-period: a pigeon lays an egg per day and continues the phenomenon for two days, thereafter ceases the laying for about ten days and the laying is then repeated in the same manner. Such a periodic laying continues for a long time. Visible coagulating point of the pigeon egg white on heating is higher than those of the chicken and the quail egg whites, and the coagulate shows an appearance of semi-transparent jelly with opalescent.

Therefore, some of the constituent proteins in pigeon egg white are supposed to be differing from the proteins in chicken egg white. The present paper describes the separation of constitutional proteins in pigeon egg white by carboxymethyl cellulose chromatography and by gradient extraction with salt, and chemical compositions of ovalbumin and ovomucoid isolated.

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Materials and methods

Materials———The eggs of pigeon (*Columba livia domestica*) were secured from the poultry farm of the Faculty of Agriculture, Kagoshima University, within 20 hours after being laid. A pair of male and female pigeon have been bred in each one cage. The weight of each egg was measured, and the egg white was separated as completely as possible from the yolk with the aid of injector, and the weights of white, yolk and shell were measured, respectively.

The white was blended with a homogenizer at a slow speed and employed as the experimental sample. While it was unused, it was stored in the freezer at $-20°C$.

Measurement of the pH of egg white———As the difference between *pH* values of two eggs laid on the first day and the next day was observed, *pH* values of both egg whites from the appointed pigeon had been separately measured during five months, from April to August.

Variation of the condition of pigeon and chicken egg whites with heating———Five or six *ml* of the homogenized egg white was poured into the pyrex tube (1.7 × 12 *cm*) with reflex, maintained in water bath, and the visible variation of the condition on heating was observed. The temperature raise was measured by the thermometer put in another tube in the same bath.

Separation of egg white proteins by CM-*cellulose column chromatography*———The separation of the constituent proteins of the pigeon egg white on CM-cellulose column was carried out by the method as described in the previous report,[7] adopting the elution by the *pH* linear gradient.

Separation of egg white proteins by gradient extraction with ammonium sulfate——— The full saturation was achieved by adding the excess ammonium sulfate to 4 *ml* of the diluted egg white solution (*C*, 4%), and 1*g* of celite powder was added on to it. The whole precipitates formed and the supernatant were poured slowly on the celite layer of the extracting apparatus as shown in the previous report.[7] The extracting procedure of precipitated proteins and the measurement of the salt concentrations in extracting solution were also performed as described previously.[7] The effluent was collected in 5 *ml* fractions with the drop-count type fraction-collector from Toyo Kagaku Co. Ltd.

Neutral sugar content in each fraction was determined by the phenol-sulfuric acid method proposed by Dubois *et al.*[8]

Isolation of ovalbumin and ovomucoid———An equal volume of saturated ammonium sulfate solution was added to the homogenized egg white, whilst stirring gently. The globulin precipitate formed was removed by centrifugation and the supernatant was employed for separating ovalbumin and ovomucoid.

Referring to the result of the gradient extraction, the separation of two kinds of proteins was done as follows. The *pH* of the supernatant was adjusted to 4.7 and followed by the slow addition of ammonium sulfate to show the concentration of 37% (*w/v*) *i.e.* 0.7 saturation, and the ovalbumin precipitate formed was obtained by centrifugation. Ammonium sulfate was subsequently added to the supernatant to have the concentration of 48%, *i.e.* 0.9 saturation and the ovomucoid precipitate produced was obtained by centrifugation.

These crude proteins were separately dissolved in water and reprecipitated under the above-mentioned condition. The precipitates were taken up in water and led to further refining. This ovalbumin aqueous solution was dialyzed against 0.025*M* sodium acetate

buffer, *pH* 4.0, and the concentration adjusted to 1%. Twenty *ml.* of ovalbumin solution was adsorbed on to a CM-cellulose column of 1.8 × 20 *cm* packed in 0.025*M* acetate buffer, *pH* 4.0. Elution was carried out by the linear increasing of the *pH* and the effluent was collected in 5 *ml.* These procedures will ensure that the product does not contain conalbumin and other proteins. The fractions corresponding to the ovalbumin, which had been well defined, were combined and dialyzed against 20% polyethylene glycol for concentrating.

After being dialyzed against the water and subsequently 0.1*M* acetate buffer, *pH* 4.50, the solution (0.64%, 10 *ml*) was applied on Sephadex G-100 column, 2.1 × 52*cm*. Eluting with the same buffer, the effluent was collected in 5 *ml* fractions. The protein and neutral sugar content in each fraction were determined, respectively. Ovalbumin fractions were put together and dialyzed against water and were lyophilized. Pigeon ovalbumin was thus isolated. CM-cellulose column chromatography of ovomucoid obtained by the salting out and the subsequent refining by gel-filtration were performed by the same procedure as those of ovalbumin.

Drawing of calibration curves of ovalbumin and ovomucoid———The absorbances of the purified ovalbumin and ovomucoid aqueous solutions with different concentrations were separately measured at 280 *nm*, and then their calibration curves were drawn, respectively.

Identification of hexose in ovalbumin and ovomucoid———Each hydrolysate of ovalbumin and ovomucoid treated with 1*N* H_2SO_4 in a sealed tube at 100°*C* for 7 *hr.*, were diluted to an appropriate volume, and the solution was passed through the Amberlite IR-120 column (2.2 × 49 *cm*), followed by the Amberlite IR-4B column (2.2 × 29 *cm*) and concentrated under the reduced pressure.

The solution was employed for the ascending paper chromatography, using buthanol-acetic acid-water (4:1:2) as solvent. Solvent was repeatedly run on a given paper thrice in order to separate hexoses, sufficiently. Saturated $AgNO_3$-aceton (1:100, *v/v*) and 0.5*N* NaOH in ethanol were used for developing the colour. The hexose amount was determined by the phenol-sulfuric acid method.

Identification and determination of hexosamine in ovalbumin and ovomucoid———The identification and determination of hexosamine in the pigeon ovalbumin and ovomucoid were separately carried out according to the Pearson's procedure[9] followed by the Elson-Morgan method modified by Boas.[10] Twenty *ml* of 4*N* HCl was added to 250 *mg* of the freeze-dried ovalbumin and to 200 *mg* of the freeze-dried ovomucoid. The mixtures were hydrolyzed in a sealed tube at 100°*C* for six hours, respectively. The filtrate of the hydrolysate was concentrated to a small volume under the reduced pressure, and then evaporated in a vacuum desiccator over sodium hydroxide particles, being kept for five days. The dried hydrolysate was dissolved and diluted to be 50 *ml* with 0.3*N* HCl. One *ml* of the solution was applied on a column (0.6 × 40 *cm*) of Amberlite CG-120 for chromatography. Using 0.3*N* HCl as the eluting agent, the effluent was collected in 1 *ml*. Subsequent procedures were conducted as described in the previous report.[11] The glucosamine amount was calculated from the equation of the calibration curve: $y = 15.1 \times 10^{-3}x$, where y is optical density at 530 *nm* and x, *r/ml*. Moreover, the determination was also performed with the convenient method devised by Koga and Fukunaga,[12] using a short column in the amino acid analysis. A known amount of authentic glucosamine and galactosamine were independently applied on to a column and eluted.

Amino acid analysis of ovalbumin and ovomucoid———Five *ml* of 12*N* HCl (special grade) was added to 5 *ml* of 1% ovalbumin aqueous solution in the pyrex tube. After

removal of air by the suction followed by the introduction of nitrogen gas, the tube was sealed. Hydrolysis was conducted for 24 and 44 hours at 110°*C*, respectively. The filtrate of the hydrolysate was concentrated to be a small volume and diluted to 100 *ml* with citrate buffer (0.2*N* as Na ion, *pH* 2.2). An aliquot of the solution was employed for the analysis, using Yanagimoto LC-5S type amino acid analyzer. Tryptophan was determined by the ultraviolet absorption method.[13]

Results and discussion

General properties of egg white———Some general properties of the pigeon egg white compared with those of the chicken's are as presented in Table 1. Comparing with the chicken white, it was ascertained that the white and yolk index, the white proportion and the electric resistance of white in the pigeon egg are larger, while the nitrogen content, smaller.

Fig. 1 shows the *pH* of the whites of many eggs from an appointed female pigeon. The *pH* of the white of an egg laid on the first day was higher than that of an egg laid on the

Table 1. General properties of the pigeon egg white compared with those of the chicken egg white

	Pigeon	Chicken (White Leghorn)
Weight of entire egg (*g*)	18.3 ~ 19.2	52.4 ~ 67.2
White/Yolk (*w*/*w*)	3.0 ~ 4.0	1.9 ~ 2.3
	Av. 3.3	Av. 2.1
White/Egg × 100 (%)	63.2 ~ 69.0	58.4 ~ 62.5
pH of egg white, 1st egg	8.35 ~ 8.60	7.80 ~ 8.30
2nd egg	7.80 ~ 8.10	
Nitrogen in dry matter (%)	12.7	14.0
Electric resistance at 15° *C*	178 *ohm*	158 *ohm*
Specific conductance at 15° *C*	5.62×10^{-3} *mho*	6.37×10^{-3} *mho*
Sp. conductance in C = 4.9%, at 15° *C*	3.38×10^{-3} *mho*	3.25×10^{-3} *mho*

Av.: Average value

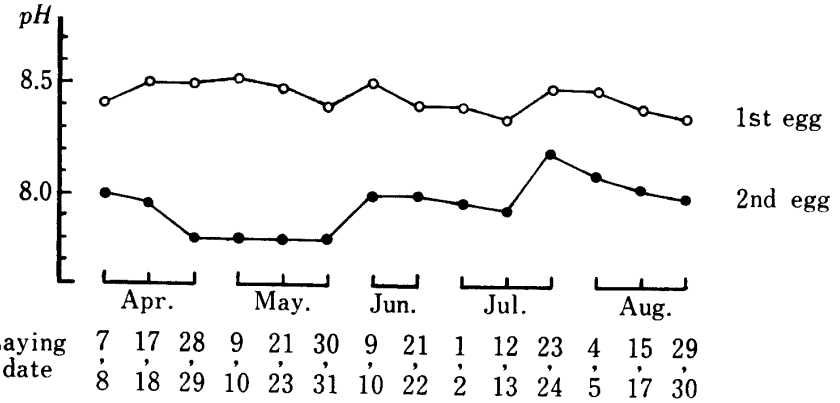

Fig. 1. pH values of the egg whites of eggs laid from an appointed female pigeon, on the first day and the next day

Table 2. Visible variation of egg white homogenate with heating

Variation	Unvariable	Slightly turbid point	Full turbid point	Coagulating point
Pigeon	50° C	60° C	63° C	66° C
Chicken	50° C	56° C	59° C	62° C

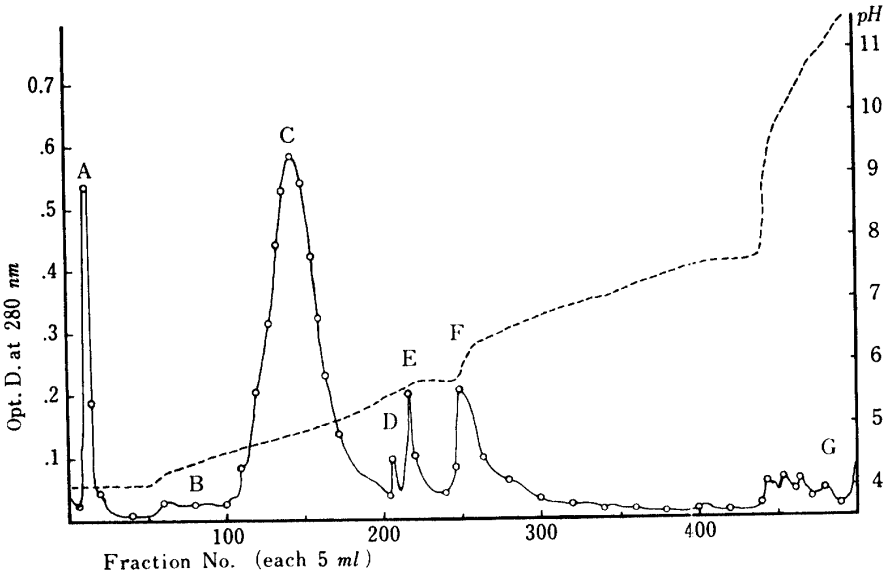

Fig. 2. Separation of the pigeon's egg white proteins by CM-cellulose column chromatography (Egg on the first day)
—○—○— Protein, - - - - - Eluting pH

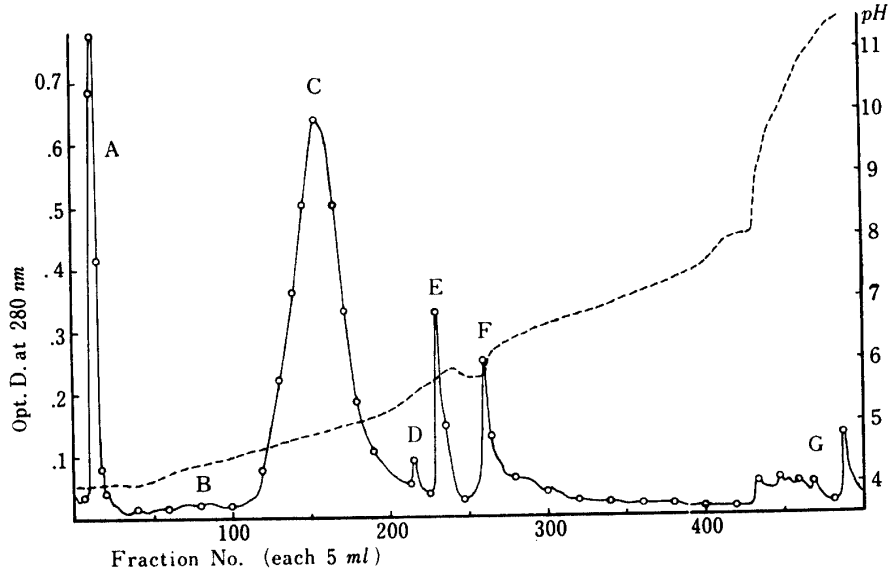

Fig. 3. Separation of the pigeon's egg white proteins by CM-cellulose column chromatography (Egg on the second day)
—○—○— Protein, - - - - - Eluting pH

Table 3. Relative protein amount and *pH* values for elution of each component of pigeon egg white separated by CM-cellulose column chromatography

Component		A	B	C	D	E	F	G	Others	Total
Pigeon egg (1st)	Protein (%)	7.0	2.8	56.7		5.5	13.0	4.6	10.4	100
	pH of eluate at peak	4.0	4.4	4.87	5.50	5.66	5.80	11.0		
Pigeon egg (2nd)	Protein (%)	8.8	2.7	59.0		7.1	9.8	3.6	9.0	100
	pH of eluate at peak	4.0	4.4	4.85	5.57	5.70	5.90	10.85		
Chicken	Protein (%)	6.6	2.8	43.9		8.7	14.1	14.3	9.6	100
	pH of eluate at peak	4.0	4.5	4.93	5.32	5.68	5.95	11.02		

A: Protein being anionic at *pH* 4.0, B: Ovomucoid fraction, C: Ovalubmin fraction (mixed with ovomucoid) D,E: Globulin fraction, F: Conalbumin fraction G: Lysozyme fraction

next day in all of the eggs laid periodically. This is an interesting phenomenon physiologically, since it may be related to the secretion of egg white in oviduct. Table 2 showed the visible variations of the appearance of pigeon egg white homogenate and the chicken's on heating. Pigeon white became slightly turbid at 60°*C*, its turbid temperature being higher than that of chicken white, and became then full turbid at 63°*C*. Coagulating temperature of the former was observed to be 66°*C*, being higher by 4°*C* than that of the latter. This suggests either the relatively much presence of uncoagulative ovomucoid or the presence of some glycoprotein having a higher coagulating point in pigeon egg white.

Separation of egg white proteins by CM-*cellulose column chromatography*———The elution diagrams of the white protein of each pigeon egg laid on the first day and on the next day with CM-cellulose column were shown in Fig. 2 and 3, respectively. Relative proportion of each component and the *pH* of the eluate at each peak were represented in Table 3, accompanied with the data from the chicken egg white.[7]

Both of the first and the second separation patterns were quite similar each other. The elution diagram reveals seven components. These were marked A, B, C, D, E, F, G in the order of their elution. Referring to the order of elution and the *p*H values of the eluate at peak described in the literature,[7,14] the resolved components were inferred as follows. Component A is the protein being anionic at *pH* 4.0; B, ovomucoid; C, ovalbumin (mixed with ovomucoid, judging from the experiment described later); D, E, globulin; F, conalbumin and G, lysozyme. Chromatographic separation of the component B corresponding to ovomucoid was not so well defined as the separation of ovomucoids of the chicken, quail and duck was in the previous report.[7,11]

Comparing with the eluate *pH* at peak of the chicken ovalbumin, the value of the pigeon ovalbumin fraction was lower, and it was also appreciated on the purified ovalbumin, later. This suggests that the isoelectric point of pigeon ovalbumin is lower than that of the chicken ovalbumin. The proportion of lysozyme is not sure, because the lyzosyme pattern is not so sharp as those of the chicken and quail. In spite of the experiments repeated several times, a defined lysozyme peak could not be obtained.

From these results, it was presumed that the lysozyme amount in pigeon white was very small.

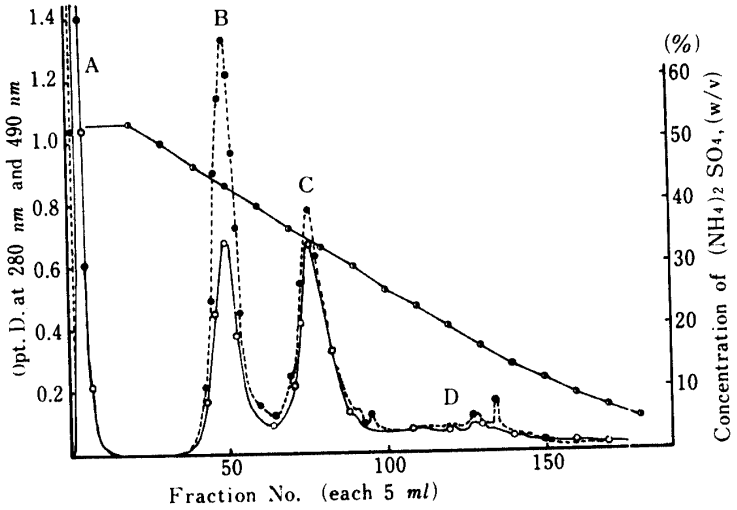

Fig. 4. Fractionation of the pigeon's egg white proteins by the gradient extraction with salt —○—○— Protein, ·····●·····●····· Neutral sugar, —◑—◑— Concentration of amm. sulfate

Table 4. Relative amount and sugar content of each component of the pigeon egg white proteins separated by gradient extraction with ammonium sulfate

Component		A	B	C	D	E	F	Others	Total
Pigeon	Concn. of salt (*w/v*, %)	53.0	47.2 ≀ 37.8	37.7 ≀ 27.0	26.6 ≀ 3.5				
	Protein proportion (%)	0 (23.7)	33.9 (25.9)	39.0 (29.8)	25.2 (19.2)	— —	— —	1.8 (1.4)	100 (100)
	S-P raito ($.10^{-2}$)	6.8	10.1	5.5	2.4	—	—	0	6.5
Chicken	Concn. of salt (*w/v*, %)	53.0	40.5 ≀ 34.0	33.8 ≀ 23.4	23.0 ≀ 19.5	19.0 ≀ 12.0	12.0 ≀ 3.5		
	Protein proportion (%)	0 (3.4)	3.4 (3.3)	86.0 (83.1)	3.1 (3.0)	4.8 (4.6)	1.8 (1.7)	1.0 (0.9)	100 (100)
	S-P ratio ($.10^{-2}$)	93	8.1	3.4	2.1	1.3	0	0	6.4

A: Mixture of free amino acid and sugar, B: Ovomucoid fraction,
C: Ovalbumin and conalbumin fraction, D, E, F: Globulin fraction
Values in parentheses were calculated, supposing that component A is a protein.

Separation of egg white proteins by gradient extraction with ammonium sulfate——As shown in Fig. 4, the fractionation of the pigeon egg white proteins by the gradient extraction method gave four components. The proportion of each component and the sugar content were represented in Table 4 together with those in chicken white. The component not precipitated with full saturation of ammonium sulfate was marked A, and each component extracted was marked B, C and E in turn. In general, as the classification of protein

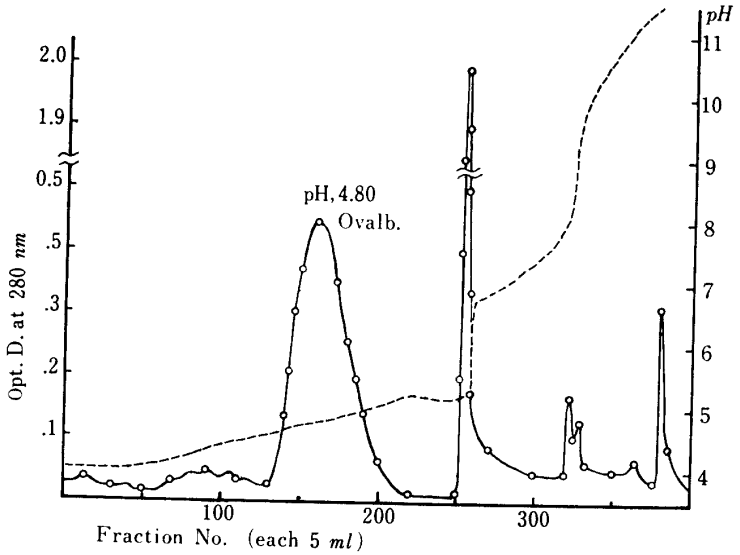

Fig. 5. Chromatography of the pigeon ovalbumin separated by the salting-out on a CM-cellulose column
—○—○—Protein, - - - - - pH

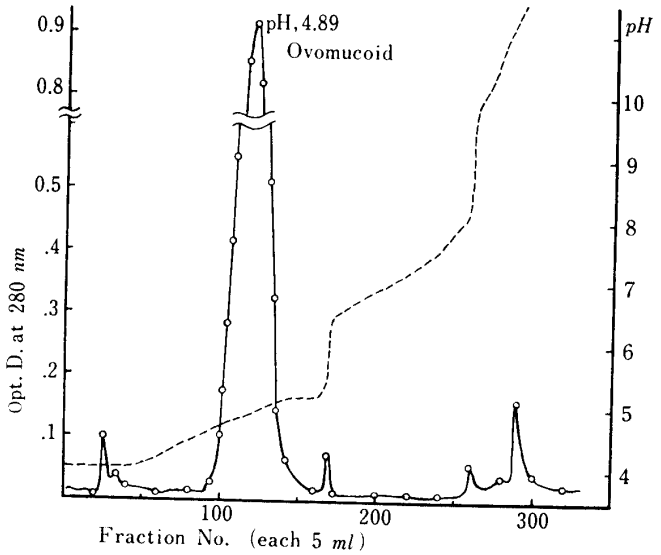

Fig. 6. Chromatography of the pigeon ovomucoid separated by the saling-out on a CM-cellulose column
—○—○— Protein, - - - - - Eluting pH

is based on the solubility, judging from the concentration of salt for extracting, it is to be inferred that component A is the mixture of free amino acid and sugar; B, ovomucoid fraction; C, ovalbumin and conalbumin mixture; D, E, F, globulin fraction, respectively. In pigeon egg white, compared with the chicken white protein, ovomucoid and globulins are relatively large and the mixture of ovalbumin and conalbumin, small, in quantity.

The sugar-protein ratio in the mixed fraction of ovalbumin and conalbumin of pigeon white was larger than that in chicken white. Relative smallness of albumins coincides with the electrophoretic pattern for the pigeon egg white by Bain and Deutsch.[1] Especially, the remarkably large amount of ovomucoid to be separated definitely with the gradient

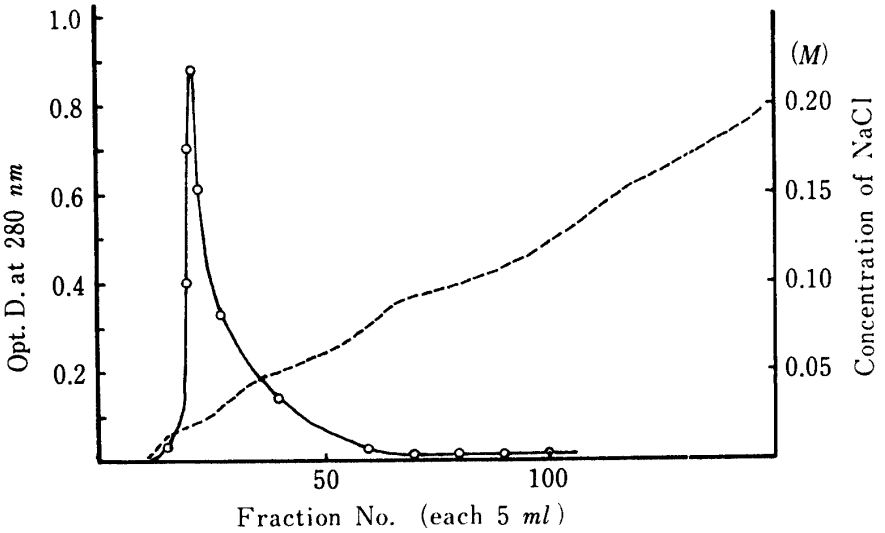

Fig. 7. Chromatography of the pigeon ovomucoid separated by the salting-out on a DEAE-cellulose column Sample: 1%, 5 *ml*, Column: 1.8 ×6 *cm*, Eluting: 0.05 *M* acetate buffer, *pH* 5.55, NaCl 0~0.2 *M* linear gradient

extraction was a feature of pigeon egg white. The sugar-protein ratio in the ovomucoid fraction of pigeon white was higher than that of the chicken, amounting to 10.1×10^{-2} in the former, 8.1×10^{-2} in the latter, respectively. Although the amount was relatively large, the separation of emu ovomucoid was not to be carried out so easily as in case of the pigeon's even with the gradient extraction.[15]

Ion exchange cellulose chromatography of ovalbumin and ovomucoid separated by the salting out———As represented in Fig. 5 the chromatography of the ovalbumin obtained by the salting out on a CM-cellulose column gave the separation to a major ovalbumin having the elution *pH* of 4.80 and several other proteins. As shown in Fig. 6 the ovomucoid obtained by the salting out separated to a major ovomucoid having one sharp peak at *pH*, 4.89 and a small amount of alien proteins. Only a little difference between the eluting *pH* values of ovalbumin and ovomucoid at their peaks was observed. Therefore, it was sure that the component *C* on the elution diagram of the pigeon white proteins with the CM-cellulose column, generally considered to be ovalbumin, contained the ovomucoid. From this result, the first employing of CM-cellulose for separating the pigeon ovomucoid from the egg white was ascertained to be inappropriate, though it was convenient for the other avian egg whites. As shown in Fig. 7, the chromatography of ovomucoid obtained by the salting out on a DEAE-cellulose column gave the single peak tailed slightly, differing from the elution pattern in the CM-cellulose chromatography.

The chromatography of crude ovalbumin with DEAE-cellulose did not give the definite resolution of ovalbumin and contaminants, either. In refining the ovalbumin and the ovomucoid obtained by the salting out, the employing of CM-cellulose was appreciated to be better than that of DEAE-cellulose.

Sephadex gel-filtration of ovalbumin and ovomucoid———Ovalbumin and ovomucoid separated by the salting out method followed by the CM-cellulose column chromatography were separately sieved on the same Sephadex G-100 column. Their elution diagrams were shown in Fig. 8 and 9, respectively. Two diagrams revealed only one sharp peak concerning

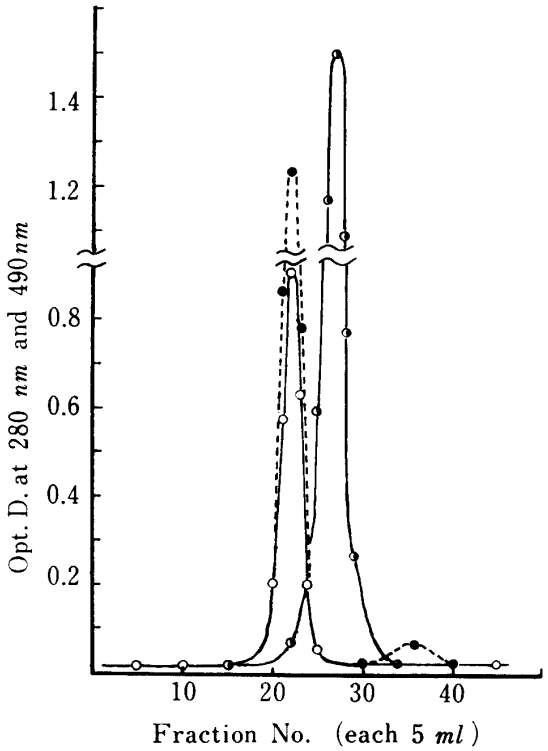

Fig. 8. Sephadex gel-filtration of the pigeon ovalbumin separated by the salting-out followed with the CM-cellulose chromatography, compared with the chicken ovalbumin purified.
—○—○— Pigeon ovalbumin (protein pattern)
----●----●---- Pigeon ovalbumin (sugar pattern)
—◑—◑— Chicken ovalbumin (protein pattern)
Sephadex G-100 column: 2.1 × 52 *cm*,
Eluting: 0.1 *M* acetate buffer, *pH* 4.50

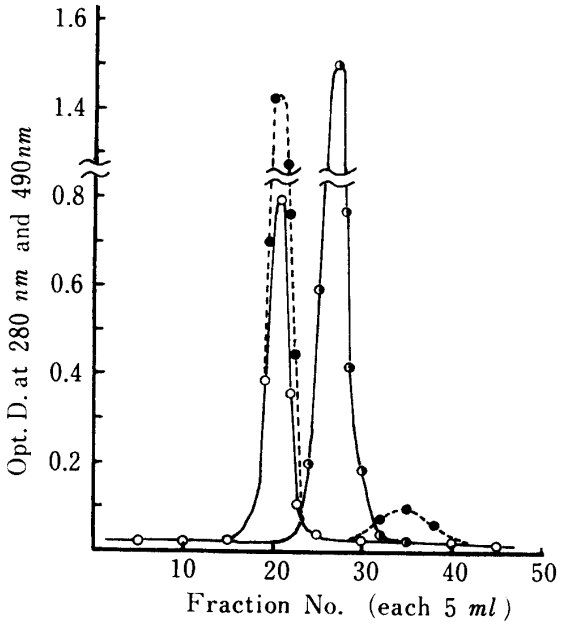

Fig. 9. Sephadex gel-filtration of the pigeon ovomucoid separated by the salting-out followed with the CM-cellulose chromatography, compared with the chicken ovalbumin purified
—○—○— Pigeon ovomucoid (protein pattern)
----●----●---- Pigeon ovomucoid (sugar pattern)
—◑—◑— Chicken ovalbumin (protein pattern)

the both of protein and sugar, respectively. A minor peak of sugar which appeared late was not the protein component but the decomposed product from the CM-cellulose.

Ovalbumin and ovomucoid were eluted at the 22nd and 21st tube from the column of the same size, respectively. Moreover, the eluate peak of chicken ovalbumin was observed at the 27th tube, under the same experimental condition. From these facts, it was ascertained that the molecular weights of the pigeon ovalbumin and ovomucoid are mutually similar, and larger than that of the chicken ovalbumin (45000). The neutral sugar contents of the ovalbumin and ovomucoid were amounting to 8.0% and 10.5%, respectively. Comparing with the hexose contents of the chicken, quail and duck ovalbumins (1.79%, 2.30% and 4.0%)[12] determined by authors previously, the hexose content of the pigeon ovalbumin was found to be remarkably large.

Hexose amount in the ovalbumin was larger than that in the albumin fraction separated by the gradient extraction method. This is due to the fact that the latter is accom-

Table 5. Visible variation of pigeon ovalbumin and ovomucoid aqueous solutions with heating

Variation	Unvariable	Slightly turbid point	Full turbid point	Coagulating point
Ovalbumin	50°C	56°C	60°C	67°C
Ovomucoid	50°C	65°C	70°C	76°C

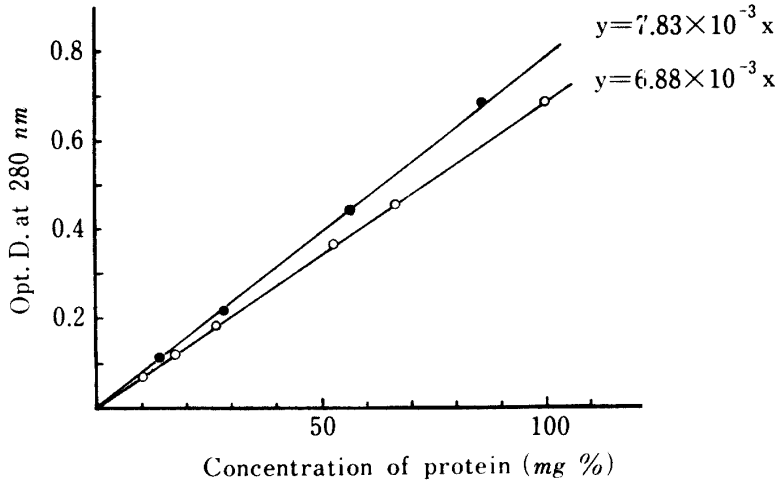

Fig. 10. Calibration curves for pigeon's ovalbumin and ovomucoid
—●—● Ovalbumin, —○—○— Ovomucoid

panied with the conalbumin containing no sugar. Hexose content of the ovomucoid from the gel-filtration was coincident with that of ovomucoid separated by the gradient method, and these values are larger than those in the chicken ovomucoid: 8.0%[16] (by Bragg *et al.*), 5.7%[17] (by Chatterjee *et al.*), 5.1%[18] (Stevens *et al.*), and 8.0 – 9.2%[19] (Kanamori *et al.*).

Visible variation of ovalbumin and ovomucoid solutions with heating———As shown in Table 5, the aqueous solutions of pigeon ovalbumin and ovomucoid got slightly turbid at 56°C and 65°C, full turbid at 60°C and 70°C, and coagulated at 67°C and 76°C, respectively. Coagulating point of the ovomucoid is higher by 9°C than that of the ovalbumin. The coagulate of the ovalbumin was white like the coagulate of the chicken egg white, while that of the ovomucoid appeared like the semi-transparent jelly with opalescent.

The glycoprotein that precipitates at salt concentration higher than the concentration at which the precipitation of ovalbumin takes place was named "ovomucoid," and it has been known to be heat uncoagulable for a long time.

However, differing from the ovomucoids in the chicken-, quail-, duck- and the other egg whites, the pigeon ovomucoid was turbid on heating, coagulated finally. Such a ovomucoid isolated by the authors has never been found in other egg whites. The appearance of the specific coagulating point of the pigeon egg white, which is much higher than that of the chicken white in the preceding description was confirmed to be owing to the presence of relatively large amount of ovomucoid having a higher coagulating point. The difference of the appearance between both coagulates of the pigeon and the chicken whites was also due to the same reason.

The calibration curves for ovalbumin and ovomucoid———The relation between the absorbance and the concentration obtained with the pigeon ovalbumin and ovomucoid

isolated separately was as shown in Fig. 10. The equations of calibration curves for the ovalbumin and ovomucoid could be represented as $y = 7.83 \times 10^{-3}x$ and $y = 6.88 \times 10^{-3}x$, respectively. That for chicken ovalbumin was $y = 6.41 \times 10^{-3}x$, where y is the optical density at 280 *nm*, and x, *mg*%.

These equations suggest that the amount of aromatic amino acid is the largest in the pigeon ovalbumin, middle in the pigeon ovomucoid and smallest in the chicken ovalbumin of all the three.

On the neutral sugar of ovalbumin and ovomucoid———The neutral sugar in the pigeon ovalbumin was appreciated to be galactose through the comparison with the authentic galactose, mannose and glucose run together on a given paper, chromatographically. The neutral sugar in the pigeon ovomucoid was ascertained to be consisting of large proportion of galactose and small proportion of mannose by the same method.

The relation of the amount between both hexoses in pigeon ovomucoid was noted to be opposite to that in chicken ovomucoid reported by the other investigators.[16,17,19]

Kanamori *et al.*[19] described that the molar ratio of mannose to galactose in chicken

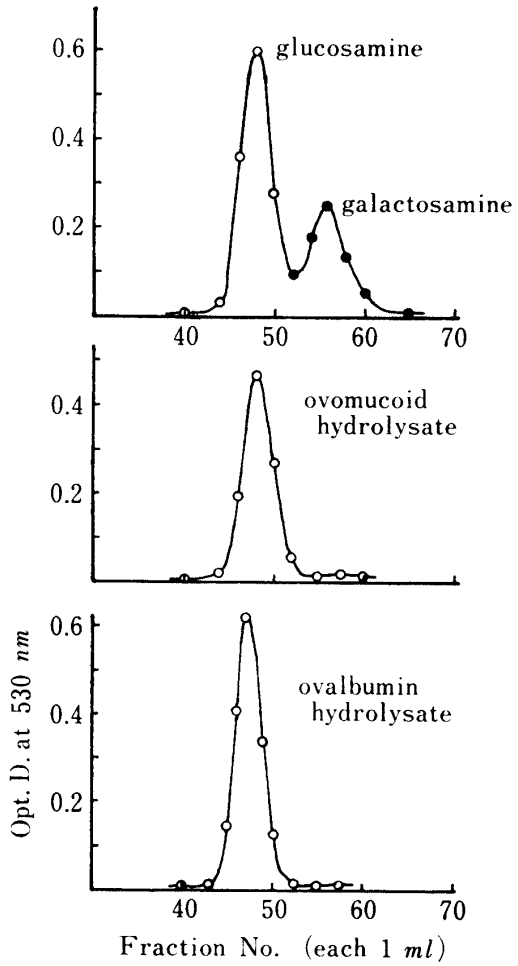

Fig. 11. Chromatography of hexosamine on a Amberlite CG-120 column. Column: 0.6 × 40 *cm*. Eluting: 0.3*N* HCl

Table 6. Amino acid compositions of pigeon ovalbumin and ovomucoid compared with those of chicken's ones (Values are grams of amino acid residues in 100 *g*. protein)

Amino acid	Pigeon ovalbumin	Chicken ovalbumin	Pigeon ovomucoid	Chicken ovomucoid*
Lysine	5.62	5.30	8.29	6.23
Histidine	1.37	1.94	0.54	2.11
Ammonia	1.10	0.75	0.82	—
Arginine	3.53	4.83	2.33	3.51
Aspartic A.	6.82	7.49	8.34	13.11
Threonine	4.61	3.09	2.76	5.27
Serine	6.16	5.91	5.25	3.89
Glutamic A.	14.66	13.46	15.28	6.87
Proline	4.37	2.98	2.33	2.67
Glycine	2.14	2.34	1.30	3.28
Alanine	4.31	5.50	3.47	2.97
Cystine/2	trace	1.28	0.50	6.38
Valine	5.35	5.56	4.77	5.67
Methionine	3.04	4.26	1.90	0.89
Isoluecine	5.38	5.77	4.12	1.29
Leucine	7.59	7.48	6.94	4.93
Tyrosine	4.22	3.38	3.79	3.90
Phenylalanine	7.39	6.78	5.58	2.79
Tryptophan	1.30	1.10	2.40	0
Total residues	88.96	89.20	79.39	75.76
Hexose	8.0	1.79	10.5	9.66
Glucosamine	2.5	1.20	3.5 (4.6)	12.09

Tryptophan value was calculated from the ultraviolet absorption determination. The value in parenthesis was obtained with amino aid analyzer.
*Calculated from the data of Osuga and Feeney (1968), Reference No. (20)

ovomucoid was amounting to ca. 4:1. As the constituent hexose in pigeon ovalbumin differs from those in the chicken and quail ovalbumins, the further minute studies may be necessary.

Identification and determination of hexosamine in ovalbumin and ovomucoid——Hexosamines of pigeon ovalbumin and ovomucoid were together identified as glucosamine from the elution diagrams shown in Fig. 11, and identified also from the chromatogram on a short column at the amino acid analysis.

According to the Elson-Morgan method modified by Boas, the glucosamine contents in ovalbumin and ovomucoid were ascertained to be 2.5% and 3.5%, respectively. Moreover, the content in ovomucoid was amounting to 4.6% according to the method with amino acid analyzer. The amount in pigeon ovalbumin is larger than those in chicken and quail ovalbumins[12], and similar to that of the duck.[12] The amount in pigeon ovomucoid is much smaller than those (13.6%,[18] 12.1%[20]) in chicken ovomucoid in the literature. This seems to be a feature of the pigeon ovomucoid. Kanamori showed that

only one among four components in chicken ovomucoid contained 5% of glucosamine, having no tryptic activity.[19]

Amino acid compositions of ovalbumin and ovomucoid———Pigeon ovalbumin and ovomucoid were isolated by the salting out followed with the CM-cellulose chromatography and the subsequent gel-filtration on a Sephadex G-100 column.

Amino acid compositions of pigeon ovalbumin and ovomucoid compared with those of chicken ovalbumin and ovomucoid were represented in Table 6.

Comparison between the composition of pigeon ovalbumin and that of the chicken's shows that threonine, glutamic acid, proline, tyrosine and phenylalanine are more in the pigeon than in the chicken, while arginine, aspartic acid, alanine, half cystine and methionine in the former are less than in the latter. The presence of a trace of half cystine is noteworthy. Total aromatic amino acid content is more in the pigeon than in the chicken and this relation agreed with the preceding descriptions as to the calibration curves.

Compared with the composition of chicken ovomucoid,[20] in pigeon ovomucoid, lysine, serine, glutamic acid, methionine, isoleucine, leucine, phenylalanine and tryptophan were observed to be more, while histidine, aspartic acid, threonine, glycine and half cystine, less in quantity. Especially, remarkable smallness of half cystine in pigeon ovomucoid was noteworthy, though the value in the chicken's was large. Total aromatic amino acid content of pigeon ovomucoid is less than those of the pigeon and chicken ovalbumins.

This relation is consistent with the description concerning the calibration. The difference between the amounts of acidic amino acid and basic amino acid was observed to be larger in the pigeon ovalbumin than in the chicken's and this relation agreed with the difference between the *p*H values of both proteins eluted from a CM-cellulose column.

The inhibitory action of pigeon ovomucoid against proteinases———The inhibitory activity of pigeon ovomucoid against bovine trypsin and chymotrypsin was estimated with the Kunitz's casein digestion method. The inhibitory activity was scarcely appreciated on some experiments under various conditions. As avian ovomucoids have been known to include the glycoprotein inhibiting trypsin or chymotrypsin, authors are now studying to clarify the reason why only the pigeon ovomucoid shows no inhibition. Fredericq *et al.*[21] found the electrophoretic heterogeneity of chicken ovomucoid in earlier days and Bier *et al.*[22] reported that different electrophoretic fractions of ovomucoid had different isoelectric points, although the fractions appeared to be similar in carbohydrate content and antitryptic activity. Melamed[23] described that differences in sialic acid content account for only part of the electrophoretic heterogeneity of chicken ovomucoid. Feeney *et al.*[4] found that preparations of ovomucoids of chicken and other ten avian species are heterogeneous when examined with gel-electrophoresis. Since Lineweaver and Murray[24] isolated ovomucoid and established that it was the component responsible for the trypsin inhibitory activity, many researches on the proteinase inhibition have been reported. Rhodes *et al.*[3] isolated the ovomucoids of eleven different avian species and divided them into four classes on the basis of their inhibitory activities against proteinases. Kanamori *et al.*[25] reported that chicken ovomucoid was fractionated by CM-cellulose chromatography into four components and that two of them were trypsin inhibitors and the other two were apoprotein of flavomucoid. Avian ovomucoids have been known to be heterogeneous and heat uncoagulable for a long time, and moreover, to be including at least an antiproteolytic glycoprotein in them. On the contrary, the pigeon ovomucoid was found not to have such chemical and biological properties by authors. Even in all cases such as the gradient extraction with salt, followed by CM-cellulose chromatography and DEAE-cellulose chro-

matography and the subsequent gel-filtration, pigeon ovomucoid appeared to be homogeneous. In general, avian ovomucoids were eluted at *p*H lower than the respective ovalbumins on CM-cellulose chromatography and could be distinguished in the elution diagrams. However, the eluting *pH* of pigeon ovomucoid was closed to that of the ovalbumin. As pigeon ovomucoid is, moreover, heat coagulable, having no inhibitory action against proteinases, and the chemical composition differs from those of the other ovomucoids, it was ascertained to be a new-typed ovomucoid.

Summary

1) The *pH* of the white of an egg laid on the first day was found to be higher than that of an egg laid on the next day in all of the eggs laid periodically by the appointed female pigeon.

2) The elution diagram of pigeon egg white proteins separated by CM-cellulose chromatography revealed seven components. Chromatographic separations of egg white proteins between two eggs laid on the first day and the next day were similar each other. Ovalbumin fraction appeared relatively large, being due to the simultaneous elution of ovomucoid on CM-cellulose chromatography. Each component corresponding to ovomucoid and lysozyme was not well resolved, respectively. Lysozyme content was inferred to be quite small. Eluting *pH* of pigeon ovalbumin fraction was lower than that of the chicken's and it was also appreciated on the isolated ovalbumin.

3) The gradient extraction of egg white proteins with ammonium sulfate showed that ovomucoid and globulin fraction are relatively large, and the mixed fraction of ovalbumin and conalbumin, small, in quantity, compared with the chicken egg white proteins. Especially, the separation of ovomucoid was well defined and the content in the whole proteins was surprisingly high, amounting to 34%, and the ratio of sugar to protein was 10.1×10^{-2}. The content of ovalbumin and conalbumin mixture was amounting to 39%.

4) Isolation of each ovalbumin and ovomucoid was achieved by the salting out of egg white followed with CM-cellulose chromatography and the subsequent Sephadex gel-filtration. It was found that the eluting *pH* of the ovomucoid is closed to that of ovalbumin on CM-cellulose chromatography. From the result of gel-filtration, the molecular weights of pigeon ovalbumin and ovomucoid were inferred to be mutually similar and larger than that of the chicken ovalbumin.

5) Heterogeneity of pigeon ovomucoid could not be observed at all throughout the refining process.

6) Pigeon ovalbumin contained 8.0% of hexose and 2.5% of glucosamine. Pigeon ovomucoid contained 10.5% of hexose and 3.5% of glucosamine. Hexose in ovalbumin was ascertained to be galactose, and that of ovomucoid, galactose and mannose by paper chromatography.

7) Comparison of the composition of pigeon ovalbumin with that of the chicken's showed that threonine, glutamic acid, proline, tyrosine and phenylalanine are more in the pigeon than in the chicken, while arginine, aspartic acid, alanine, half cystine and methionine in the former are less than in the latter. The presence of a trace of half cystine in pigeon ovalbumin was noteworthy. Compared with the composition of chicken ovomucoid, in the pigeon ovomucoid, lysine, serine, glutamic acid, methionine, isoleucine, leucine, phenylalanine and tryptophan were appreciated to be more, while histidine, aspartic acid, threonine, glycine and half cystine, less. Especially, the remarkable smallness of

half cystine was noteworthy.

8) The coagulating point of pigeon ovomucoid on heating was higher by 9°C than that of pigeon ovalbumin and the coagulate of ovomucoid appeared like the semi-transparent jelly. The heighness of the coagulating point of pigeon egg white compared with that of the chicken white was clarified to be owing to the presence of large amount of ovomucoid having a higher coagulating point in the egg white.

9) The inhibitory action of pigeon ovomucoid against proteinases was scarcely observed.

10) Pigeon ovomucoid was a homogeneous, heat-coagulable glycoprotein, having the same chromatographic behavior as the ovalbumin, and moreover, showed no inhibitory action against proteinases. The chemical composition differed from those of the other avian ovomucoid. Therefore, it was appreciated to be a new-typed ovomucoid.

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