

Chemical and Immunological Properties and Amino Acid Sequences of Three Lysozymes from Peking-Duck Egg White

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Three lysozymes (DLs-1, -2, and -3) were purified from Peking-duck egg white by adsorption on CM-Sephadex C-25 resin, followed by CM-Sephadex C-25 and Sephadex G-50 column chromatographies. The three enzymes each moved as a single band, but showed different electrophoretic mobilities, on disc-polyacrylamide gel electrophoresis at pH 4.1. Final yields of DL-1, DL-2, and DL-3 were 18.2%, 22.0%, and 6.0%, respectively, from the crude material adsorbed on CM-Sephadex resin. The enzymatic activities of DL-1, DL-2, and DL-3 were 1.53, 1.52, and 1.34 times that of hen egg white lysozyme, respectively, using *Micrococcus lysodeikticus* cell wall as a substrate at pH 6.2 and 37°C.

All the DLs lacked histidine and their amino acid compositions differed from each other by a few amino acid exchanges. The amino acid sequences of DL-2 and DL-3 differed from that of DL-1 by two displacements (Ser-37 to Gly and Gly-71 to Arg) and three displacements (Pro-79 to Arg in addition to the same substitutions), respectively. In comparison with Duck II and Duck III lysozymes from Kaki-duck (Hermann and Jollès (1970) *Biochim. Biophys. Acta* **200**, 178–179; Hermann *et al.* (1971) *Eur. J. Biochem.* **24**, 12–17) as regards amino acid sequences, Duck II is identical to DL-1 except for one displacement of Gln-57 in DL-1 to Glu in Duck II, while Duck III is rather different from our three lysozymes.

All DLs gave single precipitin lines with any rabbit antisera against DLs and each precipitin line fused completely with that of any of the DLs in Ouchterlony double diffusion tests. However, the radiobinding inhibition assay showed that some anti-DL antisera clearly discriminated fine differences among the three DLs. The displacement at residue 79 (Pro ↔ Arg) gave the clearest immunological difference among the three kinds of displacements found in DLs.

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Abbreviations used: DL, lysozymes isolated from Peking-duck egg white; HEL, hen egg white lysozyme; RCM, reduced and carboxymethylated; SDS, sodium dodecyl sulfate; TPCK, L-(1-tosylamido-2-phenyl)ethylchloromethyl ketone; PTH, phenylthiohydantoin; DNS, 1-dimethylaminonaphthalene-5-sulfonyl; AEAP-glass, N-(2-aminoethyl)-3-aminopropyl glass.

Lysozymes are among the most extensively investigated proteins in terms of the immunological properties and the antigenic determinants (1-9). The amino acid sequences have been reported for 12 species (10). Lysozyme contains mainly five regions of antigenic determinants, and Fujio *et al.* (11) summarized them as follows; region I, N- (residues 1-12) and C- (residues 123-129) terminal regions crosslinked by Cys6-Cys127; region II, a region consisting of residues 29-37 and 109-123 crosslinked by Cys30-Cys115; region III, an antiparallel β -sheet region consisting of residues 38-54 which contains no cystine bridges; region IV, first loop region (named loop I) consisting of residues 64-82 crosslinked by Cys64-Cys80; and region V, second loop region (named loop II) consisting of residues 76-96 crosslinked by Cys76-Cys94. Atassi and Lee (8) and Atassi (9) synthesized and investigated various antigenic site analogous peptides (sites 1, 2, and 3) to the antigenic determinants in HEL, which correspond to regions I, V, and II, respectively. The regions III and IV should be added to their proposed determinants in order to describe completely the antigenic determinants of HEL (4, 7).

The relationship between molecular evolution and antigenic structure among lysozymes from various sources, mainly bird egg whites (12-15), has been investigated, and the correlation of immunological differences with the amino acid substitutions, *i.e.*, factors of exposed and buried residues in lysozymes, was estimated (14, 15).

One approach to resolve the antigenic determinants on the lysozyme molecule is to synthesize the antigenic determinants or their analogs. This method has been used by Atassi and Lee (9) and Arnon *et al.* (52, 54). Immunological comparisons among lysozymes whose substitutions are restricted to only a few amino acid residues could be another approach to resolve the precise localities of antigenic sites on the lysozyme molecule. Under such circumstances, it might be relatively easy to investigate the antigenic role of a particular amino acid residue within an antigenic site of a protein, because conformational influence induced by amino acid displacement toward other portions of the protein molecule is minimized. Prager *et al.* (16) and Fainaru *et al.* (17) made use of sets of hen and bobwhite quail lysozymes and hen, bobwhite quail and turkey lysozymes, respectively, for this pur-

pose. Ibrahim *et al.* (15) made use of two lysozymes from California quail and bobwhite quail egg whites which differed by two amino acid substitutions at residues 68 (Arg and Lys) and 121 (His and Gln), respectively.

It has been reported that duck egg white contains more than two lysozyme isozymes (18-20, 23). Jollès *et al.* (21, 22) reported the amino acid sequences of two duck lysozymes, Duck II and Duck III, isolated from Kaki-duck egg white. There are six amino acid substitutions between these lysozymes (see Fig. 10). Imanishi *et al.* (20) isolated two isozymes from Peking-duck egg white, and Prager and Wilson (23) isolated three lysozymes from the same source. The amino acid compositions of lysozymes reported by the latter two groups (20, 23) differed slightly from those of Duck II and Duck III. We attempted to isolate three lysozymes from Peking-duck egg white and to investigate the amino acid sequences.

In the present paper, we report the purification and characterization of three lysozymes from Peking-duck egg white and elucidation of their amino acid sequences. We found that amino acid substitutions through the three lysozymes (DLs-1, -2, and -3) were restricted to three positions (residues 37, 71, and 79). We further found that differences of immunoreactivities of DLs against rabbit anti-DL antisera were derived from the amino acid substitutions, and the correlations between the immunological differences and amino acid displacements in DLs are discussed.

MATERIALS AND METHODS

Materials—Peking-duck eggs were purchased from Mori Farm (Kan-onji, Kagawa). The following materials were purchased from the sources indicated; TPCK-trypsin (Worthington Biochemical Co.), *Staphylococcus aureus* V8 protease (Miles Laboratories Inc.), carboxypeptidase Y (Oriental Yeast Co.), iodoacetic acid (recrystallized from chloroform) and cyanogen bromide (Nakarai Chemicals Co.), Sephadexes (Pharmacia), DEAE-cellulose DE-32 (Whatman), lyophilized cell wall of *Micrococcus lysodeikticus* and hen egg white lysozyme (six times recrystallized) (Seikagaku Kogyo Co.), ¹²⁵I-iodine (RCC Amersham), silica gel sheet F254 (Merck), polyamide sheets (Cheng Chin Trading Co.), solvents and reagents for

sequence analysis (Wako Pure Chemicals Co.), and 4 M methanesulfonic acid containing 0.2% tryptamine (Pierce). Other chemicals used were of analytical grade. Goat anti-rabbit immunoglobulin antisera were prepared in Kan-onji Institute and the immunoglobulin G fraction was purified and collected by precipitation with 50% saturated ammonium sulfate followed by Sephadex G-150 column chromatography.

Purification of Lysozymes—Duck egg white was separated from the yolk (450 eggs), homogenized slightly and adjusted to pH 6.2 with glacial acetic acid. The homogenate was mixed with CM-Sephadex C-25 resin equilibrated with 0.05 M K-phosphate (pH 6.2), and stirred for 1 h at room temperature. The suspension was decanted and the supernatant was discarded, then the resin was collected by filtration and washed thoroughly with the above buffer. The proteins adsorbed on the resin were eluted with 0.8 M K-phosphate buffer (pH 8.0). The eluate was dialyzed against deionized water and lyophilized. The crude lysozyme fraction was dissolved in distilled water (10 mg/ml) and adjusted with 5 M NH_4OH to pH 8.0. The protein solution was applied on a CM-Sephadex C-25 column (5.0 $\times$ 74 cm) equilibrated with 0.05 M ammonium carbonate (about pH 9), and the proteins adsorbed on the column were eluted with a linear gradient (9,000 ml) from 0.05 M to 0.6 M ammonium carbonate. Lysozyme fractions were collected, dialyzed against deionized water and lyophilized. Lysozyme fractions were gel-filtered on a Sephadex G-50 fine column (3.0 $\times$ 150 cm) with 0.05 M ammonium acetate (pH 5.0). The major fraction derived from the crude lysozyme fraction III was further rechromatographed on a CM-Sephadex C-25 column (4.0 $\times$ 47 cm) with a linear gradient elution (3,600 ml) from 0.2 M to 0.4 M ammonium carbonate. The concentrations of DLs and HEL were estimated on the basis of $A_{1\text{cm}}^{1\%}$ values of 28.5 and 26.7, respectively. Purity of DLs was checked by polyacrylamide gel electrophoresis at pH 4.1 (24) and SDS-polyacrylamide gel electrophoresis at pH 7.2 (25).

Lysozyme Activity—The activity was assayed by the method of Parry *et al.* (26), using lyophilized cell wall of *M. lysodeikticus*. Aliquots of the effluent from the column were added directly to the substrate solution (2 ml, 0.5 mg/ml of cell wall in 0.05 M NaCl, 0.066 M phosphate buffer, pH 6.2).

After the mixture had been incubated for 5 min at 37°C, the decrease of %T at 540 nm was measured.

Cleavage of Disulfide Bonds—Lysozymes were reduced with 2-mercaptoethanol followed by alkylation with iodoacetic acid in 1 M Tris-HCl buffer (pH 8.6) in the presence of 6 M guanidine-HCl (27). The RCM-derivatives were dialyzed against deionized water and lyophilized.

Cleavage with Cyanogen Bromide—RCM-enzyme was cleaved with cyanogen bromide (100-molar excess over methionines) in 70% formic acid at 4°C for 24 h. The mixture was thoroughly diluted with distilled water and lyophilized several times.

Proteolytic Digestion—Large cyanogen bromide peptides were digested with V8 protease in 2% ammonium bicarbonate (pH 8.0) at 37°C for 16 h (substrate-to-enzyme ratio of 50 : 1, w/w). Trypsin digestion of peptide was carried out in 2% ammonium bicarbonate (pH 8.0) at 37°C for 5 h (substrate-to-enzyme ratio of 200 : 1, mol/mol). The digests were immediately frozen and lyophilized.

Fractionation of Peptides—CNBr peptides were fractionated on a Bio-Gel P-10 column with 10% formic acid. V8 protease peptides were fractionated on a Sephadex G-50 fine column with 0.05 M NH_4OH . Further purification was performed by DEAE-cellulose (1.2 $\times$ 30 cm) chromatography with linear gradient elution (total 500 ml) from 0.05 M to 1 M ammonium bicarbonate, and by paper electrophoresis at pH 3.5 and pH 6.5. Tryptic peptides derived from the large CNBr-peptide (N-2) of RCM-DL-3 were subjected to two-dimensional peptide mapping (first, electrophoresis at pH 6.5; second, chromatography with 1-butanol-pyridine-acetic acid-water, 15 : 10 : 3 : 12, by vol.) on Whatman 3MM papers. Peptides on papers were eluted successively with 30% acetic acid and 20% pyridine.

Sequence Analysis—RCM-enzyme and peptides were sequenced by means of an LKB 4020 solid-phase sequencer, after they had been coupled to AEAP-glass by the phenylene diisothiocyanate method (28), homoserine lactone method (29), or EDC (*N*-ethyl, *N'*-(3-dimethylaminopropyl)-carbodiimide-HCl) method (30). 2-Anilinothiazolinone derivatives were converted to PTH-amino acids in 1 M HCl at 80°C for 10 min. The

manual Edman method (31) and dansyl-Edman method (32) were also used. PTH-amino acids were identified on silica gel sheets (33, 34) or polyamide sheets (35). PTH-Arg was identified on silica gel sheets (36) and confirmed by means of the phenanthrenequinone test (37).

Amino Acid Analysis—RCM-proteins were hydrolyzed in evacuated, sealed tubes with 6 M HCl containing 1% phenol at 105°C for 24, 48, and 72 h. Peptides were hydrolyzed with the same HCl solution at 105°C for 24 h. Hydrolyzates were analyzed in a Hitachi KLA-5 automated amino acid analyzer (38).

Antisera—Rabbits, weighing 2.5–3.0 kg, were obtained from Nihon Biological Products Co. (Tokyo). A dose of 1 mg of the purified DL in 0.4 ml of complete Freund's adjuvant emulsion was injected into all four foot pads. The first, second, and third booster injections (the same amount) were carried out 2 weeks, 5 weeks, and 9 weeks later, respectively. Bleedings were carried out 7 days after each booster injection and separately collected. Final blood was collected by cardiac puncture 9 days after the last injection. The antisera were purified to remove serum albumin by precipitation with 50% saturated ammonium sulfate at 4°C. The precipitated immunoglobulin fractions were redissolved in distilled water and dialyzed against 0.1 M K-phosphate (pH 7.5). The immunoglobulin fractions were stored in a freezer until immediately before use.

Labeling with ^{125}I —Duck lysozymes were labeled with ^{125}I by the iodine monochloride method (40). Lysozyme (0.65 mg) was dissolved in borate buffer (pH 8.5) and added to a mixture of ^{125}I (400 μCi) and iodine monochloride (molar ratio of 1:1–1.5). The reaction was performed for 10 min at room temperature and terminated by adding glacial acetic acid. The labeled proteins were dialyzed thoroughly against 0.1 M K-phosphate (pH 7.5). The specific radioactivities of labeled DL-1, DL-2, and DL-3 were 18,600, 16,000, and 17,600 cpm/ μg , respectively.

Immunological Methods—Double immunodiffusion on agar plates was carried out as described by Ouchterlony (41).

Inhibition of radioactive DL binding to anti-DL antibody by cold DL or HEL was performed as described previously (42). One hundred μl of ^{125}I -DL (5 $\mu\text{g}/\text{ml}$ of 0.1 M K-phosphate, pH 7.5,

containing 0.2% protamine) and the same volume of cold lysozyme (various concentrations in the same buffer) were mixed and preincubated for 30 min at 37°C. Then 100 μl of diluted rabbit anti-DL antisera, which had been diluted with the same buffer to a suitable concentration, was added to the mixture and the whole was incubated for 1 h at 37°C. Goat anti-rabbit immunoglobulin antiserum (100 μl) diluted with the above buffer was added to the mixture, which was further incubated for 1 h at 37°C, and allowed to stand overnight at 4°C. The reaction mixture was centrifuged at 3,500 rpm for 15 min, the supernatant was discarded, and the precipitate was washed with ice-cold 0.1 M K-phosphate (pH 7.5) twice. The radioactivity of the precipitate was counted with a Beckman Gamma 8000 machine.

RESULTS

Purification of Lysozyme—The fraction adsorbed on CM-Sephadex C-25 resin was fractionated into five fractions by CM-Sephadex C-25 column chromatography using concentration gradient elution with ammonium carbonate (about pH 9), as shown in Fig. 1. Lysozyme activity was found in the last three major fractions (named fractions III to V). Fraction III was gel-filtered on a Sephadex G-50 fine column and almost all of the applied fraction was recovered as a major peak (III-2), which possessed lysozyme activity, as shown in Fig. 2. As fraction III-2 was impure, as judged from disc-polyacrylamide gel electrophoresis at pH 4.1, the fraction was rechromatographed on a CM-Sephadex C-25 column and separated well into two fractions, a minor fraction (III-2-1) having no lytic activity and a major fraction (III-2-2) possessing lysozyme activity, as shown in Fig. 3.

Fractions IV and V were also gel-filtered on a Sephadex G-50 fine column, and almost all of the applied material was recovered as a single major lysozyme fraction (IV-2 and V-2, respectively), which corresponded to fraction III-2.

Fractions III-2-2, IV-2, and V-2 were homogeneous on polyacrylamide gel electrophoresis at pH 4.1 (Fig. 4), and the three fractions were named DL-1, DL-2, and DL-3, respectively, from the order of elution of the fractions from the first CM-Sephadex column. They differed from each

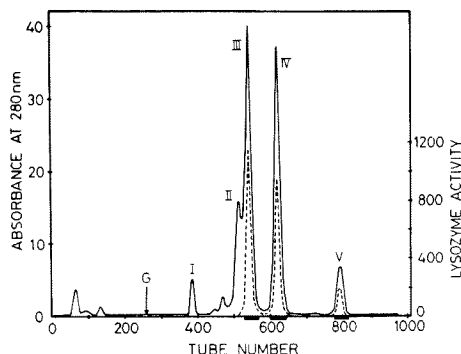


Fig. 1. Chromatography of the fraction adsorbed on CM-Sephadex C-25 resin from duck egg white. The fraction (9,800 mg) was applied to a CM-Sephadex C-25 column (5.0 $\times$ 74 cm) equilibrated with 0.05 M ammonium carbonate. The column was washed with 1,100 ml of the same buffer and then eluted with a linear gradient (total 9,000 ml) from 0.05 M to 0.6 M ammonium carbonate at a flow rate of 120 ml/h, and 10 ml-fractions were collected. The absorbance at 280 nm (—) was measured. Lysozyme activity (---) was assayed using lyophilized cell wall of *M. lysodeikticus*. The fractions marked by a bar were pooled, dialyzed against deionized water and lyophilized. G shows the start point of the gradient.

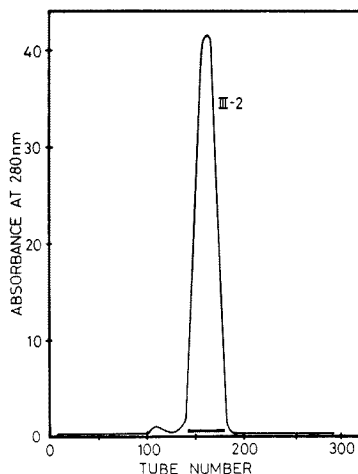


Fig. 2. Gel chromatography of fraction III in Fig. 1. Fraction III (2,550 mg) was applied to a Sephadex G-50 (fine) column (3.0 $\times$ 150 cm) equilibrated with 0.05 M ammonium acetate (pH 5.0) and eluted with the same buffer at a flow rate of 50 ml/h; 5 ml-fractions were collected. Almost all of the lysozyme activity was eluted as a major peak (III-2). The fraction marked by a bar was pooled, dialyzed against deionized water and lyophilized.

other in electrophoretic mobility when they were mixed and migrated, as shown in Fig. 4. Their mobilities increased in the order DL-1, DL-2, and DL-3, the same as that of elution from the CM-Sephadex column, which indicates that the basicity of the three DLs increased in the order of elution.

Properties—The three enzymes showed the same mobilities as hen egg white lysozyme on SDS-polyacrylamide gel electrophoresis with or without treatment with a reducing agent. Lytic activities of DLs on *M. lysodeikticus* cell wall were 1.34 to 1.53 times that of HEL. These high activities of DLs, in comparison with HEL, might be due to the increase of basic residues in the DL molecules (see Table II). Final protein recoveries of DLs from the adsorbed material are shown in Table I. DL-3 content was smaller and its activity was weaker than those of the other two DLs.

The amino acid compositions of the three DLs are listed in Table II. The three DLs all lacked histidine and were very similar in compo-

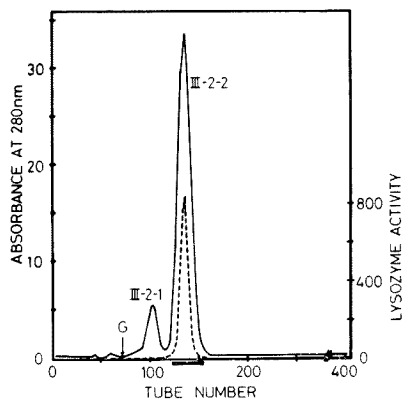


Fig. 3. Chromatography of major fraction III-2 in Fig. 2. Fraction III-2 (2,450 mg) was applied to a CM-Sephadex C-25 column (4.0 $\times$ 47 cm) equilibrated with 0.2 M ammonium carbonate and eluted with a linear gradient (total 3,600 ml) from 0.2 M to 0.4 M ammonium carbonate. The fraction marked by a bar was pooled, dialyzed against deionized water and lyophilized. The flow rate was 60 ml/h and 4 ml-fractions were collected. G shows the start point of the gradient.

TABLE I. Purification of duck lysozymes. The activity was measured using *M. lysodeikticus* cell wall as a substrate in 0.066 M phosphate buffer (pH 6.2) at 37°C.

	Weight mg	Yield %	Specific activity ^b	Total activity	Recovery %
Egg white			0.68		
Adsorbed fraction ^a	9,800	100	0.96	9,408	100
Fraction III-2	2,450	25.0	1.36	3,332	35.4
Fraction III-2-2 (DL-1)	1,780	18.2	1.53	2,723	28.9
Fraction IV-2 (DL-2)	2,160	22.0	1.52	3,283	34.9
Fraction V-2 (DL-3)	590	6.0	1.34	791	8.4

^a Crude lysozyme fraction adsorbed on CM-Sephadex C-25 resin. ^b The activity is expressed as mg of hen egg white lysozyme equivalent activity per mg of protein.

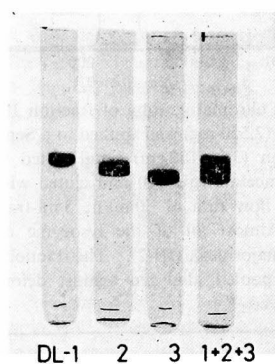


Fig. 4. Electrophoretic patterns of the three duck lysozymes. Fractions III-2-2 (DL-1), IV-2 (DL-2), and V-2 (DL-3) (each 100 μ g) were dissolved in pH 4.1 electrophoresis buffer and applied to 15% polyacrylamide gel columns. After the proteins had been electrophoresed at 2 mA/gel column for 3 h, the gels were stained with Coomassie Brilliant Blue G-250 in perchlorate and destained with 7% acetic acid. The electrophoresis direction was from top to bottom.

sition, except for a few amino acid exchanges. It is noteworthy that the contents of arginine increased by one residue in the order of DL-1, DL-2, and DL-3, while that of serine in DL-2 decreased by one residue and those of serine and proline residues in DL-3 each decreased by one residue in comparison with DL-1. These results indicated that displacements in amino acid sequences among the three DLs had occurred at restricted sites. The increase of arginine residue among DLs was consistent with the order of their mobilities on pH 4.1 electrophoresis (see Fig. 4).

TABLE II. Amino acid compositions of the three duck lysozymes. The values for residues are the average ones from three hydrolyses for 24, 48, and 72 h at 110°C, except that those for Thr and Ser were extrapolated to zero time of hydrolysis and that those for Ile, Leu, and Val were the highest values. Figures in parentheses are from the sequence determined. Half-cystine was estimated as carboxymethylcysteine, and Trp content was estimated after hydrolysis with 4 M methanesulfonic acid containing 0.2% tryptamine at 105°C for 24 h (39).

	DL-1	DL-2	DL-3	HEL
Lys	5.99 (6)	6.19 (6)	5.86 (6)	6
His				1
Arg	13.00 (13)	13.84 (14)	14.96 (15)	11
Trp	5.95 (6)	5.90 (6)	5.86 (6)	6
Asp	18.99 (19)	18.80 (19)	18.71 (19)	21
Thr	6.98 (7)	6.80 (7)	7.10 (7)	7
Ser	10.92 (11)	9.50 (10)	9.90 (10)	10
Glu	5.14 (5)	4.90 (5)	4.83 (5)	5
Pro	2.02 (2)	2.04 (2)	1.08 (1)	2
Gly	12.04 (12)	12.14 (12)	11.79 (12)	12
Ala	10.91 (11)	11.04 (11)	10.96 (11)	12
Cys	7.77 (8)	8.07 (8)	7.83 (8)	8
Val	7.10 (7)	6.89 (7)	6.64 (7)	6
Met	2.00 (2)	2.00 (2)	1.90 (2)	2
Ile	6.09 (6)	5.89 (6)	5.79 (6)	6
Leu	7.96 (8)	8.18 (8)	8.08 (8)	8
Tyr	5.04 (5)	5.02 (5)	4.98 (5)	3
Phe	0.97 (1)	1.03 (1)	1.03 (1)	3
Total	129	129	129	129

As compared with HEL, the contents of arginine, valine, and tyrosine residues in DLs were higher by a few residues, while those of aspartic acid (and its amide), alanine, and phenylalanine residues were lower by two, one and two residues, respectively. Contents of most other residues were the same as those of HEL.

Amino Acid Sequence of DL-1—After RCM-DL-1 had been coupled to AEAP-glass by the phenylene diisothiocyanate method, the immobilized polypeptide was sequenced up to 35 cycles (Fig. 5). The N-terminal and 13th lysine residues could not be identified, because they were attached to AEAP-glass via α - and ϵ -amino groups (28). RCM-DL-1 released Leu (0.95 mol/mol), Arg (0.66), and Cys(Cm) (0.12) on carboxypeptidase Y digestion (37°C, 10 min).

RCM-DL-1 was cleaved with cyanogen bromide and the digest was fractionated on a Bio-Gel P-10 column (Fig. 1S). The pass-through fraction contained the central large fragment (N-2), consisting of 93 residues, of RCM-DL-1. The second and third fractions were also concluded to contain peptide N-2, judging from their amino acid compositions and N-terminal sequence analyses by the manual Edman method (data not shown). The fourth fraction was the N-terminal fragment (N-1) consisting of 12 residues. The fifth fraction was the salt fraction. The sixth and seventh fractions contained the same fragment, the C-terminal peptide (N-3), which contained no homoserine/homoserine lactone residue.

N-2: The large CNBr peptide N-2 was sequenced up to 30 cycles after being coupled to AEAP-glass by the phenylene diisothiocyanate method (Fig. 5). N-2 was digested with V8 protease and the digest was fractionated on a Sephadex G-50 fine column (Fig. 2S). Each fraction was further fractionated by DEAE-cellulose column chromatography.

N2E2: N2E2 was sequenced up to the 17th cycle after coupling to AEAP-glass by the carbodiimide method (Fig. 5). N2E2 contained one glutamic acid (or its amide) according to its amino acid composition, but it was located as a Gln residue at the 6th position from the N-terminus. At the 13th and 17th cycles, no PTH-derivatives was extracted. We deduced that the residues at these positions were aspartic acid attached to AEAP-glass beads via β -carboxyl groups, because

two aspartic acids (or amides) remained to be identified after the amino acids identified by the above automated sequencing were subtracted from the amino acid composition of N2E2. The C-terminal Asp was confirmed by carboxypeptidase Y digestion of N2E2, but the 13th Asp was not confirmed. We concluded that V8 protease quantitatively hydrolyzed the Asp-Tyr (52-53) bond.

N2E3: This peptide was coupled to AEAP-glass by the phenylene diisothiocyanate method. Recovery of PTH-derivatives suddenly fell after the 13th cycle and only a small amount of PTH-Asp was identified. The result of sequencing of N2E3 after coupling by the carbodiimide method was the same. Therefore, N2E3 was digested with TPCK-trypsin and the digest was separated into five peptides (N2E3T1 to N2E3T5) by paper electrophoresis at pH 6.5. The isolated peptides were sequenced by the manual Edman method or the dansyl-Edman method (Fig. 5). N2E3T2 was determined to be Trp-Trp-Cys(Cm)-Asp- by the manual Edman method, but after the next cycle no PTH-derivative was recovered. Therefore, after the 4th cycle of Edman degradation was over, the residual peptide was dansylated and hydrolyzed with 6 M HCl. DNS-Asp was identified from the hydrolyzate. After redansylation of the hydrolyzate, DNS-Gly, and α,ϵ -bis-DNS-Lys (small amount) were newly identified in addition to DNS-Asp. Therefore, the sequence after the 4th residue was deduced to be Asx-Gly-Lys, in view of the amino acid composition of N2E3T2. The net charge of N2E3T2 was -1 upon pH 6.5 electrophoresis, so the 5th Asx must be in an amide form. This conclusion was in accordance with the resistance of the Asn-Gly bond to Edman degradation (43, 44).

N2E4: This peptide was coupled to AEAP-glass by the homoserine lactone method and completely sequenced up to the C-terminal homoserine.

N-3: N-3 was coupled to AEAP-glass by the phenylene diisothiocyanate method. At the 17th cycle of automated Edman degradation, the residual peptide after the 17th residue was released and extracted from the AEAP-glass after trifluoroacetic acid cleavage. The acidic aqueous phase after ethyl acetate extraction was lyophilized and the released peptide was recovered. The peptide was sequenced up to the C-terminal residue by the manual Edman method. The N-terminal Asn

and the 17th Lys were confirmed by sequencing N-3 coupled to AEAP-glass by the carbodiimide method.

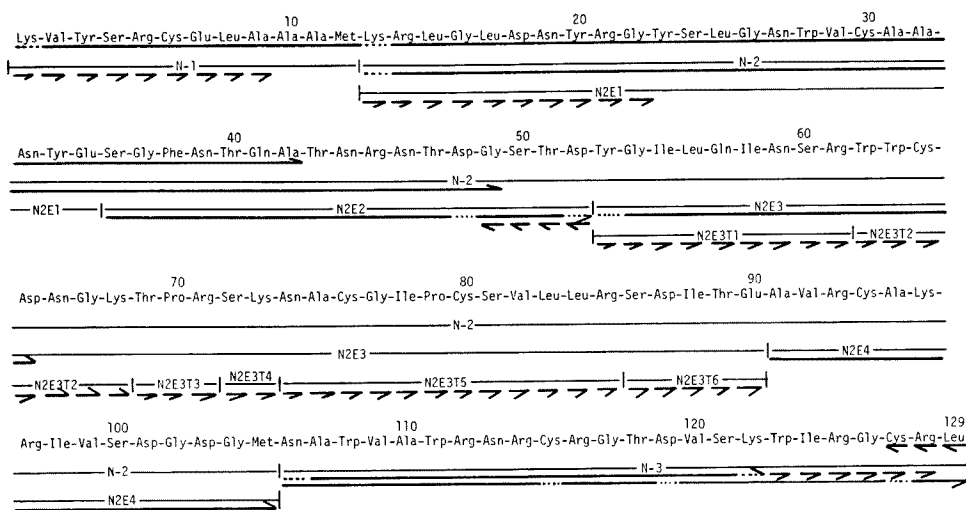
Amino acid compositions of CNBr-peptides, V8 protease peptides and tryptic peptides are shown in Table IS. The alignment of N2E1 and N2E2 was supported by the sequencing of N-2, and N2E4 was located at the C-terminus of N-2 because it contained homoserine/homoserine lactone. Therefore, N2E3 was inserted between N2E2 and N2E4. N2E3T1 and N2E3T2 were aligned according to the sequencing of N2E3. N2E3T5 was located at the C-terminus of N2E3, because this peptide had neither arginine nor lysine. The alignment of N2E3T5 and N2E3T4 was deduced by analogy with the amino acid sequences of other lysozymes, especially Duck II (21). Thus, the amino acid sequence of DL-1 was completed as shown in Fig. 5.

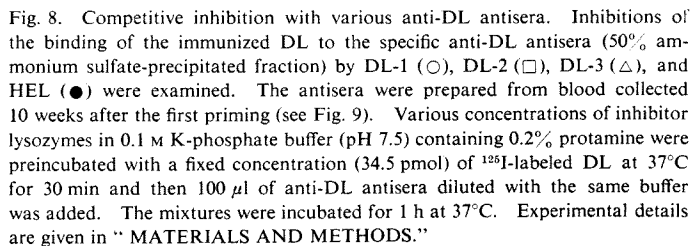
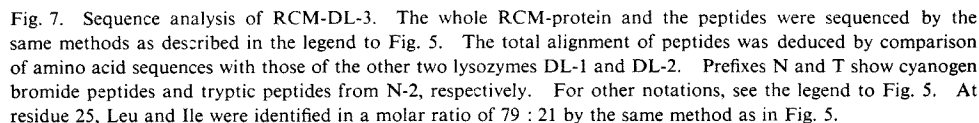
Amino Acid Sequence of DL-2—The purification procedures for CNBr-peptides from RCM-

DL-2, V8 protease peptides from N-2 fragment and tryptic peptides from peptides N2E3 were mostly the same as in the case of RCM-DL-1. The only difference was in the fractionation of peptide N2E3. The tryptic digest of peptide N2E3 was fractionated into six tryptic peptides (N2E3T1 to N2E3T6, see Table IIS) by paper electrophoresis at pH 6.5. Amino acid compositions of CNBr-peptides, V8 protease peptides and tryptic peptides are listed in Table IIS. RCM-DL-2 or the recovered peptides were sequenced by automated Edman degradations, the manual Edman method or the dansyl-Edman method in the manner used for sequence analyses of RCM-DL-1. N3E3T2 was sequenced by the same method as N2E3T2 of RCM-DL-1. RCM-DL-2 released Leu (0.85 mol/mol), Arg (0.45), and Cys (Cm) (0.09) upon carboxypeptidase Y digestion (37°C, 10 min). The results of the sequence analyses of RCM-DL-2 and its peptides are summarized in Fig. 6. The total alignment of the



Fig. 5. Sequence analysis of RCM-DL-1. The whole RCM-protein and peptides were sequenced by the automated Edman method with an LKB 4020 solid-phase sequencer using various coupling methods: phenylene-diisothiocyanate method (—), carbodiimide method (—) and homoserine lactone method (—), as well as manual Edman degradation (→) and the dansyl-Edman method (→). Carboxypeptidase Y digestion (↔) was also used. Prefixes N, E, and T show cyanogen bromide peptides, V8 protease peptides derived from N-2, and tryptic peptides derived from N2E3, respectively. Dotted lines in automated Edman methods show residues that were undetermined because they were attached to AEAP-glass. At residue 25, Leu and Ile were identified in a molar ratio of 82 : 18 by high-performance liquid chromatography of PTH-amino acids (47).





Immunological Properties—We prepared two rabbit anti-DL antisera against each DL protein, that is, D-11 and D-12 antisera for DL-1, D-21 and D-22 antisera for DL-2, and D-31 and D-32 antisera for DL-3. All antisera showed the same precipitin reaction against DL-1, DL-2, and DL-3, since fused lines were formed without any spur against the three DLs, on double diffusion on agar plates. They precisely discriminated HEL from the three DLs, since their precipitin lines against HEL were very much weaker than those against DLs, and DLs formed a strong spur over the HEL precipitin line (data not shown).

The results of competitive inhibition experiments by radioimmunoassay techniques are shown in Fig. 8. All antisera examined were prepared from blood taken 10 weeks after the first injection. The rabbits injected with the same DL produced anti-DL antibody specific to the immunized DL, but with different immunoreactivity. Every system was examined with 5 μ g/ml of ^{125} I-labeled DLs, and all diluted antisera were inhibited to the extent of about 40% by adding the same amount (5 μ g) of cold DLs used for immunization.

D-11 and D-12 antisera differed in ability to discriminate between DL-1 and DL-2. In the case of D-11 antiserum, inhibitions by DL-1, DL-2, and DL-3 were proportional to the extent of amino acid substitution. The inhibition curve by DL-3 seemed to reach a plateau at about 60%. In contrast, in D-12 antiserum, there was no difference in the degree of inhibitions by DL-1 and DL-2 over a wide concentration range of inhibitors, while the inhibition by DL-3 was lower than those of the other two DLs. These results can be tentatively interpreted as follows; D-11 antiserum contains all kinds of antibodies responsible for discrimination among the three DLs, while D-12 antiserum lacks the antibody population responsible for discrimination between DL-1 and DL-2.

D-21 antiserum was not capable of distinction between DL-1 and DL-3. D-22 antiserum distinguished the three DLs and thus seemed to contain all antibodies responsible for discrimination among the three DLs. In the latter antiserum, inhibition by DL-3 was lower than that by DL-1, although the amino acid substitutions in DL-1 and DL-3 occurred at two positions and a single position, respectively, in comparison with DL-2. This result might imply that the substitution at

position 79 had a more pronounced effect on immunoreactivity than those of the two substitutions at positions 37 and 71.

D-31 antiserum was not capable of discrimination between DL-1 and DL-2, and both inhibitions were definitely lower than that by DL-3. D-32 antiserum recognized the differences (amino acid substitutions) among the three DLs, and the inhibitions were proportional to the extent of amino acid substitutions among the DLs.

Inhibitions by HEL in all antisera were always lower than that of any DL, as would be expected from the extensive amino acid substitutions in comparison with DLs.

Figure 9 shows the variations of inhibitions of anti-DL antisera by duck lysozymes during the course of immunization. All anti-DL antisera, excepting D-22 antiserum, showed not variations of inhibition during the immunization course. These results showed that the results of experiments using anti-DL antisera at ten weeks after (see Fig. 8) might be applicable to the other (3 weeks and 6 weeks) antisera. The three anti-DL antisera (D-12, D-21, and D-31) were incapable of discrimination of differences between DL-1 and DL-2, between DL-1 and DL-3, and between DL-1 and DL-2, respectively, from the early stage of immunization.

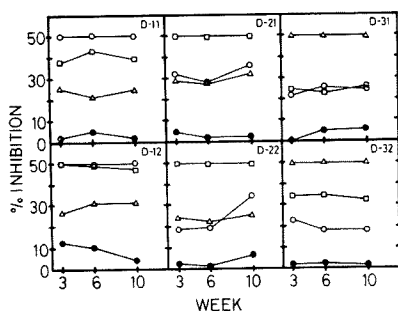


Fig. 9. Variation of competitive inhibitions with immunization course. ^{125} I-labeled DL (34.5 pmol) and various cold lysozymes (equimol) were preincubated at 37°C for 30 min and then anti-DL antisera was added and incubated for 1 h at 37°C. The details are described in "MATERIALS AND METHODS." Booster injections to rabbits were performed 2, 5, and 9 weeks after the first priming. The extents of inhibition by the immunized DLs were about 40%, but they were adjusted to 50% inhibitions. Added inhibitors were DL-1 (○), DL-2 (□), DL-3 (△), and HEL (●).

DISCUSSION

We isolated three lysozymes from Peking-duck egg white, but we could not find a histidine-containing lysozyme such as reported by Jollès *et al.* (18). The amino acid compositions of the three lysozymes were almost identical to the values reported for Duck A, Duck B, and Duck C isolated from Peking-duck egg white by Prager and Wilson (23), except that the values of serine residues of Duck B and Duck C were reported to be 10–11 and 9, respectively, while those of DL-2 and DL-3 were both 10; in addition, they did not evaluate the contents of proline, tryptophan, and cystine residues. We think that DL-1, DL-2, and DL-3 are identical to Duck A, Duck B, and Duck C, respectively, in terms of amino acid sequences. Further support for this view is provided by experiments kindly performed by Drs E.M. Prager and A.C. Wilson (personal communication, 1971) as follows; DL-1 and DL-2 migrated identically with Duck A and Duck B, respectively, in starch gel electrophoreses at pH 5.3 and 11.9. Furthermore, the results of micro-complement fixation tests using anti-Duck A and anti-Duck B antisera indicated that DL-1 and DL-2 were indistinguishable from Duck A and Duck B, respectively.

The enzymatic activities of DLs at pH 6.2 against cell wall of *M. lysodeikticus* were 1.34–1.53 times that of HEL. Jollès *et al.* (45) reported that the optimum pH values of Duck II and Duck III were 8–8.5. Although the enzymatic activities of DLs were not measured under optimum conditions, that of DL-3 was lower than those of the other two DLs. The change of Pro to Arg at position 79 may induce a decrease of enzymatic activity.

The Asn-Gly (66–67) bond caused difficulty in sequence analyses of DLs. We tried the specific cleavage of Asn-Gly bond in RCM-DLs with hydroxylamine at pH 9.5 (46). However, the cleavage yields were under 5%, as estimated by analysis of the N-terminal residues of the reacted DLs by the manual Edman method, and by high-performance liquid chromatography of PTH-amino acids (47). Position 25 in DLs was heterogeneous, and Leu and Ile existed in a molar ratio of about 4:1, as estimated by high-performance liquid chromatography (47). This heterogeneity

was not thought to have had any effect on the immunological analysis.

The three Peking-duck lysozymes differed from HEL in amino acid sequences (Fig. 10) and immunological reactivities (Fig. 8). Figure 10 compares the amino acid sequences of the three DLs from Peking-duck, Duck II and Duck III from Kaki-duck and HEL. DL-1 is identical to Duck II in primary structure, except for one difference at position 57, *i.e.*, Glu in Duck II and Gln in DL-1. DL-2 differs from DL-1 by two substitutions at positions 37 and 71. The former substitution was found in Duck III by Hermann *et al.* (22). They reported that 30% of residue 37 (Ser) in Duck III was substituted by Gly. DL-3 had one more substitution at position 79 (Pro to Arg), as compared with DL-2. The substitution at position 79 newly discovered among duck lysozymes and is not found in other avian lysozymes, though chachalaca lysozyme has a Ser residue at position 79 (12). DL-1, DL-2, and DL-3 differ from Duck III by 6, 6, and 7 residues, respectively (amide differences are omitted). Also, DL-1, DL-2, and DL-3 differ from HEL by 19, 20, and 21 residues out of 129 amino acids, respectively (differences in amide form are omitted); these displacements are found in all four antigenic determinants on the lysozyme molecule, except for the β -sheet region (residues 38–54). These findings are consistent with low immunoreactivities of anti-DL antisera to HEL (see Fig. 8).

Residue 37 is located near the β -sheet region, and residues 71 and 79 are in the loop I region and loop I and II regions, respectively. These three regions participate in the antigenic determinants of the lysozyme molecule (1–9, 11) and the three residues are exposed on the molecular surface (48), so the substitutions must be recognized by anti-DL antibodies. From the secondary structures among DLs, calculated by the method of Chou and Fasman (49, 50) (see Table III), the displacement at residue 37 (Ser to Gly) does not induce any change in the secondary structure, while those at residues 71 (Gly to Arg) and 79 (Pro to Arg) induced reduction in the formation of two β -turns in residues 71–75 and 78–81, respectively. Fainaru *et al.* (17) hypothesized that change of Arg-68 to Lys (hen to turkey lysozymes) induces a conformational change in the loop region, because of the reduction of stability of the

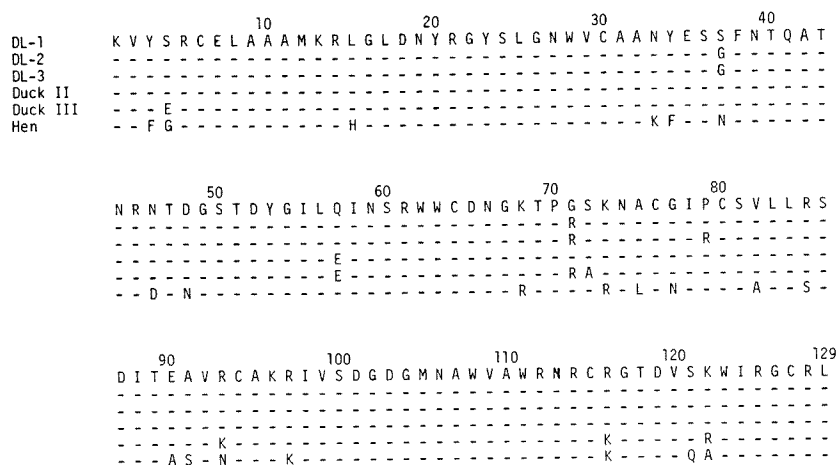


Fig. 10. Comparison of the amino acid sequences of the three lysozymes (DL-1, DL-2, and DL-3) from Peking-duck, two lysozymes (Duck II and Duck III) from Kaki-duck and hen egg white lysozyme (HEL). Bars marked in the sequences indicate that the same residues in DL-1 are located at the same positions.

TABLE III. Effects of amino acid substitutions in the three duck lysozymes.

Position	Amino acid substitution	Side chain ^a	Location ^b	Change of bulk of side chain	Conformational change predicted ^c
37	Ser → Gly	Exposed	β -Sheet	Small	No
71	Gly → Arg	Exposed	Loop I	Large	β -Turn → Random
79	Pro → Arg	Exposed	Loop I/II	Change	β -Turn → Random

^a Locations of side chains are cited from Browne *et al.* (48). ^b Location among the five antigenic determinants in HEL (11). ^c The secondary structures were calculated by the method of Chou and Fasman (49, 50).

β -bend (turn) consisting of residues 66 to 69. A similar conformational change might occur among DL molecules.

The three DLs provide simple systems for immunological analyses of antigenic determinants of the lysozyme molecule, because the amino acid substitutions are restricted to three positions through the three DLs. However, the results of the radioimmunoassay were complicated. A half of the six antisera prepared discriminated the three DLs well. Two (D-12 and D-31 antisera) of the remainder failed to distinguish between DL-1 and DL-2, and D-21 antiserum failed to differentiate between DL-1 and DL-3. Differences in immunoreactivity between the antisera from outbred rabbits immunized with the same antigen DL may be due to genetic differences among individual rabbits.

Ability to discriminate among DLs seems to depend on the presence of an antibody population specific to particular antigenic region of the molecules. We can assume that D-12 antiserum lacks residue 37- and 71-specific antibodies and that D-31 antiserum also lacks the same ones. These two antisera might be capable of discrimination among the three DLs based on distinction (amino acid displacement) at only position 79. Overlapping of the inhibition curves observed in D-21 antiserum by DL-1 and DL-3 cannot be readily elucidated, since substitutions from DL-2 to DL-1 or DL-3 occurred at different positions. The antiserum must contain at least both residue 37- and 71-specific and residue 79-specific antibodies, because the antiserum distinguished DL-2 from DL-1 and DL-3. One possible explanation is that

such phenomena depend on the content of these antibodies, but we had no evidence on this.

As mentioned in the consideration of conformational influences of substitutions (see Table III), substitution at residue 37 has relatively little effect on the immunological properties (*i.e.*, antigenic differences), and substitutions at residues 71 and 79 are predicted to induced the same conformational effect. However, substitution at residue 79 had a remarkable effect on the immunological reactivity, because DL-3 was always the most markedly distinguished from the other two DLs by all rabbit antisera tested, except D-21 antiserum. These results indicated that substitution between Pro and Arg is more distinguishable than that between Gly and Arg by anti-DL antibodies.

Antigenic determinants in the loop region in lysozyme have been investigated extensively (5, 17, 51–54). Arnon and Sela (5) and Maron *et al.* (51) isolated the loop region from HEL and studied its immunological properties. Arnon *et al.* (52) and Teicher *et al.* (54) synthesized a loop region (HEL) analog and its variants. Fainaru *et al.* (17) and Prager *et al.* (16) studied the immunological effects of amino acid displacements in the loop region on the native conformations using hen, bobwhite quail and turkey lysozymes. The above investigation indicated that changes of Pro-79 (to Ala) and Arg-68 (to Ala) had a greater effect on antigenic differences than those of Pro-70 and Arg-73, respectively (54, 17). The effect of substitution at residue 79 on antigenicity in DLs was similar to that found in these studies. However, we could not estimate the influence of change at position 71 from the results of our investigation and the findings reported above.

It is still essential to clarify the correlation between amino acid substitution and antibody population in order to completely understand the immunological significance of amino acid substitutions in cross-reactive proteins. It was reported that the loop-specific antibody accounts for 5–14% of typical anti-lysozyme antibody (55). Therefore, whenever we use anti-DL antisera preparation for investigations on the antigenic loop region, we have to consider the influences of various other antigenic site-specific antibodies. We hope to investigate further the antigenic properties of the three DLs by using the loop region specific

antibody and/or antisera in which the loop region-antibody is removed, in order to obtain simpler systems.

We are very grateful to Drs. E.M. Prager and A.C. Wilson, Department of Biochemistry, University of California, for their gift of duck lysozymes and also performing studies to compare our duck lysozymes and their duck lysozymes. We are indebted to Dr. H. Toda, Institute for Protein Research, Osaka University, for her advice and discussions on sequence analyses. Thanks also due to Mr. H. Shibata for his assistance with the purification of duck lysozymes.

REFERENCES

1. Shinka, S., Imanishi, M., Miyagawa, N., Amano, T., Inoue, M., & Tsugita, A. (1967) *Biken J.* **10**, 89–107
2. Fujio, H., Imanishi, M., Nishioka, K., & Amano, T. (1968) *Biken J.* **11**, 207–218
3. Fujio, H., Imanishi, M., Nishioka, K., & Amano, T. (1968) *Biken J.* **11**, 219–223
4. Takagaki, Y., Hirayama, A., Fujio, H., & Amano, T. (1980) *Biochemistry*, **19**, 2498–2505
5. Arnon, R., & Sela, M. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 163–170
6. Arnon, R. (1974) *Rehovot Symposium on Poly Amino Acids, Polypeptides and Proteins and Their Biological Implications*, p. 538
7. Ibrahim, I.M., Eder, J., Prager, E.M., Wilson, A.C., & Arnon, R. (1980) *Mol. Immunol.* **17**, 37–46
8. Atassi, M.Z. & Lee, C.L. (1978) *Biochem. J.* **171**, 429–434
9. Atassi, M.Z. (1978) *Immunochemistry* **15**, 909–936
10. Jollès, J., Ibrahim, I.M., Prager, E.M., Schoentgen, F., Jollès, P., & Wilson, A.C. (1979) *Biochemistry* **18**, 2744–2752
11. Fujio, H., Shinka, S., Dohi, Y., & Amano, T. (1980) *Immunology '80* (in Japanese) (Yamamura, Y., Tada, T., & Kishimoto, C., eds.) pp. 105–118, Nakayama Shoten, Tokyo
12. Jollès, J., Schoentgen, F., Jollès, P., Prager, E.M., & Wilson, A.C. (1976) *J. Mol. Evol.* **8**, 59–78
13. White, T.J., Mross, G.A., Osserman, E.F., & Wilson, A.C. (1977) *Biochemistry* **16**, 1430–1436
14. White, T.J., Ibrahim, I.M., & Wilson, A.C. (1978) *Nature* **274**, 92–94
15. Ibrahim, I.M., Prager, E.M., White, T.J., & Wilson, A.C. (1979) *Biochemistry* **18**, 2736–2744
16. Prager, E.M., Fainaru, M., Wilson, A.C., & Arnon, R. (1974) *Immunochemistry* **11**, 153–156
17. Fainaru, M., Wilson, A.C., & Arnon, R. (1974) *J. Mol. Biol.* **84**, 635–642

18. Jollès, J., Spotorno, G., & Jollès, P. (1965) *Nature* **208**, 1204–1205
19. Jollès, P., Charlemagne, D., Petit, J.F., Maire, A.C., & Jollès, J. (1965) *Bull. Soc. Chim. Biol.* **47**, 2241–2259
20. Imanishi, M., Shinka, S., Miyagawa, N., Amano, T., & Tsugita, A. (1966) *Biken J.* **9**, 107–114
21. Hermann, J. & Jollès, J. (1970) *Biochim. Biophys. Acta* **200**, 178–179
22. Hermann, J., Jollès, J., & Jollès, P. (1971) *Eur. J. Biochem.* **24**, 12–17
23. Prager, E.M. & Wilson, A.C. (1971) *J. Biol. Chem.* **246**, 523–530
24. Davis, B.J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
25. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
26. Parry, R.M., Jr., Chandan, R.C., & Shahani, K.M. (1965) *Proc. Soc. Exp. Biol. Med.* **119**, 384–386
27. Crestfield, A.M., Moore, S., & Stein, W.H. (1963) *J. Biol. Chem.* **238**, 622–627
28. Laursen, R.A., Horn, M.J., & Bonner, A.G. (1972) *FEBS Lett.* **21**, 67–70
29. Horn, M.J. & Laursen, R.A. (1973) *FEBS Lett.* **36**, 285–288
30. Previero, A., Derancourt, J., Coletti-Previero, M.-A., & Laursen, R.A. (1973) *FEBS Lett.* **33**, 135–138
31. Edman, P. & Henschen, A. (1975) in *Protein Sequence Determination* (Needleman, S.B., ed.) 2nd. Ed., pp. 232–262, Springer-Verlag, Berlin, Heidelberg, New York
32. Gray, W.R. (1972) in *Methods in Enzymology* (Hirs, C.H.W. & Timasheff, S.N., eds.) Vol. 25, pp. 333–344, Academic Press, New York and London
33. Jeppsson, J.-O. & Sjöquist, J. (1967) *Anal. Biochem.* **18**, 264–269
34. Bridgen, J., Graffeo, A.D., Karger, B.L., & Waterfield, M.D. (1975) in *Instrumentation in Amino Acid Sequence Analysis* (Perham, R.N., ed.) pp. 111–145, Academic Press, London, New York, San Francisco
35. Klube, K.D. (1974) *Anal. Biochem.* **59**, 564–573
36. Inagami, T. (1973) *Anal. Biochem.* **52**, 318–321
37. Yamada, S. & Itano, H.A. (1966) *Biochim. Biophys. Acta* **130**, 538–540
38. Spackman, D.H., Stein, W.H., & Moore, S. (1958) *Anal. Chem.* **30**, 1190–1206
39. Simpson, R.J., Neuberger, M.R., & Liu, T.Y. (1976) *J. Biol. Chem.* **251**, 1936–1940
40. MacFarlane, A.S. (1958) *Nature* **182**, 53–54
41. Ouchterlony, O. (1949) *Acta Pathol. Microbiol. Scand.* **26**, 507–515
42. Kobayashi, T., Fujio, H., Kondo, K., Dohi, Y., Hirayama, A., Takagaki, Y., Kosaki, G., & Amano, T. (1982) *Mol. Immunol.* in press
43. Groskopf, W.R., Holleman, J.W., Margoliash, E., & Klotz, I.M. (1966) *Biochemistry* **5**, 3738–3796
44. Weber, K.I. & Konigsberg, W. (1967) *J. Biol. Chem.* **242**, 3563–3578
45. Jollès, P., Bernier, I., Berthon, J., Charlemagne, D., Faure, A., Hermann, J., Jollès, J., Perin, J.P., & Saint-Blancard, J. (1974) in *Lysozymes* (Osserman, E.F., ed.) pp. 31–54, Academic Press, New York and London
46. Bornstein, P. & Bolian, G. (1970) *J. Biol. Chem.* **245**, 4854–4856
47. Bhow, A.S., Mole, J.E., Weissinger, A., & Bennet, J.C. (1978) *J. Chromatogr.* **148**, 532–535
48. Browne, W.J., North, A.C.T., Phillips, D.C., Brew, K., Vanaman, T.C., & Hill, R.L. (1969) *J. Mol. Biol.* **42**, 65–86
49. Chou, P.Y. & Fasman, G.D. (1977) *J. Mol. Biol.* **115**, 137–175
50. Chou, P.Y. & Fasman, G.D. (1978) in *Advances in Enzymology* (Meister, A., ed.) Vol. 47, pp. 45–148, Interscience Publication John Wiley & Sons, New York, Chichester, Brisbane, Toronto
51. Maron, E., Shiozawa, C., Arnon, R., & Sela, M. (1971) *Biochemistry* **10**, 763–771
52. Arnon, R., Maron, E., Sela, M., & Anfinsen, C.B. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1450–1455
53. Pecht, I., Maron, E., Arnon, R., & Sela, M. (1971) *Eur. J. Biochem.* **19**, 368–371
54. Teicher, E., Maron, E., & Arnon, R. (1973) *Immunochemistry* **10**, 265–271
55. Arnon, R. (1977) in *Immunochemistry of Enzymes and Their Antibodies* (Salton, M.J.R., ed.) pp. 1–28, John Wiley & Sons Inc., New York

Supplemental Materials

Table IS Amino acid compositions of cyanogen bromide peptides (prefix N), V8 protease peptides (prefix E) from N-2 fragment and tryptic peptides (prefix T) from peptide N2E3. Numbers in parentheses are from the sequence determined. P, S, DE and PE in purification mean Bio-Gel P-10, Sephadex G-50 fine and DEAE-cellulose column chromatographies and paper electrophoresis at pH 6.5, respectively. Peptides were hydrolyzed with 6M HCl containing 1% phenol for 24 h at 105°C. Trp content is uncorrected.

	N-1	N-2	N-3	N2E1	N2E2	N2E3	N2E4	N2E3T1	N2E3T2	N2E3T3	N2E3T4	N2E3T5
Lys	0.81 (1)	3.84 (4)	0.95 (1)	1.12 (1)		1.93 (2)	0.90 (1)		0.60 (1)	0.84 (1)		
Arg	1.03 (1)	7.16 (7)	5.04 (5)	1.98 (2)	1.14 (1)	2.13 (2)	2.00 (2)	0.76 (1)			0.97 (1)	
Trp ^a		1.71 (3)	1.73 (3)	0.37 (1)		+	(2)		+	(2)		
HsL ^a	0.37 (1)	0.56 (1)				0.69 (1)						
Cys(Cm)	1.00 (1)	5.03 (5)	2.15 (2)	1.10 (1)		2.77 (3)	1.06 (1)		1.07 (1)		1.70 (2)	
Asp		15.36 (16)	3.09 (3)	4.06 (4)	4.73 (5)	5.13 (5)	2.13 (2)	0.97 (1)	1.90 (2)		1.13 (1)	1.09 (1)
Thr		5.86 (6)	1.02 (1)		3.91 (4)	1.80 (2)				0.54 (1)		0.89 (1)
Ser	0.94 (1)	8.39 (9)	1.07 (1)	1.09 (1)	2.66 (3)	3.51 (4)	0.95 (1)	1.01 (1)	0.32		0.96 (1)	0.74 (1)
Glu	1.16 (1)	4.02 (4)	1.02 (1)	0.23	1.04 (1)	1.74 (2)	0.29	0.83 (1)				1.18 (1)
Pro		2.00 (2)				2.23 (2)				0.70 (1)	0.48 (1)	
Gly		9.81 (10)	1.96 (2)	3.12 (3)	1.01 (1)	3.77 (4)	1.82 (2)	0.95 (1)	1.32 (1)	1.26 (1)	1.04 (1)	
Ala	2.81 (3)	6.17 (6)	1.96 (2)	2.19 (2)	0.81 (1)	1.15 (1)	1.81 (2)				0.74 (1)	
Val	1.07 (1)	4.02 (4)	2.16 (2)	0.97 (1)		1.09 (1)	1.22 (2)				0.68 (1)	
Ile		4.72 (5)	1.05 (1)	0.23		3.45 (4)	0.53 (1)	1.50 (2)	0.35		1.04 (1)	0.91 (1)
Leu	0.98 (1)	4.79 (6)	1.05 (1)	2.39 (3)		2.51 (3)		0.73 (1)	0.38		1.51 (2)	
Tyr	0.88 (1)	3.56 (4)		2.86 (3)		0.84 (1)						
Phe		0.80 (1)			1.01 (1)							
Total	12	93	24	23	17	38	15	9	7	5	12	5
Yield(%)	82.4	68.1	65.9	38.7 ^d	28.5 ^d	30.3 ^d	69.5 ^d	37.0 ^e	55.0 ^e	40.0 ^e	30.0 ^e	73.0 ^e
Purification	P	P	P	S,DE	S,DE	S,DE	S,DE	PE	PE	PE	PE	PE

a Homoserine lactone. b Leu-Leu (83-84) bond is present. c Ile-Val (98-99) bond is present. d Recoveries from N-2 fragment.

e Recoveries from peptide N2E3.

Table IIS Amino acid compositions of cyanogen bromide peptides from RCM-DL-2, V8 protease peptides from N-2 fragment and tryptic peptides from peptide N2E3. Numbers in parentheses are from the sequence determined. Notations and notes are the same as those in Table IS.

	N-1	N-2	N-3	N2E1	N2E2	N2E3	N2E4	N2E3T1	N2E3T2	N2E3T3	N2E3T4	N2E3T5	N2E3T6
Lys	1.01 (1)	3.80 (4)	0.93 (1)	0.73 (1)		2.00 (2)	1.08 (1)	0.09	1.02 (1)		1.03 (1)	1.03 (1)	1.09 (1)
Arg	0.89 (1)	7.84 (8)	5.10 (5)	2.01 (2)	0.85 (1)	3.24 (3)	2.06 (2)	1.00 (1)					
Trp ^a		1.71 (3)	2.01 (3)	0.30 (1)		0.74 (2)			+	(2)			
HsL ^a	0.44 (1)	0.58 (1)				0.63 (1)							
Cys(Cm)	0.98 (1)	5.10 (5)	1.99 (2)	1.18 (1)		3.23 (3)	0.86 (1)	0.03	0.56 (1)			1.75 (2)	
Asp		15.38 (16)	3.14 (3)	3.92 (4)	4.52 (5)	5.08 (5)	2.22 (2)	1.30 (1)	1.86 (2)			1.08 (1)	1.11 (1)
Thr		5.78 (6)	0.77 (1)		3.93 (4)	2.01 (2)		0.03	0.13	0.84 (1)		0.09	0.93 (1)
Ser	0.84 (1)	7.67 (8)	0.81 (1)	0.99 (1)	2.07 (2)	3.21 (4)	1.01 (1)	1.00 (1)	0.15		1.06 (1)	0.86 (1)	0.70 (1)
Glu	0.99 (1)	3.74 (4)		1.04 (1)	1.08 (1)	1.68 (2)		1.04 (1)	0.12				1.10 (1)
Pro		2.16 (2)				2.02 (2)				0.87 (1)		0.54 (1)	
Gly		9.72 (10)	2.03 (2)	2.73 (3)	1.90 (2)	2.53 (3)	1.84 (2)	1.03 (1)	1.20 (1)			1.25 (1)	
Ala	3.10 (3)	6.04 (6)	1.88 (2)	2.11 (2)	0.71 (1)	1.14 (1)	1.78 (2)	0.05				0.97 (1)	
Val	1.10 (1)	4.14 (4)	2.05 (2)	0.93 (1)		0.80 (1)	1.22 (2)					0.61 (1)	
Ile		4.90 (5)	0.90 (1)	0.24		3.43 (4)	0.55 (1)	1.36 (2)	0.08			0.82 (1)	0.84 (1)
Leu	0.98 (1)	5.05 (6)	1.01 (1)	2.22 (3)		2.66 (3)		0.82 (1)	0.09			1.42 (2)	
Tyr	0.98 (1)	3.84 (4)		2.64 (3)		0.90 (1)		0.43 (1)					
Phe		0.99 (1)			0.93 (1)								
Total	12	93	24	23	17	38	15	9	7	3	2	12	5
Yield(%)	62.4	68.1	64.5	58.3 ^d	30.4 ^d	30.9 ^d	55.6 ^d	42.0 ^e	38.0 ^e	36.0 ^e	56.0 ^e	28.0 ^e	59.0 ^e
Purification	P	P	P	S,DE	S,DE	S,DE	S,DE	PE	PE	PE	PE	PE	PE

Table IIIS Amino acid compositions of cyanogen bromide peptides from RCM-DL-3 and tryptic peptides from N-2 fragment.

Numbers in parentheses are from the sequence determined. Numbering of tryptic peptides was based on the spot numbers on the tryptic peptide map shown in Fig. 35.

	N-1	N-2	N-3	N2T1-1	N2T1-2	N2T2	N2T3	N2T4	N2T5	N2T6	N2T7
Lys	1.00 (1)	3.96 (4)	1.00 (1)				0.46 (1)	0.23		0.20	0.58 (1)
Arg	0.98 (1)	9.05 (9)	4.70 (5)	1.00 (1)	1.05 (1)	0.50 (1)		1.06 (1)	1.23 (1)	1.00 (1)	
Trp ^a		1.30 (3)	1.48 (3)				+	(2)	+	(1)	
HsL ^a	0.42 (1)	0.35 (1)									
Cys(Cm)	0.94 (1)	4.93 (5)	1.87 (2)				0.39 (1)	0.89 (1)			
Asp		15.99 (16)	2.73 (3)	2.01 (2)			2.00 (2)	4.19 (4)	0.94 (1)	4.14 (4)	
Thr		5.72 (6)	0.92 (1)				0.22	2.02 (2)	1.12 (1)	1.83 (2)	
Ser	1.02 (1)	7.66 (8)	1.06 (1)		0.90 (1)	0.98 (1)	0.25	1.87 (2)	0.98 (1)	1.43 (2)	0.88 (1)
Glu	1.44 (1)	4.33 (4)					0.20	1.96 (2)	0.95 (1)	0.72 (1)	
Pro		0.75 (1)									
Gly		10.10 (10)	1.89 (2)	0.98 (1)			1.29 (1)	2.66 (3)	0.37	1.93 (2)	
Ala	3.00 (3)	6.18 (6)	1.60 (2)				0.26	2.32 (3)	0.77 (1)		
Val	0.92 (1)	3.99 (4)	1.42 (2)		0.84 (1)	0.51 (1)	0.14	0.72 (1)	0.94 (1)		
Ile		4.64 (5)	0.81 (1)			0.65 (1)	0.09	0.27	0.91 (1)	1.45 (2)	
Leu	0.84 (1)	6.18 (6)	0.94 (1)	2.00 (2)	1.50 (2)		0.13	0.92 (1)	0.30	0.78 (1)	
Tyr	0.88 (1)	3.88 (4)		0.87 (1)			0.21	1.62 (2)	0.15	0.84 (1)	
Phe		0.57 (1)						0.79 (1)			
Total	12	93	24	7	6	4	7	24	8	16	2
Yield(%)	77.4	65.2	63.3	70.0 ^b	70.0 ^b	8.0 ^b	9.5 ^b	12.5 ^b	57.9 ^b	35.6 ^b	7.5 ^b

a Homoserine lactone. b Recoveries from N-2 fragment.

(to be continued)

(Table IIIS cont'd)

	N2T8	N2T9	N2T10	N2T11	N2T12	N2T13	N2T14	N2T15	N2T16	N2T17
Lys	0.50 (1)		0.15	0.13	1.04 (1)	1.00 (1)	0.45 (1)	0.85 (1)		
Arg	0.86 (1)	(1)	1.02 (1)	1.00 (1)	0.99 (1)	0.84 (1)	0.99 (1)	0.23		
Trp	+	(2)								
HsL									0.46 (1)	0.56 (1)
Cys(Cm)	0.42 (1)			0.91 (1)			1.02 (1)	1.11 (1)		
Asp	2.04 (2)		0.07	1.07 (1)				0.15	2.11 (2)	2.11 (2)
Thr	0.89 (1)		0.71 (1)	0.08		0.57 (1)	0.09	0.10	0.11	
Ser	0.13		0.12	0.11		0.93 (1)	0.16	0.10	1.11 (1)	
Glu	0.20		0.07	0.06			0.08	0.05		
Pro	0.64 (1)		0.73 (1)			+	(1)			
Gly	1.07 (1)			0.96 (1)			0.14	0.27	2.30 (2)	2.03 (2)
Ala	0.18			0.85 (1)			0.90 (1)	0.78 (1)	0.25	
Val	0.13			0.04				0.17	0.51 (1)	
Ile	0.29			0.63 (1)				0.06	0.65 (1)	
Leu	0.12			0.06						
Tyr	0.14									
Phe										
Total Yield(%)	10 43.0 ^b	1 32.5 ^b	3 20.0 ^b	6 85.0 ^b	2 8.0 ^b	5 28.0 ^b	4 15.0 ^b	3 75.0 ^b	8 65.6 ^b	5 15.0 ^b

a Homoserine lactone. b Recoveries from N-2 fragment.

LEGENDS TO FIGURES (MINIPRINTS)

Fig. 1S Chromatography of cyanogen bromide peptides derived from RCM-DL-1. The CNBr-peptides were fractionated on a Bio-Gel P-10 column (2.0 x 143 cm) with 10% formic acid. The flow rate was 18 ml/h and 2.7-ml fractions were collected. The absorbance at 280 nm was measured and the fractions marked by a bar were collected and lyophilized.

Fig. 2S Chromatography of V8 protease peptides derived from large CNBr-peptide N-2 (RCM-DL-1) in Fig. 1S. The digest was fractionated on a Sephadex G-50 fine column (1.5 x 200 cm) with 0.05 M NH₄OH at a flow rate of 5 ml/h, and 3-ml fractions were collected. The effluent was monitored by measuring absorbances at 230 nm (—) and 280 nm (-----). The fractions marked by a bar were collected and lyophilized.

Fig. 3S Peptide map of tryptic peptides derived from peptide N-2 of RCM-DL-3. The tryptic digest (0.8 μmol) was first electrophoresed at pH 6.5 (pyridine-acetic acid-water, 25 : 1 : 450, by vol.) and then chromatographed with 1-butanol-pyridine-acetic acid-water (15 : 10 : 3 : 12, by vol.). The dotted position shows the position of the absent spot from the tryptic peptide map of peptide N-2 of RCM-DL-2 and spot 11 is a new spot in the tryptic map of N-2 of RCM-DL-3. The other spots were the same in both digests, except that the ninhydrin color of spot 1 was increased in comparison with that of the corresponding spot of the peptide map of RCM-DL-2. K, G, E, D and NR show the positions of Lys, Gly, Glu and Asp in pH 6.5 electrophoresis and neutral red in chromatography, respectively.

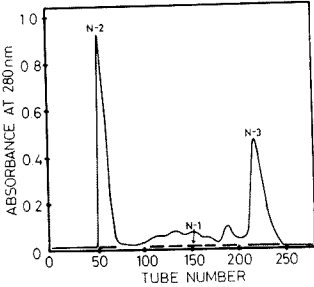


Fig. 1S

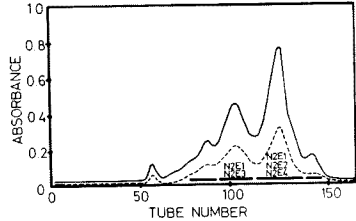


Fig. 2S

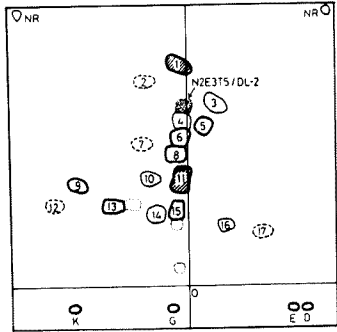


Fig. 3S