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Isolation of Ovomucoid-like Globulin, a Trypsin Inhibitor from Quail Ovoglobulin Fraction and Its Some Characteristics

Katsuya KOGA, Takao FUKUNAGA and Yasushi SUGIMOTO*

(*Laboratory of Animal Biochemistry*)

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Introduction

Since Lineweaver and Murray[20], in earlier days, isolated ovomucoid from hen egg white and established that it was the component responsible for the trypsin inhibitory activity, a number of reports and reviews on the heterogeneity of the ovomucoid have been published[5,12,13,14,15,21,24,25].

Feeney et al.[12] asserted that the heterogeneity of ovomucoid appeared to be a general phenomenon in avian egg whites. They observed the electrophoretic heterogeneity of the Japanese quail ovomucoid prepared by CM-cellulose chromatography following trichloroacetic acid-acetone treatment of egg white, but did not achieve the fractionation of ovomucoid. Although Asao et al.[3,4] 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. Koga et al.[18] separated crude ovomucoid from the quail egg white by salting-out with ammonium sulfate and were able to divide it into three protein components by DEAE-cellulose chromatography followed by CM-cellulose chromatography, and furthermore, found that one component of those was a glycoprotein inhibiting trypsin only and the other two had the capacity of combining with riboflavin.

A new proteinase inhibitor differing from ovomucoid was firstly separated from hen ovoglobulin fraction by Matsushima and named "ovoinhibitor"[22,23]. It was capable of inhibiting not only bovine trypsin but also proteinases of fungal and bacterial origins. Later on it was found to be capable of inhibiting the bovine chymotrypsin[27]. Physical properties and chemical composition of the chicken ovoinhibitor were clarified by Tomimatsu et al.[28].

Since we isolated an undescribed trypsin inhibitor differing from both ovomucoid and ovoinhibitor from quail ovoglobulin fraction, we describe some chemical characteristics of the inhibitor in the present paper.

Experimental Materials and Methods

Materials—The eggs of the Japanese quail (*Coturnix coturnix japonica*) were secured from the 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 pooled egg whites were blended with a homogenizer at a slow speed and used as the experimental samples.

* Present address: Seinan Women Junior College, Kokura-kitaku Kitakyushu 803.

Separation of pseudoglobulin from egg white—To the homogenized egg white was added an equal amount of saturated ammonium sulfate solution, whilst being stirred gently. The formed globulin precipitate was recovered by centrifuging, and then it was suspended with 0.5 saturated ammonium sulfate solution to remove the involved albumin and conalbumin, and then centrifuged. The globulin fraction was dissolved in a small amount of water and dialyzed against water until ammonium sulfate was completely removed. The pseudoglobulin aqueous solution was lyophilized.

Separation and isolation of ovomucoid-like globulin—The naming of "ovomucoid-like globulin" (abbreviation: OMLG) was done from the standpoint fixed in the later description. The lyophilized powder was dissolved in 0.025 M sodium acetate buffer, pH 4.0 to be 1.5%. Twenty ml of the solution was applied onto a CM-cellulose (from Brown Co.) column of 1.8×20 cm equilibrated with the same acetate buffer. Chromatographic elution was performed by the linear increasing of the pH level from 4.00 to 5.60, according to the descriptions in the previous report[16]. The effluent was collected at each 5 ml. The fractions corresponding to ovomucoid from the eluting pH level[16] were combined and concentrated through Amicon ultra-filtration cell, Model 202.

Rechromatography of a portion of this solution was done on the same column and on DEAE-cellulose column (1.8×7 cm) to confirm the absence of the other proteins. After being dialyzed against water and subsequently against 0.1 M acetate buffer, pH 4.50, the solution (2%, 5 ml) was applied on a Sephadex G-100 column of 2.2×55 cm. Eluting with the same buffer, the effluent was collected at each 5 ml. Neutral sugar and protein contents in each fraction were respectively determined. Ovomucoid-like globulin refined by Sephadex G-100 gel-filtration was used as the sample in analyzing chemical compositions, assaying proteinase inhibitory activity and researching a few properties.

Estimation of molecular weight—Estimation of the molecular weight of OMLG was done by the Sephadex gel-filtration method proposed by Whitaker[29] and Andrews[2]. The column from Shōei Works Co. and Sephadex G-100 from Pharmacia, Uppsala, Sweden were employed. The gel-chromatography was performed by making use of the up-flow system of 0.05 M carbonate buffer (pH 10.0, $\mu=0.10$) with flow rate of 1 ml per min.

Analysis of chemical composition—The hydrolysis of duplicate samples for the amino acid analysis was performed in every sealed tube containing nitrogen gas substituted for the air, at 110°C for 24 and 40 hr. The analysis was carried out with a Yanagimoto LC-5S type amino acid analyzer. The experimental detail was described in the previous report[17]. Tryptophan content was determined by the ultraviolet absorption method[1]. Neutral sugar determination was performed by the phenol-sulfuric method[10]. The calculation of the content was done in accordance with the equation for mannose represented in our previous report[18]. Hexosamine and N-acetylneuraminic acid were analyzed by using Shimazu GC-4B gas chromatograph, referring to the report of Bolton et al.[7]. Additionally, the determination of hexosamine content was carried out according to the Pearson's procedure[26] followed by the Elson-Morgan method modified by Boas[6].

Assay of inhibitory activity of OMLG against various proteinases—The assay of the inhibitory activity of OMLG against various proteinases with casein substrate was performed, essentially according to the descriptions of Laskowski (Kunitz method)[19]. Proteinases employed were bovine trypsin (crystallized, 2 times), bovine α-chymotrypsin (crystallized, 3 times) from Miles Co.; Bacillus alkaline protease (*Bac. subtilis* origin, crystallized, 2 times), pronase-P (*Streptomyces griseus* origin) and fungal semi-alkaline protease (*Aspergillus melleus* origin, crystallized, 2 times) from Seikagaku Kōgyo Co., Japan. The reacting pH values were as follows: 7.60 of 0.1 M

phosphate buffer for trypsin and chymotrypsin, 8.60 of the same buffer for Bac. alkaline protease, 8.48 of 0.2 M Tris-HCl buffer for pronase-P and 8.40 of 0.1 M phosphate buffer for semi-alkaline protease. Preincubation for forming the complex of each proteinase and OMLG was performed at 37°C for 30 min and the reaction after addition of Hammarsten casein was done at 37°C for 10 min. The subsequent assay procedure was done according to the descriptions in our previous report[18].

Effects of acid-, alkali- and heat-treatments on the stability of OMLG—One ml of OMLG (2.2%) aqueous solution was added to 1 ml of 6 N HCl, allowed to stand for 30 min and 60 min at room temperature, neutralized with 3 N NaOH, and diluted to 200 ml with 0.1 M phosphate buffer solution, pH 7.60. To the respective 1 ml of 0.2 N, 0.4 N and 0.6 N NaOH was added 1 ml of OMLG (2.2%) aqueous solution. They were allowed to stand for 30 min and 60 min at room temperature, and thereafter they were neutralized respectively with 0.1 N, 0.2 N and 0.3 N HCl and diluted to 200 ml with the same buffer as described above. The aliquots of the dilutions were assayed for the remaining antitryptic activities. OMLG samples were dissolved in water, 0.1 M phosphate buffer solutions of pH 6.0 and 8.0 to be 2%; these samples were respectively incubated at 90°C and 95°C for some different times. After the appropriate dilution, differential spectra of the treated samples to the untreated one were measured over the range from 240 nm to 320 nm by using Hitachi 101 type Spectrophotometer. The remaining inhibitory activities were, moreover, assayed.

Reduction of OMLG with mercaptoethanol—Twenty-five ml of phosphate buffer solution (0.1 M, pH 7.60) containing OMLG 80 mg, urea 4 M and mercaptoethanol 0.5 M was vigorously stirred in a stoppered vessel of 50 ml volume, and after sufficient introduction of nitrogen gas, it was allowed to stand for 3 days at room temperature. To block the reactivity of the produced sulfhydryl groups, monoiodoacetic acid (2.4 g) was added on it and kept for 12 hr at room temperature. The reagents, excepting OMLG, were removed by being dialyzed against 0.1 M phosphate buffer solution, pH 7.60. The solution was assayed for antitryptic activity.

Estimation of sulfhydryl group—Estimation of the sulfhydryl groups in OMLG treated with the diluted alkali was performed by the common method with the Ellman reagents [5′-dithiobis-(2-nitrobenzoic acid)][11].

Results and Discussion

CM-cellulose chromatography of pseudoglobulin from the quail egg white gave the elution of three protein components (designated as A, B and C) as shown in Fig. 1. Component A unadsorbed on the CM-cellulose was considered to be attributed to the over-charge. The eluting pH values of component B and C were separately 4.45 and 4.80. From the respect of these values, the former and the latter were ascertained to have been corresponding, respectively, to ovomucoid and ovalbumin in the chromatogram[16] of egg white proteins of the quail on CM-cellulose column. The eluting pH value of component B was, moreover, close to that of a trypsin inhibitor in the quail ovomucoid[18]. Component B was found to possess the inhibitory activity against bovine trypsin. Melamed[24] described that ovomucoid could be precipitated out of solution upon saturation with ammonium sulfate, after precipitation of globulins, ovalbumin and conalbumin with ammonium sulfate, ethanol, aceton or trichloroacetic acid.

Based on the definition of globulins, component B is to be looked upon as the one belonging to globulins, because of its being precipitated by a half-saturation with ammonium sulfate. However, concerning the chromatographic behavior, it was observed to be coincident with ovomucoid, showing an antitryptic activity. Hence we named the protein "ovomucoid-like globulin" (abbrevia-

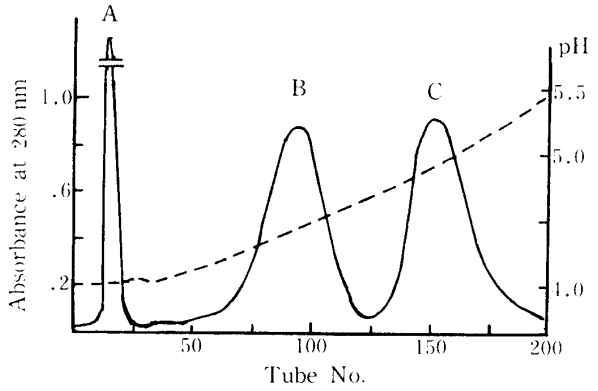

Fig. 1. CM-cellulose column chromatography of pseudoglobulin fraction from quail egg white

Column: 1.8×20 cm, Buffer: 0.025 M acetate (pH 4.00)–0.025 M sodium acetate (to pH 5.50), Fraction size: 5 ml, Flow rate: 1.0 ml/min, ——: Protein, ·····: pH

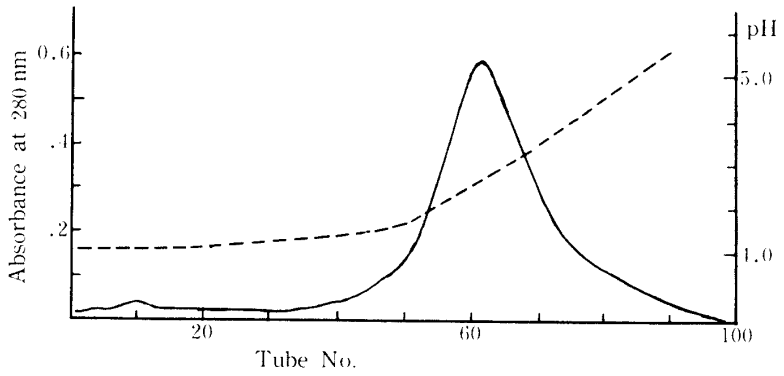

Fig. 2. Rechromatography of component B in Fig. 1 on CM-cellulose column

Sample: 1%, 10 ml, Column: 1.8×20 cm, Buffer: Same as that in Fig. 1, Fraction size: 5 ml, ——: Protein, ·····: pH

tion: OMLG).

Rechromatographies of component B on CM-cellulose column and on DEAE-cellulose column gave individually a single component free from the other protein.

The eluting pH value of the protein from CM-cellulose column was 4.45 (Fig. 2), and the protein was eluted from DEAE-cellulose column at the concentration of 0.09 mole of NaCl in Tris-HCl buffer, pH 8.0 (Fig. 3). As shown in Fig. 4, Sephadex gel-filtration of OMLG separated by the first CM-cellulose chromatography was again noted to be homogeneous with respect to the protein.

But a small amount of degraded substances of CM-cellulose which were colorable with the phenol-sulfuric reagent were eluted behind OMLG. OMLG could, therefore, be isolated from pseudoglobulin by CM-cellulose chromatography, followed by Sephadex gel-filtration.

Molecular weight—The molecular weight of OMLG was estimated to be 36000 by the gel-filtration on Sephadex G-100 column (Fig. 5), which was slightly smaller than that of a trypsin inhibitor in the quail ovomucoid reported by authors[18].

Chemical composition—Concerning the chemical composition, OMLG contained large amount of aspartic acid, considerable amount of lysine, threonine, glutamic acid, cystine, valine and

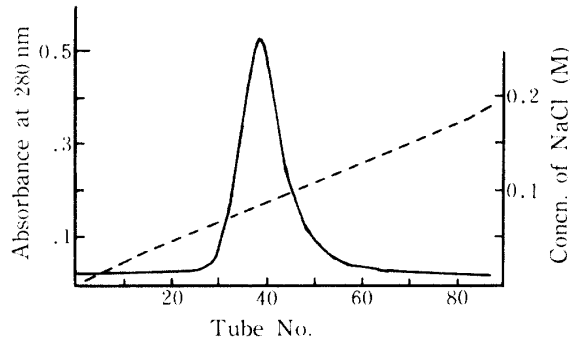

Fig. 3. DEAE-cellulose column chromatography of component B in Fig. 1

Column: 1.8×7 cm, Buffer: 0.025 M Tris-HCl, pH 8.0, with a linear gradient of concentration of NaCl (zero to 0.20 M), Fraction size: 5 ml, ——: Protein, ·····: Concentration of NaCl

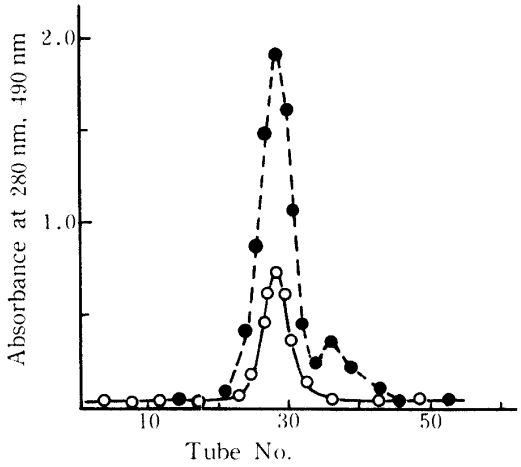

Fig. 4. Sephadex G-100 gel-filtration of OMLG obtained by the first CM-cellulose chromatography

Sample: 2%, 5 ml, Column: 2.2×55 cm, Buffer: 0.1 M acetate, pH 4.50, Fraction size: 5 ml, Flow rate: 0.3 ml/min, ---○---○---: Protein, —●—●—: Neutral sugar

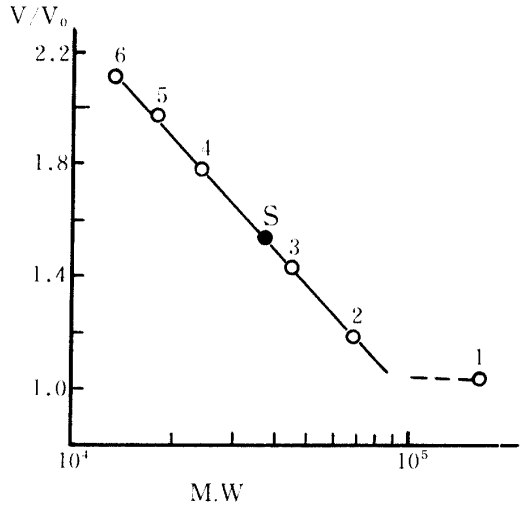

Fig. 5. Relationship between elution-volumes and molecular weights of authentic proteins and quail OMLG

1	*r*-Globulin (Human)	M. W 16×10^4
2	Serum albumin (Bovine)	6.7×10^4
3	Ovalbumin (Hen)	4.5×10^4
4	Chymotrypsinogen A (Bovine)	2.3×10^4
5	Myoglobin (Whale muscle)	17800
6	Cytochrome C (Horse heart)	12400
S	OMLG	
V	Elution volume of each protein	
V_0	Elution volume of blue dextran	

phenylalanine, and slight amount of alanine, methionine, isoleucine and tryptophan (Table 1). Excepting several amino acids, the composition was similar to that of the component I (trypsin

inhibitor) from the quail ovomucoid[18]. Especially, the existence of quite a small amount of methionine, isoleucine and tryptophan was a characteristic common to the compositions of the other trypsin inhibitors of plant and animal origins.

The majority of the neutral sugar in OMLG was identified with mannose, and the minor sugar

Table 1. Chemical composition of quail OMLG compared with that of trypsin inhibitor in the quail ovomucoid

Composition	OMLG	T·I in OM	Composition	OMLG	T·I in OM
Lysine	5.83	7.60	Valine	5.50	6.48
Histidine	2.07	trace	Methionine	1.57	1.55
Arginine	2.68	3.78	Isoleucine	1.32	1.57
Aspartic A.	11.84	14.38	Leucine	3.20	3.86
Threonine	5.40	6.59	Tyrosine	4.61	4.19
Serine	3.17	3.84	Phenylalanine	5.29	5.04
Glutamic A.	7.71	9.36	Tryptophan	0.57	0.80
Proline	2.59	3.25	Hexose	7.80	5.66
Glycine	3.13	3.92	Glucosamine	3.20 (2.90*)	2.41
Alanine	1.27	1.68			
Cystine/2	6.70	6.99	NANA	1.00	1.32

Amino acid compositions were represented as grams of the residues in 100 g protein. Tryptophan amount was determined by the ultraviolet absorption method.[1]

NANA: N-acetylneuraminic acid, T·I: Trypsin inhibitor, OM: Ovomucoid

* This value was determined according to the Pearson's procedure[26] followed by the Elson-Morgan method modified by Boas.[6]

was identified with fucose by the gas chromatography. The contents of total neutral sugar (7.8%) and glucosamine (3.2%) of OMLG were larger than those of the trypsin inhibitor from the quail ovomucoid[18], and were also different from those of hen ovomucoid[8,15] and ovoinhibitor[9]. It remains unexplained why OMLG was precipitated by half saturation of egg white with ammonium sulfate, in spite of the considerable largeness of carbohydrate content.

The equation of calibration curve obtained from the various concentrations of OMLG aqueous solutions was $y=5.04\times10^{-3}x$, where y is the absorbance at 280 nm, x is the concentration (mg %). The precise concentration was determined from the dry weight. The equation for a trypsin inhibitor from the quail ovomucoid was $y=4.78\times10^{-3}x$. The difference between the two coefficients was affirmed from the aromatic amino acid contents of the two kinds of proteins.

Antitryptic activity and stability—The antitryptic activity of OMLG with casein substrate appeared linearly with the increase of OMLG amount in the earlier stage, as shown in Fig. 6. Inhibitory activity was expressed as a decreasing of tryptic activity (A_{280nm}) per mg of OMLG in one ml of assay solution: Specific inhibitory activity$=I_0-I_{0.1}$/Inhibitor (mg in 1 ml of assay solution), where I_0 is the tryptic activity in the case when no inhibitor was added to the assay medium; $I_{0.1}$ is the activity noted when 0.1 ml of inhibitor was added to it. Specific inhibitory activity of OMLG against bovine trypsin was 43, being about 1.7 times as much as that of a trypsin inhibitor from the quail ovomucoid[18]. Although quail OMLG strongly inhibited bovine trypsin, it did not inhibit at all bovine chymotrypsin, bacterial alkaline protease, pronase-P and fungal semi-alkaline protease. The treatment of OMLG with 3 N HCl for 30 min or 60 min at room temperature gave little variation of the antitryptic activity as shown in Fig. 7. The treatment of

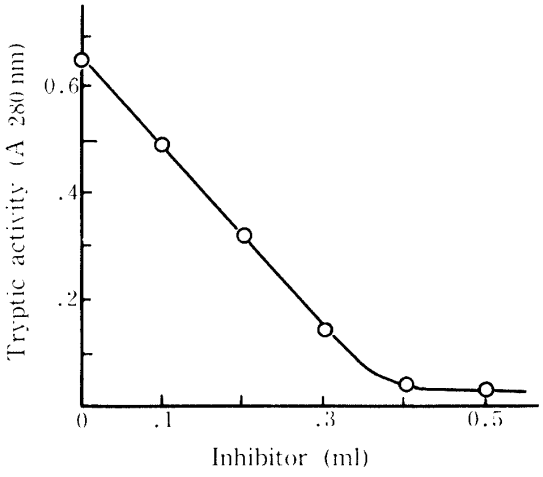

Fig. 6. Inhibition of OMLG against bovine trypsin
Enz., $A_{280\,nm}$=0.065, 2 ml
Buffer: 0.1 M phosphate, pH 7.60
OMLG, $A_{280\,nm}$=0.080, 0~0.5 ml
Substrate: 5% casein, 1.5 ml
Reaction volume: 5 ml
Stopper: 10% trichloroacetic acid 3 ml

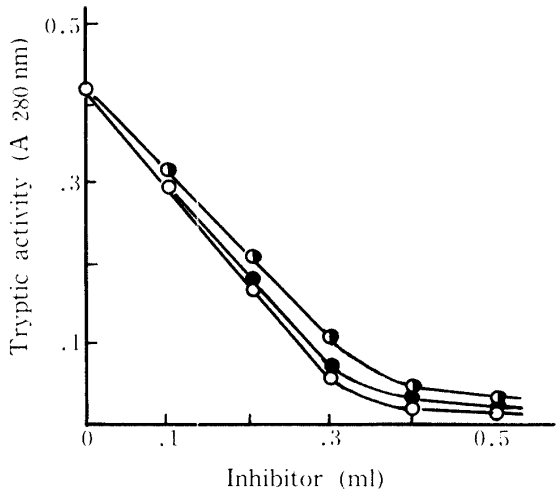

Fig. 7. Antitryptic activities of OMLG treated with 3 N HCl at room temperature
—○—○—: Native, —●—●—: Treated for 30 min, —◑—◑—: Treated for 60 min

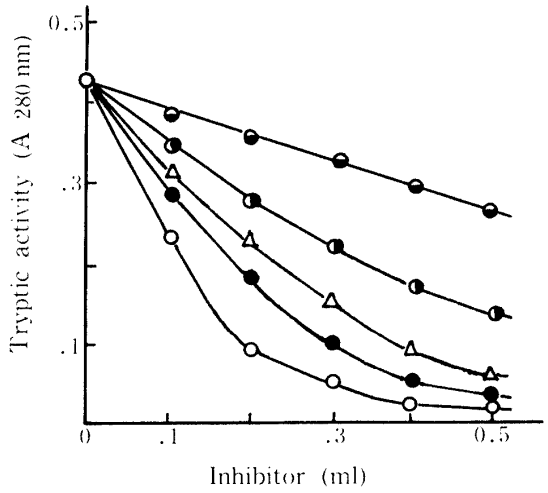

Fig. 8. Antitryptic activities of OMLG after heated in aqueous solution
—○—○—: Native, —●—●—: 90°C, 30 min, —△—△—: 90°C, 50 min, —◑—◑—: 95°C, 30 min, —◒—◒—: 95°C, 80 min

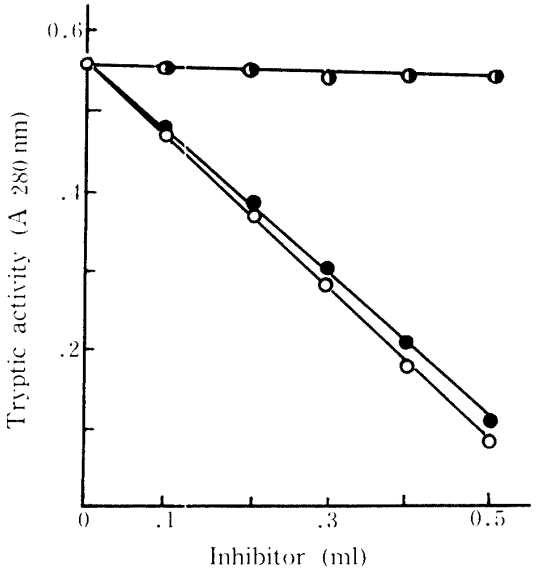

Fig. 9. Antitryptic activities of OMLG after heated in 0.1 M phosphate buffer of pH 6.0 or 8.0
—○—○—: Native, —●—●—: 90°C, 15 min or 30 min at pH 6.0, —◑—◑—: 90°C, 15 min at pH 8.0

it with 0.1 N, 0.2 N NaOH for 30 min or 60 min gave also quite little variation (Table 2). On the contrary, the treatment of it with 0.3 N NaOH for 60 min at room temperature reduced its activity by 35%. Antitryptic activities of OMLG heated in aqueous solution were represented in Fig. 8 and Table 3.

The activity was decreased with the rising of temperature and the prolongation of the heating time. Heating for 50 min at 90°C gave a decreasing-rate of 35% and that for 80 min at 95°C gave a value of 80%. Antitryptic activity of OMLG remained unchanged when it was heated for 15 min or 30 min at 90°C, at pH level of 6.0, whereas the activity disappeared upon being heated for the

Table 2. Relative antitryptic activity of OMLG treated with the diluted sodium hydroxide solution at room temperature

Native OMLG	0.1 N NaOH		0.2 N NaOH		0.3 N NaOH	
	30 min	60 min	30 min	60 min	30 min	60 min
100	100	94	100	94	100	65

Table 3. Relative antitryptic activity of OMLG upon heating in aqueous solution

Native OMLG	90°C, 30 min	90°C, 50 min	95°C, 30 min	95°C, 80 min
100	67	65	37	20

same periods of time at pH 8.0 (Fig. 9).

Even on being heated under the conditions of aqueous or slightly acidic solution, OMLG was rendered neither coagulable nor turbid. This phenomenon may be due to the comparative largeness of carbohydrate and disulfide linkage contents in OMLG. Because, it is well known that hen- and quail-ovalbumins containing a small amount of carbohydrate and disulfide linkage are heat-coagulable.

As shown in Fig. 10 and Fig. 11, the blue shift was scarcely observed upon heating the aqueous solution of OMLG or slightly acidic solution of it and the increase in absorption was observed. Denatured blue shift was, however, observed over the range from 280 nm near 290 nm upon heating the slightly basic solution of OMLG. Antitryptic activity of OMLG was entirely lost by being reduced with mercaptoethanol (Fig. 12). This result demonstrates that disulfide linkages contained largely in the quail OMLG contribute primarily to the stability of the conformation.

The absence of sulfhydryl group in the native OMLG was ascertained by using the Ellman reagent[11]; but when OMLG was heated in a slightly basic region at 90°C, the appearance of sulfhydryl group in it increased with the passage of heating time (Table 4). This suggests a partial splitting of disulfide linkage by alkali-hydrolysis. From these results, it was supposed that heating of OMLG under slightly basic condition caused a considerable conformational change which was due to a partial splitting of disulfide linkages in OMLG molecule and brought about the loss of

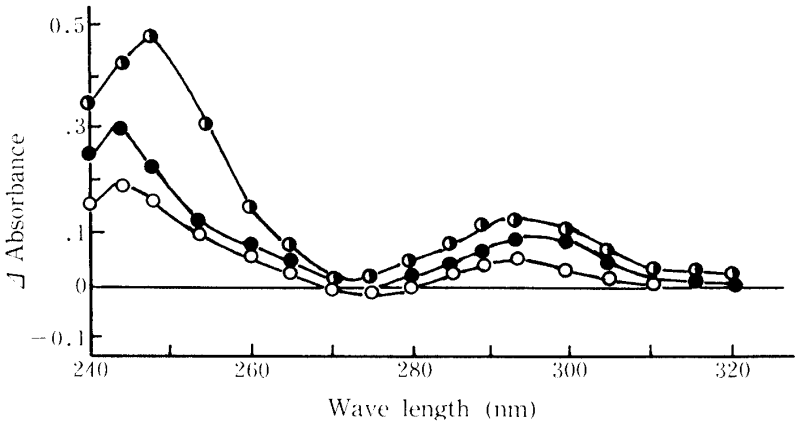

Fig. 10. Differential spectra between OMLG heated in aqueous solution and native OMLG

—○—○—: 90°C, 30 min, —●—●—: 90°C, 50 min, —◗—◗—: 95°C, 80 min

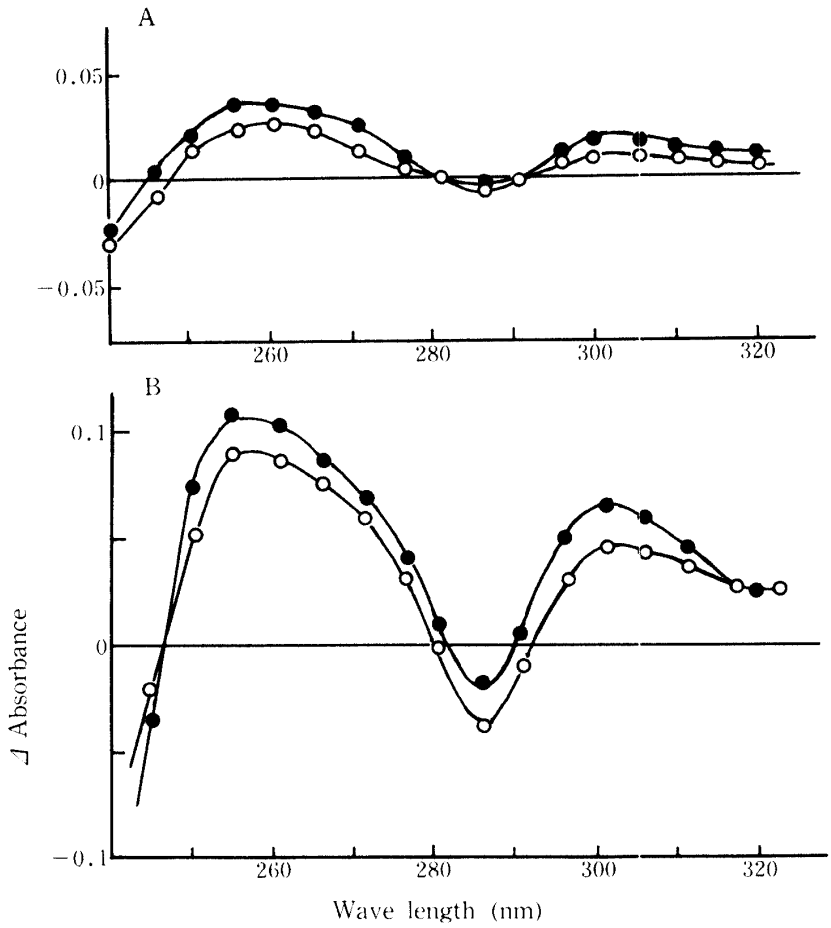

Fig. 11. Differential spectra between OMLG heated in slightly acidic (pH, 6.0) or basic (pH, 8.0) solution and native OMLG

A: Heating at pH 6.0, B: Heating at pH 8.0, —○—○—: 90°C, 15 min, —●—●—: 90°C, 30 min

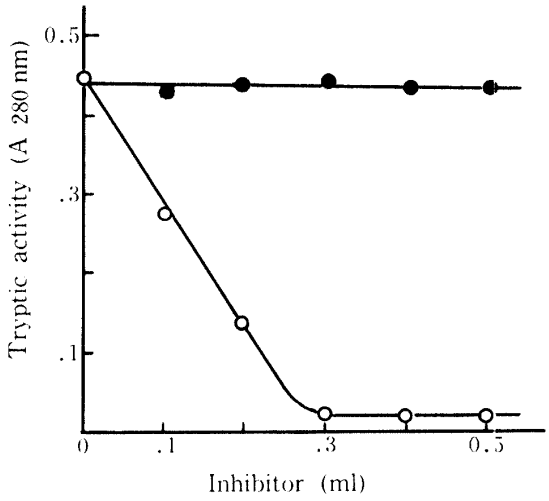

Fig. 12. Antitryptic activity of OMLG reduced with mercaptoethanol

—○—○—: Native OMLG, —●—●—: Reduced OMLG

antitryptic activity.

The present study demonstrates that the direct separation of ovomucoid from the quail egg

Table 4. Variation of sulfhydryl group contents of OMLG upon heating in slightly basic (pH 8.0) solution.

(mole/mole)

Native OMLG	90°C, 15 min	90°C, 30 min
0	0.17	0.30

white by CM-cellulose chromatography is improper. From the previous and present studies, authors propose that at least two kinds of glycoproteins inhibiting only trypsin exist in the quail egg white.

Summary

A glycoprotein, chromatographic behavior of which on the CM-cellulose column was coincident with that of quail ovomucoid, was isolated from the ovoglobulin fraction of the quail egg white, and so it was named "ovomucoid-like globulin" (OMLG). Quail OMLG contained the largest amount of aspartic acid, considerable amount of lysine, threonine, glutamic acid, cystine, valine and phenylalanine, and slight amount of alanine, methionine, isoleucine and tryptophan, having the molecular weight of 36000.

Neutral sugar and glucosamine contents were larger than those of a trypsin inhibitor separated from the quail ovomucoid by authors (1984). Although quail OMLG strongly inhibited bovine trypsin, it did not inhibit bovine chymotrypsin, bacterial and fungal proteinases. Antitryptic activity of OMLG was approximately 1.7 times as much as that of a trypsin inhibitor from the quail ovomucoid.

Antitryptic activity of OMLG upon being heating in aqueous solution was decreased with the rising of temperature and the prolongation of heating time, without bringing about coagulation or turbidity. Heating for 50 min at 90°C gave the decreasing-rate of 35%.

Antitryptic activity of OMLG was completely lost upon being heated for 30 min, at pH 8.0, 90°C, accompanying with a blue shift in absorbance. On the contrary, the activity of OMLG remained unchanged upon being heated for 30 min, at pH 6.0, 90°C. Antitryptic activity was entirely lost by being reduced with mercaptoethanol. From the previous (1984) and present studies, authors propose that at least two kinds of glycoproteins inhibiting only trypsin exist in the quail egg white.

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