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ANTIGENIC STRUCTURES OF HEN EGG WHITE LYSOZYME

II. SIGNIFICANCE OF THE N-AND C-TERMINAL REGION AS AN ANTIGENIC SITE

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SUMMARY An immunologically active peptide was isolated by limited digestion of hen egg white lysozyme (HL) with pepsin. It consisted of two peptides, i.e. Lys^{1} to Asn^{27} and Ala^{122} to Leu^{129}, and the two peptides were linked together by a single S-S bond (Cys^{6} to Cys^{127}). Equilibrium dialysis studies demonstrated the specific binding of the peptide by anti-HL antibody. The average association constant of the peptide by one batch of purified anti-HL antibody was 1.78×10^{5} at 10°C, and the percentage of antibody directed to this peptide in 7S anti-HL antibody was evaluated as 47%. The peptide did not show any precipitin reaction with rabbit HL antisera.

The peptide also inhibited the enzymic activities of hen and duck egg white lysozymes when *M. lysodeikticus* was used as a substrate. The specific binding of the peptide by *M. lysodeikticus* was observed.

The nature of the antigenic determinant involved in the peptide and the possible mechanism of enzymic inhibition by the peptide were discussed.

INTRODUCTION

Proteins have very complex structures, and there is not enough information available on the antigenic determinants in proteins to understand their nature. To simplify the problems involved, hen egg white lysozyme (HL) was chosen as antigen because it has a relative low molecular weight and is easily purified. Available information on the three dimensional arrangement of the amino aicd residues in the crystalline lysozyme molecule (PHILLIPS, 1966; PHILLIPS, 1967; BLAKE *et al.*, 1967) provides a valuable basis for understanding the spatial arrangement of the antigenic determinants in the molecule.

In previous reports the preparations of four immunologically active peptides from HL and their characterizations were described (SHINKA *et al.*, 1962; SHINKA *et al.*, 1967). They all consisted of 51 amino acid residues and were derived from the region from Gln^{57} to Ala^{107} of HL.

This paper describes a new peptide with antibody-and bacterial substrate-binding activities. It consisted of 35 amino acid residues

located at the N-and C-terminal region of HL. Equilibrium dialysis studies showed the significance of the antigenic determinant involved in this peptide.

MATERIALS AND METHODS

1. *Preparation of peptides*

Six times recrystalized HL (Lot No. 4112) was purchased from Seikagaku-Kogyo Co. Ltd. and stored at −20°C. Limited digestion of HL with pepsin was carried out as described in a previous report (SHINKA *et al.*, 1967) with a slight modification. Twice recrystalized pepsin was purchased from Worthington Biochemical Corporation. A 1% solution of crystalline HL in 0.1 N HCl was adjusted to pH 1.62 with sodium hydroxide and warmed to 40°C. Then 5 mg of crystalline pepsin were added to 10 g of HL. The digestion was carried out for one hour. Then the digest was adjusted to pH 5.0 with sodium hydroxide and immediately lyophilized and stored at −20°C until use. Before use it was passed through a Sephadex G-50 column in the initial buffer used for CM-cellulose chromatography at 4°C to remove pepsin. The eluate from the Sephadex G-50 column was applied to a CM-cellulose column. The chromatographic procedure was as described in the previous report (SHINKA *et al.*, 1967).

An example of CM-cellulose chromatography of the peptides is shown in Fig. 1.

2. *Preparation of duck egg white lysozyme (DL)*

Duck egg white lysozyme was purified as described in the previous report (IMANISHI *et al.*, 1966) with some modification. Crude DL which was eluted from IRC-50 column was dialyzed against 0.2 M phosphate buffer, pH 7.16. Approximately 100 mg

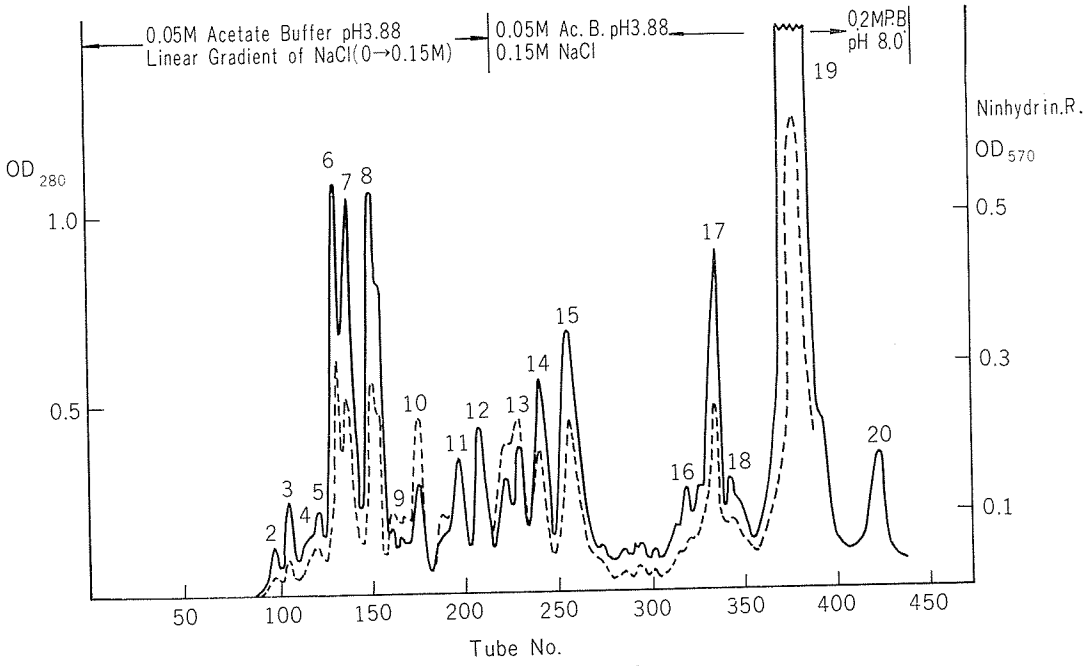

FIGURE 1 CM-cellulose chromoatography of peptic digest of HL.

One gram of pepsin digest was applied to a CM-cllulose column (3×40 cm, 0.5 meq./g. 200–400 mesh) at 25°C. The column was washed with 0.05 M acetate buffer, pH 3.88, and eluted with a linear gradient to 0.15 M NaCl in 0.05 M acetate bufferf pH 3.88 followed by a gradient from 0.15 M NaCl, 0.05 M acetate buffer, pH 3.88 to 0.2 M phosphate buffer, pH 8.0.

Twenty ml. fractions were collected.

——— OD at 280 mμ

..... OD of ninhydrin reaction at 750 mμ

of crude DL were applied on a SE-Sephadex column (1.5×47 cm) which was equilibrated with 0.2 M phosphate buffer, pH 7.16, and eluted with the same buffer. Three ml fractions were collected and the optical density of each tube was measured at 280 mμ. The acidic and neutral proteins were eluted just after the void volume of the column, followed by three components. The second of these components was the major one and was used as DL in the following experiments.

3. *Production of antisera*

Antisera were obtained from male and female albino rabbits weighing between 2 and 3 kg. HL was purified by SE-Sephadex (2.3 meq per g. Pharmacia) chromatoraphy in 0.2 M sodium phosphate buffer, pH 7.16 at 25°C. The chromatcgraphic pattern was essentially the same as that on the IRC-50 column (HIRS *et al.*, 1953). Only the main component was used for the immunization. After chromatography a solution of the purified HL (10 mg/ml) in 0.15 M NaCl, 0.02 M sodium phosphate buffer, 0.02% sodium azide, pH 6.0, was emulsified with an equal volume of complete Freund's adjuvant. Two kinds of immunization schedule were used.

Schedule A: Each rabbit received an initial dose of 2 mg of antigen injecting equal doses into the four footpads. A booster dose of 2 mg was given in the same way 5 weeks after the first injection. Then 8 and 10 days after the boc̣ster injection 40 ml of blood were taken from each rabbit by cardiac puncture and after 12 days the rabbit was exanguinated.

Schedule B: The initial injection was carried out as in shcedule A. But booster injections of 2 mg of HL were given four times 5, 10, 15 and 20 weeks after the initial injection in the same way as the initial injection. Blood was taken 8, 10 and 12 days after the last injection.

Qualitative precipitin tests were positive for every serum and the sera from 8 rabbits in each group were pooled. More than 99% of the added ^{125}I–HL (KUWAHARA *et al.*, 1966) was always precipitated at the equivalence point when the pooled antisera (Schedule A or B) were tested by the quantitative precipitin reaction.

4. *Purification and properties of 7S anti-HL antibody*

The specific 7S anti-HL antibody preparations were purified from the two kinds of pooled rabbit HL antisera for equilibrium dialysis experiments. HL antiserum #P24 was prepared by immunization schedule A and HL antiserum #P28 was obtained by schedule B.

Anti-HL antibody was precipitated from the pooled serum with the equivalent amount of HL and the mixture was incubated for one hour at 37°C and then for 2 days at 4°C. Specific precipitates were thoroughly washed at 4°C with 0.15 M NaCl, 0.02 M sodium phosphate, pH 6.0 (PBS). Two hundred mg of specific precipitate were dissolved into 50 ml of 0.2 N acetic acid and incubated at 37°C for one hour with stirring. The clarified HL and anti-HL antibody mixture was passed through a Sephadex G-150 column (5.5×90 cm) saturated with 0.2 N acetic acid. Chromatography was carried out at 25°C and 10 ml fractions were collected. The OD of each fraction was measured at 280 mμ. Three peaks were found. Although the properties of the material in the first peak are still under investigation, this material is presumably a kind of high molecular weight antibody. The second peak was the 7S antibody and the third was identified as dissociated HL from its lytic activity against *M. lysodeikticus*. It was noticed that 0.1 N acetic acid only dissociated about half the added HL from the specific precipiate prepared from HL antiserum #P28 (schedule B) although the same solvent dissociated HL prepared from HL antiserum #P24 (schedule A) almost completely. Therefore 0.2 N acetic acid was used routinely for dissociation of the antigen-antibody complex. The recovery of 7S anti-HL antibody from HL antiserum #P24 (schedule A) was 87% and that from HL antiserum #P28 (schedule B) was 67% of percipitable antibody in each serum. The recoveries of the dissociated HL from the specific precipitates were more than 90% in both cases, but the amount of material in the first peak from the Sephadex G-150 chromatogram with HL antiserum #P28 was almost twice that with HL antiserum #P24. The 7S fraction was dialysed against 100 volumes of PBS, pH 6.0, at 4°C for 3 days changing the buffer twice a day. The dialyzate was concentrated by pressure dialysis. The stock solutions of the purified antibody preparations containing 5 to 10 mg protein per ml were kept at −20°C until use.

Sedimentation velocity experiments on purified anti-HL antibodies were carried out in a Hitachi analytical centrifuge (Model UCA-IA). The two preparations of the purified HL antibody showed

essentially the same pattern and were identified as 7S class antibody by calculation of the sedimentation coefficient.

5. *7S normal rabbit γ globulin (7S NRG)*

Globulin fractions were precipitated from normal rabbit serum by half saturation of ammonium sulfate and purified further by gel filtration on a column of Sephadex G-150. The 7S fraction of globulin was applied to a DEAE-cellulose column equilibrated with 0.05 M phosphate buffer, pH 7.5, and eluted with the same buffer.

6. *Determination of peptide concentration*

The concentrations of solutions of peptide were determined by the biuret reaction (KABAT and MAYER, 1961). The biruet reaction was standardized with a solution of peptide in which the nitrogen had been determined by the Kjeldahl-Nesseler method (YOKOI and AKASHI, 1955). The calculated N content of peptide 17 was 16.5% according to the results of amino acid analysis.

7. *Amino acid analysis*

The amino acids in peptide 17 were analyzed as described in cur previous report (M. IMANISHI *et al.*, 1966). A Yanagimoto amino acid analyzer, Model LC-5, was used.

8. *Molecular weight measurement*

The molecular weight of peptide 17 was estimated by the method of Archibald (ARCHIBALD, 1947; KLAINER and KEGELES, 1956). A Hitachi analytical ultracentrifuge was used and runs were performed at 23,100 rpm and at 20.0°C. The molecular weight was calculated by data obtained from meniscus analysis.

9. *Preparation of [1-^{14}C] acetyl peptide 17*

Five μM of peptide 17 (20 mg) were dissolved into 4 ml of 1.0 M sodium acetate (pH 8.0). A half mc (27.1 μM) of [1-^{14}C] acetic anhydride (Daiichi-Kagaku, Co. Ltd.) in 0.5 ml of benzene was layered on the top of the cooled peptide solution at 4°C. The reaction was allowed to continue for 48 hours at 4°C. The bottom layer was put on to a Sephadex G-25 column (3×60 cm) equilibrated with 50% (v/v) acetic acid at 25°C and eluted with the same solvent. Ten ml fractions were collected. The OD at 280 mμ of each was measured. Twenty μl of each fraction were put into 10 ml of Bray solution (BRAY, 1960) and the radioactivity was counted in a Packard liquid scintillation counter (Model 3003). The fractions containing peptide were pooled. Acetic acid was removed by rotatory evaporator under reduced pressure and lyophilization.

An average of approximately 0.2 moles of acetyl group were introduced per mole of peptide 17 under these conditions. The [1-^{14}C] acetyl peptide 17 was diluted with cold peptide 17 for the equilibrium dialysis experiment. The final solution had a specific activity of 1.84×10^{6} cpm/μM.

10. *Equilibrium dialysis*

A dialysis cell like that described by KARUSH and KARUSH (1968) was used. One ml of peptide solution of 1.3×10^{-5} M to 1×10^{-4} M was put into one cell and an equal volume of purified anti-HL antibody was put into the other cell. The antibody concentration was usually ca. 2×10^{-5} M and its exact concentration was estimated by Kjeldahl-Nessler analysis of the stock solution taking the molecular wieght as 150,000. The solvent was PBS, pH 6.0, containing 0.02% sodium azide.

As a semipermeable membrane, so called gel-cellophane was used throughout the experiment. The pore size of gel-cellophane was such that serum albumin passed through while γ G-globulin did not. The gel-cellophane used in this experiment was a gift from Dai-Seru Co. Ltd. A preliminary experiment showed that 36 hours were necessary to attain equilibrium of peptide 17 at 10°C.

The equilibrium dialysis cell was rotated at 5 rpm in a water bath at 10°C. The free peptide concentration was estimated by Lowry's modification (LOWRY *et al.*, 1951) of the Folin reaction and also by counting radioactivity. Sample solution (0.1 ml) was put into 10 ml of Bray solution and counted in a Packard liquid scintillation counter (Model 3003). A correction was made for the casing adsorption of the peptide in each experiment. A concentration of free peptide of as low as 5×10^{-8} M could be measured accurately by counting radioactivity.

11. *Enzyme assay*

Two kinds of substrate were used for enzyme assay.

Method I: *Micrococcus lysodeikticus* was used as a substrate and the reduction of its OD at 540 mμ was measured as described previously (SHINKA *et al.*, 1962).

Method II: glycol chitin (HAMAGUCHI and FUNatsu, 1959) was used as a substrate. The glycol chitin used was a gift from Dr. Matsushima. Gly-

col chitin (184 μg) in 200 μl of veronal buffer saline (μ=0.15), pH 6.0, was mixed with various amounts of HL (0.02 to 0.4 mM) in 225 μl of the buffer. The mixture was incubated at 37°C for 30 minutes. After incubation at 37°C the reaction was stopped by cooling the mixture in an ice bath. The reducing activity of the mixture was measured by the method of PARK and JOHNSON (1949).

12. *Chitin*

Chitin was purchased from Eastman Organic Chemicals Co. and treated as follows. Frist it was ground and sieved and the fine particles were treated twice with 5 N NaOH in a cold room followed each time by wahsing with deionized water. Then the chitin was treated with 85% formic acid in the cold and washed with water. Finally the powder was wahsed with acetone and dried at 60°C under reduced pressure.

RESULTS

1. *Purification of peptide 17 by gel filtration*

Further purification of peptide 17 was carried out by gel filtration on Sephadex G-50 in 10% (v/v) acetic acid. The eluates obtained by CM-cellulose chromatography were desalted by the method of Dixon (1959) and concentrated in a rotatory evaporator. The temperature of the water bath was kept at below 37°C and the pressure inside the flask at 15 mm Hg. A typical elution pattern of crude peptide 17 is shown in Fig. 2(a).

The peptide Fr. 17, eluted from the CM-cellulose column, was usually found to be contaminated with the intact HL when examined by high voltage electrophoresis at pH 3.6. Therefore it was further purified by repeated gel filtration on a Sephadex G-50 column, as shown in Fig. 2(b).

The fractions were pooled as indicated in the figure and concentrated by rotatory evaporator under reduced pressure and lyophilized. The material thus obtained was used as the preparation of peptide 17 in the following experiments.

2. *High voltage paper electrophoresis of peptide 17*

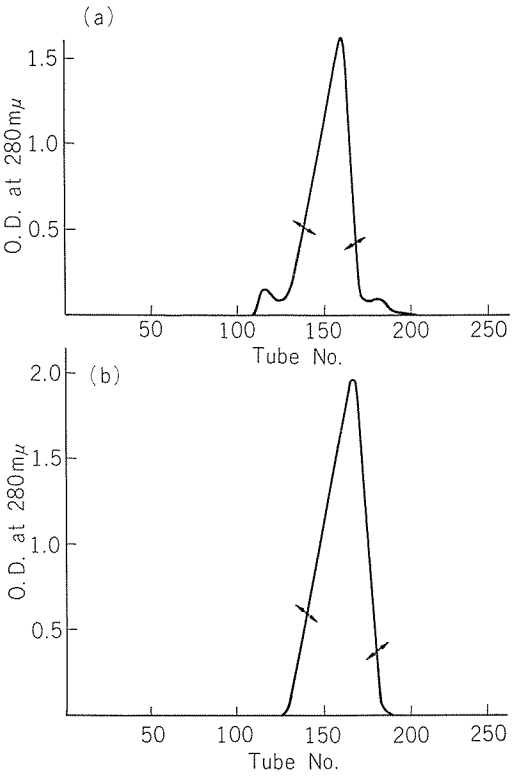

FIGURE 2 (a) Gel filtration of crude peptide fraction 17 on a Sephadex G-50 column. 160 mg of peptide Fr. 17 eluted from a CM-cellulose column were dissolved into 12.5 ml of 10% (v/v) acetic acid and applied to a Sephadex G-50 column (5.5×92cm) operated at 25°C and 10 ml fractions were collected. Fractions were pooled as indicated by arrows. (b) Refiltration of peptide 17 on a Sephadex G-50 column. 217 mg of peptide 17 in 12.5 ml of 10% acetic acid were applied to a similar column as in (a). Experimental details as in (a).

Electrophoresis was carried out as described previously (SHINKA *et al.*, 1967) to assure the purity of the peptide. The peptide 7a (SHINKA *et al.*, 1967) was always run in the electrophoresis for comparison.

Samples of 2.5 mg of peptide 7a and peptide 17 were each dissolved in 100 μl of pyridine-acetate buffer, pH 3.6 (RYLE *et al.*, 1955) and applied to Toyoroshi filter paper #51 (5× 60 cm) as 1 cm wide bands. A voltage of 3,000 v was applied between the ends of the

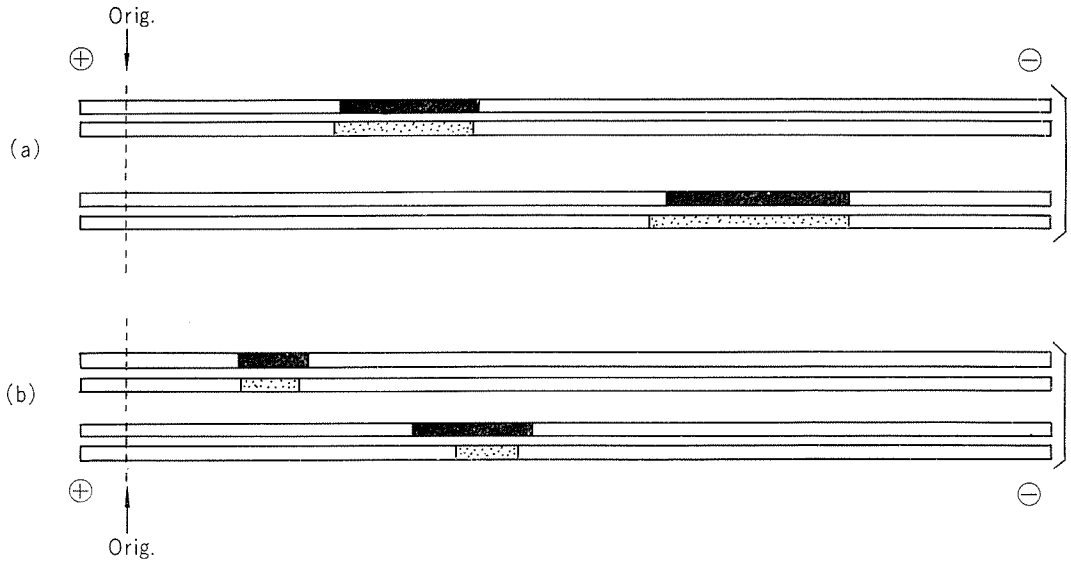

FIGURE 3 High voltage paper-electrophoresis of peptide 17
(a) Electrophoretic pattern of peptide 17 (bottom) and 7a (top) at pH 3.6 using 3000 volts for 135 miuntees.
(b) Electrophoretic pattern of peptide 17 (bottom) and 7a (top) at pH 6.5 using 3000 volts for 60 minutes.
▬ stained with ninhydrin reagent. ▭ stained with Ehrlich reagent.

paper for 135 min. Two 0.5 cm wide strips of each sample were cut out. The peptides were located by staining with ninhydrin and Ehrlich reagent. The homogeneities of the two peptides were also checked at pH 6.5 (pyridine-acetate buffer; RYLE *et al.*, 1955) using 3,000 v for 60 min. The results are shown in Fig. 3.

The peptides each showed a single band at both pH's values. Peptide 17 has a relatively high positive charge as can be deduced from its behavior on CM-cellulose chromatography.

3. *Amino acid compositon of peptide 17*

The amino acid composition of peptide 17 expressed as moles of each amino acid residue per mole of peptide is shown in Table 1.

Glycine was taken as a standard to calculate the amino acid composition. The minimum molecular weight of peptide 17 was calculated as 4,000 from the results of amino aicd analysis. The third column of Table 1 shows the integer value of each amino acid deduced from the analysis. The amino acid composition in the regions from Lys^{1} to Asn^{27} and from Ala^{122} to Leu^{129} of HL was calculated from the data given by CANFIELD and LIU (1965) and is shown in the fourth column of Table 1. The values are consistent with each other. Therefore, it is concluded that peptide 17 consisted of two peptides linked by a S-S bond. The characteristics of the amino acid composition of peptide 17 are as follows. It contained one mole of histidine which was the only histidine residue in HL. It contained one of the two methionine residues of the original molecule. One of the six tryptophan residues in the original HL was located in this region. Peptide 17 had no proline or threonine. From its amino acid composition peptide 17 seems to be rather basic and this is consistent with the results of high voltage electrophoresis and CM-cellulose chromatoraphy of the peptide.

4. *Ultracentrifugal study of peptide 17*

The molecular weight of peptide 17 was measured by ARCHIBALD's method (1947). Runs

TABLE 1 *Amino acid composition of peptide 17*

Amino acid residue	Hydrolysis for		Integer value	Lys^{1}-S-Asn^{27} [c]
	24 hrs[a]	72 hrs[a]		Leu^{129}-S-Ala^{122}
Lysine	2.45	2.26	2	2
Histidine	1.10	1.02	1	1
Ammonia	4.68	2.73		2
Arginine	5.23	5.07	5	5
Aspartic acid	3.31	3.28	3	3
Threonine	0.23	0.15	0~1	0
Serine	1.03	0.74	1	1
Glutamic acid	1.23	1.20	1	1
Proline	0	0	0	0
Glycine	5.00	5.00	5	5
Alanine	3.76	3.63	4	4
Half-cystine	1.68	2.03	2	2
Valine	1.30	1.37	1~2	1
Methionine	0.78	1.10	1	1
Isoleucine	0.84	0.94	1	1
Leucine	3.78	3.81	4	4
Tyrosine	1.85(1.7)[b]	1.93	2	2
Phenylalanine	1.12	1.14	1	1
Tryptophan	(0.9)[b]		1	1
Total			35	35

a Values are expressed as molar ratios, assuming there are 5 glycine per mole of peptide 17.

b Tryptophan contents were estimated by the U. V. absorption method (Goodwin and Morton, 1946).

c Moles of amino acids in the correspoinding region of HL calculated from the data of Canfield and Liu (1965).

were made at two concentrations of the peptide in PBS, pH 6.0. Results are shown in Fig. 4.

At lower concentrations of peptide(4.85 mg/ml), the molecular weight is evaluated to be 3,900 as can be seen in the figure. This is consistent with a minimum molecular weight of 4,000 calulated from the results of amino acid analysis. At higher concentrations of peptide (9.9 mg/ml), dimers tended to form as can be seen from its higher apparent molecular weight at the start of the run, but the value became closer to 4,000 later in the run.

5. *Inhibition of HL and DL activities by peptide 17*

No lytic activity of peptide 17 could be detected even at a concentration of 200 μM with *M. lysodeikticus* as substrate, although as little as 0.02 μM of HL could be detected under our experimental conditions. But when peptide 17 was mixed with HL or DL, the lytic activities of the two enzymes were inhibited considerably.

Various amounts of peptide 17 (25 mμM to 150 mμM) were mixed with a constant amount of HL (0.41 mμM) or DL (0.37 mμM) in 2 ml of PBS, pH 6.0. Two ml of *M. lysodeikticus* suspension in PBS, pH 6.0, containing 1.2 mg/ml, were added to each mixture. The lytic activity of the mixture was measured as described previously (SHINKA *et al.*, 1967). The degree of inhibition reached 50% against either enzyme, as seen in Fig. 5.

Peptide 17 did not show any enzymic activity

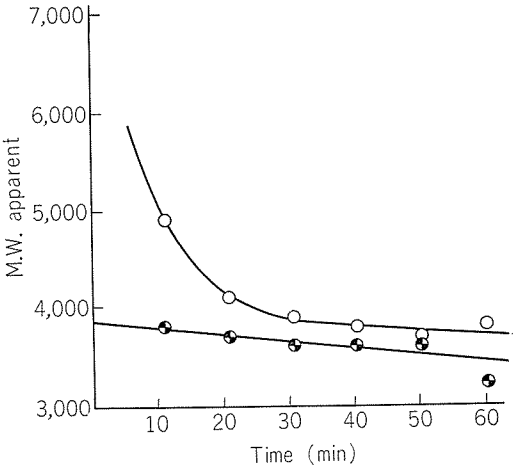

FIGURE 4 Molecular weight measurements of peptide 17 by the method of ARCHIBALD. Runs were made at two concentrations of peptide 17 (○———○: 9.9 mg/ml and ⊖———⊖: 4.85 mg per ml). Apparent molecular weights were calculated after various durations of centrifugation.

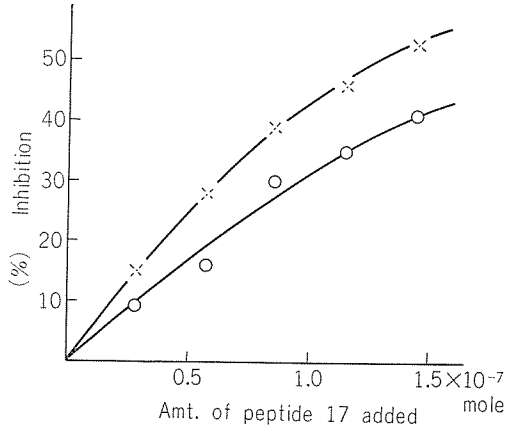

FIGURE 5 Inhibition of enzymic activites of HL and DL by peptide 17. Percentage inhibition of HL activity (○———○); Percentage inhibition of DL activity (×———×). See text for details of experiments.

even at a concentration of 266 mμM per ml, although as low as 0.16 mμM of HL could be detected under our experimental conditions using glycolchithin as substrate. The inhibitory activity of peptide 17 on the activity of

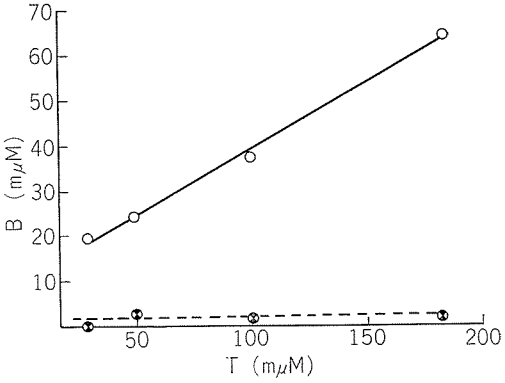

FIGURE 6 Binding of peptide 17 by *M. lysodeikticus* cells and chitin at 37°C.
T: Total amount of peptide 17 added minus amount of peptide adsorbed to centrifuge tube.
B: Amount of peptide bound per 2.4 mg of *M. lysodeikticus* cells (○———○) or per 2.0 mg of chitin (⊖———⊖).

HL with glycolchitin as substrate was also tested. But no noticable inhibition could be detected even with a 400 molar excess of peptide 17 over HL.

To elucidate why peptide 17 was only inhibitory with *M. lysodeikticus* as substrate, the binding of the peptide by *M. lysodeikticus* and chitin was studied. Mixtures of various amounts of peptide 17 (30 mμM to 180 mμM) and a constant amount of *M. lysodeikticus* cell (2.4 mg) or chitin (2 mg) suspension in 2 ml of PBS, pH 6.0, were put into conical centrifuge tubes. Controls contained peptide alone and *M. lysodeikticus* or chitin alone in PBS, pH 6.0. Each tube was incubated at 37°C for 30 minutes with constant shaking and centrifuged at 3,000 rpm for 20 min at room temperature. The peptide concentration of each supernatant was determined by Lowry's modification of the Folin reaction. Liberation of Folin positive materials from *M. lysodeikticus* cells or chitin was negligible, and the correction for casing adsorption, expressed as a percentage of the peptide added, ranged from 2 to 8% under the experimental conditions. As shown in Fig. 6, the amount of peptide 17 adsorbed on *M. lysodeikticus* cells increased linearly with in-

crease in the peptide concentration in the reaction mixture.

More than 70 percent of the peptide 17 in the system was adsorbed onto *M. lysodeikticus* cells under certain experimental conditions. On the other hand, there was no detectable adsorption of peptide 17 by chitin. These results were consistent with the fact that peptide 17 inhibited the enzmic activities of HL or DL only when *M. lysodeikticus* was used as substrate.

6. *Binding of peptide 17 by purified anti-HL antibody*

The binding activities of peptide 17 by two batches of purified anti-HL antibody were measured by equilibrium dialysis. The amounts of bound peptide (B) was plotted against the sum of free and bound peptide in the chamber containing antibody (G). This plot is shown in Fig. 7. As a control the binding of peptide 17 by 7S normal rabbit γ-globulin (7S NRG) is also shown. At equilibrium the free peptide concentration was measured both by counting ^{14}C and by the Folin reaction. As seen in Fig. 7, the values obtained by the two methods were in agreement. There was no appreciable amount of non-specific binding between peptide 17 and 7S NRG.

To evaluate the maximal amount of bound peptide in the system, the reciprocals of G were plotted against the amounts of bound peptide as shown in Fig. 8. The B value obtained by extrapolation of the reciprocal of G to zero, corresponds to the maximal amount of bound peptide in the system. If the valency of antibody is taken as two, the moles of antibody directed to peptide 17 in the system can be calculated by dividing the maximal moles of bound peptide by two. Thus the amount of antibody directed to peptide 17 in the purified anti-HL antibody #P28 preparation was calculated as 9.2×10^{-9} moles per ml. This value corresponds to 47 percent of the total anti-HL antibody in the system.

A SCATCHARD plot (1949) of the data on binding is given in Fig. 9, where " r " is the average number of peptide molecules bound per antibody molecule at the free peptide concentration " c " at equilibrium. The average association constant (K_0) was taken as the re-

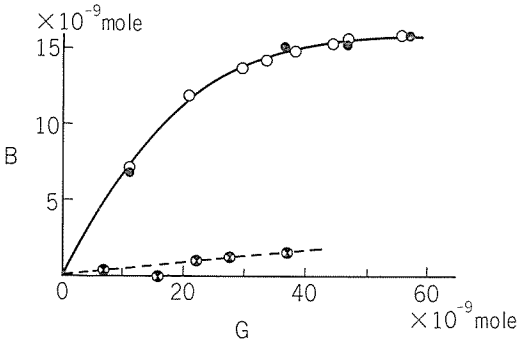

FIGURE 7 Binding of peptide 17 by the purified fied anti-HL antibody #p 28 at 10°C.
B: Amount of bound peptide
G: Sum of free and bound peptide in the chamber containing antibody.
Values obtained by ^{14}C-counting (○———○) and values obtained by Folin reaction (●———●); binding of peptide 17 by 7S NRG measured by counting of ^{14}C (◉———◉).

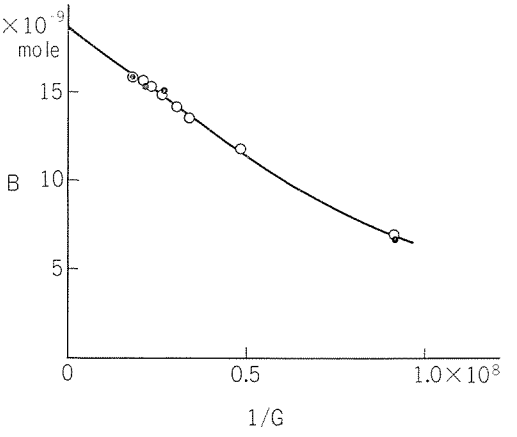

FIGURE 8 Estimation of maximal amount of bound peptide 17 using constant amount of purified anti-HL antibody #P 28.
B and G: See footnote of Fig. 7; values obtained by counting of ^{14}C (○———○) and values obtained by Folin-reaction (●———●).

reciprocal of the free peptide concentration corresponding to half saturation of the antibody, i.e., $r=1$ (KARUSH and KARUSH, 1968).

The total range of r covered in the experiments was from 0.6 to 1.7. The average association constants of the binding of peptide 17 by two batches of purified anti-HL antibody are listed in Table 2 together with the percentage of the fraction of antibody directed to this peptide.

The two batches of antibody differ in antibody affinity and in the percentage of antibody fraction directed to peptide 17. This could be due to the difference in the immunization schedule (EISEN and SISKIND, 1964; FUJIO and KARUSH, 1966). The antibody (#P24) in the early phase of immunization has a lower affinity for peptide 17 than that (#P28) in a late phase of immunization. Both purified antibody preparations were obatined from large pools of rabbit HL-antisera, but this point must be studied further.

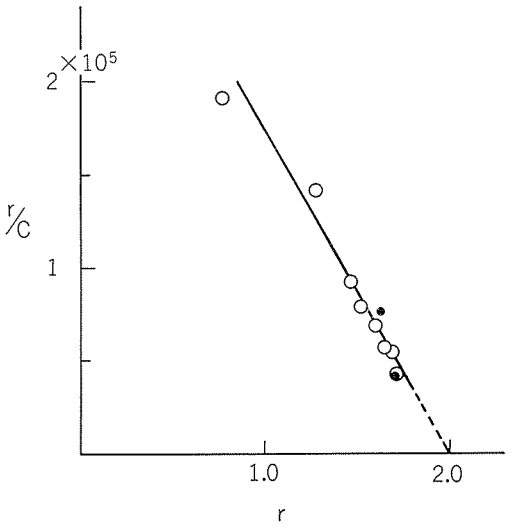

FIGURE 9 Schatchard plot of binding between peptide 17 and purified HL antibody #P 28.
r: moles of bound peptide per mole of antibody
c: concentration of free peptide
Values of r and c were obtained by counting of ^{14}C (○———○) and by Folin-reaction (●———●).

TABLE 2 *Contents of anti-peptide 17 in HL antibody and mean association constants (Ko) of the interaction between HL antibody and peptide 17.*

Purified HL antibod	Interval[a] (weeks)	Content of anti-peptide 17[b] (%)	Ko (l/mole)
#P24	5	22	5.8×10^4
#P28	20	47	1.8×10^5

a The interval between the first and last injections is given in the table. See text for details of immunization schedule.
b The content of the antibody fraction was estimated by equilibrium dialysis and expressed as a percentage of the amount of purified anti-HL 7S antibody.

DISCUSSION

In our previous report (SHINKA *et al.*, 1967) we described four immunologically active peptides obtained by limited digestion of HL with pepsin. They all seemed to bear the same immunological specificity.

A new peptide, peptide 17, was also isolated from the digest of HL with pepsin from which peptides 6, 7a, 7b and 8 had been isolated. It has a basic nature as can be seen by its behavior on CM-cellulose chromatography and high voltage paper electrophoresis. Amino acid analysis indicated that ptptide 17 was derived from the N-and C-terminal regions of the HL molecule. It consists of two peptides, Lys^1 Asn^{27} and Ala^{122} to Leu^{129}, linked together by a S-S bond (Cys^6 to Cys^{127}).

The minimum molecular weight of peptide 17, calculated from amino acid analysis, was 4,000 which was consistent with the results of ultracentrifugal analysis.

Peptide 17 inhibits the enzymic activities of HL and DL when *M. lysodeikticus* is used as substrate. Moreover, peptide 17 binds with *M. lysodeikticus* cells. On the other hand, the protion of HL corresponding to peptide 17 was not found to be a binding site of substrate by PHILLIPS *et al.* (JOHNSON and PHILLIPS, 1965;

PHILLIPS, 1966), who studied the complex of crystalline HL and the oligomer of N-acetylglucosamine by X-ray analysis. The reason for this difference is unknown, but one possible explanation is that the binding site of the oligomer of N-acetylglucosamine in HL is different from that of *M. lysodeikticus*. A more probable explanation of the enzymic inhibition by peptide 17 is that peptide 17 can bind rather nonspecifically with *M. lysodeikticus* cells due to its positive charge and in so doing it changes the surface of the cells in some way so that they become less accessible to the actions of HL and DL. This possiblity may be the reason why more than a 300 molar excess of peptide 17 over HL or DL was necessary to cause 50% inhibition.

Peptide 17 did not show any precipitin reaction when up to 300 μgN of it were mixed with 100 μl of various batches of rabbit HL antisera. The immunologic activity of peptide 17 can be easily demonstrated in the studies on the binding of the peptide with purified rabbit anti-HL antibody using equilibrium dialysis. Rabbit HL antisera always contained an appreciable portion of antibody directed to peptide 17. In one batch of antisera (#P28), 47% of the 7S antibody had a specificity directed to this portion of HL. Therefore the portion of HL corresponding to peptide 17 seems to be the dominant antigenic site in the HL molecule.

In contrast, it is relatively hard to detect the specific inhibition of the precipitin reaction between HL and rabbit HL antiserum by peptide 17. However, it can be observed in some batches of rabbit HL antisera (FUJIO *et al.*, 1968b) with relatively large amounts of the peptide. One factor affecting the manifestation of precipitation inhibition would be the ratio of the affinity of the antibody fraction in the reaction with the peptide 17 to that in the reaction with native HL. We are trying to isolate the specific antibody directed to peptide 17 to make this comparison. The mean association constant of peptide 17 in reaction with the corresponding antibody fraction in anti-HL antibody is reasonably high and comparable with those of other protein antigens in reaction with the corresponding antibodies (SINGER, 1965). In addition, the binding of peptide 17 by its corresponding antibody fraction was proved to be specific, because the peptide did not show any noticeable degree of binding by 7S normal rabbit γ-globulin.

The binding specificity of peptide 17 with the corresponding antibody fraction in HL antibody was found to be independent of that of peptide 7a, as seen in the following paper (FUJIO *et al.*, 1968b).

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