AMINO ACID SEQUENCE OF JAPANESE QUAIL EGG-WHITE LYSOZYME 2. TRYPTIC AND CHYMOTRYPTIC PEPTIDES OF THE CYANOGEN BROMIDE FRAGMENTS

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AMINO ACID SEQUENCE OF JAPANESE QUAIL EGG-WHITE LYSOZYME

2. TRYPTIC AND CHYMOTRYPTIC PEPTIDES OF THE CYANOGEN BROMIDE FRAGMENTS

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Summary

The three fragments (CNBr-I, II, and III) obtained by cleavage with cyanogen bromide from Japanese quail egg-white lysozyme [EC.3.2.1.17] were subjected to proteolytic hydrolysis. CNBr-I was digested with chymotrypsin, CNBr-II with trypsin and chymotrypsin, and CNBr-III with trypsin. The resulting peptides were isolated by the uses of a ion exchange chromatography on Dowex 1–X2 or Dowex 50–X2 and a gel filtration on Bio-Gel P–10. The amino acid sequence of the peptides was determined by Edman degradation. The six amino acid interchanges between hen and Japanese quail lysozyme are established to be tyrosine for phenylalanine at residue 3, lysine for asparagine at 19, glutamine for arginine at 21, valine for glycine at 102, histidine for aspartic acid at 103, and asparagine for glutamine at 121.

In the preceding paper (1), three fragments were obtained from quail lysozyme by cleavage with cyanogen bromide. The amino acid sequence of the N-terminal region of these fragments was detected by Edman degradation technique. The present paper reports in detail that further sequence analyses of three fragments were advanced by the isolation and characterization of tryptic and/or chymotryptic peptides of the fragments.

Materials and Methods

Lysozyme was isolated from egg-white of Japanese quail (*Coturnix coturnix*) by means of the described method in the preceding paper (*1*). Fragments of CNBr-I, II, and III were prepared by cyanogen bromide cleavage and purified as described in the preceding paper (*1*). Trypsin and chymotrypsin were products from Worthington Biochemical Corporation. 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

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P-10 (100–200 mesh) was a product from Bio-Rad Laboratories. Dowex 50–X2 and 1–X2 were products from DOW Chemical Corporation.

Hydrolyses of Fragments with Trypsin and Chymotrypsin — CNBr-I (10 mg/ml) was dissolved in 1% NH_4HCO_3 and digested with chymotrypsin (5% of weight of CNBr-I) at 37° for 4 hr. CNBr-II and III were dissolved in 1% NH_4HCO_3 (10 mg/ml) and digested with trypsin at 37° for 4 hr in the substrate to the enzyme ratio of 50:1 and 15:1, respectively.

CNBr-II was dissolved in water and the solution was adjusted to pH 8.0 with 1 N NH_4OH. Chymotrypsin was added twice in a final concentration of the substrate to the enzyme ratio of 50:1 by weight. The reaction was carried out at 25° for 6 hr. The pH of the reaction mixture was held constant with the addition of 0.1 N NH_4OH using a pH stat (Radiometer TTT1, Copenhagen). After 4 hours all hydrolysates were freezed-dried.

Chromatographic Fractionation of Peptides — Chymotryptic peptides from CNBr-I were fractionated on a column (0.9×50 cm) of Dowex 50–X2. The column was equilibrated with 0.1 M pyridine-formic acid buffer of pH 3.1. The column was run at 42°. Elution was carried out at a flow rate of 20 ml/hr with linear gradient composed of 150 ml of 0.1 M pyridine-formic acid buffer, pH 3.1, and 150 ml of 2.0 M pyridine-acetic acid buffer, pH 5.0. The column was stripped with 4.0 M pyridine-acetic acid buffer of pH 5.0. Fractions of 3.0 ml were collected.

Chymotryptic peptides from CNBr-II were fractionated on a column (0.9×150 cm) of Dowex 1–X2. The peptides on the column were developed with the starting solvent of 3% pyridine at flow rate of 30 ml/hr at 26°. Exponential gradient system was applied for elution to obtain sharp separation of the peptides. In chromatography, after 0.1 effluent liter was collected, a reservoir which contained the second solvent of 0.05 M acetic acid was connected to a mixing chamber filled with 0.3 liter of 3% pyridine. The content of the reservoir was substituted for the solvents of 0.1, 0.5, 2.0, and 8.0 M of acetic acid at 0.15, 0.15, 0.10, and 0.15 effluent liters, respectively. The column was stripped with 8.0 M acetic acid. The eluent was collected in 2.0 ml fractions.

Tryptic peptides from CNBr-II were fractionated on a column (0.9×150 cm) of Dowex 1–X2 at 26°. The column was equilibrated with 3% pyridine and elution was carried out at flow rate 45 ml/hr with two gradient system. In the first system three cylindrical vessels of an equal diameter were connected in series, in a mixing vessel and the first reservoir were placed 200 milliliters of 3% pyridine, respectively, and in the last reservoir was placed 200 ml of 0.2 M acetic acid. In the second system two vessels were connected in series, 200 ml of 0.2 M acetic acid was placed in a mixing vessel and 200 ml of 8.0 M acetic acid was added from a reservoir. Fractions of 3.0 ml were collected.

Tryptic peptides from CNBr-III were fractionated on a column (0.8×55 cm) of Dowex 1–X2 at 26°. Elution was carried out at flow rate 20 ml/hr with two

gradient system. In the first system three cylindrical vessels of an equal diameter were connected in series, in a mixing vessel was placed 120 ml of 3% pyridine, in the first reservoir was placed 120 ml of deionized water and in the last reservoir was placed 120 ml of 0.5 M pyridine acetate buffer of pH 5.0. In the second system two vessels were connected in series, 100 ml of 0.5 M pyridine acetate buffer of pH 5.0 was placed in a mixing vessel and 100 ml of 8.0 M acetic acid was added from a reservoir.

All of the elution pattern was monitored by absorbance at 570 nm in an Autoanalyzer (Technicon Chromatography Corp.). The fractions were pooled and peptides were recovered after removal of the solvent by rotary evaporation or lyophilization. The isolated fractions were checked for purity by high-voltage paper electrophoresis at pH 3.65 or 6.5. The amino acid compositions of the peptides fractionated by column chromatography and the peptide fragments obtained by further splitting procedures were analyzed by an amino acid analyzer Beckman/Spinco Model MS after 24 hr hydrolysis as described in the preceding paper (*1*). The amino acid sequence analysis of peptides was performed by Edman degradation technique (*2*). In some cases the subtractive procedure (*3*) was employed.

Results and Discussion

Isolation of Chymotryptic Peptides from CNBr-I — The lyophilized digestion mixture of CNBr-I was fractionated on a column of Dowex 50–X2 with a linear gradient composed of pyridine-formic acid and pyridine-acetic acid. The separation

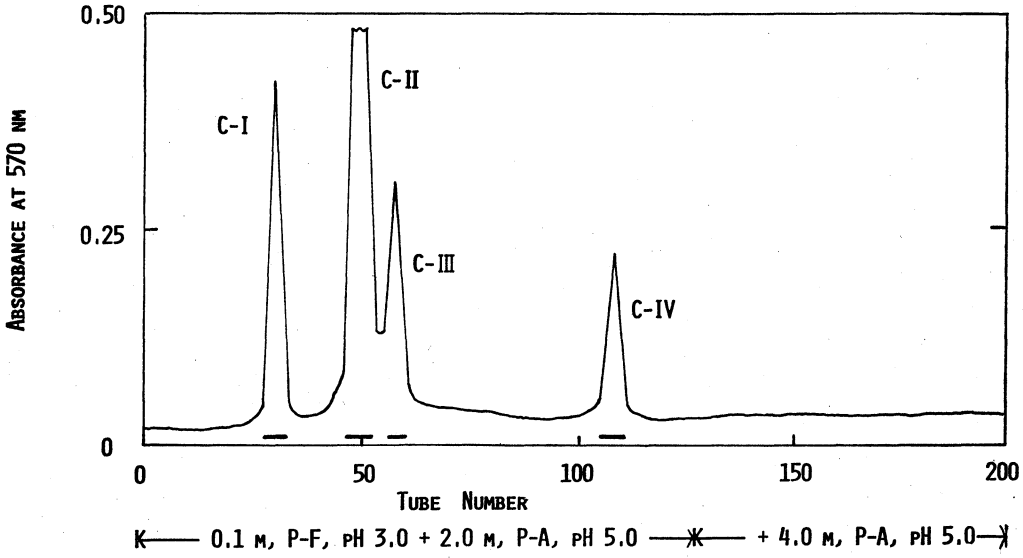

Fig. 1. Fractionation of the chymotryptic peptides of CNBr-I (4.3 μmoles) on a column (0.9× 50 cm) of Dowex 50–X2.
The elution of peptides was performed with pyridine-fromic acid(P-F) and Pyridine-acetic acid (P-A) buffers at 42° at a flow rate of 20 ml per hour. The details are described in the text. Fractions were pooled as indicated the solid bars.

Table I. Amino acid composition of chymotryptic peptides of CNBr-I.

Amino Acid	C–1	C–2	C–3
Lysine	1.02(1)		
Histidine			
Arginine		0.91(1)	
CM-Cysteine		1.00(1)	
Aspartic acid			
Threonine			
Serine			
Glutamic acid		0.97(1)	
Proline			
Glycine		0.95(1)	
Alanine			2.45(3)
Valine	1.00(1)		
Isoleucine			
Leucine		0.91(1)	
Tyrosine	1.01(1)		
Phenylalanine			
Homoserine and lactone			1.00(1)
Total	3	5	4
% yield	58	46	55
Fraction no.	C-IV	C-III	C-I

Values are given in residues per mole. The assumed integral values are indicated in parentheses.

pattern is shown in Fig. 1. Each fraction was pooled according to the solid bars and the peptides were recovered after rotary evaporation. Each fraction was checked for purity by high-voltage paper electrphoresis. The three pure chymotriptic peptides are listed in Table I. Pure peptides are designated by Arabic number in the order that they occur in the fragment, starting at the amino terminus, and fractions from the original Dowex 50–X2 column chromatography are designated by Roman numerals in order of elution. Fraction C-III and C-IV were subjected to a subtravtive Edman degradation.

Fraction C-I

Peptide C-3; (Ala, Ala, Ala, H-Ser)

This fraction contained a single pure peptide which was composed of three residues of alanine and one of homoserine and its lactone. Assuming that homoserine is the carboxy-terminal residue, this peptide was not submitted to sequence analysis.

Fraction C-III

Peptide C-2; Gly-Arg-CM·Cys (Glx, Leu)

This fraction contained a single pure peptide comprising 5 residues. The first three residues were obtained by subtractive Edman degradation.

1st Step: Arg, 1.03, CM·Cys, 1.00, Glu, 1.10, Gly, 0.02, Leu, 1.00

2nd Step: Arg, 0.01, CM·Cys, 0.88, Glu, 0.85, Gly, trace, Leu, 1.00

3rd Step: Arg, trace, CM·Cys, 0.11, Glu,1.00, Gly, trace, Leu, 1.00

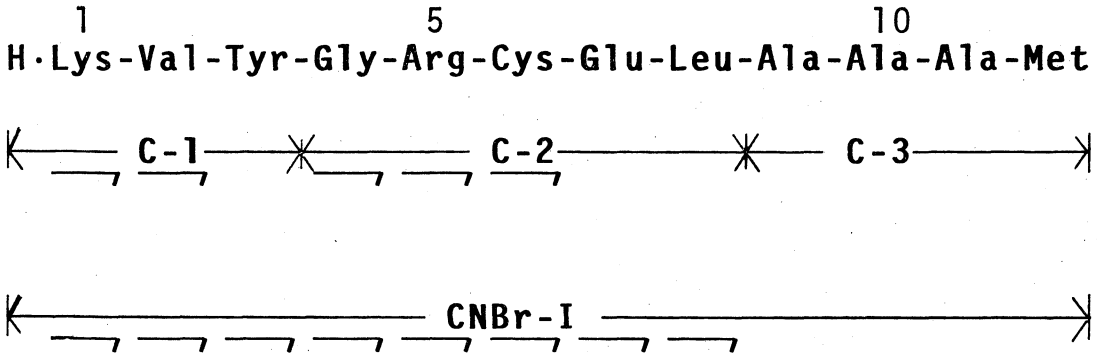

Fig. 2. Amino acid sequence of CNBr-I.
The arrow (⟶) denotes sequence determined by Edman procedure. Residue number corresponds to that of the whole molecule. The results of fragment CNBr-I is quoted from the preceding paper (*1*).

Fraction C-IV

Peptide C-1; Lys-Val-Tyr

This fraction also contained a single pure peptide. Two rounds of Edman degradation were sufficient to give the complete sequence of peptide.

1st Step: Lys, 0.09, Val. 0.84, Tyr, 1.00

2nd Step: Lys, 0.02, Val, 0.09, Tyr, 1.00

The tentative amino acid sequence of CNBr-I was presented in preceding paper (*1*), therefore, CNBr-I was not subjected to further proteolysis by other protease to obtain a overlap peptide. The amino acid sequence of CNBr-I is shown in Fig. 2.

Isolation of Tryptic Peptides from CNBr-II —The lyophilized digestion mixture of CNBr-II was fractionated on a column of Dowex 1–X2 with two gradient system.

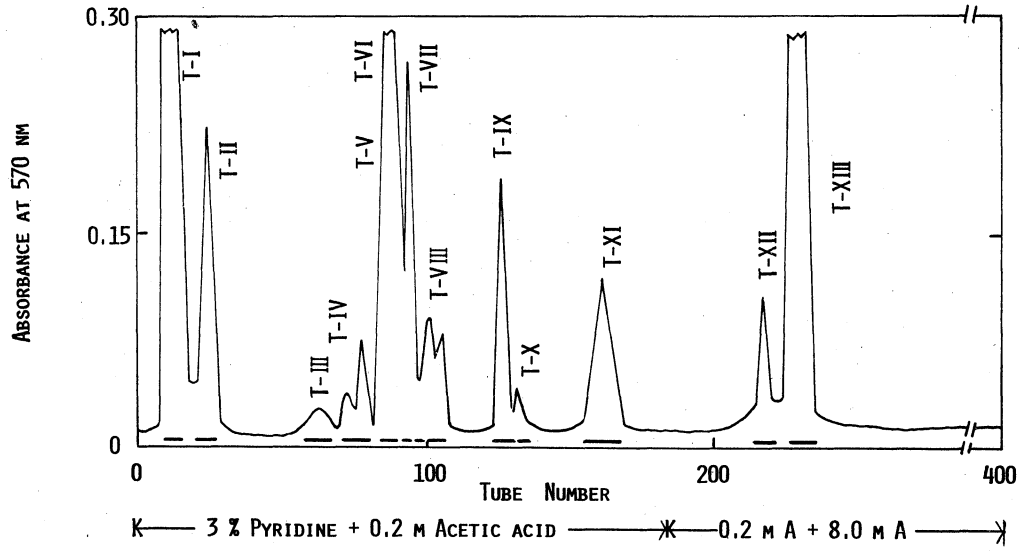

Fig. 3. Fractionation of the tryptic peptides of CNBr-II (6.6 μmoles) on a column (0.9×150cm) of Dowex 1–X2.
The details are described in the text.

Table II. Amino acid composition

Amino Acid	T–1	T–2	T–3	T–4	T–5	T–6
Lysine	1.00(1)	0.90(1)		1.04(1)		1.11(1)
Histidine				0.92(1)		
Arginine		1.00(1)	1.00(1)			
CM-Cysteine						1.13(1)
Aspartic acid				1.10(1)		0.91(1)
Threonine						
Serine						0.89(1)
Glutamic acid					0.98(1)	
Proline						
Glycine				1.00(1)	1.00(1)	1.00(1)
Alanine						2.38(2)
Valine						0.93(1)
Isoleucine						
Leucine				0.95(1)		1.09(1)
Tyrosine					1.82(2)	
Phenylalanine						
Tryptophan						0.54(1)
Homoserine and lactone						
Total	1	2	1	5	4	10
% yield	43	34	9	51	30	46
Fraction no.	T-I-2	T-I-8	T-I-3-1	T-V-2	T-XIII-2	T-XI

The pattern depicted in Fig. 3 was obtained. Each fraction was pooled according to the solid bars. The peptides were recovered after rotary evaporation and was examined for purity by high-voltage paper electrophoresis. The further purification of peptides from this chromatogram, in most cases, was necessary. The purification of impure pools were carried out by chromatography on columns of Dowex 50–X2 (or 1–X2) or Bio-Gel P–10. The amino acid composition of the pure peptides is summarized in Table II.

Fraction T-I

Fraction T-I was subfractionated on Dowex 50-X2. The separation pattern is

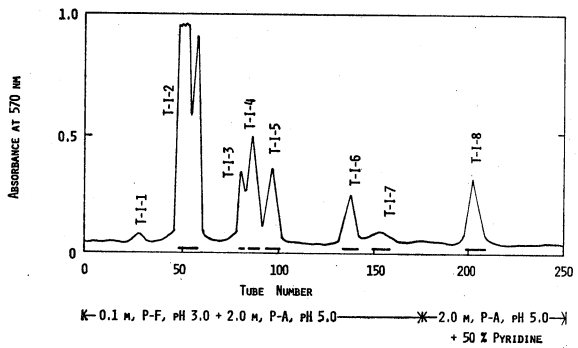

Fig. 4. Fractionation of CNBr-II-T-I on a column (0.9×50cm) of Dowex 50–X2. The chromatography was carried out at 42° at a flow rate of 25 ml per hour. Fractions of 2.5 ml were collected.

of the tryptic peptides of CNBr-II.

T–7	T–8	T–9	T–10	T–11	T–12	T–13	T–14
				0.96(1)	1.00(1)	0.65(1)	
						0.73(1)	0.93(1)
1.10(1)	1.00(1)	0.89(1)	0.92(1)				
		1.00(1)		3.20(3)			
2.85(3)	4.40(4)	2.00(2)		3.93(4)		1.00(1)	1.02(1)
1.80(2)	2.00(2)		0.86(1)	0.93(1)			
1.16(1)	2.20(2)		0.93(1)	3.60(4)		0.89(1)	0.88(1)
1.88(2)	1.00(1)						
			0.89(1)	0.96(1)			
	2.20(2)	1.00(1)	1.00(1)			1.00(1)	1.00(1)
1.00(1)				3.04(3)			
				1.00(1)		1.50(2)	1.22(2)
	1.80(2)			2.00(2)		0.65(1)	0.51(1)
	1.20(1)			3.00(3)			
	0.80(1)						
1.95(2)							
		0.83(2)					
						1.10(1)	0.93(1)
12	16	7	5	23	1	9	8
35	5	6	63	9	43	4	37
T-VII	T-XIII-5-4	T-XIII-5-5	T-I-3-2	T-X-III-5-3	T-I-2	T-IV-3	T-IX-2
	T-XII-1-1		T-I-4				T-IX-3

shown in Fig. 4.

Fraction T-I-2

Peptide T-1 and T-12; Lys

Only lysine was identified, after acid hydrolysis. Electrophoretic mobility at pH 3.7 was identical with that of authentic lysine.

Fraction T-I-3

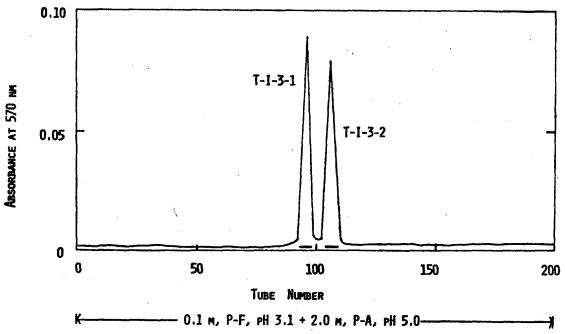

Fig. 5. Factionation of CNBr-II-T-I-3 on a column (0.9×50 cm) of Dowex 50–X2. The elution of peptides was performed with three chamber system (in both the mixing chamber and the first reservoir chamber were placed 0.1 M pyridine-formic acid buffer of pH 3.1, respectively, in the second reservoir chamber was placed 2.0 M pyridine-acetic acid buffer of pH 5.0). This chamber system was used throughout these chromatographic methods on Dowex 50–X2. The chromatography was carried out at 42° at a flow rate of 34 ml per hour. Fractions of 3.4 ml were collected.

This fraction was found to be impure and was subsequently fractionated on Dowex 50–X2. Two peptides were obtained from this chromatography as shown in Fig. 5.

Fraction T-I-3-1

Peptide T-3; Arg

Only arginine was identified, after acid hydrolysis.

Fraction T-I-3-2

Peptide T-10; Thr-Pro-Gly (Ser, Arg)

Peptide T–10 was subjected to three rounds of Edman degradations. From the specificity of trypsin, arginine is expected to be carboxy-terminus.

Fraction T-I-8

Peptide T-2; (Lys, Arg)

The sequence of the peptide has been already elucidated by Edman degradation of fragment CNBr-II as described in the preceding paper (*1*).

Fraction T-IV

Three peaks were appeared after Dowex 50–X2 purification as shown in Fig. 6. Fraction T-IV-3 contained a pure peptide, T-13.

Fraction T-IV-3

Peptide T-13; (Lys, Ile, Val, Ser, Asx, Val, His, Gly, H·Ser) This peptide has exactly the same composition as T-14, except for the presence of lysine.

Fraction T-V

One pure peptide was obtained after Dowex 50–X2 purification as shown in Fig. 7.

Fraction T-V-2

Peptide T-4; (His, Gly, Leu, Asx, Lys)

The sequence of the peptide has been already elucidated by Edman degradation of CNBr-II as described in the preceding paper (*1*).

Fraction T-VII

Peptide T-7; Phe-Glu-Ser-Asn-Phe-Asn-Thr (Glx, Ala, Thr, Asx, Arg)

This fraction contained only a single peptide, T–7. The seven residues were

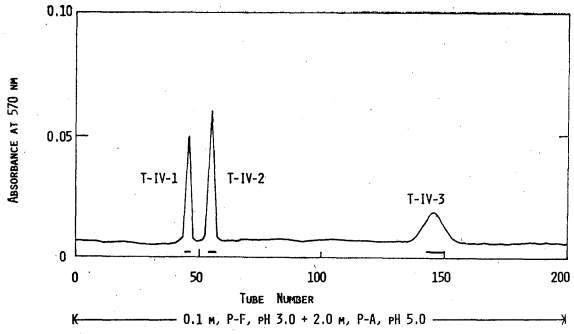

Fig. 6. Fractionation of CNBr-II-T-IV on a column (0.9 ×50cm) of Dowex 50–X2. The chromatography was carried out at 42° at a flow rate of 20 ml per hour. The effluent was collected in 2.0 ml fractions.

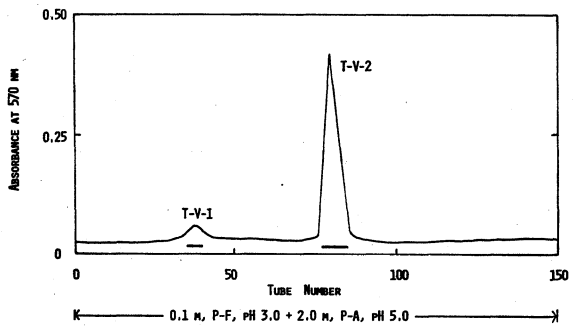

Fig. 7. Fractionation of CNBr-II-T-V on a column (0.9×50cm) of Dowex 50–X2. The chromatography was carried out at 42° at a flow rate of 25 ml per hour. Fractions of 2.5 ml were collected.

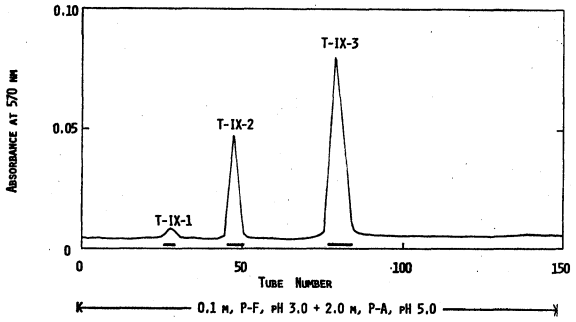

Fig. 8. Fractionation of CNBr-II-T-IX on a column (0.9×50cm) of Dowex 50–X2. The chromatography was carried out at 42° at a flow rate of 25 ml per hour. Fractions of 2.5 ml were collected.

identified by Edman degradation.

Fraction T-IX

Subfractionation of this fraction was carried out on Dowex 50–X2, and the separation pattern is shown in Fig. 8

Fraction T-IX-2 and *T-IX-3*

Peptide T-14; Ile-Val-Ser-Asp-Val-His (Gly, H·Ser)

The composition of fraction T-IX-2 was identical with T-IX-3. These peptides have exactly the same composition as T-13, except for the absence of lysine, and were subjected to six Edman degradation. Assuming that homoserine is the carboxy-terminal residue, this information gives the complete structure.

Fraction T-XI

Peptide T-6; Ser-Leu-Gly-Asn-Trp-Val (CM·Cys, Ala, Ala, Lys)

This fraction contained a single peptide comprising 10 residues. The six residues were identified by Edman degradation. The first three residues have been elucidated by Edman degradation of CNBr-II in the preceding paper (*1*).

Fraction T-XIII

Subfractionation of this fraction was carried out on Dowex 1–X2 as shown in Fig. 9. Fraction T-XIII-5 was further purified by gel filtration on Bio-Gel P–10

as shown in Fig. 10.

Fraction T-XIII-2

Peptide T-5; (Tyr, Glx, Gly, Tyr)

The peptide was neutral on high-voltage paper electrophoresis at pH 6.5, indicating the Glx residue to be glutamine. The peptide was produced by cleavage at tyrosine residue in high yield. Any other peptide produced by the other abnormal cleavage was not observed in the tryptic peptides. The sequence of the peptide has been elucidated by Edman degradation of CNBr-II in the preceding paper (1).

Fraction T-XIII-5-3

Peptide T-11; Asn-Leu-CM·Cys-Asn-Ile-Pro-CM·Cys-Ser-Ala-Leu (Leu, Ser, Ser, Asx, Ile, Thr, Ala, Ser, Val, Asx, CM·Cys, Ala, Lys)

Peptide was subjected to ten Edman degradations.

Fraction T-XIII-5-4

Peptide T-8; Asn-Thr-Asp-Gly (Ser, Thr, Asx, Tyr, Gly, Ile, Leu, Glx, Ile, Asx, Ser, Arg)

Peptide was subjeced to four Edman degradations.

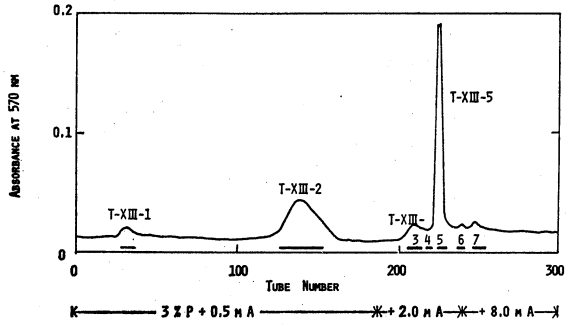

Fig. 9. Fractionation of CNBr-II-T-XIII on a column (0.9×50cm) of Dowex 1–X2. The elution of peptides was performed with an exponential gradient system as described in Fig. 11. The chromatography was carried out at 25° at a flow rate of 20 ml per hour. Fractions of 2.0 ml were collected.

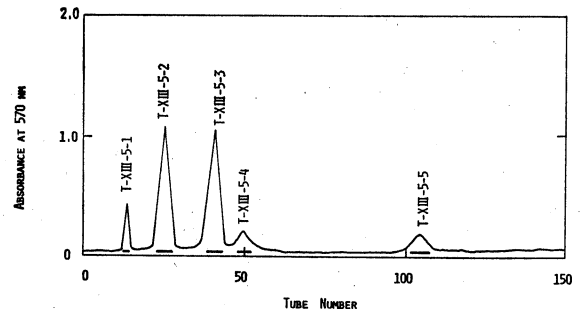

Fig. 10. Gel filtration of CNBr-II-T-XIII-5 on a column (1.3×105cm) of Bio-Gel P-10. The column was developed with 1.0 M formic acid at a flow rate of 20 ml per hour. Fractions of 2.0 ml were collected and monitored by ninhydrin method after alkaline hydrolysis.

Fraction T-XIII-5-5

Peptide T-9; Trp-Trp-CM·Cys-Asn-Asp (gly, Arg)

Edman degradation revealed the sequence of 5 residues from the amino terminus. From the specificity of trypsin, arginine is expected to be carboxy-terminus.

Isolation of Chymotryptic Peptides from CNBr-II – The elution pattern of chymotryptic peptides from Dowex 1–X2 is shown in Fig. 11. Each fraction was pooled and examined for purity by high-voltage paper electrophoresis. Impure pools were purified on chromatography of Dowex 1–X2 or 50–X2 or gel filtration on Bio-Gel P-10. The amino acid composition of the pure peptides is summerized in Table III.

Fraction C-I

This fraction was purified on Dowex 50–X2 as shown in Fig. 12.

Fraction C-I-3 and *C-I-4*

Peptide C-11; CM·Cys-Ala-Lys-Lys-Ile-Val (Ser, Asx, Val, His, Gly, H·Ser)

Both fraction C-I-3 and C-I-4 were found to possess a single peptide which had an identical composition, respectively. Peptide C-11 was subjected to six Edman degradations.

Fraction C-II

Peptide C-7; Gln-Ile-Asn (Ser, Arg, Trp, Trp)

This fraction contained a single peptide. The initial three residues were identified by Edman degradation.

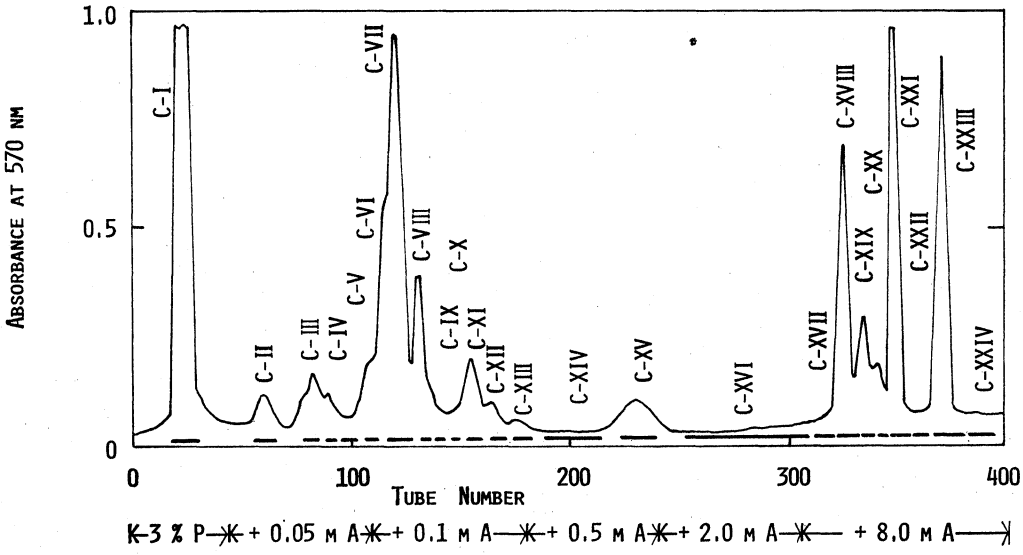

Fig. 11. Fractionation of the chymotryptic peptides of CNBr-II. The peptides from 128 mg of the fragment CNBr-II were loaded on a column (0.9 ×150cm) of Dowex 1–X2. The details are described in the text.

Table III. Amino acid composition of

Amino Acid	C-1	C-2	C-3	C-4	C-5
Lysine		0.90(1)			
Histidine					
Arginine				0.91(1)	1.04(1)
CM-Cysteine		0.86(1)			
Aspartic acid			1.10(1)	4.50(5)	2.82(3)
Threonine				3.50(4)	2.08(2)
Serine			0.97(1)	0.90(1)	1.12(1)
Glutamic acid	1.03(1)		1.00(1)	1.12(1)	
Proline					
Glycine	1.00(1)			1.15(1)	1.00(1)
Alanine		1.86(2)		1.00(1)	
Valine		0.86(1)			
Isoleucine					
Leucine					
Tyrosine	0.94(1)			0.87(1)	1.08(1)
Phenylalanine		1.00(1)	0.95(1)		
Tryptophan					
Homoserine and lactone					
Total	3	6	4	15	9
% yield	26	31	43	3	24
Fraction no.	C-XV	C-VII-4	C-XXI-1	C-XIX-1-2	C-XXI-3

Fraction C-VII

This fraction was purified on Dowex 50–X2 as shown in Fig. 13.

Fraction C-VII-3

Peptide C-6; Gly-Ile-Leu

Two rounds of Edman degradation gave the complete structure.

Fraction C-VII-4

Peptide C-2; Val-CM·Cys-Ala-Ala (Lys, Phe)

The first four residues were obtained by Edman degradation.

Fraction C-VII-6

Peptide C-11

This fraction contained the same peptide found in the fraction C-I-3 and C-I-4.

Fraction C-VIII

Subfractionation of this fraction was carried out on Dowex 50–X2 as shown in Fig. 14.

Fraction C-VIII-3

Peptide C-8; (CM·Cys, Asx, Asx, Gly, Arg, Thr, Pro, Gly, Ser, Arg, Asx, Leu)

Fraction C-XV

Peptide C-1; (Glx, Gly, Tyr)

This fraction contained a single peptide. The peptide was basic on high-voltage paper electrophoresis at pH 6.5, indicating the Glx residue to be glutamine.

chymotryptic peptides of CNBr-II.

C–6	C–7	C–8	C–9	C–10	C–11
					2.10(2)
					0.80(1)
	1.06(1)	1.60(2)			
		0.83(1)	1.75(2)		0.83(1)
	1.11(1)	2.55(3)	1.15(1)	1.80(2)	1.30(1)
		0.86(1)		1.10(1)	
	0.92(1)	0.83(1)	0.99(1)	2.26(3)	1.07(1)
	0.86(1)				
		0.78(1)	0.85(1)		
1.12(1)		2.00(2)			1.35(1)
			1.05(1)	1.00(1)	1.00(1)
				0.95(1)	1.70(2)
1.00(1)	1.00(1)		1.00(1)	0.94(1)	0.87(1)
0.98(1)		1.00(1)	1.45(2)		
	0.83(2)				
					0.87(1)
3	7	12	9	9	12
16	19	15	58	40	21
C-VII-3	C-II	C-VIII-3	C-XXIII	C-XVIII	C-I-3
					C-I-4
					C-VII-6

Fraction C-XVIII

Peptide C-10; Ser-Ser-Asp-Ile-Thr-Ala (Ser, Val, Asx)

This fraction also contained a single peptide. The first six residues were obtained by Edman degradation.

Fraction C-XIX

This fraction was purified by gel filtration on Bio-Gel P–10 as shown in Fig. 15. Fraction C-XIX-1 was further purified by a high-voltage paper electrophoresis at pH 3.65.

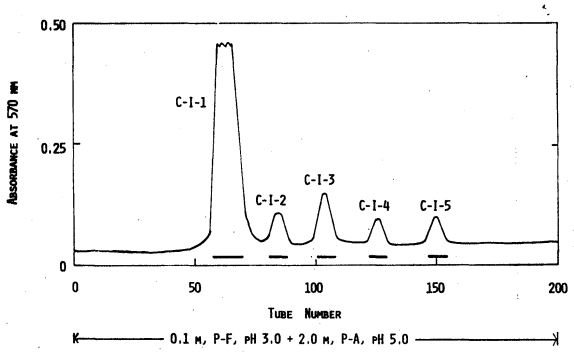

Fig. 12. Fractionation of CNBr-II-C-I on a column (0.9×50cm) of Dowex 50–X2. The chromatography was carried out at 28° at a flow rate of 25 ml per hour. Fractions of 2.5 ml were collected.

Fraction C-XIX-1-2

Peptide C-4; Asn-Thr-Gln-Ala-Thr-Asn (Arg, Asx, Thr, Asx, Gly, Ser, Thr, Asx, Tyr)

The first six residues were obtained by Edman degradation.

Fraction C-XXI

Subfractionation of this fragment was carried out on Dowex 50–X2 and the separation pattern is shown in Fig. 16.

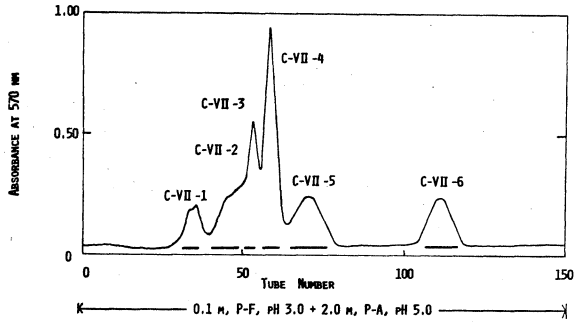

Fig. 13. Fractionation of CNBr-II-C-VII on a column (0.9×50cm) of Dowex 50–X2. The chromatography was carried out at 30° at a flow rate of 25 ml per hour. Fractions of 2.5 ml were collected.

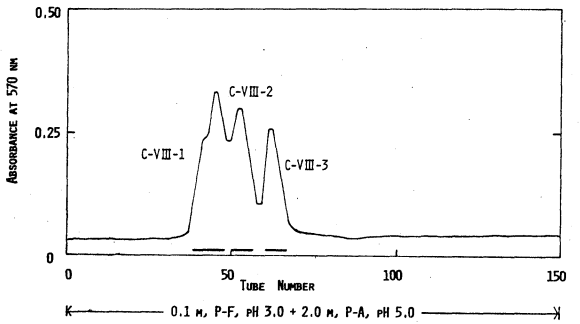

Fig. 14. Fractionation of CNBr-II-C-VIII on a column (0.9×50cm) of Dowex 50–X2. The chromatography was carried out at 40° at a flow rate of 25 ml per hour. Fractions of 2.5 ml were collected.

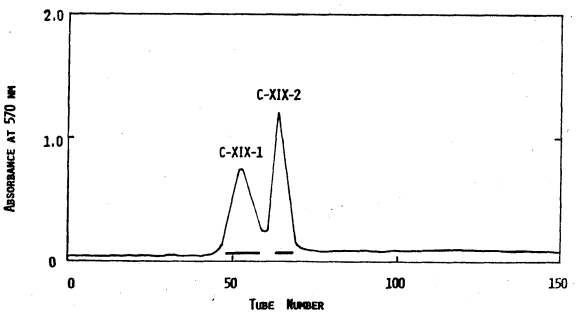

Fig. 15. Gel filtration of CNBr-II-C-XIX on a column (1.5×95cm) of Bio-Gel P-10. The details are described in the Fig. 10.

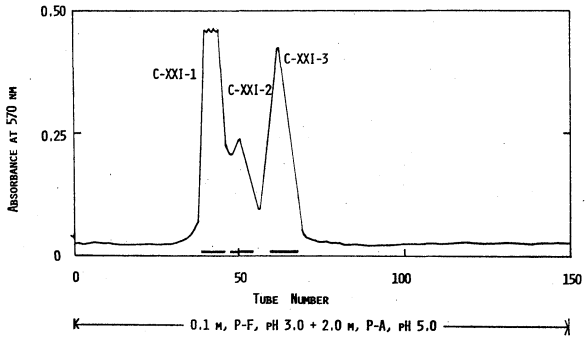

Fig. 16. Fractionation of CNBr-II-C-XXI on a column (0.9×50cm) of Dowex 50–X2. The chromatography was carried out at 30° at a flow rate of 25 ml per hour. Fractions of 25 ml were collected.

Fraction C-XXI-1

Peptide C-3; (Glx, Ser, Asx, Phe)

Fraction C-XXI-3

Peptide C-5; Arg-Asn-Thr-Asp-Gly (Ser, Thr, Asx, Tyr)

Peptide C–5 was found as corresponding to the C-terminal region of peptide C–4. The first five residues were obtained by Edman degradation.

Fraction C-XXIII

Petpdie C-9; CM·Cys-Asn-Ile-Pro-CM·Cys (Ser, Ala, Leu, Leu)

This fraction contained a single peptide. The first five residues were obtained by Edman degradation.

Alignment of Tryptic and Chymotryptic Peptides from CNBr-II — The tentative alignment of the tryptic and chymotryptic peptides is shown in Fig. 17. The residue number corresponds to that of whole molecule of lysozyme. In each line, the tryptic peptides are positioned above and the chymotryptic peptides below the sequence. The alignment of the first 14 residues, position 13–26, of fragment CNBr-II have been indicated in the preceding paper (*1*). Peptides, T–1, T–2 (T–1 plus T–3), T–3, T–4, T–5 and the first three residues of T–6 were involved in this 14 residues. Peptide C–2 was positioned at the carboxy-terminus of T–6 on the basis of its composition. Carboxy-terminal Lys of T–6 was deduced from the four rounds Edman degradation of C–2. By subtraction of the assigned residues (29–33) from C–2, phenylalanine was assigned to carboxy-terminus of C–2. Only peptide T–7 had the amino terminal Phe, therefore, peptide T–7 must follow peptide T–6. T–7 was completely determined by Edman degradations of T–7, C–4 and C–5. The alignment of T–7 and T–8 is supplied by C–4. The first four residues (46–49) of T–8 were obtained by Edman degradation of T–8 and C–5, but the four residue segment (50–53) was not ordered from these peptides. The alignment at positions, 60–61, 67—68, and 72–73 were placed tentatively from the specificity of trypsin. The alignment of T–8 and T–9 is supplied by C–7, and that of T–9, T–10 and T–11 is supplied by C–8. The carboxy-terminal Leu of C–9 was deduced by subtracting the

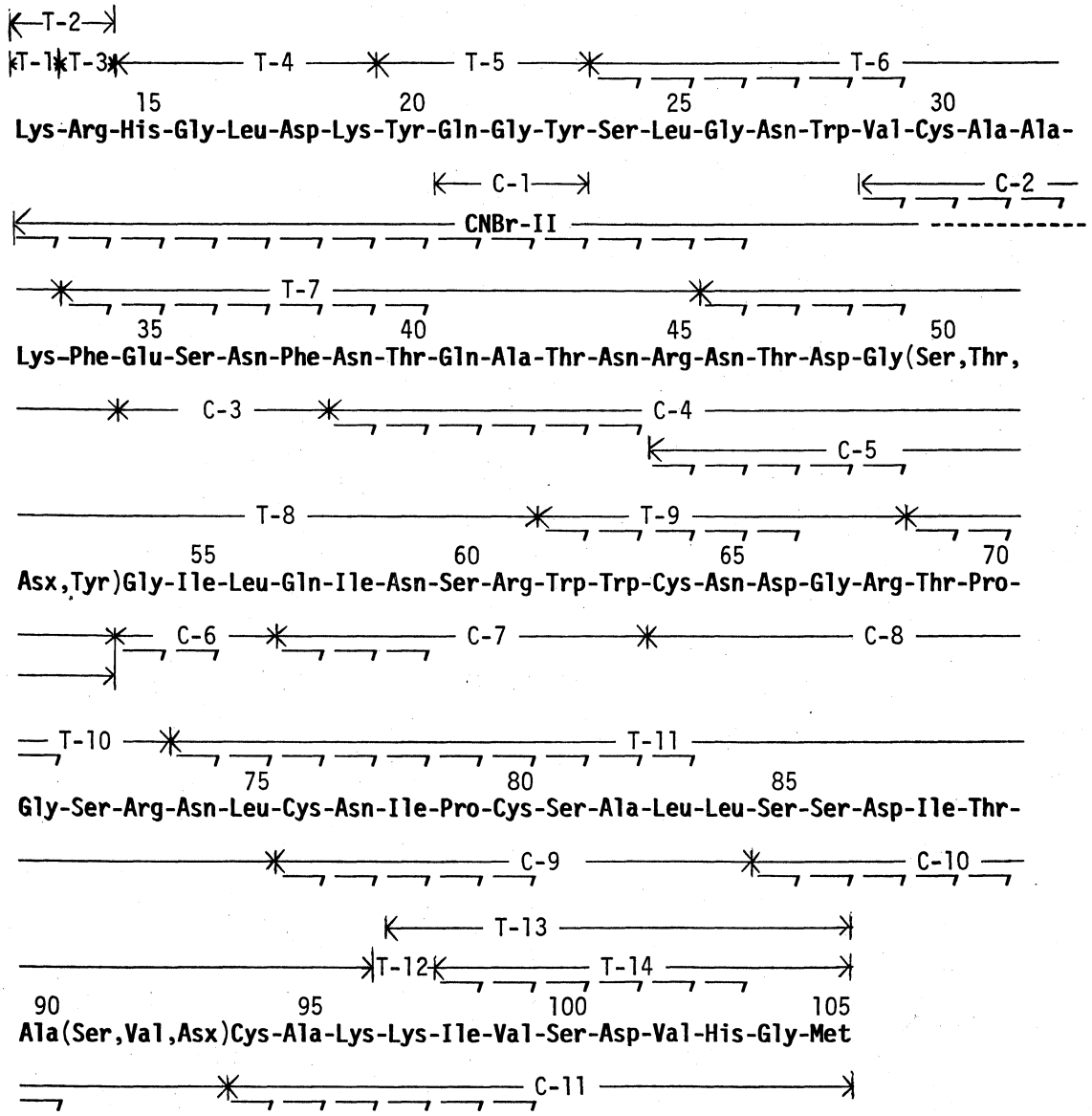

Fig. 17. Tentative alignment of the tryptic and chymotryptic peptides of CNBr-II.

The tryptic peptides are indicated double-headed arrows above the residues, while the chymotryptic peptides are listed in the same manner below the residues. The results of CNBr-II is quoted from the preceding paper (*1*). The small single-headed arrows refer to assignment by Edman degradation. Residue number corresponds to that of the whole molecule. Residues within parentheses were deduced from amino acid analyses.

assigned residues (76–83) from the composition of C–9. The remainder of T–11 was deduced from C–10 and C–11, but three residue segment (91–93) was not determined. The alignment of T–11 and T–13 is supplied by C–11.

Isolation of Tryptic Peptides from CNBr-III — The lyophized digestion mixture of CNBr-III was fractionated on a column of Dowex 1–X2 with two gradient systems as shown in Fig. 18. Each fraction was pooled and examined for purity by high-voltage paper electrophoresis. Impure pools, T-III, T-V and T-VI, were purified on

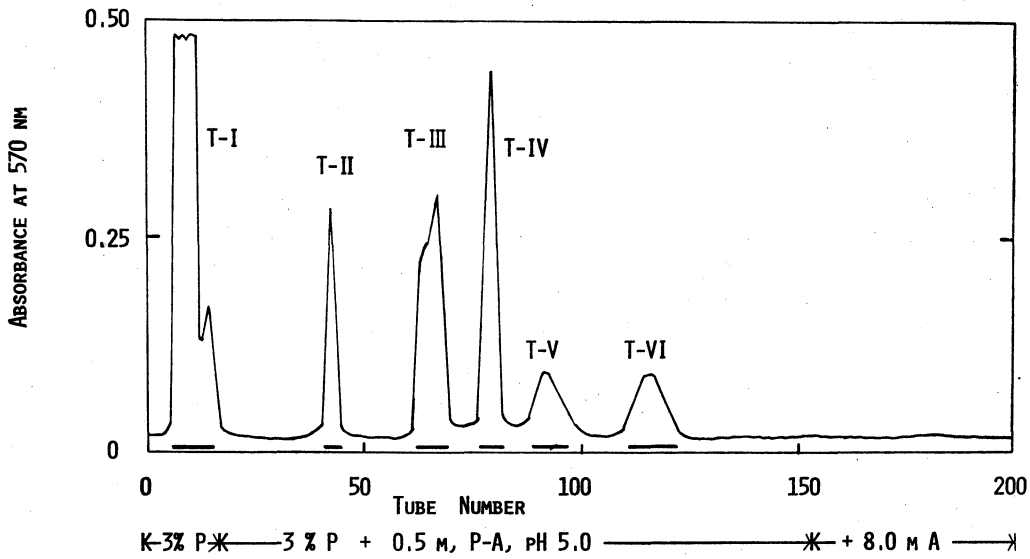

Fig. 18. Fractionation of the tryptic peptides of CNBr-III (3.5 μmoles) on a column (0.8×55 cm) of Dowex 1–X2.
Every 3.0 ml was collected at a flow rate of 30 ml per hour. The details are described in the text.

Table IV. Amino acid composition of tryptic peptides of CNBr-III.

Amino Acid	T–1	T–2	T–3	T–4	T–5	T–6	T–7	T–8
Lysine			1.00(1)	0.96(1)				
Histidine								
Arginine	1.00(1)	1.08(1)		0.96(1)	1.04(1)	1.08(1)	1.20(1)	
CM-Cysteine			1.24(1)	0.96(1)		1.10(1)	1.05(1)	
Aspartic acid	1.06(1)	1.00(1)		2.00(2)	1.85(2)			
Threonine				0.94(1)	0.90(1)			
Serine								
Glutamic acid								
Proline								
Glycine				0.96(1)	1.00(1)	1.00(1)	1.00(1)	
Alanine	2.06(2)			1.00(1)	0.98(1)			
Valine	1.00(1)			1.20(1)	0.92(1)			
Isoleucine				0.87(1)	0.83(1)			
Leucine							1.06(1)	1.00(1)
Tyrosine								
Phenylalanine								
Tryptophan	1.36(2)			0.47(1)	0.52(1)			
Homoserine and lactone								
Total	7	2	2	11	9	3	4	1
% yield	11	68	48	19	31	25	31	17
Fraction no.	T-VI-5	T-I	T-IV	T-VI-3	T-V-1	T-III-2	T-III-3	T-II

columns of Dowex 50–X2 or Bio-Gel P–10. The amino acid composition of the pure peptides is summarized in Table IV.

Fraction T-I

Peptide T-2; (Asx, Arg)

This fraction contained a single pure peptide T-2. The peptide was basic on high-voltage paper electrophoresis at pH 6.5, indicating the Asx residue to be asparagine.

Fraction T-II

Peptide T-8; Leu

This fraction contained only leucine residue. Since CNBr-III had one mole residue of leucine, it could be estimated free leucine.

Fraction T-III

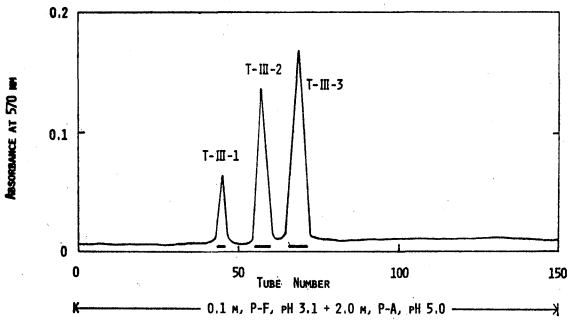

Fig. 19. Fractionation of CNBr-III-T-III on a column (0.9×50cm) of Dowex 50–X2. The chromatography was carried out at 42° at a flow rate of 20 ml per hour. Fractions of 2.0 ml were collected.

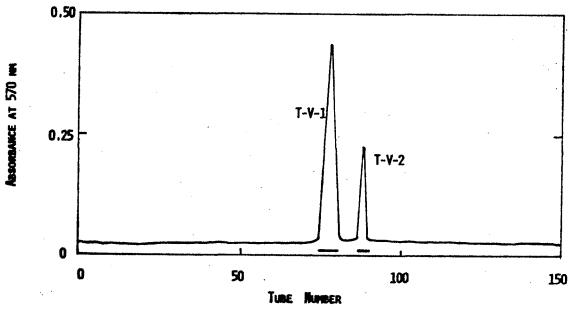

Fig. 20. Gel filtration of CNBr-III-T-V on a column (1.0×100cm) of Bio-Gel P-10. The details are described in the Fig. 10.

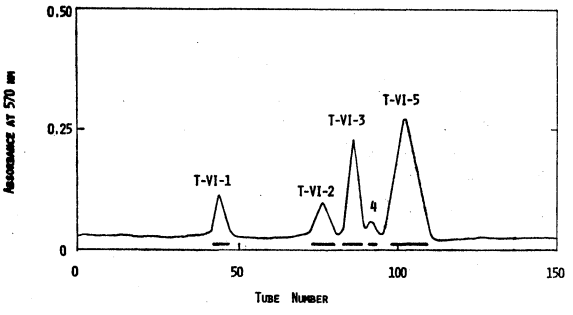

Fig. 21. Gel filtration of CNBr-III-T-VI on a column (1.0×100cm) of Bio-Gel P–10. The details are described in the Fig. 10.

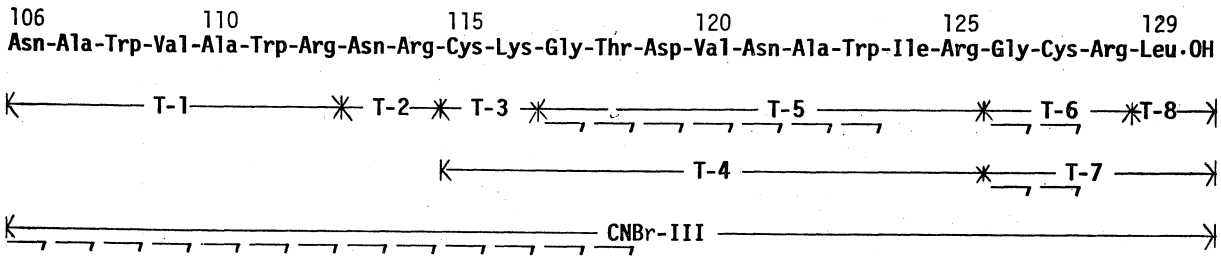

Fig. 22. Amino acid sequence of CNBr-III.
The arrow (⟶) denotes sequence determined by Edman procedure. The results of CNBr-III is quoted from the preceding paper (*1*). Residue number corresponds to that of the whole molecule.

Subfractionation of this fraction was carried out on Dowex 50–X2 as shown in Fig. 19 and two peptides were obtained.

Fraction T-III-3

Peptide T-7; Gly-CM·Cys (Arg, Leu)

Fraction T-III-2

```
                    1                                   10
Hen                 Lys-Val-Phe-Gly-Arg-Cys-Glu-Leu-Ala-Ala-Ala-Met-Lys-Arg-His-
Japanese Quail      Lys-Val-Tyr-Gly-Arg-Cys-Glu-Leu-Ala-Ala-Ala-Met-Lys-Arg-His-

                         20                                  30
Gly-Leu-Asp-Asn-Tyr-Arg-Gly-Tyr-Ser-Leu-Gly-Asn-Trp-Val-Cys-Ala-Ala-Lys-Phe-
Gly-Leu-Asp-Lys-Tyr-Gln-Gly-Tyr-Ser-Leu-Gly-Asn-Trp-Val-Cys-Ala-Ala-Lys-Phe-

                              40                                  50
Glu-Ser-Asn-Phe-Asn-Thr-Gln-Ala-Thr-Asn-Arg-Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr-
Glu-Ser-Asn-Phe-Asn-Thr-Gln-Ala-Thr-Asn-Arg-Asn-Thr-Asp-Gly(Ser,Thr,Asx,Tyr)

                                   60                                  70
Gly-Ile-Leu-Gln-Ile-Asn-Ser-Arg-Trp-Trp-Cys-Asn-Asp-Gly-Arg-Thr-Pro-Gly-Ser-
Gly-Ile-Leu-Gln-Ile-Asn-Ser-Arg-Trp-Trp-Cys-Asn-Asp-Gly-Arg-Thr-Pro-Gly-Ser-

                                        80                                  90
Arg-Asn-Leu-Cys-Asn-Ile-Pro-Cys-Ser-Ala-Leu-Leu-Ser-Ser-Asp-Ile-Thr-Ala-Ser-
Arg-Asn-Leu-Cys-Asn-Ile-Pro-Cys-Ser-Ala-Leu-Leu-Ser-Ser-Asp-Ile-Thr-Ala(Ser,

                                             100                                 110
Val-Asn-Cys-Ala-Lys-Lys-Ile-Val-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala-Trp-Val-Ala-
Val,Asx)Cys-Ala-Lys-Lys-Ile-Val-Ser-Asp-Val-His-Gly-Met-Asn-Ala-Trp-Val-Ala-

                                                  120                                 129
Trp-Arg-Asn-Arg-Cys-Lys-Gly-Thr-Asp-Val-Gln-Ala-Trp-Ile-Arg-Gly-Cys-Arg-Leu
Trp-Arg-Asn-Arg-Cys-Lys-Gly-Thr-Asp-Val-Asn-Ala-Trp-Ile-Arg-Gly-Cys-Arg-Leu
```

Fig. 23. The amino acid sequences of hen and Japanese quail lysozymes.
The sequence of hen lysozyme was taken from the paper by Canfield (*4*). The positions of the undetermined sequence of quail lysozyme are shown in parentheses. Asx is used when the acid or the amide form of aspartic acid residue has not been established. The underlined residues are different from the corresponding amino acid of hen lysozyme.

Peptide T-6; Gly-CM·Cys-Arg

Peptide T–6 was identified as corresponding to peptide T–7, with one less residue. Each two rounds of Edman degradation of both T–6 and T–7 were sufficient to structure of these peptide, respectively.

Fraction T-IV

Peptide T-3; (CM·Cys, Lys)

This fraction contained a single pure peptide.

Fraction T-V

This fraction was purified on Bio-Gel P–10 as shown in Fig. 20.

Fraction T-V-1

Peptide T-5; Gly-Thr-Asp-Val-Asn-Ala-Trp (Ile, Arg)

Fraction T-VI

Subfractionation of this fraction was carried out on Bio-Gel P–10 as shown in Fig. 21. Two peptides were obtained in major yield. By inspection of the composition, T–4 and T–5 appear to be derived from the same portion of the molecule.

Fraction T-VI-3

Peptide T-4; (CM·Cys, Lys, Gly, Thr, Asx, Val, Asx, Ala, Trp, Ile, Arg)

Fraction T-VI-5

Peptide T-1; (Asx, Ala, Trp, Val, Ala, Trp, Arg)

The first 13 residues of CNBr-III were determined by Edman degradation as described in the preceding paper (*1*). By the citation of these results the tryptic peptides of CNBr-III except peptide T-7 could be aligned consistently, while, peptide T-7 must be the carboxy-terminal peptide of CNBr-III. The amino acid sequence of CNBr-III is shown in Fig. 22.

Amino Acid Sequence of Japanese Quail Egg-White Lysozyme— The tentative amino acid sequence of Japanese quail egg-white lysozyme accounts exactly for its amino acid composition. The amino acid interchanges between hen and Japanese quail lysozymes is established at the residue Nos. 3, 19, 21, 102, 103, and 121 as shown in Fig. 23.

References

(*1*) M. Kaneda, I. Kato, N. Tominaga, K. Titani, and K. Narita, *Rept. Fac. Sci. Kagoshima Univ.* (*Math. Phys. & Chem.*), **8**, 71 (1975)

(*2*) S. Iwanaga, P. Wallén, N.J. Gröndahl, A. Henschen, and B. Blombäck, *European J. Biochem.*, **8**, 189 (1969)

(*3*) W. Konigsberg and R.J. Hill, *J. Biol. Chem.*, **237**, 2547 (1962)

(*4*) R.E. Canfield and A.K. Liu, *J. Biol. Chem.*, **240**, 1997 (1965)