BIKEN JOURNAL Vol. 10, 89-107, 1967

CHEMICAL STUDIES ON ANTIGENIC DETERMINANTS OF HEN EGG WHITE LYSOZYME. I

SOHEI SHINKA, MICHIKO IMANISHI, NOBUYUKI MIYAGAWA and TSUNEHISA AMANO

Department of Immunochemistry, Research Institute for Microbial Diseases, Osaka University, Osaka

MASAYORI INOUYE and AKIRA TSUGITA

Laboratory of Molecular Genetics, Osaka University Medical School, Osaka

(Received March 20, 1967)

SUMMARY Four immunologically active peptides were purified from a peptic digest of hen egg white lysozyme and their chemical structures were elucidated. They were all located between Gln^{57} and Ala^{107}, but each had a cleavage in the loop between Cys^{80} and Cys^{94}. The chemical structure of one of these was elucidated by sequential analysis of the peptides formed by tryptic digestion.

The four peptides obtained by pepsin digestion seemed to bear at least one antigenic determinant. They inhibited the precipitin reaction of the homologous system by a maximum of about 25 per cent, but did not inhibit the neutralization by antibodies.

A peptide obtained by trypsin degradation, consisting of three peptide sequences Trp^{62}-Arg^{68}, Asn^{74}-Leu^{83} and Leu^{84}-Lys^{96} linked by two disulfide bonds, Cys^{64}-Cys^{80} and Cys^{76}-Cys^{94}, was also found to bear weak but definite immunological activity.

INTRODUCTION

Hen egg white lysozyme (HL) is a very suitable protein antigen to study, because, 1) its molecular weight (14,300) is one of the lowest of all antigenic proteins, 2) it is very stable, 3) its amino acid sequence has already been established by JOLLÈS *et al.* (1963, 1964, 1965) and independently by CANFIELD and his coworkers (1963, 1965), 4) its tertiary structure was elucidated by X-ray analysis by BLAKE *et al.* (1965), the amino acid residues playing roles in fixing the substrate on the molecule (mainly Trp^{62}) (HAYASHI *et al.*, 1965; BLAKE *et al.*, 1965; PHILLIPS, 1966) and in hydrolyzing a glucoside linkage (Glu^{35} and Asp^{52}) (BLAKE *et al.*, 1965; PHILLIPS, 1966) were suspected, and 5) its enzymic activity can be neutralized completely by antibodies when cells of *Micrococcus lysodeikticus* are used as substrate. Therefore, this enzyme has been analyzed immunochemically in this laboratory (FUJIO *et*

al., 1959, 1962; SHINKA *et al.*, 1962; SHINKA, 1963). In these studies, SHINKA *et al.* (1962) described two peptides which were split from HL by pepsin and capable of inhibiting the precipitin reaction of the HL~ anti-HL system but not neutralization. In addition, these peptides could not be precipitated by HL antiserum. These peptides were shown to be derived from HL and not from traces of contaminating protein, and through these studies the presence of non-neutralizing but precipitating antibodies was demonstrated.

Studies were continued to isolate pure peptides with immunological activity. However the peptides isolated by Amberlite IRC-50 (XE-64) chromatography had molecular weights of about 20,000 as estimated by Archibald's method (ARCHIBALD, 1947; KLAINER and KEGELES, 1956). This indicates that they were polymerized, because the molecular weight of HL is 14,300. Accordingly, attempts were made to purify immunologically active peptides in a non-polymerized form. For this purpose, chromatographic procedures were carried out as far as possible at acidic pH (RYLE and SANGER, 1955; SPACKMAN *et al.*, 1960), and four immunologically active peptides were isolated. They all inhibited the precipitin reaction but not neutralization reaction, and their chemical structures were elucidated. , Four other immunologically inactive peptides were also isolated and their structures were clarified.

MATERIALS AND METHODS

1. *Hen egg white lysozyme (HL)*

Isoelectric crystals of HL were obtained from hen egg white according to the method of ALDERTON and FEVOLD (1946). For pepsin digestion, four times recrystallized HL was used, and for immunizing rabbits, HL was recrystallized three times and then purified further by IRC-50 (XE-64) column chromatography according to the method of HIRS *et al.* (1953).

2. *Antisera*

HL antisera were prepared as described in our previous report (FUJIO *et al.*, 1959). Ten mg of HL with incomplete Freund's adjuvant were injected into a rabbit subcutaneously once a month and immunization was continued for 3–4 months. Rabbits were bled 2 weeks after the last injection. Batches No. 332 and 166 of antiserum were employed in this study; the total precipitated protein N per ml of antiserum at the equivalence point and the amounts of HL added as protein N were 508 μg N : 30 μg N (No. 332) and 460 μg N : 27 μg N (No. 166). The antisera were inactivated at 56°C for 30 minutes and serum lysozyme was removed by treatment with Bentnite (Wako Pure Chemicals) according to the method of INAI *et al.* (1958).

Antiserum for bovine serum albumin (BSA) (Armour, powder) was obtained in a similar manner to HL antisera. The total precipitated protein N per ml of antiserum at equivalence was 630 μg and the amount of BSA added was 52 μg N.

3. *Proteolytic enzymes*

Crystalline pepsin was prepared from crude commercial pepsin (Difco) according to the method of NORTHROP (1930) and recrystallized 5 times. The activity was estimated in international units.

Crystalline trypsin (batch No. TRSF 839) and carboxypeptidases A and B (CoA-DFP, CoB-DFP) were obtained from Worthington Biochemical Corp.

4. *Sephadex and carboxymethyl (CM) cellulose*

Sephadex G-75 (medium) and G-25 (fine) were purchased from Pharmacia Co. and CM cellulose (200–400 mesh) from Serva Co.

5. *Quantitative precipitin reaction*

The experiments were performed according to the method of HEIDELBERGER and KENDALL (1935) and precipitated protein N was estimated by direct Nesslerization (YOKOI and AKASHI, 1955).

6. *Quantitative inhibition of precipitin reaction*

Various amounts (1–40 μg N) of each peptide dissolved in 0.2 ml of phosphate buffered saline (pH 6.0) were added to aliquots of 0.1 ml of HL antiserum (batch No. 332) in conical tubes and a control tube containing saline in place of peptide solution was also set up. After incubation at 37°C for 30 minutes, 50 μl of buffered saline containing 3 μg N of HL (equivalent to 0.1 ml of antiserum) were added. The tubes were incubated at 37°C for 1 hour, then at 4°C for 48 hours and then centrifuged in the cold.

After washing the precipitates twice with 2.0 ml of chilled buffered saline, they were dissolved with 50 μl of 1 N NaOH and the total volume was adjusted to 3.0 ml. Optical densities of the solutions were read at 280 mμ and the degree of inhibition was calculated by reference to the control.

The inhibitory effects of the peptides which did not reduce the amount of protein N precipitated were further examined by estimating the delay in the time of developement of turbidity at 475 mμ in a Gilford automatic recording spectrophotometer (Model 2000). In this experiment batch No. 166 of HL antiserum was used.

7. *Estimation of HL activity and inhibitory effects of peptides on the neutralizing activity of HL antiserum*

Both estimations were carried out as described in our previous report (SHINKA *et al.*, 1962).

In estimating the inhibitory effect, solutions of various concentrations of a peptide were incubated at 37°C for 60 minutes with HL antiserum and then the maximal amount of HL which was completely neutralized by the antiserum was added. After incubation at 37°C for 60 minutes, HL activity was estimated.

8. *Amino acid analysis*

The amino acid composition of the peptides was analyzed in a Beckman-Spinco amino acid analyzer, Model 120B, by the procedures of TSUGITA and FRAENKEL-CONRAT (1962) following the instructions for the analyzer. Hydrolysis was carried out at 105°C±1°C for 24 hours. The molar ratios of amino acids were calculated on the basis of Glu or Gly.

Tryptophan was determined spectrophotometrically by the method of GOODWIN and MORTON (1946).

Half cystine was analyzed after performic acid oxidation (HIRS, 1956).

9. *Terminal amino acid sequence*

The N-terminal sequence was analyzed by the phenyl isothiocyanate (PTC) method and phenyl thiohydantoin amino acids were identified by paper chromatography with solvent F according to the method of SJÖQUIST (1953, 1956).

The C-terminal sequence was analyzed with carboxypeptidase (enzyme/peptide ratio=1 : 50) and the amino acids reliesed were measured in the analyzer.

10. *Paper-electrophoresis*

High voltage electrophoresis was carried out to purify the peptides using the apparatus described by KATZ *et al.* (1959) with pyridine-acetate buffer. Toyo-roshi No. 51A paper was used.

11. *Optical rotatory dispersion (ORD)*

ORD was determined in a Rudolph 200 S photoelectric polarimeter at room temperature in the range of 578–365 mμ using a mercury arc lamp as a light source.

12. *Determination of peptide concentration*

Peptides were determined by absorption at 280 mμ and/or at 570 mμ after the ninhydrin reaction. The latter was carried out according to the method of COCKING and YEMM (1955). Concentrations of pure peptides were determined by UV absorption or as protein N by direct Nesslerization (YOKOI and AKASHI, 1955).

13. *Desalting*

Peptide solutions were desalted according to the method of DIXON (1959) using an IRC-50 (CG-50) column. Peptides were eluted with 50 per cent acetic acid and then lyophilized.

14. *Molecular weights*

Molecular weights were estimated by ultracentrifugation according to Archibald's method (ARCHIBALD, 1947; KLAINER and KEGELES, 1956). Runs were carried out at 27,160 rpm and calculations were made with data obtained from the meniscus and assuming the partial specific volume to be 0.7.

RESULTS

1. *Peptic digestion of HL and isolation of peptides*

As described in our previous report (SHINKA *et al.*, 1962), HL was digested by pepsin at pH 1.62. To 1000 ml of 1.21 per cent HL solution, dissolved in 0.1 N HCl and adjusted to pH 1.62 with concentrated NaOH, were added 3.3 ml of pepsin solution containing 5200 I.P.U. $I^{cas275}_{\gamma\text{-}Tyr}$ and the mixture was incubated at 40°C for 1 hour. No appreciable change of pH was found during the incubation.

After digestion, the pH was adjusted to 5.0 with ammonium hydroxide and the solution was lyophilized.

Then 2.1 gr of the lyophilized material were dissolved in 1000 ml of 0.05 M acetate buffer at pH 3.85 and applied to a CM cellulose column (3×40 cm) equilibrated with the same buffer. The column was washed with 2500 ml of the same buffer. Elution was performed at 25°C at a flow rate of 320 ml per hour with 6000 ml of 0.05 M acetate buffer at pH 3.85 containing NaCl in a linear concentration gradient up to 0.15 M and then with a linear gradient mixture of 2000 ml 0.05 M acetate buffer at pH 3.85 containing 0.15 M NaCl and 2000 ml of 0.2 M phosphate buffer at pH 8.0. The fraction volumes were 18.8 ml and the peptides were estimated by their O.D. at 280 mμ and by the ninhydrin reaction. As shown in Fig. 1, about 20 peaks were observed and the last component which had HL activity was regarded as intact HL. However, the other fractions were devoid of enzyme activity. The reproducibility of the chromatographic pattern was confirmed in repeated runs.

Preliminary tests were carried out on the immunological activity of each fraction. An aliquot of each fraction (Frs. 6~15) was lyophilized and dissolved to saturation in 1.0 ml of saline containing 0.02 M phosphate buffer at pH 6.0. The solution was applied to a Sephadex G-25 column (1×30 cm) equilibrated with the same buffered saline and fraction volumes of 2.8 ml were collected. Then

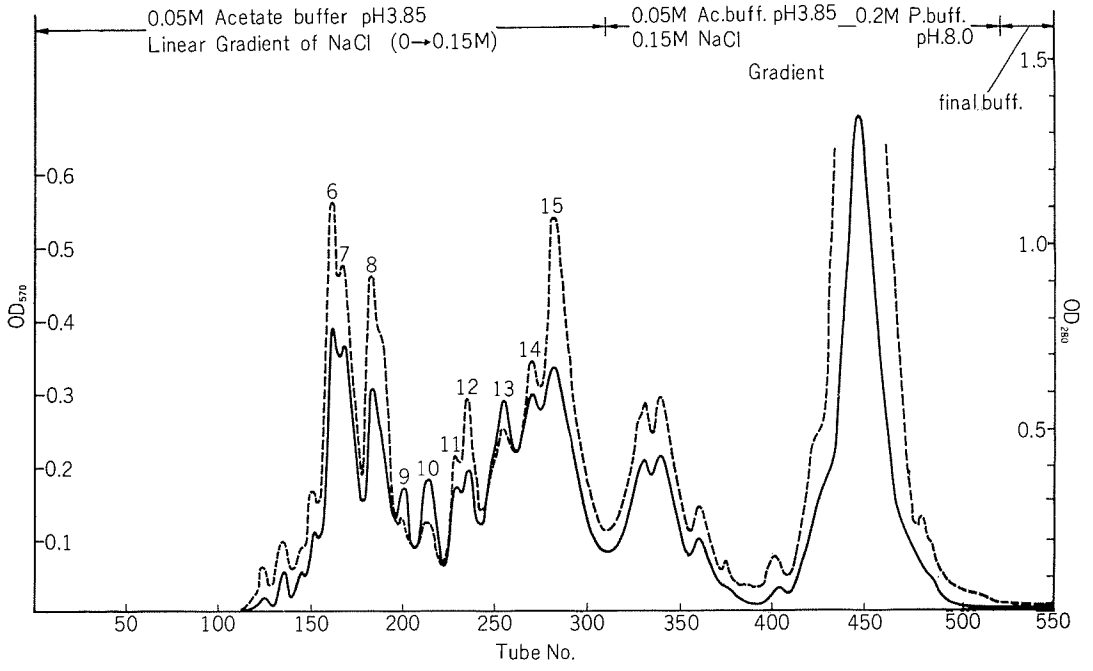

FIGURE 1 CM-cellulose chromatography of peptic digest of HL.
column size : 3×40 cm ;
fraction volume : 18.8 ml per tube ;
temperature : 25°C
——— OD at 570 mμ (ninhydrin reaction)
· · · · OD at 280 mμ

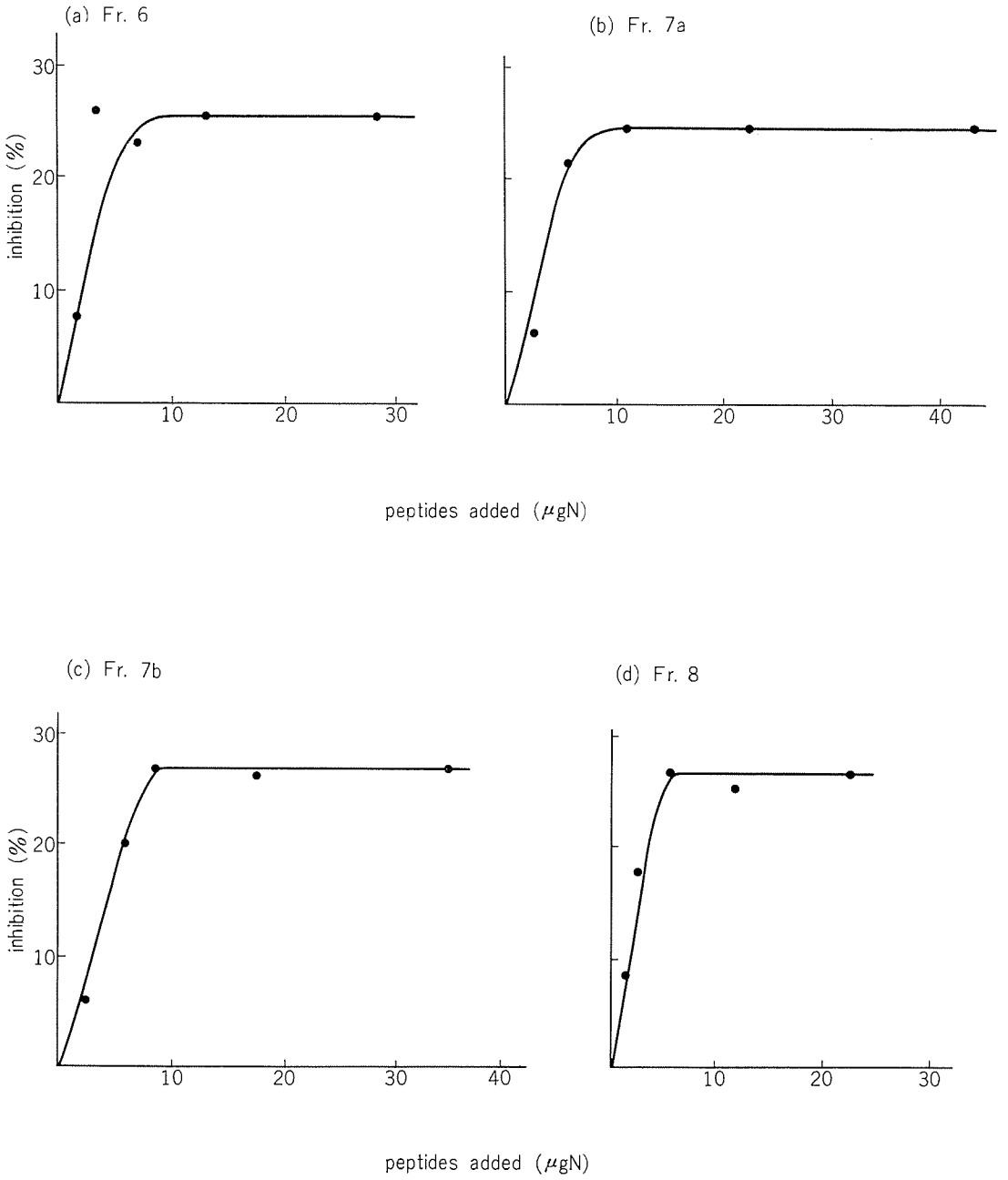

FIGURE 2 a, b, c, d
Quantitative precipitation inhibition of HL~anti-HL system by peptic peptide of HL.
Various amounts of peptic peptides were added to a series of tubes containing 3 μg N of HL and 0.1 ml of HL antiserum (No. 332).

0.3 ml of the peak component was added to the HL~anti-HL system and degree of inhibition of the precipitin reaction was measured. Frs. 6, 7 and 8 were found to be immunologically active but Frs. 9, 10, 11, 12, 13, 14 and 15 were inactive. The other fractions have not yet been examined.

The contents of the tubes containing each immunologically active fraction (Frs. 6, 7 and 8) were each pooled and diluted 3 times with distilled water and each solution was applied to a CM cellulose column (1.5×30 cm) equilibrated with 0.05 M acetate buffer at pH 3.85. After washing with the same buffer, elution was carried out with 0.05 M acetate buffer at pH 5.0 containing NaCl in a linear concentration gradient up to 0.1 M. After chromatography, several minor components were found in addition to the main components. The same procedure was repeated once or twice more, and each fraction was desalted according to Dixon's method. In the case of Fr. 7, two main components (Fr. 7a and 7b) were separated, and Frs. 7a and 7b were both purified further by rechromatography. In some batches of peptic digests of HL, Fr. 7b could not be found, though the experimental conditions were the same as deserибed above.

Thus purified Frs. 6, 7a, 7b and 8 were obtained, each with a yield of about 3 per cent, and this value corresponded to about 9 per cent molar recovery per mole of HL if the molecular weights of these fractions are assumed to be 5338. (This value was obtained from data on amino acid analysis, as described below).

2. *Immunological activity of Frs.* 6, *7a, 7b and* 8

The peptides (Frs. 6, 7a, 7b and 8) gave no precipitin reaction with HL antisera in tubes or in agar gel and had neither lysozyme activity nor "enzymoid" activity, which competitively inhibited HL activity in the presence of substrate (FRIEDEN, 1956). However, they were active in inhibiting the precipitin reaction of the HL~anti-HL system. Therefore, quantitative studies were made on the inhibitory activity of each peptide.

The results are shown in Figs. 2a, 2b, 2c and 2d. As can be seen, the four peptides likewise inhibited about 25 per cent of the precipitable protein N, and the least amounts of each peptide giving maximal inhibition were 12 (Fr. 6), 10 (Fr. 7a), 10 (Fr. 7b) and 7 (Fr. 8) μgN, respectively. The ratios of these amounts to the antigen used (3 μgN) were 4 (Fr. 6), 3.3 (Fr. 7a), 3.3 (Fr. 7b) and 2.3 (Fr. 8), and the molar ratios were 10.8, 8.9, 8.9 and 6.2.

Tests showed that the inhibitory activities of these peptides were not additive. This suggests that these peptides all contain the same antigenic determinant(s). The specificity of the inhibitory activities of these peptides were confirmed by the fact that they had no inhibitory effects on the precipitin reaction of bovine serum albumin (BSA) with its homologous antiserum, in which they were tested at the equivalence point (10.4 μgN of BSA and 0.2 ml of antiserum). These results indicate that these peptides bear one (or two) antigenic determinant(s).

As the HL activity can be completely neutralized by HL antiserum, the inhibitory effects of these four peptides were also examined in the HL~anti-HL system, in which HL was neutralized completely with the least amount of antiserum. However, these four peptides had no inhibitory effects on neutralizing antibodies even when added in about 1000 fold molar excess of HL.

3. *Chemical structures of peptides* 6, *7a, 7b and* 8

To elucidate the chemical structures of the four peptides, the amino acid composition and the N- and C-terminal amino acids were analyzed. The results are shown in Tables 1 and 2. As can be seen, these peptides lacked His, Tyr and Phe. In considering the results and the amino acid sequence of HL found by JOLLÈS (1964, 1965) and CANFIELD (1965) shown in Fig. 3, the four peptides can be considered to correspond to the peptide, Gln^{57}~

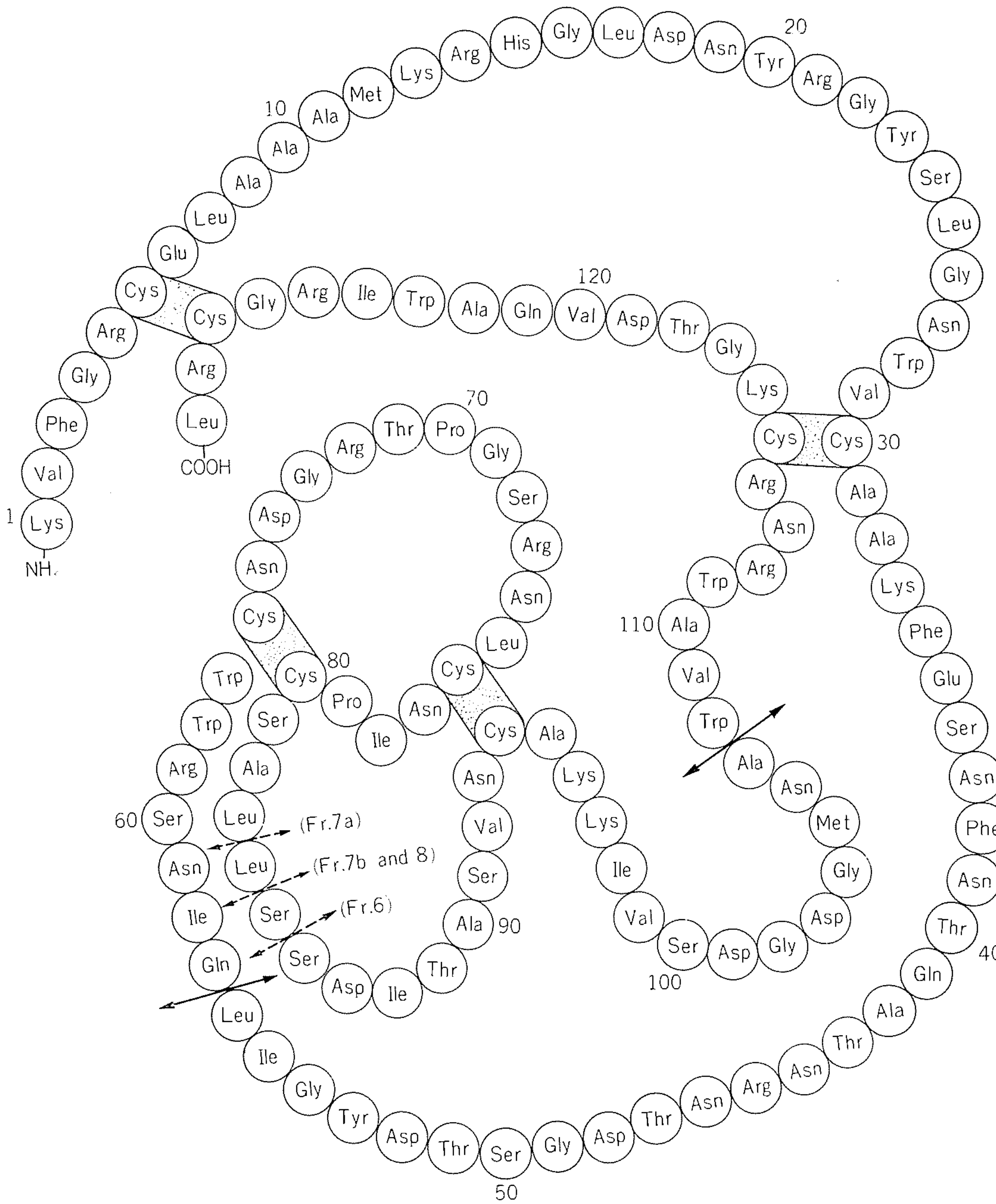

FIGURE 3 Amino acid sequence of HL (Jollès and Canfield).
Arrows in full line indicate the location of peptic peptides which have immunologic activity, and those in broken line indicate the locations of suspected cleavages in those peptides.

TABLE 1 *Amino acid compositions of active fragments isolated from a peptic digest of HL*

Amino acid	Fr. 6*	Fr. 7a**	Fr. 7b*	Fr. 8*	Gln^{57}~Ala^{107}+
Lys	2.0	2.6	1.9	1.6	2
His	—	—	—	—	—
Arg	3.3	3.1	3.3	3.5	3
Asp	10.2	10.1	10.4	10.4	10
Thr	1.7	2.1	1.9	1.9	2
Ser	6.0	6.8	6.9	6.8	7
Glu	1.0	0.8	1.0	1.0	1
Pro	2.2	1.9	2.8	2.1	2
Gly	4.1	4.0	4.3	4.3	4
Ala	3.7	3.9	3.6	4.1	4
1/2 Cys	3.1	3.0	3.6	2.7	4
Val	1.5	1.6	1.6	1.9	2
Met	0.9	0.5	0.9	1.0	1
Ile	2.9	3.6	3.5	3.4	4
Leu	2.9	2.9	3.2	2.8	3
Tyr	—	—	—	—	—
Phe	—	—	—	—	—
Trp^{++}	2	not done	2	not done	2

* Number of amino acids per mole of peptide calculated assuming each peptide contained one mole of glutamic acid.
** Number of amino acids per mole of peptide expressed assuming the peptide has one mole of glycine.
\+ Number of amino acids in the Gln^{57}~Ala^{107} peptide calculated according to the data of Jollès and Canfield.
++ Tryptophan contents were estimated by the UV absorption method.

Ala^{107}, which consists of 51 amino acids with two disulfide bonds. From this structure, the molecular weight can be assumed as 5338. This seems reasonable, because the molecular weight of Fr. 7, after purification by repeated chromatography, was approximately 7000 as measured by Archibald's method.

From the findings on the terminal amino acids, though these are somewhat inconclusive, these peptides seem to have a cleavage in the Cys^{76}~Cys^{94} loop, *i.e.* between Ser^{85} and Ser^{86} in Fr. 6, between Leu^{83} and Leu^{84} in Fr. 7a, and between Leu^{84} and Ser^{85} in both Frs. 7b and 8.

The secondary structure of Fr. 6 was studied further by estimating the optical rotatory dispersion. From the plot according to the method of MOFFITT-YANG (1956) shown in Fig. 4, the following values were obtained: $b_0 = -128$ and $a_0 = -657$. These values show that the

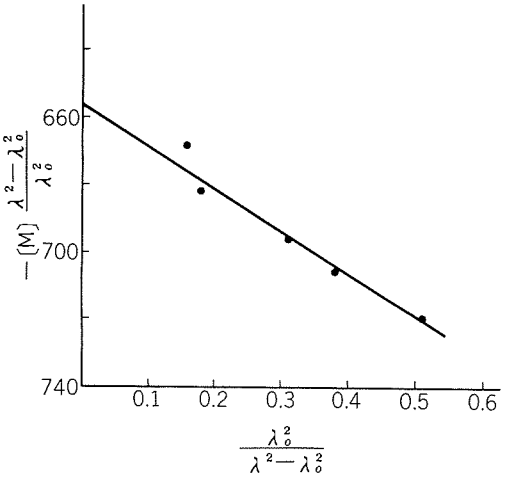

FIGURE 4 MOFFITT-YANG plot of optical rotatory dispersion of an active fragment (Fr. 6).

TABLE 2 *Terminal structures of active fragments isolated from a peptic digest of HL*

Amino acid	Fr. 6		Fr. 7a		Fr. 7b		Fr. 8	
	PTC	$Cp_{A.B}$	PTC	$Cp_{A.B}$	PTC	$Cp_{A.B}$	PTC	$Cp_{A.B}$
Lys								
His								
Arg								
Asp	P-2*							
Thr								
Ser		0.8	P-2*	0.6		0.8		0.7
Glu								
Pro								
Gly								
Ala		1.0		1.0		1.5		1.3
1/2 Cys								
Val								
Met		0.5		0.1		0.2		0.1
Ile	P-2*		P-2*					
Leu		0.6	P-1	0.9		1.6		1.3
Tyr								
Phe								
Gln	P-1		P-1		P-1		P-1	

PTC: PTH amino acids identified by the PTC method. P-1 indicates the N-terminal amino acid and P-2 indicates the 2nd amino acid from the N-terminal.
$Cp_{A.B.}$: Moles of amino acid per mole of peptide which were hydrolyzed by carboxypeptidase A and B.
* Uncertain.

ordered structure is still retained in Fr. 6, as discussed below.

4. *Tryptic digestion of Fr. 7a and fractionation of the digest*

To confirm the chemical structure of Fr. 7a, this material digested further with trypsin and the digest was fractionated by Sephadex G-25 chromatography and high voltage paper-electrophoresis. Four peptides were isolated as expected from the substrate specificity of trypsin and the chemical structure of Fr. 7a.

To a solution of 70 mg of Fr. 7a in 20 ml of distilled water were added 2 mg of crystalline trypsin, and the mixture was incubated at 37°C for 24 hours with a few drops of toluene and chloroform added as sterilizers. The pH was maintained at 8.0 by adding ammonium hydroxide at 1 hour intervals for the first 9 hours. After incubation, the solution was lyophilized.

Then 26 mg of the digest were dissolved in 2 ml of 10 per cent acetic acid and the solution was applied on a Sephadex G-25 column (3 $\times$102 cm) equilibrated with 10 per cent acetic acid. As shown in Fig. 5a, four components (7T-1, 7T-2, 7T-3 and 7T-4) were separated. Fr. 7T-4 was found to be mainly lysine and to have been derived from Lys^{97} as could be expected. Frs. 7T-1, 7T-2 and 7T-3 were lyophilized and repurified by gel-filtration. The results are presented in Figs. 5b, 5c and 5d. Only Fr. 7T-1 was purified by this procedure. As Frs. 7T-2 and 7T-3 could not be purified as single peaks, in each main fractions were pooled and lyophilized, and they were further purified by paper-electrophoresis at pH 6.5 (67 volts/cm for 40 minutes) and pH 3.6 (50 volts/cm for 45 minutes), respectively. The buffers consisted of pyridine-acetic acid-

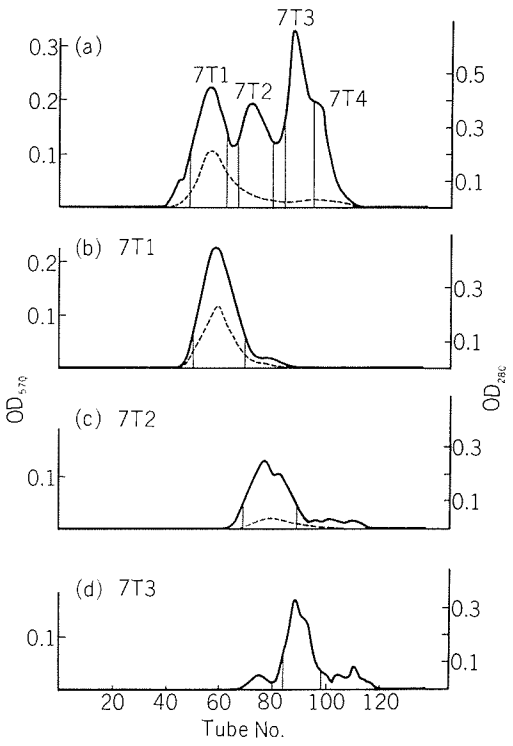

FIGURE 5
(a) Chromatography of tryptic peptides of Fr. 7 on Sephadex G-25.
(b) Rechromatography of 7T1 shown in (a)
(c) Rechromatography of 7T2 shown in (a)
(d) Rechromatography of 7T3 shown in (a)
column size: 3×102 cm; eluted with 10% acetic acid; fraction volume: 5 g; elution rate: 50 ml per hour.
——— OD at 570 mμ (ninhydrin reaction)
· · · · OD at 280 mμ

water (10: 0.4: 90 v/v at pH 6.5; 1: 10: 89 v/v at pH 3.6). The fractions, dissolved in buffer, were applied on a line, 11 cm long, on the filter paper (15×60 cm). After the run, 2 strips of 1 mm width were taken and examined with ninhydrin and the arginine reaction, respectively. The results are shown in Figs. 6a and 6b. Fr. 7T-2 was shown to contain two components (a and b) and Fr. 7T-3 seven components (a~g).

5. *Amino acid analyses of tryptic peptides from Fr. 7a*

Analyses of the amino acid composition and the N- and C-terminal amino acids were carried out on each peptide fraction obtained from the tryptic digest of Fr. 7a. From the analytical data and the assumed chemical structure of Fr. 7a, only Frs. 7T-1 (peptide B), 7T-2a (D), 7T-3b (A) and 7T-3f (C) were considered to consist of single components, as shown in Tables 3 and 4, and the yields of peptides A, B, C and D were, respectively, 29, 33, 31 and 17 moles per cent. The chemical structures of these peptides were concluded to be as shown in Table 5 and were as expected from the chemical structure of Fr. 7a and the substrate specificity of trypsin. These data also confirmed the presumed structure of Fr. 7a with a cleavage between Leu^{83} and Leu^{84}.

Of the other fractions, Fr. 7T-2b was found to be the same as B, Fr. 7T-3a seemed to be a mixture of A, B and D, while Frs. 7T-3c, 7T-3d and 7T-3e were mixtures of A and C, and Fr. 7T-3g was free lysine.

6. *Immunological activities of tryptic peptides from Fr. 7a*

The immunological activities of the tryptic peptides from Fr. 7a were investigated to see where the antigenic determinant(s) was located in the peptide Gln^{57}~Ala^{107}. The HL antiserum used in this experiment (batch No. 166) was not inhibited by Fr. 7a in the precipitin reaction with homologous antigen but Fr. 7a delayed the development of turbidity. Therefore, the effects of the tryptic peptides in delaying development of turbidity were examined.

An appropriate amount of tryptic peptide, dissolved in 0.3 ml of phosphate buffered saline at pH 6.0, was incubated with 0.3 ml of 1: 3 diluted HL antiserum in a cuvette at 15°C for 30 minutes. The control contained the same amount of saline without added tryptic peptide. Then, an amount of HL equivalent to the antiserum (3.6 μgN) dissolved in saline was added and the turbidities at 475 mμ were

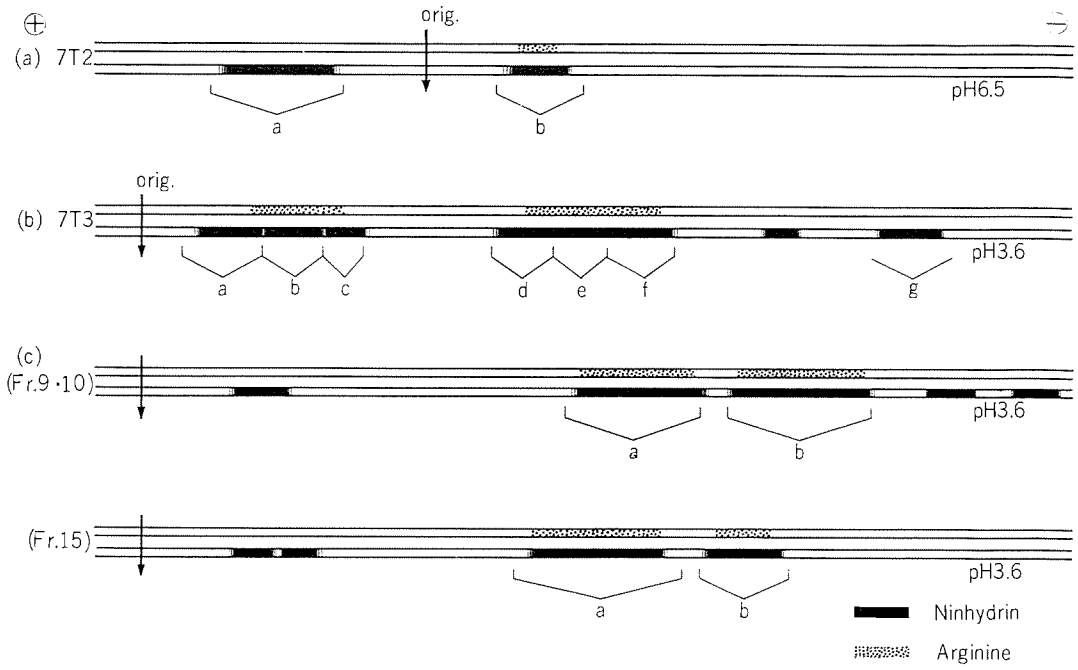

FIGURE 6 High voltage paper-electrophoresis of peptides.
(a) and (b). Electrophoretic patterns of tryptic peptides of Fr. 7 (7T2 and 7T3) which were purified by chromatography on Sephadex G-25.
(c) and (d). Electrophorectic patterns of petic peptides (Fr. 9·10 and Fr. 15) which were separated by CM-cellulose chromatography.

recorded automatically at intervals in a Gilford spectrophotometer. A cell containing 0.9 ml of 1: 9 diluted antiserum served as the reference.

As shown in Fig. 7a, Fr. 7a caused marked delay in development of turbidity when 16 fold the molar equivalent was used. Of the tryptic peptides, only B (Fig. 7c) and D (Fig. 7e) were active. B caused a delay when about 170 fold the molar amount was used and D also had some delaying effect. Both A (Fig. 7b) and C (Fig. 7d) were inactive. To study the specificity in causing the delay, Fr. 7a was tested in a system of BSA and its antiserum, where it had no effect. This indicates that the delay of increase in turbidity is caused by a weak but definitely specific inhibition of the precipitin reaction and that the peptide B contributes to form one determinant group.

7. *Isolation of immunologically inactive peptic peptides and their chemical structures*

Though Frs. 9, 10, 11, 12, 13, 14 and 15 of the peptic digest were inactive in inhibiting the precipitin reaction, as described above, studies were made on the chemical structures of Frs. 9, 10 and 15.

Frs. 9 and 10 were each chromatographed on CM-cellulose in the same way as the active peptides, but no clear cut separation was achieved. Therefore, the two main fractions were pooled (Fr. 9·10) and the same chromatography was repeated. Then the main fractions were again pooled, desalted and lyophilized. The residue was dissolved in 10 per cent acetic acid and submitted to gel-filtration on

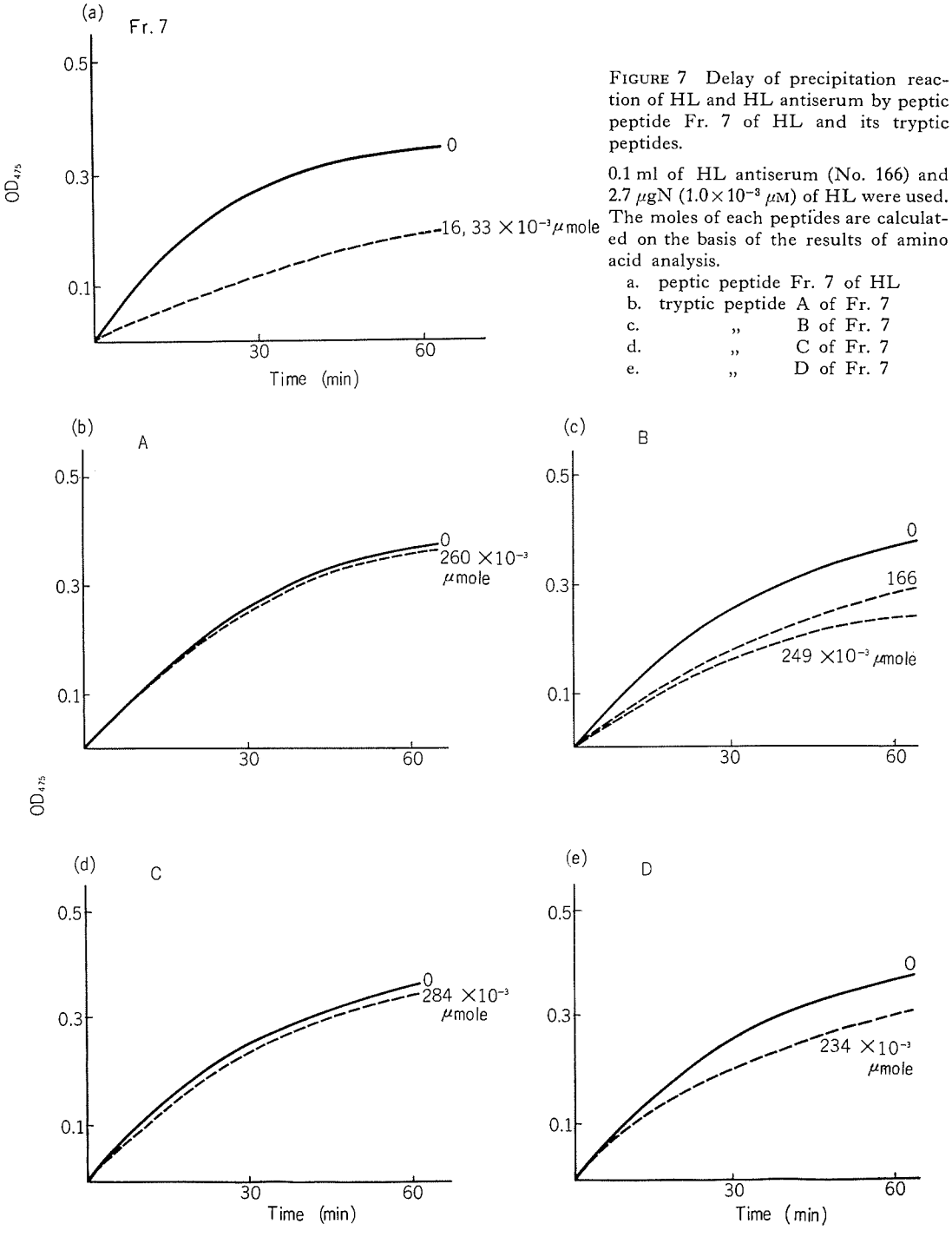

FIGURE 7 Delay of precipitation reaction of HL and HL antiserum by peptic peptide Fr. 7 of HL and its tryptic peptides.

0.1 ml of HL antiserum (No. 166) and 2.7 μgN (1.0×10^{-3} μM) of HL were used. The moles of each peptides are calculated on the basis of the results of amino acid analysis.

a. peptic peptide Fr. 7 of HL
b. tryptic peptide A of Fr. 7
c. „ B of Fr. 7
d. „ C of Fr. 7
e. „ D of Fr. 7

TABLE 3 *Amino acid compositions of peptides isolated from a tryptic digest of Fr. 7*

Amino acid	B* (7T1)	D** (7T2-a)	A*** (7T3-b)	C* (7T3-f)
Lys	1.2			
His				
Arg	1.0		1.3	0.9
Asp	5.4	2.9	1.2	
Thr	0.9			0.9
Ser	3.4	0.9	1.2	0.9
Glu			1.0	
Pro	0.9			1.1
Gly	1.0	2.0		1.0
Ala	2.8	1.0		
1/2 Cys	3.0			
Val	0.5	0.5		
Met		1.0		
Ile	1.8	0.4		
Leu	2.5			

* Number of amino acids per mole of peptide calculated assuming each peptide contained one mole of glycine.

** Number of amino acids per mole of peptide calculated assuming the peptide has two moles of glycine.

*** Number of amino acids per mole of peptide given assuming the peptide has one mole of glutamic acid.

Sephadex G-25 (1.5×104 cm column) equilibrated with 10 per cent acetic acid. The main fraction was lyophilized. The residue (4.6 mg) was purified by paper-electrophoresis using 10 per cent pyridine-acetate buffer at pH 3.6 at 50 volts/cm for 60 minutes. Two components were separated as shown in Fig. 6c (Frs. 9·10a and 9·10b). Fr. 15 was directly desalted, and 20 mg of the residue was submitted to gel-filtration by the above procedures. Ten mg of the main component were purified by paper-electrophoresis using the method described above. Two components were obtained (Frs. 15a and 15b) as shown in Fig. 6d. The yields of these fractions were 1.1 (Fr. 9·10a), 1.0 (Fr. 9·10b), 1.4 (Fr. 15a) and 1.4 μ mole (Fr. 15b).

The amino acid composition, and N- and C-terminal amino acids of each fraction are shown in Tables 6 and 7. In comparing these data with the structure of HL, the locations of the peptides in HL could be assumed to be as shown in Table 8. These four peptides were immunologically inactive and did not even delay the increase in turbidity when tested with batch No. 166 of HL antiserum.

DISCUSSION

One of the most important problems in immunochemistry is to elucideate the chemical structures of antigenic determinants of a protein antigen. For this purpose, elucidation of the chemical structures of partially degraded peptides bearing antigenic determinants is the most promising approach, although this method has limitations, because some of the determinants disappear during digestion.

Small active peptides have been obtained from fibrous proteins such as silk fibroin (LANDSTEINER, 1942; CEBRA, 1961) and oxidized ribonuclease (BROWN, 1962), and their chemical structures have been clarified.

Although a number of globular protein antigens, such as bovine serum albumin (PORTER, 1957), human serum albumin (LAPRESLE *et al.*, 1957, 1960), diphtheria toxin (RAYNAUD *et al.*, 1959), ribonuclease (SINGER *et al.*, 1959) and thyroglobulin (METZGAR *et al.*, 1962) yielded fragments by hydrolysis which are capable of combining with antibodies evoked by intact antigens, these fragments seem to have more than two antigenic determinants (polyvalent), because they gave precipitates with homologous antisera.

Non-precipitable fragments of globular protein antigens capable of inhibiting immunological reactions were isolated by PRESS and PORTER (1962) from human serum albumin, by ANDERER (1963) and his colleagues (1963, 1965) from tabacco mosaic virus, by BENJAMINI *et al.* (1964, 1965, YOUNG *et al.*, 1966; STEWART *et al.*, 1966) from tabacco mosaic virus protein, and by CRUMPTON and WILLKINSON (1965) from sperm-whale myoglobin.

SHINKA *et al.* (1962) also obtained two non-

TABLE 4 *Terminal structures of peptides isolated from a tryptic digest of Fr. 7*

Amino acid	B		D		A		C	
	PTC	$Cp_{A.B}$	PTC	$Cp_{A.B}$	PTC	$Cp_{A.B}$	PTC	$Cp_{A.B}$
Lys		1.1						
His								
Arg		0.4				0.6		0.8
Asp								
Thr							P-1	
Ser		0.2		0.4		0.3		
Glu								
Pro							P-2	
Gly						+		
Ala		0.9		0.6		+		
1/2 Cys								
Val			P-2	0.1				
Met				0.2				
Ile			P-1			+		
Leu	P-1	1.0		0.2				
Gln					P-1			
Asn	P-1							
Trp	P-1							

PTC and $Cp_{A.B}$: Abbreviations as in Table 2. + Trace

TABLE 5 *Amino acid sequence of peptides isolated from a tryptic digest of Fr. 7*

Peptide	Amino acid sequence	Mol. wt.*
A	H·Gln[57]-Ile-Asn-Ser-Arg[61]·OH	617
	H·Trp[62]-Trp-Cys-Asn-Asp-Gly-Arg[68]·OH	
B	OH·Leu[83]-Ala-Ser-Cys-Pro-Ile-Asn-Cys-Leu-Asn[74]·H	3270
	H·Leu[84]-Ser-Asp-Ile-Thr-Ala-Ser-Val-Asn-Cys-Ala-Lys[96]·OH	
C	H·Thr[69]-Pro-Gly-Ser-Arg[73]·OH	517
D	H·Ile[98]-Val-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala[107]·OH	978

* Calculated from the amino acids contents of the peptides.

precipitable fragments on peptic digestion of HL and both inhibited the precipitin reaction of HL with homologous antisera but did not inhibit neutralizing antibody activity. As these fragments were still impure and their molecular weights, estimated by Archibald's method, were about 20,000, which is larger than that of the starting material, HL, studies have been continued to isolate pure, unpolymerized peptides, with antigenic determinant(s) to elucidate the location of the determinant on the enzyme molecule. The primary structure of HL was determined by JOLLÈS *et al.* (1964, 1965) and independently by CANFIELD (1965), and BLAKE *et al.* (1965) also clarified the tertiary structure and suggested that Glu^{35} and Asp^{52} participate in the active center of the enzyme. These findings greatly facilitated our studies. However the authors did not use their methods of splitting off the peptides. Thus,

TABLE 6 *Amino acid compositions of inactive fragments isolated from a peptic digest of HL*

Amino acid	Fr. 9·10-a*	Fr. 9·10-b**	Fr. 15-a***	Fr. 15-b***
Lys	1.1	1.0	1.9	0.9
His	1.0	1.2		
Arg	1.9	2.1	2.2	2.5
Asp	3.0	2.0	2.2	
Thr			1.0	
Ser	0.9			
Glu			1.0	1.0
Pro				
Gly	3.0	2.0	1.3	2.1
Ala	0.9	1.0	3.9	1.7
1/2 Cys			1.5	1.5
Val			2.6	1.0
Met	1.0	1.0		
Ile				0.8
Leu	1.8	1.0		1.6
Tyr	1.7	1.1		
Phe			0.8	1.0

* Number of amino acids per mole of peptide assuming the peptide has three moles of glycine.
** Number of amino acids per mole of peptide assuming the peptide has 2 moles of glycine.
*** Number of amino acids per mole of peptide assuming each peptide contains one mole of glutamic acid.

JOLLÈS employed pepsin under different conditions from ours and CANFIELD reduced and alkylated HL before digesting it.

To avoid polymerization of the liberated peptides, separation procedures were carried out as far as possible at acidic pH, and there was no polymerization as shown by the observation that the molecular weight of Fr. 7 was approximately 7,000 by Archibald's method.

The immunological behaviors of Frs. 6, 7a, 7b and 8 indicate that these peptides bear at least one determinant and that this is the same in the different fractions, because the peptides were non-precipitable and inhibited the precipitin reaction of the HL~anti-HL system and because the inhibitory activities of the peptides were not additive. However, a non-precipitable antigen need not necessarily be univalent. If one postulates that infinitely large antigen-antibody complexes precipitate (under critical conditions) and determinants of a protein antigen differ in specificity, divalent antigens would not be precipitated because the critical conditions are only attained when the two kinds of antibody are at the same concentration and this special type of antiserum would not be found in practice (AMANO *et al.*, 1962 and unpublished).

Analyses of the N- and C-terminal amino acids of Frs. 6, 7a, 7b and 8 indicated that they all contained two N- and C-terminals, and there was concluded to be a fission of the loop of Cys^{76} ~ Cys^{94} in each pepaide. This assumption in the case of Fr. 7a was justified by the chemical structures of the tryptic peptides from Fr. 7a, because, considering the substrate specificity of trypsin, no leucine should be detected on terminal analysis of peptide B if Fr. 7a has an intact Cys^{76}~Cys^{94} loop. No peptide with the same amino acid sequence with an intact Cys^{76}~Cys^{94} loop has yet been obtained.

The data on the optical rotatory dispersion of Fr. 6 indicate that 20 per cent of the peptide bonds of Fr. 6 are in the α-helix structure. According to BLAKE *et al.* (1965), Fr. 6 should have two α-helices, *i.e.* Cys^{80}~Ser^{85} and Ser^{91} ~ Val^{109}, if there is no cleavage between Ser^{85} and Ser^{86}, and the helical content of this structure would account for 26 per cent of the total. These values are in fairly good agreement, or the difference may be explained by partial destruction of the α-helix of Cys^{80}~Ser^{85} in Fr. 6 by cleavage between Ser^{85} and Ser^{86}. However, the a_0 value obtained from our experiment is highly negative which is difficult to understand.

The fact, that the tryptic peptide B caused delay in development of turbidity of the HL~anti-HL system, indicates that the location of one antigenic determinant of the peptide is Gln^{57}~Ala^{107} around two disulfide bonds (Cys^{64}–Cys^{80} and Cys^{76}–Cys^{94}). If another determinant is present, it must disappear during tryptic digestion and subsequent procedures.

TABLE 7 *Terminal structures of inactive fragments isolated from a peptic digest of HL*

Amino acid	Fr. 9·10-a		Fr. 9·10-b		Fr. 15-a		Fr. 15-b	
	PTC	$Cp_{A.B}$	PTC	$Cp_{A.B}$	PTC	$Cp_{A.B}$	PTC	$Cp_{A.B}$
Lys						0.7	P-1*	
His								
Arg				0.6				0.8
Asp								
Thr								
Ser								
Glu								
Pro								
Gly		0.3		0.5				
Ala	P-1	0.2	P-1			2.5		1.8
1/2 Cys								
Val					P-1		P-2	
Met	P-2		P-2		P-2*			
Ile							P-2	
Leu								1.2
Tyr		0.3		0.5				
Phe						0.8		
Gln						0.7		
Asn		0.6						
Trp					P-1*		P-1	

PTC and $Cp_{A.B}$: Abbreviations as in Table 2. * Uncertain.

TABLE 8 *Amino acid sequence of inactive fragments isolated from a peptic digest of HL*

Peptide	Amino acid sequence	Mol. wt.*
Fr. 9·10-a	H·Ala[11]-Met-Lys-Arg-His-Gly-Leu-Asp-Asn-Tyr-Arg-Gly-Tyr-Ser-Leu-Gly-Asn[27]·OH	1952
Fr. 9·10-b	H·Ala[11]-Met-Lys-Arg-His-Gly-Leu-Asp-Asn-Tyr-Arg-Gly[22]·OH	1418
Fr. 15-a	H·(Trp[28]) Val-Cys-Ala-Ala-Lys-Phe[34]·OH H·(Trp[108]) Val-Ala-Trp-Arg-Asn-Arg-Cys-Lys-Gly-Thr-Asp-Val-Gln-Ala[122]·OH	
Fr. 15-b	H·Lys[1]-Val-Phe-Gly-Arg-Cys-Glu-Leu-Ala-Ala[10]·OH H·Trp[123]-Ile-Arg-Gly-Cys-Arg-Leu[129]·OH	1995

* Calculated from the amino acid contents of the peptides.
( ) Uncertain.

The four other peptides in the peptic digest were immunologically inactive. This may mean that most of the four antigenic determinants, concluded to be present from quantitative analysis of the precipitin reaction, disappeared during peptic digestion. The immunological activities of these four fractions are now under study by the procedure of equilibrium dialysis.

The most important peptide, Glu^{35}~Leu^{56}, containing Glu^{35} and Asp^{52} which are thought to be active centers of the enzyme, is still missing. With regard to neutralizing antibodies, two possibilities can be considered: 1)

all the neutralizing antibodies are directed to the active center of the enzyme, and 2) they are directed to the active center and also to the determinant(s) containing the amino acid(s) which participates in fixing the substrate on the enzyme molecule. The amino acid residues which participate in fixing the substrate, Asn^{59}, Trp^{62}, Trp^{63}, Asp^{101} and Ala^{107} (PHILLIPS, 1966), are all included in Frs. 6, 7a, 7b and 8, and hence these peptides should inhibit neutralizing antibodies if the latter possibility was the case. As described above, these peptides did not inhibit the neutralizing activity of HL antisera. However, the former possibility is not necessarily justified from the results, because inhibition of neutralizing antibodies would require much closer fitting to antibody combining sites than the latter possibility.

ACKNOWLEDGEMENTS

The authors express their deep gratitude to Professor Kozo Hamaguchi of Kwansei Gakuin University, Faculty of Science, for estimating optical rotatory dispersion. They also thank Miss Kimie Watanabe of the Laboratory of Molecular Genetics, Osaka University Medical School, for operating the amino acid analyzer.

REFERENCES

ALDERTON, G. and FEVOLD, H. L. (1946). Direct crystallization of lysozyme from egg white and some crystalline salts of lysozyme. *J. Biol. Chem.*, **164**, 1–5.

AMANO, T., SHOJI, I., TOKUNAGA, T. and SATO, S. (1962). A new statistical-mechanical theory on the antigen-antibody reaction. *Biken J.*, **5**, 259–296.

ANDERER, F. A. (1963). Versuche zur Bestimmung der serologischen determinanten Gruppen des Tabakmosaikvirus. *Z. Naturforsch.*, **18**b 1010–1014.

ANDERER, F. A. und HANDSCHUH, D. (1963). Einfluss der Tertiärstruktur auf die serologischen Eigenschaften des Tabakmosaikvirus. *Z. Naturforsch.*, **18**b, 1015–1021.

ANDERER, F. A. and SCHUMBERGER, H. D. (1965). Properties of different artificial antigens immunologically related to tabacco mosaic virus. *Biochim. Biophys. Acta*, **97**, 503–509.

ARCHIBALD, W. J. (1947). A demonstration of some new methods of determining molecular weights from the data of the ultracentrifuge. *J. Phys. Colloid Chem.* **51**, 1204–1214.

BENJAMINI E., YOUNG, J. D., SHIMIZU, M. and LEUNG, C. Y. (1964). Immunochemical studies on the tabacco mosaic virus protein. I. The immunological relationship of the tryptic peptides of tabacco mosaic virus protein to the whole protin. *Biochem.*, **3**, 1115–1120.

BENJAMINI, E., YOUNG, J. D., PETERSON, W. J., LEUNG, C. Y. and SHIMIZU, M. (1965). Immunochemical studies on the tobacco mosaic virus protein. II. The specific binding of a tryptic peptide of protein with antibodies to the whole protein. *Biochem.*, **4**, 2081–2085.

BLAKE, C. C. F., KOENIG, D. F., MAIR, G. A. NORTH, A. C. T., PHILLIPS, D. C. and SARMA, V. R. (1965). Structure of hen egg-white lysozyme. A three-dimensional fourier synthesis at 2 Å resolution. *Nature*, **206**, 757–763.

BROWN, R. K. (1962). Studies on the antigenic structure of ribonuclease. III. Inhibition by peptides of antibody to performic-acid-oxidized ribonuclease. *J. Biol. Chem.*, **237**, 1162–1167.

CANFIELD, R. E. (1963). The amino acid sequence of egg-white lysozyme. *J. Biol. Chem.*, **238**, 2698–2707.

CANFIELD, R. E. and LIU, A. K. (1965). The disulfide bonds of egg white lysozyme (muramidase). *J. Biol. Chem.*, **240**, 1997–2002.

CEBRA, J. J. (1961). Studies on the combining sites of the protein antigen silk fibroin. III. Inhibition of the silk fibroin-antifibroin system by peptides derived from the antigen. *J. Immunol.*, **86**, 205–214.

COCKING, E. C. and YEMM, E. W. (1954). Estimation of amino acids by ninhydrin. *Biochem. J.* **58**, xii.

CRUMPTON, M. J. and WILLKINSON, J. M. (1965). The immunological activity of some of the chymotryptic peptides of sperm-whale myoglobin. *Biochem. J.*, **94**, 545–556.

DIXON, H. B. F. (1959). A method of desalting certain polypeptides. *Biochim. Biophys. Acta*, **34**,

251–253.

FUJIO, H., KISHIGUCHI, S., SHINKA, S., SAIKI, Y. and AMANO, T. (1959). Immunochemical studies on lysozyme. I. Comparative studies of lysozyme and lysozyme methyl ester. *Biken's J.* **2**, 55–76.

FUJIO, H., SAIKI, Y., IMANISHI, M., SHINKA, S. and AMANO, T. (1962). Immunochemical studies on lysozzyme. III. Cross reaction of hen and duck lysozymes and their methyl esters. *Biken J.*, **5**, 201-226.

FRIEDEN, E. H. (1956). Enzymoid properties of lysozyme methyl ester. *J. Am. Chem. Soc.*, **78**, 961-965.

GOODWIN, T. W. and MORTON, R. A. (1946). The spectrophotometric determination of tyrosine and tryptophan in proteins. *Biochem. J.*, **40**, 628-632.

HAYASHI, K., IMOTO, T., FUNATSU, G. and FUNATSU, M. (1965). The position of the active tryptophan residue in lysozyme. *J. Biochem.*, **58**, 227-235.

HEIDELBERGER. M. and KENDALL, F. E. (1935). A quantitative theory of the precipitin reaction. III. The reaction between crystalline egg albumin and its homologous antibody. *J. Exptl. Med.*, **62**, 697-720.

HIRS, C. H. W., MOORE, S. and STEIN, W. H. (1953). Chromatographic studies on lysozyme. *J. Biol. Chem.*, **200**, 507-514.

HIRS, C. H. W. (1956). The oxidation of ribonuclease with performic acid. *J. Biol. Chem.*, **219**, 611-621.

INAI, S., KISHIMOTO, S., HIRAO, F. and TAKAHASHI, H. (1958). Studies on serum lysozyme. II. Variations of the serum lysozyme level. Presented at the 11th meeting of Kansai branch of the society of Japanese Bacteriogists, on October, 19, 1958.

JOLLÈS, J., JAUREGUI-ADELL, J. and JOLLÈS, P. (1963). La structure chimique du lysozyme de blanc d'oeuf de poule: Étude détailée. *Biochim. Biophys. Acta*, **78**, 668-689.

JOLLÈS, P., JAUREGUI-ADELL, J. and JOLLÈS, J. (1964). Le lysozyme de blanc d'oeuf de poule: disposition des ponts disulfures. *Compt. Rend. Acad. Sci.*, **258**, 3926–3928.

JAUREGUI-ADELL, J., JOLLÈS, J. and JOLLÈS, P. (1965). The disulfide bridges of hen's egg-white lysozyme. *Biochim. Biophys. Acta*, **107**, 97–111.

KATZ, A. M., DREYER, W. J. and ANFINSEN, C. B. (1959). Peptide separation by two-demensional chromatography and electrophoresis. *J. Biol. Chem.*, **234**, 2897–2900.

KLAINER, S. M. and KEGELES, G. (1956). The molecular weights of ribonuclease and bovine plasma albumin. *Arch. Biochem. Biophys.*, **63**, 247–254.

LANDSTEINER, K. (1942). Serological reactivity of hydrolytic products from silk. *J. Exptl. Med.*, **75**, 269–276.

LAPRESLE, C. and DURIEUX, J. (1957). Étude de la dégradation de la sérum albumine humaine par un éxtrait de rate de lapin. III. Madifications immunologiques de l'albumine en fonction de stade dégradation. *Ann. Inst. Pasteur*, **92**, 62–73.

LAPRESLE, C. and WEBB, T. (1960). Étude de la dégradation de la sérum albumine humaine par un éxtrait de rate de lapin. *Ann. Inst. Pasteur*, **99**, 523–532.

METZGER, H., SHARP, G. C. and EDELHOCH, H. (1962). The properties of thyroglobulin. VII. The immunologic activity of thyroglobulin fragments. *Biochem.*, **1**, 205–214.

MOFFITT, W. and YANG, J. T. (1956). The optical rotatory dispersion of simple polypeptides. I. *Proc. Natl. Acad. Sci.*, **42**, 596–603.

NORTHROP, J. H. (1930). Crystalline pepsin. I. Isolation and test of purity. *J. Gen. Physiol.*, **13**, 739–766.

PHILLIPS, D. C. (1966). The three-dimensional structure of an enzyme molecule. *Scientific American*, **215**(5), 78–90.

PORTER, R. R. (1957). The isolation and properties of a fragment of bovine serum albumin which retains the ability to combine with rabbit antiserum. *Biochem. J.*, **66**, 677–686.

PRESS, E. M. and PORTER, R. R. (1962). Isolation and chracterization of fragments of human serum albumin containing some of the antigenic sites of the whole molecule. *Biochem. J.* **83**, 172-180.

RAYNAUD, M. et RELYVELD, E. H. (1959). La réaction toxine-antioxine diphthérique. *Ann. Inst. Pasteur*, **97**, 673–678.

RYLE, A. P. and SANGER, F. (1955). Disulphide interchange reactions. *Biochem. J.*, **60**, 535–540.

SHINKA, S., IMANISHI, M., KUWAHARA, O. and AMANO, T. (1962). Immunochemical studies on lysozyme. II. On the non-neutralizing antibodies. *Biken. J.*, **5**, 181–200.

SHINKA, S. (1963). Symposium on "Immunochemistry of biologically active proteins." III. Immunochemical studies on egg-white lysozyme. Abstract of the Proceedings of the 16th General Assembly of the Japan Medical Congress. April 1963, Osaka, p. 396.

SINGER, S. J. and RICHARDS, F. M. (1959). The in-

teraction of subtilisin-modified ribonuclease with antisera against native ribonuclease. *J. Biol. Chem.*, **234**, 2911–2914.

SJÖQUIST, J. (1953). Paper strip identification of phenyl thiohydantoins. *Acta Chem. Scand.*, **7**, 447–448.

SJÖQUIST, J. and EDMAN, P. (1956). Identification and semiquantitative determination of phenyl thiohydantoins. *Acta Chem. Scand.*, **10**, 1507–1509.

SPACKMAN, D. H., STEIN, W. H. and MOORE, S. (1960). The disulfide bonds of ribonuclease. *J. Biol. Chem.*, **235**, 648–659.

STEWART, J. W., YOUNG, J. D. RENJAMINI, E., SHIMIZU, M..and LEUNG, C. Y. (1966). Immunochemical studies on tobacco mosaic virus protein, IV. The automated solid-phase synthesis of a decapeptide of tobacco mosaic virus protein, and reaction with antibodies to the whole protein. *Biochem.*, **5**, 3396–3400.

TSUGITA, A. and FRAENKEL-CONRAT, H. (1962). The composition of proteins of chemically evoked mutants of TMV RNA. *J. Mol. Biol.*, **4**, 73–82.

YOKOI, H. and AKASHI, S., (1955). An improved Kjeldahl-Nessler method. *Nagoya Med. J.*, **3**, 91–104.

YOUNG, J. D., BENJAMINI, E., SHIMIZU, M. and LEUNG, C. Y. (1966). Immunochemical studies on tobacco mosaic virus protein. III. The degradation of an immunologically active tryptic peptide of tobacco mosaic virus protein and the reactivity of the degradation products with antibodies to the whole protein. *Biochem.*, **5**, 1481–1488.