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AMINO ACID SEQUENCE OF JAPANESE QUAIL EGG-WHITE LYSOZYME

1. CHARACTERIZATION OF THE FRAGMENTS OBTAINED BY CLEAVAGE WITH CYANOGEN BROMIDE

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Summary

Amino acid analysis of lysozyme [EC 3.2.1.17] from Japanese quail (*Coturnix coturnix*) egg-white indicated that the numbers of 129 amino acid residues are identical with that of hen egg-white lysozyme, but following 8 residures are different from hen lysozyme; Lys_{+1}, His_{+1}, Arg_{-1}, Asx_{-1}, Gly_{-1}, Val_{+1}, Tyr_{+1}, and Phe_{-1}.

Quail lysozyme has been subjected to cleavage by cyanogen bromide in 70% formic acid and followed by reduction and S-carboxymethylation. Three peptide fragments, designated CNBr-I, II, and III, have been obtained by gel filtration on Bio-Gel P–10. Amino acid analyses of the fragments indicated that CNBr-I, CNBr-II, and CNBr-III are composed of 12, 93, and 24 amino acid residues, respectively.

The amino acid sequences from the amino terminal region of these fragments and the reduced and alkylated whole protein were established by the Edman degradation technique.

The primary structures of several avian egg-white lysozyme have been so far determined. Hen egg-white lysozyme is one of a few enzymes whose primary and three dimensional structure have been completely determined by the chemical (*1–3*) and the X-ray crystallographic (*4–7*) 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.

Previously, we have reported a tenative amino acid sequence of Japanese quail egg-white lysozyme (*8*). The present paper reports in detail the preparation of the fragments obtained by cleavage with cyanogen bromide and the amino acid sequences of amino terminal region of these fragments.

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Abbreviation: CM, carboxymethyl.

Materials and Methods

Quail eggs (*Coturnix coturnix*) were obtained from the Orita's Quail Farm, Kagoshima. Cyanogen bromide, trifluoroacetic acid and phenyl isothiocyanate were obtained from Nakarai Chemicals, Ltd., Kyoto. β-Mercaptoethanol was purchased from Wako Pure Chemical Industries, Ltd., Osaka. Bio-Gel P–10 (100–200 mesh) was a product from Bio-Rad Laboratories.

Assay of Enzymatic Activity – The activity of lysozyme was measured by the method of Jollés by use of *Micrococcus lysodeikticus* as a substrate (*9*).

Amino Acid Analyses — Reduction and S-carboxymethylation of lysozyme was performed by the procedure of Crestifield et al. (*10*). Acid hydrolysis of lysozyme was carried out by the method of Moore and Stein (*11*, *12*). For the determination of tryptophan, lysozyme was hydrolyzed with 4N barium hydroxide in a evacuated sealed tube at 110° for 48 hours (*13*). Amino acid analyses were performed with a use of the Beckman/Spinco Model MS amino acid analyzer.

Cyanogen Bromide Cleavage — Approximately 300 mg of purified quail lysozyme was dissolved in 30 ml of 70% formic acid. Cyanogen bromide (300 mg) was added and the mixture was allowed to stand at 27° overnight in the dark. The reaction was terminated by the addition of 5 volumes of water/volume of the reaction mixture and the solution was lyophilized. Reduction and S-carboxymethylation of the cyanogen bromide treated lysozyme were carried out by the method of Crestfield et al. (*10*).

Isolation of Fragments — After the termination of the alkylation, the reaction mixture was immediately placed on the top of a Bio-Gel P–2 column (2×100 cm) equilibrated with 1.0 M acetic acid in the dark. Eight ml of fractions were collected and the absorbancy at 280 nm was measured for each tube and 0.5 ml aliquots from every tube were analyzed by the ninhydrin method after alkaline hydrolysis. All of the protein appeared as a single peak at the column void volume. The appropriate fractions were pooled and lyophilized. In order to separate the individual peptide fragments, 100 mg of lyophilized material was dissolved in a small volume of 1.0 M formic acid and applied to a column (2×100 cm) of Bio-Gel P–10 equilibrated with 1.0 M formic acid. Five ml fractions were collected and detected by measuring in the above ninhydrin method. The fractions containing the peptide fragments were pooled and lyophilized, respectively.

Sequencial Analysis — The N-terminal groups were determined by the Edman degradation technique (*14*). The identification of PTH-amino acid was made by silica-gel thin-layer chromatography.

Results and Discussion

Preparation of Quail Egg-White Lysozyme — Homoginized egg-white (800 ml) from 2 to 4 day-old eggs was diluted with an equal volume of deionized water, and brought to 0.1 M NaCl solution adding solid sodium chloride and adjusted to pH

4.0–4.5 with 1 N HCl. After standing 4° overnight, the precipitate was centrifuged and discarded. To the supernatant readjusted to pH 6.5–7.0 was added CM-cellulose (wet weight, 200 g) which was previously activated and adjusted to pH 7.0. After stirring at room temperature for a few hours, CM-cellulose was filtered off using a glass filter and washed with 0.1 M NaCl solution sufficiently. Lysozyme was eluted twice with 300 ml of 0.8 M NaCl solution and the combined eluate was dialyzed against deionized water. The dialyzate was centrifuged to remove insoluble materials, and lyophilized. Approximately 1.1 g of the crude enzyme was obtained.

One g of the lyophilized crude enzyme was dissolved in 40 ml of 2.5% NaCl solution at room temperature. The solution was adjusted to approximately pH 4 with 0.5 N HCl and again returned to pH 8.3 with 0.5 N NaOH and centrifuged to remove insoluble material formed. The clear solution was kept at about 8°, after about three days the hexagonal crystals of lysozyme appeared. After two times recrystallization under the same condition, about 200 mg of lyophilized quail lysozyme was obtained. The crystallized quail lysozyme showed a single symmetrical boundary with $s_{20,w}$ value of 1.67 on the ultracentrifugation analysis and the high lytic activity against *Micrococcus lysodeikticus* cells. These orders are similar to those of the hen egg-white lysozyme.

Amino Acid Composition of Quail Lysozyme— The analytical results for the acid and alkaline hydrolysate are summerized in Table I. The results are expressed

Table 1. Amino acid composition of quail egg-white lysozyme.

Amino Acid	Found	Integral	Differernce from Hen(c)
Lysine	6.8	7	+1
Histidine	1.8	2	+1
Arginine	9.7	10	−1
CM-Cysteine	8.0	8	
Aspartic acid	20.3	20	−1
Threonine	6.7	7	
Serine	8.8	10(b)	
Glutamic acid	4.7	5	
proline	2.1	2	
Glycine	10.8	11	−1
Alanine	11.8	12	
Valine	6.7	7	+1
Methionine	1.9	2	
Isoleucine	5.7	6	
Leucine	8.0	8	
Tyrosine	4.1	4	+1
Phenylalanine	2.0	2	−1
Tryptophan	5.9(a)	6	
Total		129	0

(a) Tryptophan was determined on alkaline hydrolysate (*13*).
(b) This value was corrected by as described in the text.
(c) The contents of hen egg-white lysozyme were used the value according to Canfield (*2*).

as residue per molecule based a value of leucine as 8.0. The value of serine residue was corrected for decompotion during hydrolysis by the citation of the factor obtained from the hen lysozyme. Total amino acid residues of quail lysozyme (129) is identical with that of the hen lysozyme, but quail lysozyme contains one more residue each of valine, tyrosine, histidine, and lysine, and one less residue of aspartic (or asparagine), glycine, phenylalanine, and arginine than hen lysozyme.

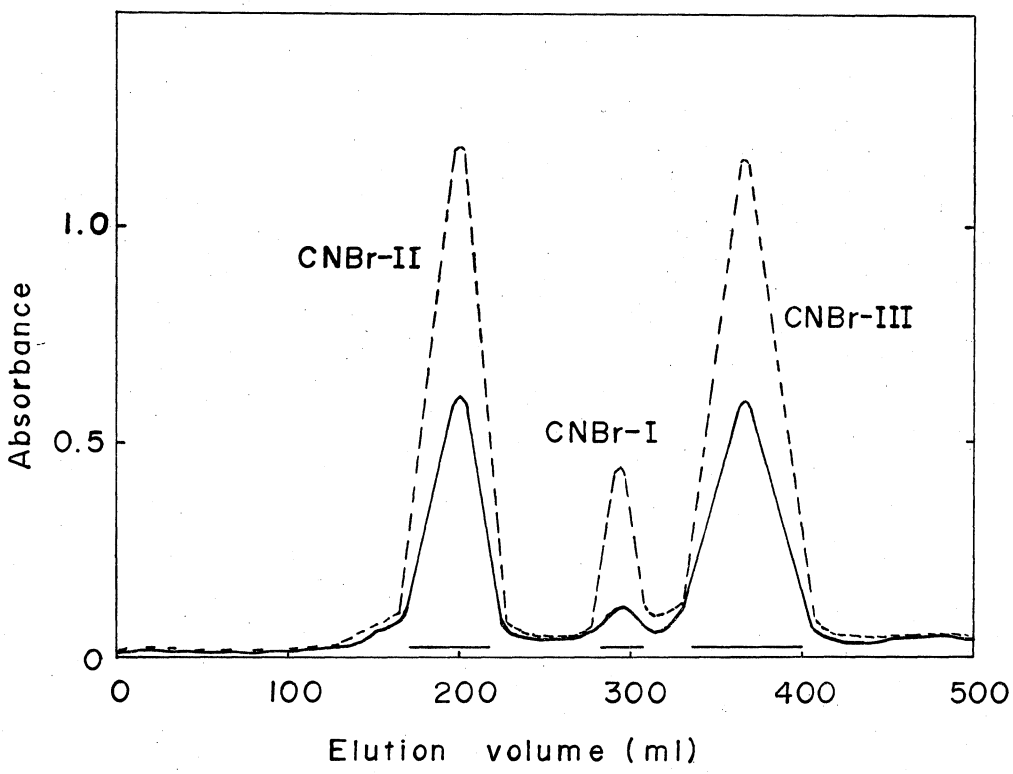

Fig. 1. Gel filtration of fragments produced by reduction and S-carboxymethylation of cyanogen bromide treated quail lysozyme.
Fragment mixture (100 mg) was applied to a column (2.0×100 cm) of Bio-Gel P–10 and chromatographed with 1.0 M formic acid. The flow rate was approximately 40 ml/hour and 5 ml fractions were collected. Absorbancy of the fractions was measured at 280 nm (——) and aliquots of each fraction were tested for ninhydrin after alkaline hydrolysis at 570 nm (- - - -). The roman numerals I, II, and III represent the three fragments obtained, which were numbered from the N-terminal end of the quail lysozyme molecule.

Characterization of Fragments— The separation of the three CNBr-fragments of quail lysozyme by gel-filtration on Bio-Gel P–10 is shown in Fig. 1. In accordance to expectation three distinct components were detected by two measurements with both absorbance at 280 nm and ninhydrin method. The amino acid composition of three components are expressed on the basis of leucine residue/molecule (CNBr-I: 1, CNBr–II:6, CNBr-III:1).

The comparison of the amino acid compositions of the three components with that of hen lysozyme indicated that the first peak was corresponding to the middle

polypeptide of hen lysozyme (residues, 13–105), the second, the N-terminal dodecapeptide, and the last peak, the carboxy-terminal peptide (residues, 106–129) with a small amount of substitution, respectively. Three peptide fragments have been designated in the corresponding to that of hen lysozyme, CNBr-II, CNBr-I, and CNBr-III in order of the elution. The amino acid compositions of the three fragments are shown in Table II. The total amino acid residues of three peptides except undetermined tryptophan is compatible with that of the whole molecule. CNBr-I and II contained detectable quantities of homoserine and its lactone.

Table II. Amino acid composition of CNBr-fragments.

Amino Acid	CNBr-I		CNBr-II		CNBr-III		Total Fragments	Quail Lysozyme
	Found	Different from Hen	Found	Different from Hen	Found	Different from Hen		
Lysine	0.8(1)		5.4(5)	+1	1.0(1)		7	7
Histidine			1.8(2)	+1			2	2
H·Serine H·Serine. L	1.1(1)		1.2(1)				2	2
Arginine	0.9(1)		5.5(5)	−1	3.5(4)		10	10
CM-Cysteine	0.9(1)		5.2(5)		2.2(2)		8	8
Aspartic acid			16.0(16)	−2	3.4(4)	+1	20	20
Threonine			5.8(6)		0.9(1)		7	7
Serine			9.1(10)				10	10
Glutamic acid	1.0(1)		3.9(4)	+1		−1	5	5
Proline			2.3(2)				2	2
Glycine	1.0(1)		8.1(8)	−1	2.0(2)		11	11
Alanine	2.7(3)		6.3(6)		2.9(3)		12	12
Valine	0.9(1)		3.4(4)	+1	1.8(2)		7	7
Isoleucine			4.6(5)		0.8(1)		6	6
Leucine	1.0(1)		6.0(6)		1.0(1)		8	8
Tyrosine	0.8(1)	+1	3.0(3)				4	4
Phenylalanine		−1	2.0(2)				2	2
Tryptophan (a)			(3)		(3)		6	6
Total	12		93		24		129	129

(a) Taken from the accompanying paper (M. Kaneda et al.)

Sequence Analyses

S-CM-Quail Lysozyme, Lys-Val-Tyr-Gly-Arg-CM·Cys-Glu-Leu···

Five mg of the S-CM-quail lysozyme was used for step wise degradation. During 8 consecutive degradations, one main amino acid derivative was released in each cycle. The average repetitive yield, during the first 4 steps, was 80%, thereafter the yield decreased. Eight residues were determined.

CNBr-I, Lys-Val-Tyr-Gly-Arg-CM·Cys-Glu-Leu (Ala_3, H·Ser)

CNBr-I fragment (0.48 μmole) was submitted to sequence analysis. The average yield in each degradation cycle during the first 4 steps was 85%, thereafter a greater decrease in yield was observed. The first 8 residues were determined and identical to the sequence of the N-terminal of the S-CM-quail lysozyme, while CNBr-I must occupy the N-terminal portion on the whole protein. Alignment of the fragments is as

follows, CNBr-I-II-III. After the 8th step of the degradation of CNBr-I, the amino acid residues of remaining peptide were presumed to be 3 residues of alanine and one of homoserine or its lactone from the amino acid composition of this fragment, therefore, the amino acid sequence of the remaining peptide could be given as follows, Ala-Ala-Ala-Met.

CNBr-II, Lys-Arg-His-Gly-Leu-Asp-Lys-Tyr-Gln-Gly-Tyr-Ser-Leu-Gly · · ·

Five mg of the CNBr-II fragment was used and 14 consecutive residues were obtained. The average repetitive yield in the first 7 degradation cycles was 90%, thereafter a greater decrease in yield was observed.

CNBr-III, Asn-Ala-Trp-Val-Ala-Trp-Arg-Asn-Arg-CM·Cys-Lys-Gly-Thr · · ·

The CNBr-III fragment (0.63 μmole) was used. The average repetitive yield in the first 7 degradation cycles was 85%, thereafter a greater decrease in yield was observed. Thirteen residues were determined. Thus, the two parts of amino acid sequence of quail lysozyme were elucidated, one of the part occupy from the N-terminal to 26th residue and the other from 105th to 118th residue as shown in Fig. 2.

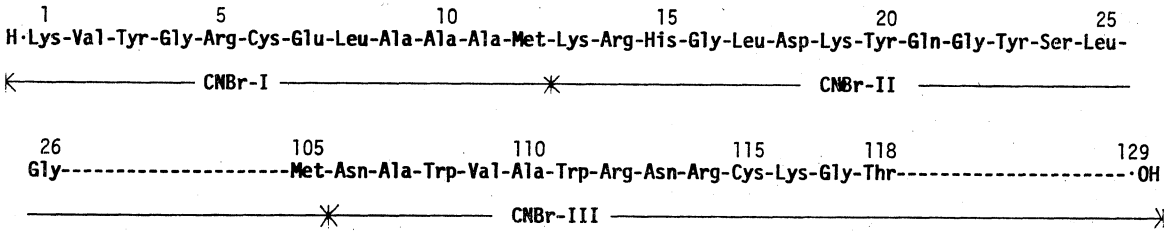

Fig. 2. Tentative alignment of CNBr-fragments of quail lysozyme.

In comparison with hen lysozyme, the following three interchanges have been so far established, namely, the third phenylalanine residue of hen lysozyme substituted for tyrosine residue, 19th asparagine substituted for lysine, and 21st arginine for glutamine, respectively.

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