STUDIES ON GOOSE EGG-WHITE LYSOZYME (1) Purification and Fragmentation by Cyanogen Bromide

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STUDIES ON GOOSE EGG-WHITE LYSOZYME

(1) Purification and Fragmentation by Cyanogen Bromide

By

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Summary

Goose (*Cygnopsis cygnoides*) egg-white lysozyme [EC3.2.1.17] was isolated and subjected to cleavage by cyanogen bromide in 70% formic acid. The lysozyme was cleaved to three fragments, designated FI, FII, and FIII. One (FIII) of the fragments was obtained in the pure state by gel-filtration. Following 25 amino acid were contained in one molecule of the fragment FIII; Asx_5, Thr_2, Glx_2, Gly_2, Ala_3, Val_2, Ile_1, Tyr_3, Lys_1, His_2, Arg_1, and $H\cdot Ser_1$.

Introduction

The primary structures of several avian egg-white lysozymes — hen (*1*, *2*), Japanese quail (*3*), duck-II (*4*), turkey (*5*), and Bobwhite quail (*6*) — have been so far determined. Hen lysozyme is one of a few enzymes whose primary and three dimensional structure have been completely determined by the chemical and the X-ray crystallographic analyses. It would be expected that the structural study on lysozyme from other species provides further information concerning the relationship of structure to function, and also the chemical basis of evolutionary changes in this enzyme.

The studies on avian egg-white lysozymes (*7*, *8*, *9*) suggest that the primary structure of goose lysozyme may be radically different from the known avian lysozymes. This lysozyme is very labile at higher temperatures, even at acidic pH (*10*).

In the present work, goose egg-white lysozyme has been isolated under conditions milder than the method of Canfield et al. (*11*) in a chromatographic techniques, and in order to obtain the informations of the primary structure two methionine residues conteined in lysozyme have been allowed to cleave by cyanogen bromide.

Materials and Methods

Egg — Goose eggs (*Cygnopsis cygnoides*) were bought from Takeyama's Avian Farm, Kagoshima city. All eggs were refrigerated within 24 hours after laying.

BrCN — Cyanogen bromide was obtained from Nakarai Chemicals Ltd., Kyoto and used without further treatment. The reagent was stored at 5°C when not use.

Gel-filtration — Bic-Gel P–10 (100–200 mesh, BIO·RAD) was used. The dry gel was allowed to divide into two groups and to swell in eluents, respectively (0.1 M NaCl and 1.0 M acetic acid). The column operations were performed at room temperature.

Lysozyme activity — Lysis of *Micrococcus lysodeikticus* was measured according to the procedure of Jollés (*12*).

Cleavage with BrCN — Native goose lysozyme (30 mg) was dissolved in 5 ml of 70% formic acid and 40 mg of BrCN were added to the solution. The mixture was incubated at 30°C for 24 hours, then diluted with 10 volumes of water, and lyophilized directly. The dried material was resuspended in water and lyophilized in order to ensure complete removal of BrCN.

Amino acid analysis — The lysozyme was hydrolyzed with distilled hydrochloric acid in a carefully evacuated sealed tube at 110°C for 24 hours. The hydrochloric acid in the hydrolysate was removed over potassium hydroxide pellets *in vacuo*. The residue was dissolved in 1.1 ml of 0.2 M citrate buffer of pH 2.2. An aliquot (0.5 ml) of the solution was analyzed by a Hitachi Model 034 analyzer according to the direction of Moore et al. (*13*, *14*).

Results and Discussion

Isolation of Goose Egg-White Lysozyme — The egg-white were separated from the yolk and homogenized with to be suppressing froth, 0.7 liter of the homogenized egg-white were obtained from ten eggs and diluted with 0.02 M phosphate buffer of pH 7.0* to 3 liter of final volume. The solution was adjusted to pH 4.5 with 30% acetic acid and stirred quietly at room temperature for a few minutes. After standing over ten minutes, the resulting aggregated precipitate was filtered off with gauze. The filtrate was again returned to about pH 7 with 2.5 N NaOH and added to CM-cellulose (wet weight 150 g) which was pre-equilibrated to pH 7.0, after stirring at 5°C for a few hours. The CM-cellulose was filtered off using a Büchner's funnel and washed sufficiently with the buffer. After two times repeating batch wise absorption, the combined CM-cellulose was packed to a column (6.5×40 cm). Elution was carried out by stepwise elution. The elution pattern is shown in Fig. 1. The lysozyme activity was only found in the last peak and the fraction of the peak were pooled, to the fractions eluted was added $(NH_4)_2SO_4$ to saturation. After being kept overnight at 5°C, the resulting precipitate was collected by centrifugation, then the precipitate was dialyzed** against 0.2 M NaCl in the buffer overnight and dialysate was centrifuged to remove the insoluble materials. The supernatant was applied to the top of a column of CM-cellulose. The chromatography was carried out with a linear gradient of NaCl molality changed from 0.2 to 0.5 M in the buffer. The elution pattern is shown in Fig. 2. The lysozyme fractions were pooled. To this eluate was added $(NH_4)_2SO_4$ to 0.8 saturation. After being kept overnight at 5°C, the resulting precipitate was collected by centrifugation. The precipitate was dialyzed against distilled water and lyophilized. The dried material was dissolved in 0.1 M NaCl and applied to a Bio-Gel P–10 column equilibrated with the 0.1 M NaCl. The results are

* This buffer was used throughout these experiments with various indicated modifications in the NaCl concentration.

** The dialyzing tube was previously boiled in 10 % $NaHCO_3$ for 10 minutes.

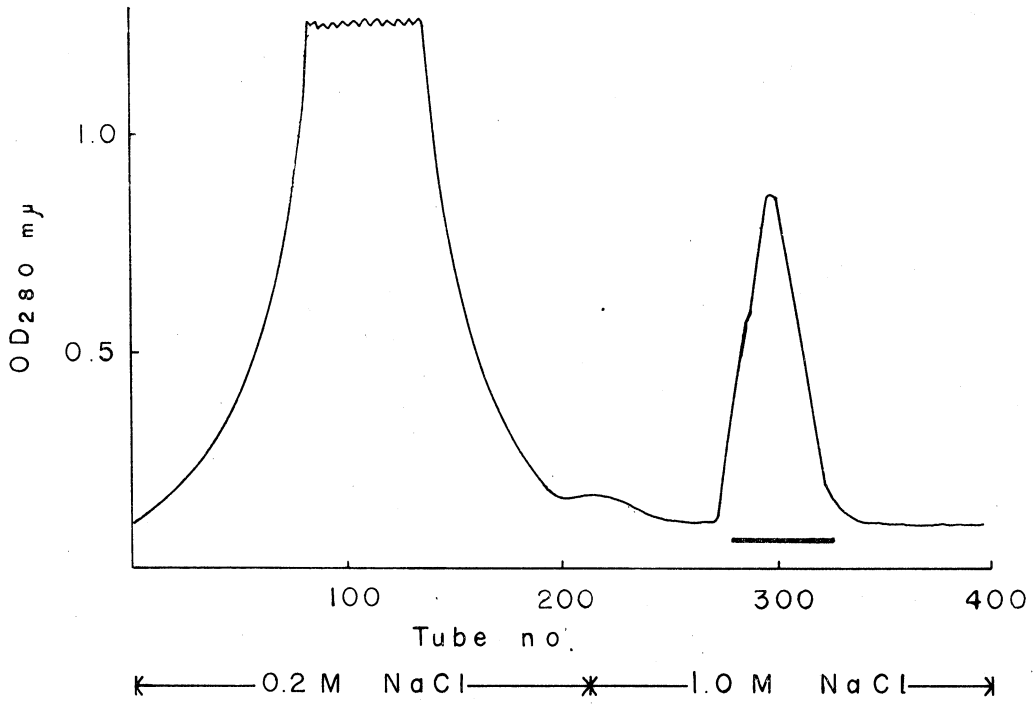

Fig. 1. Fractionation of goose egg-white proteins on CM-cellulose.

The column (6.5×40 cm) was washed with 0.2 M NaCl, after the first large peak was eluted, the column was strriped with 1.0 M NaCl all containing 0.02 M phosphate buffer of pH 7.0 at room temperature. Fractions of 15 ml were collected. No lysozyme activity was found in the first peak. Lysozyme fractions were pooled as indicated by the solid bar.

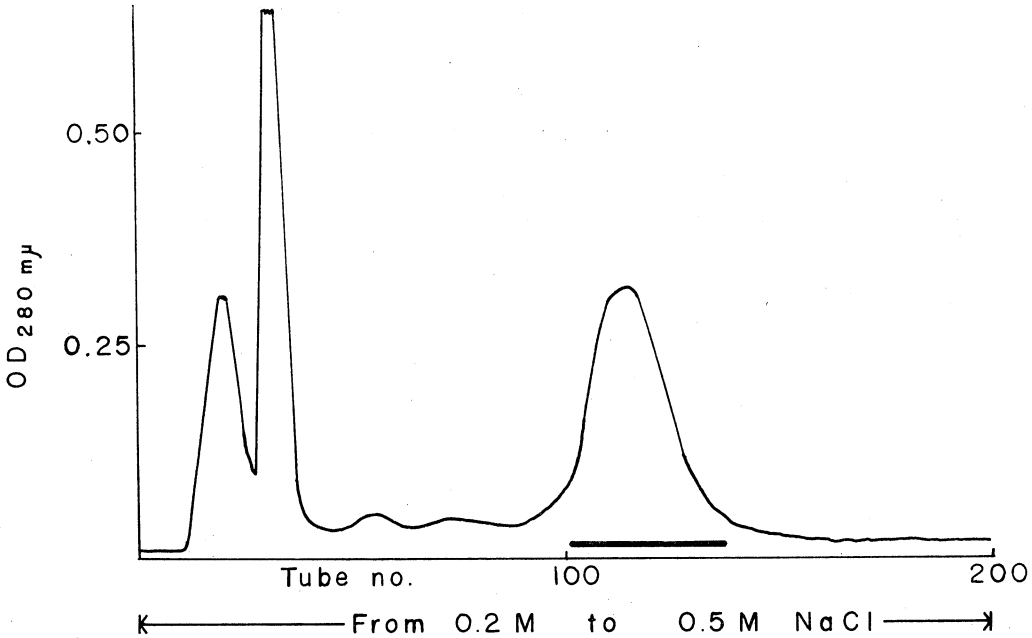

Fig. 2. Fractionation of crude goose lysozyme on a 3.5×50 cm column of CM-cellulose.

The column was developed with a linear gradient of NaCl molarity changed from 0.2 to 0.5 M in 0.02 M phosphate buffer pH 7.0 at room temperature. Fractions of 15 ml were collected. No lysozyme activity was found in the first two peaks. Lysozyme fractions were pooled as indicated by the solid bar.

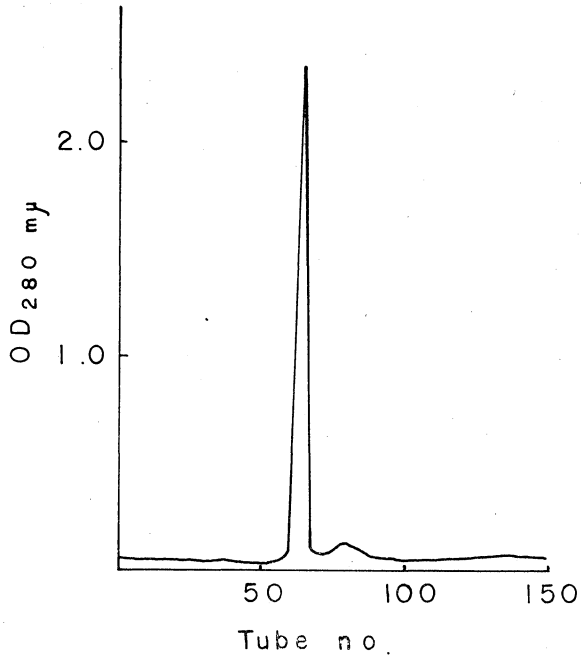

Fig. 3. Gel-filtration of goose lysozyme on a 2.5×90 cm column of Bio-Gel P-10.
The lysozyme was eluted with 0.1 M NaCl at a flow rate 60 ml per hour. Fractions of 6.3 ml were collected.

shown in Fig. 3. The lysozyme activity of the last minor peak was not found. The lysozyme fractions were pooled and lyophilized after dialysis against distilled water. About 25 mg of goose egg-white lysozyme were obtained. The lysozyme showed to be composed of homogeneous and the migration pattern similar to hen lysozyme on electrophoresis of polyacrylamide gel at pH 4.

Amino Acid Composition of Goose Lysozyme — The analytical results for the acid hydrolysate are summerized in Table I. The results are expressed as residue per molecule based the value of tyrosine as 6.0. Comparison of the amino acid composition of goose lysozyme determined in the present studies and in other laboratories is shown. In general, fairly good agreement can be seen.

Characterization of Fragments — The separation of the BrCN fragments of goose lysozyme by gel-filtration on Bio-Gel P-10 is shown in Fig. 4. Three distinct components were detected by two measurements with both absorbance at 280 mμ and ninhydrin method. The three fragments were designated F_I, F_{II}, and F_{III} corresponding to their order of elution. The fractions were pooled according to the solid bars.

Fraction (F_I+F_{II}): This fraction was taken to dryness by lyophilization and then dissolved in 1.0 M acetic acid. But clear solution could not be obtained by a usual method, therefore, attempts to separate these fragments were unsuccessful.

Fragment F_{III}: Pure material was obtained from the fraction. Amino acid composition of F_{III} is shown in Table II. The total number of amino acid in F_{III} has been caluculated to be 25. In comparison with whole molecule, it is observed that the fragment F_{III} lack the residue of serine, proline, cysteine, leucine, phenylalanine, and

Table I. Amino acid composition of goose egg-white lysozyme.

Amino acid	Moles per mole protein	Assumed no. of residue	(15) Jollés	(11) Canfield
Aspartic acid	14.8	15	16−17	13−14
Threonine	7.7	8-9	10−11	8−9
Serine	5.5	6-7	8	6−7
Glutamic acid	10.2	10	10−11	10
Proline	2.5	2-3	3	2−3
Glycine	14.3	14	14−15	14
Alanine	10.1	10	10±1	10
Cystine	nd		4	3−4
Valine	7.3	7	6−7	7
Methionine	2.1	2	2	2
Isoleucine	8.6	8-9	7−8	9
Leucine	4.8	5	5−6	4−5
Tyrosine	6.0	6	5−6	6
Phenylalanine	2.0	2	2	2
Tryptophan	nd		2	2−3
Lysine	10.6	10-11	10	11
Histidine	2.7	3	3	3−4
Arginine	5.8	6	7±1	6−7
Total			129±5	118−127

No correction is made for the values of threonine and serine for their decompositions during acid hydrolysis.

nd: not determined.

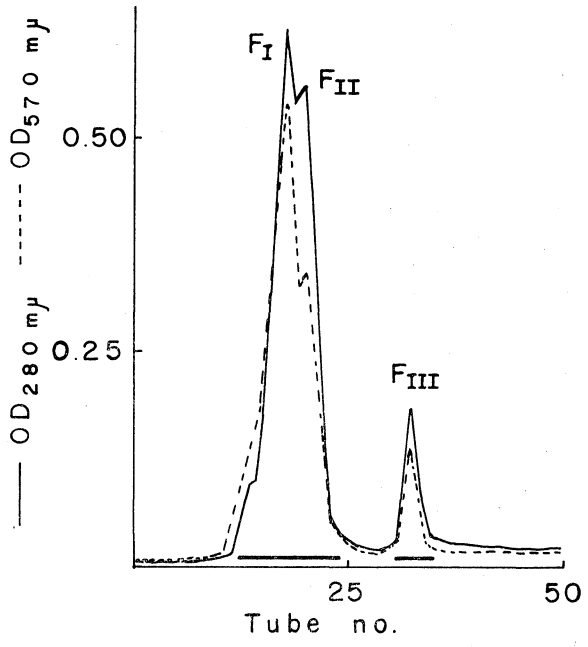

Fig. 4. Gel-filtration of the products by the action of BrCN on goose lysozyme.

30 mg of the products applied to a Bio-Gel P–10 column (1.5×85 cm) equilibrated with 1.0 M acetic acid. The products were eluted with 1.0 M acetic acid at a flow rate 20 ml per hour. Fractions of 5.1 ml were collected and monitored by measurement the absorbancy at 280 mμ and 570 mμ for ninhydrin after alkaline hydrolysis.

Table II. Amino acid composition of fragment FIII.

Amino acid	Moles per mole peptide	Assumed no. of residue	Amino acid	Moles per mole peptide	Assumed no. of residue
Aspartic acid	5.2	5	Leucine		0
Threonine	1.9	2	Tyrosine	2.9	3
Serine		0	Phenylalanine		0
Glutamic acid	2.1	2	Tryptophan		0[a]
Proline		0	Lysine	0.8	1
Glycine	2.2	2	Histidine	1.9	2
Alanine	3.3	3	Arginine	0.7	1
Cystine		0	Homoserine +lactone	0.3	1
Valine	1.8	2			
Isoleucine	1.0	1	Total		25

a: by alkaline spectra.

tryptophan. Fragment F_{III} must be the N-terminal or the middle portion of the whole molecule because it contains homoserine and its lactone as the constituent. By cyanogen bromide treatment, goose lysozyme was cleaved to three fragments. This phenomenon has not been observed in the known avian lysozyme. Consequently, it is deduced that the disulfide bridge (16) contained in the molecule is partial to one or two of the fragments.

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