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Author(s)	Fujio, Hajime; Martin, Rosario Esuguerra; Ha, Yun-Mun et al.
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ANTIGENIC STRUCTURES OF HEN EGG-WHITE LYSOZYME. IV. ANTIGENICITY OF THE SECOND LOOP

HAJIME FUJIO, ROSARIO ESUGUERRA MARTIN[1], YUN-MUN HA, NOBUO SAKATO and TSUNEHISA AMANO

Department of Immunology, Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka

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SUMMARY The peptide containing the second loop (sequence 76–94) of hen egg-white lysozyme (HL) was separated by tryptic digestion of citraconylated HL or of cyanogen bromide treated HL. The peptide ($P_{loop\ II}$) consisted of sequence 62–68 and of sequence 74–96. About 5 to 10% of rabbit anti-HL antibody reacted with $P_{loop\ II}$ and the specificity of $P_{loop\ II}$ was distinct from that of the first loop (64–80). The association constant of HL with rabbit anti-$P_{loop\ II}$ antibody was estimated by equilibrium dialysis at 10 C as 1.1×10^5 (L/M). The rabbit anti-$P_{loop\ II}$ antibody could neutralize the enzymic activity of HL when *M. lysodeikticus* was used as substrate but not when hexa-*N*-acetyl chitohexaitol was used as substrate.

INTRODUCTION

The secondary structure of HL has two special regions, since the main peptide chain forms loops in two regions.

Peptide 8 (sequence 57–107) has been shown to be antigenic (Shinka et al., 1967) and this region contains both loops. In peptide 8, the first loop (sequence 64–80) was found to be intact, but the second loop (sequence 76–94) is split between residues 84 and 85. Therefore, it is possible that the antigenic activity of the second loop may be lost in peptide 8, because according to Sachs et al. (1972a) most of the antibodies directed to protein antigen seem to recognize the native conformation.

The antigenic activity of the first loop has been clearly established (Arnon and Sela, 1969). To examine the antigenicity of the second loop attempts were made to isolate it intact. From the known sequences of HL (Canfield and Liu, 1965), trypsin should be the most suitable enzyme for this purpose. To make HL susceptible to trypsin, it was either pretreated with cyanogen bromide to split the two methionine bonds or its free amino groups were citraconylated by the method of Dixon and Perham (1968). Both methods were satisfactory to obtain $P_{loop\ II}$ (sequence 62–68 linked by Cys^{64}-Cys^{80} with sequence 74–96). The antigenicity of the second loop was then examined and found to differ from that of the first loop.

1 Present address: Bureau of Research and Laboratories, San Lazaro Compound, Sta. Cruz, Manila, Philippines

MATERIALS AND METHODS

1. *Hen egg-white lysozyme (HL)*

Six times recrystallized HL was purchased from Seikagaku Fine Biochemicals Co. Ltd. This preparation was used without further purification for preparing the active peptide. For preparation of immunizing antigen, crystalline HL was further purified on SE-Sephadex C-25 in 0.2 M sodium phosphate buffer, pH 7.16 and then passed through QAE-Sephadex C-25 in 0.005 M sodium phosphate buffer, pH 8.0.

2. *Cyanogen bromide treatment of HL*

Crystalline HL were treated by cyanogen bromide by the method of Gross and Witkop (1962).

3. *Treatment of HL with citraconic anhydride*

Reversible blocking of amino groups was done by the method of Dixon and Perham (1968).

To find suitable conditions for complete citraconylation of HL, various amounts of citraconic anhydride (at molar ratios of anhydride to amino groups of 5 to 15) were added in several portions to HL solution (10 mg/ml) at room temperature and the pH of the solution was kept at 8.0 by addition of 4 M NaOH. The reaction was continued for one hr. Each sample was dialyzed against 100 volumes of precooled 0.5% sodium bicarbonate in a cold room overnight. The nitrogen content and free amino group of each sample were measured. More than 99% of the amino groups in HL were masked, when the anhydride was used in 15 molar excess over amino groups. Therefore, all citraconylated HL (CHL) preparations used in the following experiments were prepared using this concentration of anhydride.

4. *TPCK-trypsin*

Twice crystallized trypsin was purchased from Worthington Biochemical Corporation (U.S.A.) and treated with *N*-tosyl-*L*-phenylalanine chloromethyl ketone (TPCK) by the method of Shaw (1967).

5. *Estimation of free amino groups*

The method used was essentially the same as that of Satake et al. (1960). Solutions of various kinds of protein were heated with 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) in 2% $NaHCO_3$ at 40 C for 2 hr. Sodium dodecyl sulphate were used to dissolve the 2, 4, 6-trinitrophenyl protein (TNP-protein) following the method of Habeeb (1966) and the optical density of the solution was measured at 340 nm. The molar extinction coefficient of mono TNP-peptide was taken as 1.05×10^4 (Satake et al., 1960).

6. *Purification of peptides*

Tryptic digests of HL were purified by SE-Sephadex C-25 chromatography and gel-filtration on Bio-Gel P-10. Peptides were desalted as described previously (Sakato et al., 1972).

7. *Deionization of urea*

Just before use, 10 M urea solution was passed through a column containing a mixture of equal volumes of Amberlite IR-120 (H^+-form) and Amberlite IRA-400 (OH^--form). The eluate were examined with an electric conductivity meter to check that the conductivity was less than 1 mho at 20 C.

8. *Amino acid analysis*

The amino acid compositions of the peptides were determined by the method of Spackman et al. (1958) using a Nihon-Denshi amino acid analyzer, Model JLC-3BC. Peptides were hydrolyzed by treatment with constant boiling 5.7 N HCl at 105±1 C for 24 and 72 hr. The contents of half cystine were taken as the values obtained by amino acid analyses of acid hydrolyzates of peptides without correction. Performic acid oxidation was not performed.

9. *Antisera*

Two mg of highly purified HL with complete Freund's adjuvant were injected into rabbits. Booster injections of 2 mg of HL were given once every five weeks, the last being given after 20 weeks. Blood was taken 7 and 9 days after the last injection and the rabbits were exsanguinated 11 days after the last injection. Sera from 10 rabbits were pooled.

10. *Purification of the peptide specific antibody fraction*

Each peptide was coupled to Sepharose 4B as described previously (Fujio et al., 1971). Rabbit antisera were passed through the peptide-Sepharose 4B immunoadsorbent at 2 C in the presence of 0.01 M EDTA. The column was thoroughly washed with 0.135 M NaCl, 0.01 M EDTA, pH 7.5. The antibody-protein was eluted with 0.2 N acetic acid at 2 C and immediately neutralized with 1.0 M K_2HPO_4. This purification of antibody protein

with immunoadsorbent was usually repeated to obtain a highly purified antibody fraction.

The 7S fraction of antibody was further separated on a Sephadex G-150 column.

11. *Equilibrium dialysis*

HL was labelled with ^{125}I by the iodine monochloride method of McFarlane (1958). Equilibrium dialysis was performed at 10 C for at least 45 hr, as described previously (Fujio et al., 1971).

12. *Enzymic activity*

The lysozyme activities of various preparations of peptides and the inhibitions of the enzymic activity of lysozyme by various antibody species were measured with either *M. lysodeikticus* or hexa-*N*-acetyl-chitohexaitol as substrate, as described in our previous report (Imanishi et al., 1969).

TABLE 1. *Amino acid composition of P_{Ic}*

Amino acid	Hydrolysis for[a]		Ile^{58}–Lys^{97}[b]
	24 hr	72 hr	
Lys	1.66	1.89	2
His	0	0	0
Arg	2.70	2.93	3
Asp	6.55	6.70	7
Thr	1.91	1.84	2
Ser	5.99	5.89	6
Glu	0	0	0
Pro	1.90	2.13	2
Gly	2.00	2.00	2
Ala	2.77	2.77	3
1/2 Cys	3.61	3.02	4
Val	0.91	0.94	1
Met	0	0	0
Ile	2.90	2.89	3
Leu	2.85	2.83	3
Tyr	0	0	0
Phe	0	0	0
Trp	Nd	Nd	2
Total			40

[a] Values are expressed as molar ratios, assuming there are 2 glycine residues per mole of P_{Ic}.

[b] Moles of amino acids in the corresponding HL calculated from the data of Canfield and Liu (1965).

13. *Preparations and characterizations of various HL-fragments*

P_8 (sequence 57–107) was prepared as described before (Shinka et al., 1967) and further purified on SE-Sephadex C-25 in 0.08 M sodium acetate buffer, pH 5.0, in the presence of 6 M urea at 2 C. P_{17} (sequence 1–27 linked by Cys^6-Cys^{127} with sequence 122–129) was prepared as described previously (Fujio et al., 1968).

P_{IC} (sequence 58–97) was prepared by limited digestion of HL with thermolysin as described before (Sakato et al., 1972) and purified on SE-Sepha-

TABLE 2. *The amino acid compositions of two peptides obtained from reduced and alkylated P_{Ic}*

Amino acid	P_{Ic}–1[b]	Ile^{58}–Leu^{83}[c]	P_{Ic}–2[b]	Leu^{84}–Lys^{97}[c]
Lys	0	0	1.88	2
His	0	0	0	0
Arg	2.59	3	0	0
CM-Cys	3.14	3	1.20	1
Asp	4.70	5	2.13	2
Thr	1.04	1	0.98	1
Ser	2.57	3	2.83	3
Glu	0	0	0	0
Pro	1.70	2	0	0
Gly	2.00	2	0	0
Ala	1.04	1	1.90	2
Val	0	0	1.00	1
Met	0	0	0	0
Ile	1.86	2	1.14	1
Leu	1.64	2	1.42	1
Tyr	0	0	0	0
Phe	0	0	0	0
Trp	Nd	2	Nd	0
Total		26		14

[a] When the reduced and alkylated P_{Ic} was subjected to gel-filtration on a Bio-Gel P-10 column, two fractions were obtained, named P_{Ic}–1 and P_{Ic}–2 in order of elution.

[b] Values are expressed as molar ratios, assuming one valine residue per mole of P_{Ic}–1 and two glycine residues per mole of P_{Ic}–2.

[c] Moles of amino acid in the corresponding regions of HL, calculated from the data of Canfield and Liu (1965).

dex C-25 in 0.17 M sodium acetate buffer, pH 5.0 in the presence of 6 M urea. The results of amino acid analysis of this peptide are shown in Table 1.

The results indicate that P_{IC} represents the sequence 58–97 of HL. To test the possibility of peptide bond cleavage within the loop region, the peptide was reduced with 2-mercaptoethanol and alkylated with iodoacetic acid. The reduced and alkylated P_{IC} was separated into two fractions on a Bio-Gel P-10 column in 25% acetic acid. The amino acid compositions of these two fractions are given in Table 2.

The results indicate that the peptide was cleaved between residues 83 and 84.

14. *Disc electrophoresis of peptides*

The electrophoretic homogeneity of each peptide was tested by disc electrophoresis in 20% acrylamide in acidic media. The method of Elson and Jovin (1969) was modified to use in acidic media. In brief, 20 g of acrylamide and 3 g of bis-acrylamide were dissolved in 50 ml of deionized 8 M urea (solution A). One ml of *N*, *N*, *N'*, *N'*-tetramethylenediamine, 13.2 ml of glacial acetic acid and 24 g of purified urea were mixed and adjusted to 50 ml with deionized water (solution B). Then 800 mg of ammonium persulphate and 24 g of purified urea were dissolved in deionized water and the solution was adjusted to 50 ml (solution C). Two parts of solution A were mixed with one part of solution B and one part of solution C. Polyacrylamide gel columns were prepared in 5×80 mm glass tubes. Upper reservoir contained 0.015 M acetic acid in 7 M deionized urea and the lower reservoir 0.015 M acetic acid. The polyacrylamide column was pretreated at 3.5 ma per tube for one hr. Samples of 20 μg of each peptide in 10% sucrose in 8 M urea were applied to the tubes. Electrophoresis was carried out at 2 C, applying 2 ma per tube for 15 min and then 3.5 ma per tube for one hr. The gels were stained (Maron et al., 1971) with 0.25% Coomassie blue in methanol-water-acetic acid (5: 5: 1, v/v/v).

15. *Miscellaneous procedures*

After chromatography, peptides were located by measuring the optical density at 280 nm and also by the ninhydrin reaction. For the latter, a sample of 0.3 ml of each fraction was mixed with one ml of ninhydrin reagent (Moore et al., 1954) and boiled for twenty min. After cooling, 4 ml of 50% ethanol were added and optical density was measured at 570 nm.

Peptides were reduced at 20 C by treatment with 0.1 M 2-mercaptoethanol, 0.005 M EDTA, 0.5 M Tris-HCl buffer, pH 8.5 for one hr, keeping the solution at pH 8.3 by adding 2 M Tris solution. Ten per cent excess (on a molar basis) of recrystallized iodoacetic acid was added to the reduced peptide solution and the pH was kept at 8.3 by adding 2 M Tris. Alkylation was continued for one hr at 20 C. The reagents were then removed by gel-filtration on Bio-Gel P-10 column.

The concentration of peptide, HL and purified antibody were estimated by the biuret reaction (Kabat and Mayer, 1961). The biuret reaction of each protein was standardized using a solution of each protein in which the nitrogen had been determined by the Kjeldahl-Nessler method (Yokoi and Akashi, 1955).

RESULTS

1. *Tryptic digestion of citraconylated HL*

Tryptic digestion of CHL was carried out using solid trypsin. Fifty mg of TPCK-trypsin coupled to Sepharose 4B were added to 100 ml of CHL solution (10 mg/ml) in 0.5% sodium bicarbonate at 37 C. One drop of toluene was added as a preservative and the suspension was stirred gently with a magnetic stirrer. CHL solution alone in the same solvent was also incubated at 37 C. Duplicate 1 ml aliquots of each sample were taken 0, 1, 2, 4, 6 and 16 hr after addition of trypsin and the supernatant of each sample was analyzed by the Kjeldahl-Nessler and TNBS methods. The free amino groups of the tryptic digest of CHL are shown in Fig. 1.

Approximately 11 amino groups per mol of CHL were detected after digestion for 4 hr under these condition. The number of amino groups released by trypsin is consistent with the number of arginine residues per mole of HL. Therefore it seems probable that all the arginyl bonds of CHL were split. Tryptic digestion of CHL in following experiments was performed for 16 hr under the conditions described above, except that sodium bicarbonate was replaced by 0.5% ammonium bicarbonate. The ammonium bicarbonate was

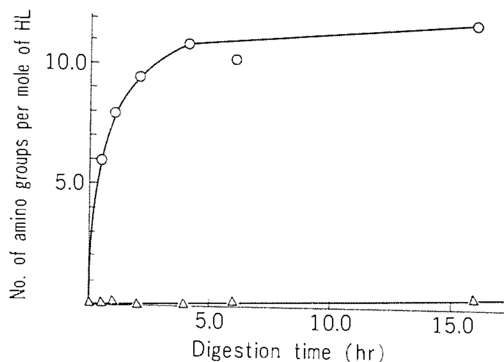

FIGURE 1. *Tryptic digestion of citraconylated HL. Citraconylated HL (CHL) was digested with TPCK-trypsin coupled to Sepharose 4B at 37 C. Free amino groups released were estimated by the TNBS method (Satake et al., 1960) and expressed as the numbers of amino groups per mole of HL taking the molecular weight of HL as 14,300.* ○——○, *CHL with TPCK-trypsin;* △——△, *CHL alone (control).*

removed by repeated lyophilization.

Deblocking of citraconyl groups was carried out by suspending the tryptic digest of CHL (10 mg/ml) in 0.1 N acetic acid at 25 C overnight and stirring it with a magnetic stirrer. The suspension was very turbid. The acetic acid was removed by lyophilization. Estimation of total nitrogen and free amino groups in this deblocked tryptic digest of CHL indicated that 7 amino groups per mole of CHL were uncovered by this treatment.

The lysyl bonds of HL were broken during a second digestion as follows. One g of deblocked tryptic digest of CHL was dissolved into 100 ml of 0.5% sodium bicarbonate. The solution was slightly turbid. One drop of toluene was added a preservetive and the solution was incubated at 37 C. After 0, 1, 2, 4, 6 and 24 hr of incubation, 10 mg of TPCK-trypsin were added successively. Duplicate 1 ml aliquots were taken after 1, 2, 4, 6, 24 and 32 hr of incubation just before addition of TPCK-trypsin. Trypsin activity was stopped by mixing with equal weight of soy bean trypsin inhibitor. The mean numbers of amino groups per mole of HL at each digestion time were 21.3 (1 hr), 23.6 (2 hr), 25.9 (4 hr), 27.1 (6 hr), 26.5 (24 hr) and 27.5 (32 hr). If TPCK-trypsin split only arginyl and lysyl bonds of HL, 24 amino group per mole of HL should appear after the second digestion of HL by trypsin. The value obtained by the TNBS method after incubation for 4 hr was slightly higher than this calculated value, as shown above. This discrepancy may be due to the tendency of TNP amino acids and TNP derivatives of small peptides to give higher molar extinction coefficients than large peptides. In any case, the number of amino groups per unit of protein seemed to reach a constant level after 6 hr of digestion. In following experiments, the first digest of CHL was dissolved in 0.5% of ammonium bicarbonate and TPCK-trypsin, at one hundredth the concentration of substrate was added after 0, 1, 2 and 4 hr of incubation at 37 C. The second digestion was continued for 16 hr.

2. *Purification and identification of the tryptic peptides of citraconylated HL*

The second digest of approximately 4 g of HL was lyophilized three times to remove ammonium bicarbonate and then dissolved in 5 liter of 0.02 N acetic acid. A minute amount of insoluble material was removed by filtration through Toyo-filter paper, No. 131. The filtrate was applied to a SE-Sephadex C-25 column (3×40 cm) which had been quilibrated to 0.02 N acetic acid. The column was developed using a 3 chamber varigrad with 3 liters of 0.05 M sodium acetate buffer (pH 3.88), 0.07 M sodium acetate buffer (pH 4.50) and 0.15 M sodium acetate buffer (pH 5.50), respectively in the chambers. Chromatography was performed at 25 C and fractions of 20 g were collected. As a preservative 0.05% chloreton were added to all buffers.

The elution pattern is shown in Fig. 2. Amino acid analysis of each peak indicated that fraction 10 of Fig. 2 contained the second loop of HL. This fraction was rechromatographed on SE-Sephadex C-25 in 0.03 M sodium acetate buffer, pH 4.5, in the presence of 6 M urea.

The chromatographic pattern of Fr. 10 on

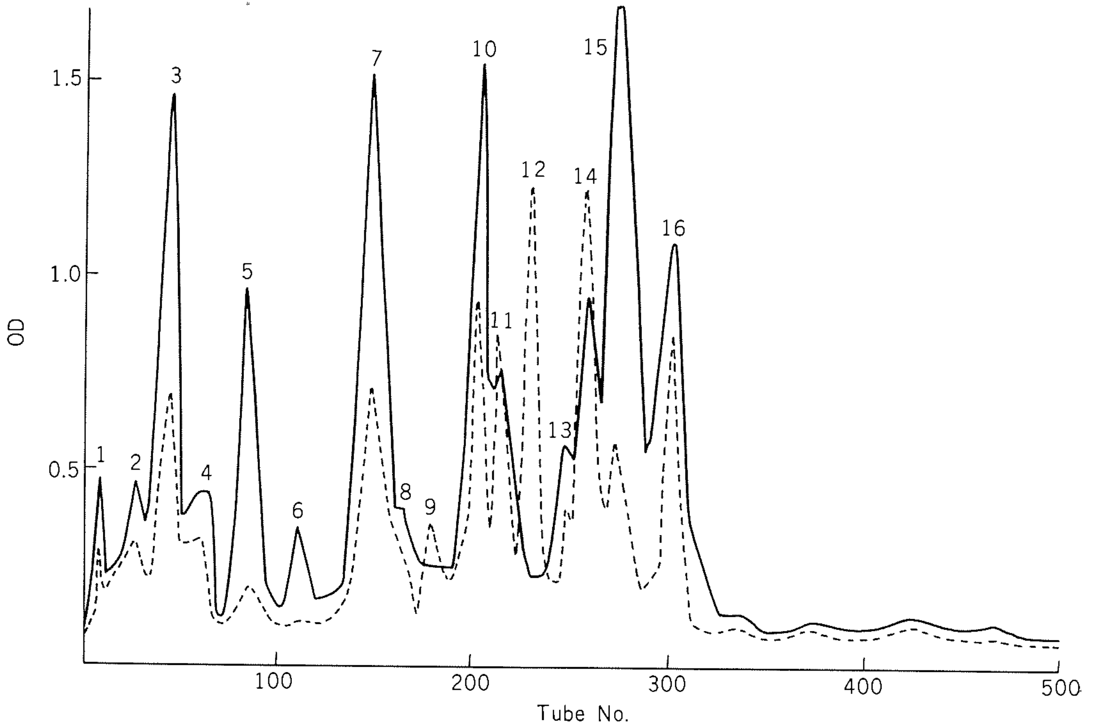

FIGURE 2. *Chromatography of the second tryptic digest of CHL on a SE-Sephadex C-25 column (3×40 cm). Approximately 4 g of the digest were applied and 20 g fractions were collected at 25 C. The solid line represents the optical density at 280 nm and the dotted line that of the ninhydrin reaction at 570 nm (0.3 ml aliquots of each fraction were used for the ninhydrin reaction).*

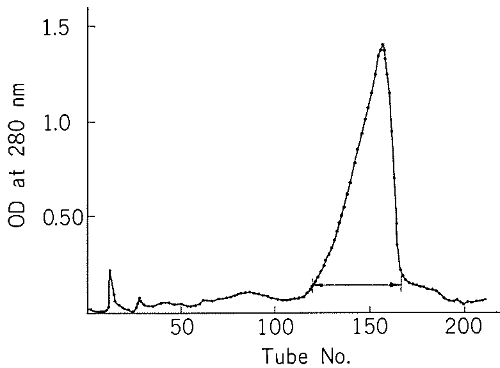

FIGURE 3. *Rechromatography of Fr. 10 from on a SE-Sephadex C-25 column (2×40 cm). A sample of 130 mg was applied and the column was eluted with 0.03 M sodium acetate buffer, 6 M urea, pH 4.5 at 25 C. Fractions of 10 g were collected.*

rechromatography is shown in Fig. 3. The main component was collected as shown by arrows in the figure. This fraction was desalted as described in the Materials and Methods and further purified by gel-filtration on Bio-Gel P-10 (3×155 cm) in 25% (v/v) acetic acid. This gel-filtration step was repeated. The gel-filtration effectively removed a minute amount of contaminating intact HL. The lytic activity of the purified peptide was examined using 5 mg per ml of peptide solution but no lytic activity could be detected even with a high concentration of the peptide. Therefore, contamination of the peptide with intact lysozyme amounts to less than one ten thousandth the weight of peptide.

The results of amino acid analysis, given in Table 3, show that the peptide consists of sequences 62–68 and 74–96 of HL ($P_{loop\ II}$). Next

the possibility of peptide bond cleavage in the second loop region of $P_{loop\ II}$ was tested. Thirty mg of $P_{loop\ II}$ were reduced and alkylated (RA-$P_{loop\ II}$) as described in the Materials and Methods. The RA-$P_{loop\ II}$ was fractionated on a Bio-Gel P-10 column (3×160 cm) in 25% (v/v) acetic acid. The UV absorption of each fraction at 280 nm and its reaction with ninhydrin were examined. The fraction eluted first (RA-$P_{loop\ II}$-1) had no UV absorption at 280 nm but gave a positive ninhydrin reaction.

TABLE 3. *Amino acid composition of $P_{loop\ II}$*

Amino acid	Found[a]		Calcd.[b]
	$P_{loop\ II}$-A[c]	$P_{loop\ II}$-B[d]	
Lys	1.07	1.05	1
His	0	0	0
Arg	1.12	1.18	1
Thr	0.94	0.92	1
Ser	3.57	3.63	4
Glu	0.04	0.06	0
Pro	0.98	0.95	1
Gly	1.00	1.00	1
Ala	2.91	2.88	3
1/2 Cys	2.63	3.11	4
Val	1.08	1.05	1
Met	0	0	0
Ile	1.97	1.92	2
Leu	2.92	2.89	3
Tyr	0	0	0
Phe	0	0	0
Trp	(1.9)[e]	(1.8)[e]	2

[a] $P_{loop\ II}$ was hydrolyzed for 24 hr in constant boiling HCl (5.7 N). Values are expressed as molar ratios, assuming there is one glycine residue per mole of $P_{loop\ II}$.

[b] Moles of amino acids in the sequences (62–68 and 74–96) of HL were calculated from the data of Canfield and Liu (1965) and listed in the Table for comparison.

[c] $P_{loop\ II}$-A was prepared by tryptic digestion of CHL.

[d] $P_{loop\ II}$-B was prepared by tryptic digestion of CBHL.

[e] Tryptophan contents estimated by the UV absorption method (Goodwin and Morton, 1946).

On the other hand, the second fraction (RA-$P_{loop\ II}$-2) had UV absorption at 280 nm and also gave a positive ninhydrin reaction. The results of amino acid analyses of these two fractions are shown in Table 4.

The results indicate that there was no peptide bond cleavage in the loop region of $P_{loop\ II}$ and the amino acid composition of $P_{loop\ II}$ was reconfirmed.

The recovery of $P_{loop\ II}$ peptide from the second digest of CHL was tested using the chromatographic conditions described in Fig. 3. Approximately 15% of the HL can be recovered as $P_{loop\ II}$. The theoretical recovery

TABLE 4. *Amino acid compositions of two peptides obtained from reduced and alkylated $P_{loop\ II}$*[a]

Amino acid	RA-$P_{loop\ II}$-1	Asn^{74}-Lys^{96}[b]	RA-$P_{loop\ II}$-2	Trp^{62}-Arg^{68}[b]
Lys	1.04	1	0	0
His	0	0	0	0
Arg	0	0	1.07	1
Asp	3.87	4	1.89	2
Thr	0.92	1	0	0
Ser	3.62	4	0	0
Glu	trace	0	0	0
Pro	0.96	1	0	0
Gly	trace	0	1.00	1
Ala	2.93	3	0	0
CM-Cys	3.12	3	1.05	1
Val	1.00	1	0	0
Met	0	0	0	0
Ile	1.94	2	0	0
Leu	2.88	3	0	0
Tyr	0	0	0	0
Phe	0	0	0	0
Trp	Nd	0	Nd	2

[a] Each peptide was hydrolyzed with 5.7 N HCl at 105 C for 24 hr. Values are expressed as molar ratios, assuming there is one valine residue per mole of RA-$P_{loop\ II}$-1 and one glycine residue per mole of PA-$P_{loop\ II}$-2.

[b] Moles of amino acid in the corresponding regions of HL were calculated from the data of Canfield and Liu (1965).

is 22.9% taking the minimal molecular weight of $P_{loop\ II}$ as 3,270.

The homogeneity of the purified $P_{loop\ II}$ was tested by disc electrophoresis.

As shown in Fig. 4 $P_{loop\ II}$ gave a single band.

FIGURE 4. *Disc electrophoresis of purified $P_{loop\ II}$ in in acid-urea gel 20 μg of $P_{loop\ II}$ were applied. A current of 2 ma per tube was applied for 15 min and then 3.5 ma per tube for one hr. The anode is at the top.*

3. *Tryptic digestion of cyanogen bromide treated HL (CBHL)*

Bonavida et al. (1969) thoroughly investigated the effect of cyanogen bromide treatment on HL. The results indicated that HL became partially susceptible to trypsin digestion after cyanogen bromide treatment. Therefore, we tested the possibility of obtaining $P_{loop\ II}$ from a tryptic digest of CBHL.

Preliminary experiments indicated that the following experimental conditions were satisfactory to obtain complete cleavage of the methionyl bonds in HL. One g of HL was dissolved in 50 ml of 70% formic acid and 500 mg of cyanogen bromide were added. The mixture was stirred at room temperature for 16 hr. Amino acid analysis of the resulting CBHL showed that more than 99% of the methionine residues of HL were lost by treatment with cyanogen bromide. CBHL in 70% formic acid was diluted ten-fold with deionized water and lyophilized. The susceptibility of CBHL to trypsin was tested by repeated addition of TPCK-trypsin (1: 100) to a 1% solution of CBHL in 0.5% sodium bicarbonate after 0, 1, 2, 4 and 6 hr of incubation at 37 C. Total nitrogen and free amino group analyses of the series of samples taken at intervals just before addition of trypsin indicated that digestion for at least 6 hr at 37 C with repeated additions of TPCK-trypsin was neccessary to achieve complete digestion of CBHL. Table 5 shows results of estimations of the free amino groups of CBHL and of its tryptic digest.

When the tryptic digest of CBHL were chromatographed on SE-Sephadex C-25 in 0.03 M sodium acetate buffer, pH 4.5, in the presence of 6 M urea, a peptide fraction was eluded in the same position as that of $P_{loop\ II}$. This peptide could not be differentiated by disc electrophoresis from $P_{loop\ II}$ obtained by tryptic digestion of citraconylated HL. The amino acid composition of this peptide, shown in Table 3, is also similar to that of $P_{loop\ II}$ obtained from CHL. The recovery of this peptide from the tryptic digest of CBHL was 18%, or about 80% of the theoretical value.

4. *Test for the specificity of rabbit (R) anti-$P_{loop\ II}$ by immunoadsorbents*

A solution of 100 mg of purified $P_{loop\ II}$ in 20 ml of 0.1 M sodium phosphate buffer, pH 7.0 was stirred with 15 ml of activated Sepha-

TABLE 5. *Release of free amino groups by cyanogen bromide treatment of HL and by tryptic digestion of CBHL*

Preparation	No. of amino groups per 14,300 g	
	Found[a]	Calcd.
HL	7.1	7
CBHL	9.5	9[b]
Tryptic digest of CBHL (6 hr)	30.2	26[c]

[a] The TNBS method was used and the numbers of amino groups were calculated taking the molar extinction coefficient of mono TNP-peptide as 1.05×10^4.

[b] Calculated assuming that HL was split at 2 methionyl bonds.

[c] Calculated assuming that HL was split at 2 methionyl, 11 arginyl and 6 lysyl bonds.

rose 4B at 2 C overnight. In this way 59 mg of $P_{loop\ II}$ were coupled to Sepharose. Then 200 ml of rabbit anti-HL antiserum containing 0.01 M EDTA were applied to the immunoadsorbent at 2 C. After extensive washing of the immunoadsorbent with 0.135 M NaCl, 0.01 M EDTA, pH 7.5, 50.9 mg of protein were eluted on further treatment of the immunoadsorbent with 0.1 M citrate buffer, pH 2.3 (Sachs et al., 1972b). The first filtrate from the immunoadsorbent, was again treated with the same immunoadsorbent, but no appreciable amount of protein could be eluted. As a control, 200 ml of normal rabbit serum containing 0.01 M EDTA were passed through the same immunoadsorbent and treated in exactly the same way as in the case of anti-HL antiserum. About 7.5 mg of protein were eluted. This nonspecific adsorption corresponds to 15% of the antibody fraction. Therefore the antibody fraction obtained by the first treatment with immunoadsorbent was subjected to a second treatment with the same immunoadsorbent. Only 75% of the initial antibody fraction was adsorbed and eluted. But when this antibody fraction after two treatments was tested for binding with HL using equilibrium dialysis, it was shown that at least 90% of the protein could bind with HL. Therefore, antibody preparations were all treated with immunoadsorbent twice before use in following experiments. The antibody content which reacted with the $P_{loop\ II}$ region of HL (R anti-$P_{loop\ II}$) was calculated as 0.19 mg per ml of this rabbit serum (R anti-HL #P53) from the amount of protein after two treatments with $P_{loop\ II}$ immunoadsorbent. Since the precipitable antibody in the rabbit anti-HL antiserum used in this experiment was evaluated as 1.89 mg/ml by the modified method of quantitative precipitin analysis (Fujio et al., 1971), 10.1% of this R antibody reacted with the $P_{loop\ II}$. Several other rabbit anti-HL antisera were also tested and in these samples antibody content which reacted with the $P_{loop\ II}$ ranged from 5 to 10% of the antibody precipitable by HL. In the following experiments R anti-$P_{loop\ II}$ antibody was prepared from R anti-HL antiserum #P53.

Since the amino acid sequence of $P_{loop\ II}$ (62–68 and 74–96) overlaps that of P_8 (sequence 57–107) and both peptides were shown to have binding activities with anti-HL antibody, the specificity of the R anti-$P_{loop\ II}$ antibody was tested with P_8 immunoadsorbent, prepared as described previously (Fujio et al., 1971). The capacity of the P_8 immunoadsorbent was found to be 23 mg of anti-P_8 antibody protein. Ten mg of purified R anti-$P_{loop\ II}$ antibody were applied to the P_8 immunoadsorbent and the washing and elution procedures described above were performed. More than 90% of the protein was not adsorbed and only 5% of the applied protein were eluted with acid. It is not clear whether the protein elute with acid is adsorbed nonspecifically or whether it is a specific antibody to P_8, since due to shortage of material the specificity of this protein could not be tested. Nevertheless, it is clear that there is an antibody fraction which reacts with $P_{loop\ II}$ and that it's specificity is distinct from that of anti-P_8. The result is consistent with the results of equilibrium dialysis, described in a later section.

5. *Equilibrium dialysis of rabbit anti-$P_{loop\ II}$ antibody with ^{125}I-HL and its inhibition by various peptides*

The specificity of R anti-$P_{loop\ II}$ antibody, first tested with immunoadsorbent, as described above, was studied in more detail by equilibrium dialysis.

The 7S fraction of R anti-$P_{loop\ II}$ antibody was separated by gel-filtration on a Sephadex G-150 column and used in the following experiment.

Fifty μl of the purified R anti-$P_{loop\ II}$ antibody (17.2 μM) were dialyzed against equal volumes of various concentrations of ^{125}I-HL (12 μM to 340 μM) at 10 C for 55 hr. The results of this experiment are shown as a Scatchard plot of r/c against r, in Fig. 5 in which r represents the moles of HL bound per mole of antibody and c represents the concentration of free ^{125}I-HL. The extrapolated value of

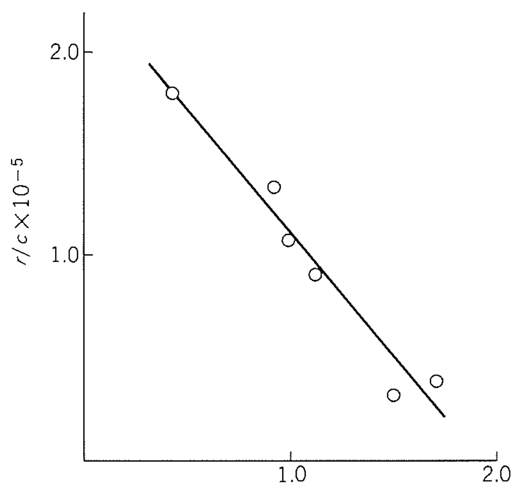

FIGURE 5. *Binding of purified rabbit anti-$P_{loop\ II}$ antibody with ^{125}I-HL at 10 C. Values are shown as a Scatchard plot. r, moles of ^{125}I-HL bound per mole of antibody, taking the molecular weight of antibody as 150,000 and that of HL as 14,300; c, concentration of free ^{125}I-HL* (M).

r is approximately 1.9. This suggests that approximately 90 to 100% of the R anti-$P_{loop\ II}$ antibody can bind with HL. The K_A value of binding of rabbit anti-$P_{loop\ II}$ antibody was found to be 1.1×10^5 (L/M) at 10 C. Next the inhibitory activities of a wide variety of peptides, which were, or were not structurally related to $P_{loop\ II}$, were tested against the binding of ^{125}I-HL with anti-$P_{loop\ II}$ antibody. Various concentrations (1×10^{-4} to 4×10^{-3} M: final concent) of peptides in PBS, pH 6.0 were mixed with 1.36×10^{-4} M (final concn) of ^{125}I-HL and the mixtures were dillyzed against purified anti-$P_{loop\ II}$ antibody (1.72×10^{-5} M) at 10 C. The results are shown in Table 6.

To check the possibility of modification of the binding property of HL with R anti-$P_{loop\ II}$ antibody by iodination of HL, the inhibitory activity of unlabelled HL was tested. The K_I of unlabelled HL was evaluated as 1.6×10^5, which was close to the K_A obtained by direct binding of ^{125}I-HL with R anti-$P_{loop\ II}$ antibody.

Among the peptides tested, only the $P_{loop\ II}$ showed detectable inhibition, but the K_I of the $P_{loop\ II}$ was only one tenth of that of intact HL. P_8 and P_{IC}, which were shown to have sequences overlapping that of $P_{loop\ II}$ but with scissions in the peptide bond of second loop of HL, did not inhibit the binding, even at the highest concentrations tested. In addition, RA-$P_{loop\ II}$-1, which is the extended form of the second loop of HL, also had no effect on the binding of ^{125}I-HL with R anti-$P_{loop\ II}$ antibody. These results clearly indicate that the second loop of HL is one of the determinant groups of HL and that the complete loop is necessary for its activity.

P_{17} (Fujio et al., 1968), which is a determinant group located in the N- and C-terminal region of HL, quite far from the second loop of HL, also had no effect on the binding of ^{125}I-HL with R anti-$P_{loop\ II}$ antibody.

TABLE 6. *Inhibition of the binding of ^{125}I-HL to rabbit anti-$P_{loop\ II}$ antibody by various peptides*

Peptide	K_I[a] (L/M)
HL[b]	1.6×10^5
$P_{loop\ II}$	1.4×10^4
P_8	$<5 \times 10^2$
P_{1c}	$<5 \times 10^2$
RA-$P_{loop\ II}$-1[c]	$<1 \times 10^3$
P_{17}	$<5 \times 10^2$

[a] Association constant of inhibitor (Karush, 1959),

$$K_I = \frac{(r/r'-1)(1+K_A c)}{(i)}$$

The K_A value of the binding of R anti-$P_{loop\ II}$ antibody with ^{125}I-HL was 1.1×10^5 (L/M); r, moles of antigen bound per mole of antibody in the absence of inhibitor; r', moles of antigen bound per mole of antibody in the presence of inhibitor; (i), equilibrium concentration of inhibitor.

[b] Unlabelled hen egg-white lysozyme.

[c] This peptide was poorly soluble so the experiment was carried out with the highest concetration possible.

6. *Inhibitory activity of R anti-$P_{loop\ II}$ antibody on the enzymic activity of HL*

If the anti-$P_{loop\ II}$ antibody interacts with the native form of HL, the enzymic activity of HL should be influenced by the binding of this antibody, especially when the size of the substrate is large. In addition, it has been shown that the efficiency of neutralization of HL activity by one kind of antibody is strongly dependent on the specificity of this antibody, especially when the size of substrate is small (Fujio et al., 1971). Therefore it seemed interesting to see the efficiency of neutralization of the enzymic activity of HL by R anti-$P_{loop\ II}$ antibody.

Fig. 6 depicts the neutralization of enzymic activity of HL by increasing amounts of R anti-$P_{loop\ II}$ antibody with *M. lysodeikticus* as substrate. For comparison, the neutralization of the enzymic activity of HL by R anti-HL antibodies, prepared by dissociation of specific precipitates, as described before (Fujio et al., 1968), is also shown in Fig. 6. Neutralization by R anti-$P_{loop\ II}$ antibody was slightly less efficient than that by R anti-HL antibody.

Fig. 7 shows results on the neutralization of the enzymic activity of HL by increasing amounts of anti-$P_{loop\ II}$ antibody with hexa-N-acetyl-chitohexaitol (Imanishi et al., 1969) as substrate. R anti-P_8 antibody was also prepared with P_8-Sepharose immunoadsorbent from the same batch of R anti-HL antiserum used for preparation of R anti-$P_{loop\ II}$ antibody. Results of neutralization experiments using R anti-HL antibody and R anti-P_8 antibody with hexa-*N*-acetyl-chitohexaitol as substrate are also plotted in Fig. 7 for comparison.

R anti-$P_{loop\ II}$ antibody did not neutralize the enzymic activity of HL at all when a small sized substrate was used. In contrast, R anti-P_8 antibody neutralized the enzymic activity of HL even when a small sized substrate was used. The results again indicate that the anti-$P_{loop\ II}$ antibody can also be differentiated from the anti-P_8 antibody by its behavior in neutralization of enzymic activity. The results are

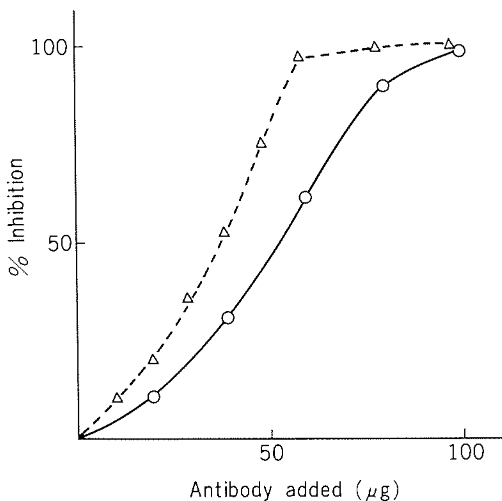

FIGURE 6. *Neutralization of the enzymic activity of HL with increasing amounts of anti-$P_{loop\ II}$ antibody. M. lysodeikticus was used as substrate. HL solution (2.6 μg) in 0.025* M *sodium diethyl-barbiturate-HCl, pH 6.0 was mixed with various amounts (10 to 100 μg) of R anti-$P_{loop\ II}$ (○——○) or R anti-HL (△······△) antibody. The percent inhibition of the enzymic activity is plotted against the amount of antibody added.*

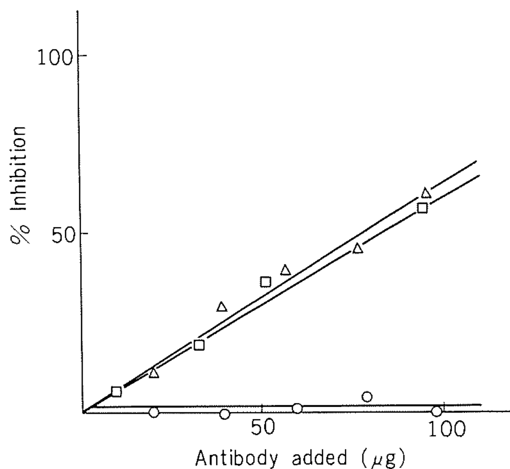

FIGURE 7. *Neutralization of the enzymic activity of HL by increasing amounts of anti-$P_{loop\ II}$ antibody. Hexa N-acetyl-chitohexaitol (Imanishi, et al., 1969) was used as substrate. HL solution (4.3 μg) in 0.025* M *sodium diethyl-barbiturate-HCl, 0.1* M *NaC1, pH 6.0 was mixed with various amounts (10 to 100 μg) of antibody. The percent inhibition of enzymic activity is plotted against the amount of antibody added. ○——○, R anti-$P_{loop\ II}$; □——□, R anti-P_8; △——△, R andti-HL antibody.*

consistent with those obtained from experiments with immunoadsorbents and equilibrium dialysis, described in the previous section, which showed that the specificity of the $P_{loop\ II}$ antibody is distinct from that of P_8 antibody.

DISCUSSION

The reversible blocking of the amino groups of HL have been studied by Habeeb and Atassi (1970) and recently a preliminary report by Atassi et al. (1973) indicated that a peptide (sequence 62–68 linked by Cys^{64}-Cys^{80} with sequence 74–96) has antigenic activity. We also independently studied the tryptic digestion of citraconylated HL and of cyanogen bromide treated HL (Fujio et al., 1973). In our studies, the antigenicity of the second loop of HL were carefully tested, especially in relation to the antigenicity of the first loop of HL.

The antigenicity of the first loop (sequence 64–80) of HL has been thoroughly investigated (Arnon and Sela, 1969; Maron et al., 1971; Teicher et al., 1967) since P_8 (sequence 57–107) was found to be antigenic (Shinka et al., 1967). The conformational factors of this determinant seem to be very important in keeping the loop peptide in an antigenetically active state.

In this paper, the second loop of HL was isolated and its antigenicity was studied. The results of binding experiments using immunoadsorbent and equilibrium dialysis indicated that the specificity of the second loop is distinct from that of the first loop. The opening of the loop by reduction and alkylation or any split of the loop destroys the antigenic activity of this determinant.

The data in experiments with immunoadsorbent and equilibrium dialysis were obtained using the $P_{loop\ II}$ obtained from CHL. The $P_{loop\ II}$ obtained from CBHL was also tested in both ways and gave essentially the same results as the $P_{loop\ II}$ from CHL. Recovery of $P_{loop\ II}$ from the tryptic digest of cyanogen bromide treated HL was better than that from the tryptic digest of CHL. The reason for this is uncertain, but it is possible that citraconylation does not proceed completely homogeneously, as described by Habeeb et al. (1970), who suggested the partial citraconylation of serine and threonine residues.

Recently, Reichlin (1972) made the interesting observation that when a rabbit was immunized with human haemoglobin, only the regions of the haemoglobin molecule with different amino acid sequences from that of rabbit haemoglobin were antigenic. These results strongly suggest that a factor governing the antigenicity of a certain region of the molecule is the structural relationship between the antigen molecule and its equivalent in the host. This concept was also discussed in our previous report to explain the possible immunogenicity of the catalytic site of HL against rabbits (Sakato et al., 1972). At present the complete amino acid sequence of rabbit lysozyme is unknown, but it would be interesting to compare the amino acid sequence of the second loop of HL with that of the corresponding region of human leukemia lysozyme (Canfield et al., 1971). Comparison of the amino acid sequence from 74 (Asn) to 96 (Lys) of HL with the corresponding region of human lysozyme, shows that 10 of 23 amino acid residues are different. Nine of 10 amino acid residues are located on the surface of the molecule, according to Browne et al. (1969). Therefore, it seems possible that the second loop region of HL may be antigenic in mammals. More direct comparison must be made of the amino acid sequences of the immunogen and of the equivalent protein in the host.

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