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FURTHER STUDIES ON THE SPECIFICITY OF THE N- AND C-TERMINAL ANTIGENIC DETERMINANT OF HEN EGG-WHITE LYSOZYME

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SUMMARY The specificity of the N- and C-terminal antigenic determinant (P_{17}: sequence Lys[1]–Cys[6]–Asn[27], Trp[123]–Cys[127]–Leu[129]) of hen egg-white lysozyme (HL) was studied in more detail. In a Scatchard plot of the binding of ^{14}C-acetyl HL with guinea pig purified anti-P_{17} antibody experimental values bent sharply near $r=1$. This suggests the presence of two antibody populations with different affinities for HL or possible steric hindrance in the binding of a second HL molecule to the second binding site of the antibody molecule.

The antigenic activities of various peptides were tested by measuring their inhibition of the binding of ^{14}C-acetyl-P_{17} with the anti-P_{17} antibody. Only p_{17} and P_{17t} (sequence Lys[1]–Cys[6]–Homoser[12], Trp[123]–Cys[127]–Leu[129]) were inhibitory, with K_I values of 2.0×10^4 and 8.1×10^3, respectively. These results indicate that the direct binding site of P_{17} to anti-P_{17} antibody may be located in the terminal portion of P_{17} (sequence Lys[1]–Cys[6]–Homoser[12], Trp[123]–Cys[127]–Leu[129]) while the rest of P_{17} may be important in maintaining the conformation of this determinant.

The single disulphide bond involved in this determinant is essential for manifestation of immunological activity.

INTRODUCTION

Previously, we reported (Fujio et al., 1968), that the N- and C-terminal portion (Sequence Lys[1]–Cys[6]-Asn[27], Trp[123]–Cys[127]–Leu[129]) of HL was an important antigenic determinant of HL with respect to its specific interaction with circulating antibody against native HL. However, the molecular weight of the N- and C-terminal peptide of HL (P_{17}) is 4,000. Thus, if the size of antigenic determinant is in the order of 6 or 7 amino acid residues (Kabat, 1968) P_{17} could consist of two or more antigenic determinants.

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On the other hand, Miyagawa et al. (1973) reported that P_{17} is also an important determinant for the delayed hypersensitivity of HL in guinea pig. Thus it seemed necessary to study the exact localation of the immunological specificity of this determinant directed to circulating antibody within the N- and C-terminal region of HL in comparison with the specificity of delayed hypersensitivity.

In this work 2 peptides were prepared by splitting the methionyl bond of P_{17}, which is located in approximately the middle of this determinant. An overlapping peptide, composed of the middle portion of the N-terminal peptide of P_{17}, was also prepared directly from a peptic digest of HL. Finally, P_{17} was split into an N-terminal peptide and C-terminal peptide, by reduction and alkylation of the single disulphide bond. The binding properties of these peptides with purified guinea pig anti-P_{17} antibody, were examined to obtain detailed information on the immunological specificity of N- and C-terminal antigenic determinant of HL.

MATERIALS AND METHODS

1. *Hen egg-white lysozyme*

Six times recrystallized HL was purchased from Seikagaku Fine Biochem. Co. Ltd. It was used without further purification for preparing the N- and C-terminal peptide (P_{17}) of HL and derivative peptides. For preparation of immunizing antigen, crystalline HL was further purified by passage through SE-Sephadex C–25 in 0.2 M sodium phosphate buffer, pH 7.16 and then through QAE-Sephadex C–25 in 0.005 M phosphate buffer, pH 8.0 to remove possible contaminating acidic proteins of egg-white.

2. *Desalting of peptides*

Peptides were desalted by the method of Dixon (1959) as described previously (Sakato et al., 1972).

3. *Preparation of peptide 17 (P_{17})*

Twice crystallized pepsin was purchased from Worthington Biochem. Corp. (U.S.A.) and used without further purification.

The previous method for preparing P_{17} (Fujio et al., 1968) was modified as follows. Digestion with pepsin was carried out as described before except that the ratio of pepsin to HL was 1 : 1,000. The digestion was stopped by adjusting the mixture to pH 7.0 with 4 N NaOH.

Five g of lyophilized peptic digest were dissolved into 1 liter of deionized water and adjusted to pH 4.0 with glacial acetic acid. The solution was applied to a CM–23 (Whatman) column (3×40 cm) at 25 C and P_{17} was eluted with a linear gradient formed using 3 liters each of 0.15 M NaCl, 0.05 M sodium acetate buffer, pH 3.88 and 0.12 M sodium phosphate buffer, pH 8.0. For further purification 500 mg of crude P_{17} were applied to a CM–23 column (3×40 cm) at 25 C and eluted with a linear gradient formed using 3 liters each of 0.12 M sodium phosphate buffer, pH 6.0 and 0.18 M sodium phosphate buffer, pH 6.0. All chromatographic procedures were carried out at 25 C and 0.05% chloreton was used as a preservative. The elution pattern obtained on rechromatography is shown in Fig. 1.

The main fractions of eluate were pooled and desalted. For further purification 400 mg of P_{17} in 20 ml of 1.0 N acetic acid were applied to a Bio-Gel P–10 (100–200 mesh) column (5.5×90 cm) equilibrated with the same solvent and fractions of 20 g were collected. Usually a single peak was ob-

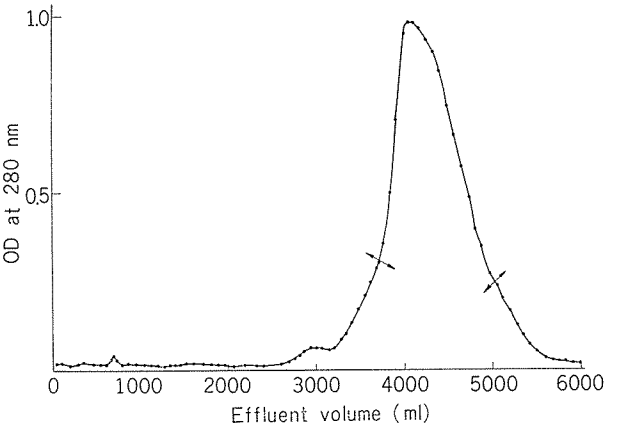

FIGURE 1. *Rechromatography of P_{17} on a CM–23 column (3× 40 cm) at 25 C. 500 mg. of crude P_{17} were applied and the peptide was eluted with a linear gradient formed using 3 liters volumes of 0.12 M sodium phosphate buffer, pH 6.0 and of 0.18 M sodium phosphate buffer, pH 6.0. Arrows indicate the fractions collected.*

tained. This gel filtration procedure was repeated to minimize contamination with intact HL. To test the contamination of P_{17} with intact HL, P_{17} was subjected to a third gel filtration on a small column (2×140 cm) and the fraction corresponding to intact HL was concentrated. The lytic activity of the concentrated fraction was measured as described previously (Shinka et al., 1967). The results indicated that after the second gel filtration P_{17} preparations always contained less than 0.01% of intact HL.

4. *Preparation of peptide 17 m (P_{17m})*

The material in the peptic digest of HL eluted from the CM-23 column with 0.15 M NaCl, 0.05 M sodium acetate buffer, pH 3.88 was used as starting material for preparation of P_{17m}. The eluate was diluted 2-fold with deionized water and adjusted to pH 4.0 with glacial acetic acid. The solution was applied to a SE-Sephadex C-25 (1×35 cm) equilibrated with 0.05 M sodium acetate buffer, pH 3.88. All chromatographic and gel filtration procedures were carried out at 25 C with 0.05% chloretone added to the buffers as preservative. A linear gradient, formed using 1 liter of 0.14 M sodium acetate buffer, pH 5.5, was used. Material eluted in a volume of 1,200 ml to 1,620 ml was collected. This fraction was rechromatographed on a SE-Sephadex C-25 column (1×35 cm) using the same gradient as in the first chromatography. Fig. 2 shows the elution pattern of the peptide on rechromatography.

The peptide was desalted and purified further by gel fiftration on a Bio-Gel P-10 (100-200 mesh) column (3×155 cm) in 25% (v/v) acetic acid. The gel filtration pattern is shown in Fig. 3. The second peak was collected and acetic acid was removed by repeated evaporation in a rotatory evaporator under reduced pressure. The peptide was finally lyophilized. Results of amino acid analysis of this peptide are shown in Table 1.

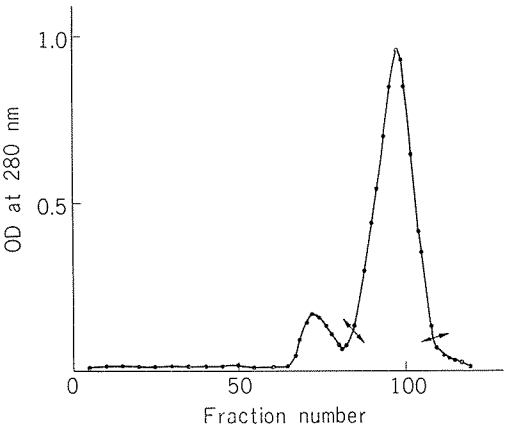

FIGURE 3. *Purification of P_{17m} by gel filtration through a Bio-Gel P-10 column (3×155 cm) in 25% v/v) acetic acid at 25 C. 130 mg of P_{17m} obtained by rechromatography on SE-Sephadex C-25 were applied. Fractions of 8 g were collected. Arrows indicate the fractions collected.*

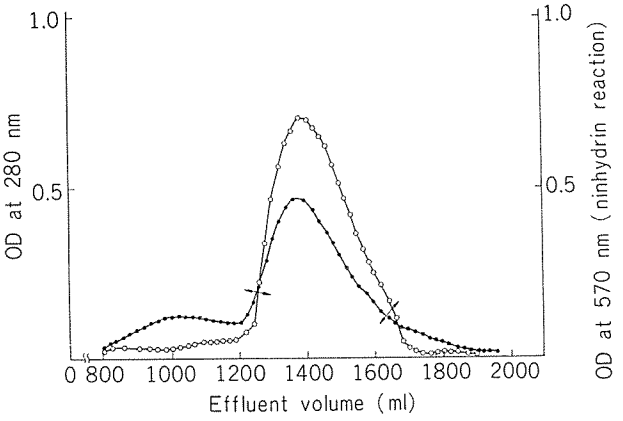

FIGURE 2. *Rechromatography of P_{17m} on a SE-Sephadex C-25 column (1×35 cm) at 25 C. 300 mg of crude P_{17m} were applied and the peptide was eluted with a linear gradient formed using 1 liter volumes of 0.14 M sodium acetate buffer, pH 5.5 and 0.16 M sodium acetate buffer, pH 5.5. Arrows indicate the fractions collected.* ●——●, *OD at 280 nm;* ○——○, *OD at 570 nm (ninhydrin reaction).*

The results indicate that this peptide is composed of the Ala^{11}-Gly^{22} portion of HL and corresponds to Fr. 9-10-b in the previous report (Shinka et al., 1967). Since this peptide is located in the middle of P_{17}, it is designated as P_{17m} in this paper.

5. *Cyanogen bromide cleavage of P_{17}*

P_{17} was treated with cyanogen bromide by the method of Gross and Witkop (1962) as modified by Seers, Craven and Anfinsen (1965). A sample of 100 mg of P_{17} in 50 ml of 70% formic acid was mixed with 500

TABLE 1. *Amino acid composition of* P_{17m}

Amino acid[a]	For residues Ala^{11}–Gly^{22}	
	found[b]	calculated[c]
Lys	1.07	1
His	1.06	1
Arg	2.06	2
Asp	2.17	2
Gly	2.00	2
Ala	1.07	1
Met	0.86	1
Leu	1.06	1
Tyr	1.18	1

[a] Only amino acid residues found at concentrations of more than 0.03 mole per mole of peptide are listed.

[b] The peptide was hydrolyzed for 24 hr in constant boiling HCl (5.7 N). Values are expressed as molar ratios, assuming that there are two glycine residues per mole of peptide.

[c] Moles of amino acids in the sequence Ala^{11}–Gly^{22} of HL were calculated from the data of Canfield and Liu (1965).

mg of cyanogen bromide. The reaction mixture was stirred with a magnetic stirrer at 40 C for 20 hr. Then it was concentrated in a rotatory evaporator under reduced pressure and subjected to gel filtration on a Bio-Gel P-10 column (3×145 cm) in 25% (v/v) acetic acid to remove cyanogen bromide.

6. *Reduction and alkylation of* P_{17}

Dithiothereitol was added to at final concentration of 0.01 M to a solution of 50 mg of P_{17} in 5 ml of 0.4 M Tris-HCl buffer, pH 8.5 containing 0.004 M EDTA and the reaction mixture was stirred at 25 C for 1 hr. Then recrystallized iodoacetic acid in NaOH was added to a final concentration of 0.022 M and the mixture was kept at pH 8.3 by addition of Tris for 1 hr at 25 C. Then further dithiothreitol was added to give a slight excess over iodoacetic acid. The reaction mixture was subjected to gel filtration in 25% (v/v) acetic acid to separate the peptides.

7. *Amino acid analysis*

The amino acid compositions of the peptides were determined by the method of Spackman et al. (1958) using a Yanagimoto amino acid analyzer, Model LC-5. Peptides were hydrolyzed by treatment with constant boiling 5.7 N HCl at 105±1 C for 24 hr. Values for the contents of half cystine obtained on analysis of acid hydrolyzates of the peptides were not corrected. Performic acid oxidation was not performed.

8. *Antisera*

Non-inbred guinea pigs, weighing 250 to 300 g, were obtained from a single commercial farm. A dose of 500 μg of highly purified HL in 0.4 ml of complete Freund's adjuvant was injected into all four foot pads of the guinea pigs. A booster injection of 500 μg of HL in incomplete Freund's was given 5 weeks later. Blood was taken by cardiac puncture 9 days after the last injection. Sera from 20 guinea pigs were pooled.

9. *Quantitative precipitin reaction*

Volumes of 100 μliters of guinea pig anti-HL antiserum were mixed with increasing amounts of purified HL. The quantitative precipitin reaction was carried out in the presence of 0.01 M EDTA, as described previously (Fujio et al., 1971). The antibody content of pooled guinea pig anti-HL antiserum was estimated as 6.00 mg per ml.

10. *Immunoadsorbents*

Sepharose activated with cyanogen bromide was used following the method of Axen, Porath and Ernback (1967).

Six ml of Sepharose 4B (Pharmacia, Sweden) were washed thoroughly with deionized water and suspended in 6 ml of cyanogen bromide solution (50 mg/ml). The suspension was adjusted to pH 11.0±0.5 at 20 C for 8 min and then thoroughly washed first with chilled deionized water and then with 60 ml of chilled 0.1 M sodium phosphate buffer, pH 6.5. The activated Sepharose 4B cake was suspended in 10 ml of peptide solution (10 mg/ml) and the suspension was gently stirred at 4 C for 22 hr. The immunoadsorbent was then washed once with 20 ml of 0.1 M sodium bicarbonate and 3 times with 20 ml volumes of 1.0 N acetic acid and finally treated with 0.05 M monoethanolamine at pH 8.0. By this method, approximately 40 mg of P_{17} were coupled to 6 ml of Sepharose and 12 mg of P_{17t} were coupled to 3 ml of Sepharose.

11. *Labelling of HL and* P_{17}

One-^{14}C acetic anhydride (12.9 mCi/mmole) was

purchased from Daiichi Pure Chemicals Co. Ltd.

After purification by chromatography, HL (2.1 μmole) was dissolved in 3 ml of dimethylsulfoxide and 60 μliters of 1-^{14}C-acetic anhydride (3.1 μmole) were added. The mixture was stood at room temperature for 2 hr with occasional shaking (once every 15 min). Then it was subjected to gel filtration on a Sephadex G-25 fine (Pharmacia, Sweden) column (3×77 cm) equilibrated with 0.15 NaCl, 0.02 M sodium phosphate, pH 6.0 (PBS). The protein fractions of the eluate were pooled, concentrated by lyophilization and extensively dialyzed against PBS. The specific activity of the preparation was 7,248 cpm/nmole and it contained approximately 0.64 acetyl groups per mole of HL.

Highly purified P_{17} (7.5 μmole) in 3 ml of dimethylsulfoxide was mixed with 220 μliters of 1-^{14}C-acetic anhydride (11.4 μmole) for 2 hr. The reaction mixture was then subjected to gel filtration on Sephadex G-15 (3×140 cm) which had been saturated with 10% (v/v) acetic acid. The peptide fractions were collected and concentrated in a rotatory evaporator under reduced pressure. Acetic acid was removed by repeated evaporation under nitrogen gas. Finally the ^{14}C-acetyl-P_{17} was lyophilized and dissolved in PBS. The specific activity of the labelled peptide was 9,680 cpm/nmole and it contained 0.86 acetyl groups per mole of P_{17}.

12. *Preparation of P_{17} specific antibody fraction from guinea pig anti-HL antiserum*

A sample of 120 ml of pooled guinea pig anti-HL antiserum was mixed with 12 ml of 0.1 M EDTA, cooled in an ice bath and applied to the P_{17}-Sepharose (40 mg of P_{17} coupled) column (2×2 cm) as described in section 9 of the Materials and Methods. The immunoadsorbent was kept at 4 C and the antiserum was applied at a rate of 12 ml per hr. Then the column was washed with 120 ml of 0.135 M NaCl, 0.01 M trisodium EDTA, pH 7.5 (SEDTA). The unbound fraction of antiserum (120 ml) and the washing fluid (120 ml) were pooled and concentrated to the original volume (120 ml) by pressure dialysis. This fraction was again subjected to adsorption on the immunoadsorbent, as described in the latter part of this section. The immunoadsorbent was then washed with SEDTA (approximately 300 ml) until the OD of the washing fluid at 280 nm was less than 0.01. The P_{17} specific antibody (anti-P_{17} antibody) was recovered by elution with 2 ml of 3×10^{-3} M HL in SEDTA at 4 C and then with 48 ml of SEDTA. These elution procedures were repeated 3 times and all the eluates were pooled. The eluates were concentrated by pressure dialysis to approximately 5 ml and applied to a Sephadex G-150 column (3×90 cm) equilibrated with 0.1 N acetic acid. The column was kept at 25 C and fractions of 5 g were collected. The gel filtration pattern is shown in Fig. 4.

The first fraction seems to be so called 19 S antibody, but this fraction only amounted to about 1.1% of the total anti-P_{17} antibody, and it was not studied further. The second fraction was pooled as indicated by the arrows in the Figure and immediately neutralized with NaOH. The antibody protein was concentrated to 5 mg per ml and extensively dialyzed against PBS. This antibody fraction was found to be so-called 7 S globulin by gel filtration on Sephadex G-200 in neutral buffer. The unbound fraction of anti-HL antiserum was applied to the same immunoadsorbent and treated in exactly the same way as in the first adsorption and elution of antibody. The second fraction of 7 S antibody eluted was combined with the first fraction and used as anti-P_{17} antibody in the following experiments. Approximately 80 mg of anti-P_{17} antibody were obtained from 120 ml of guinea pig anti-HL antiserum. This represents 11.1% of the total precipitable anti-

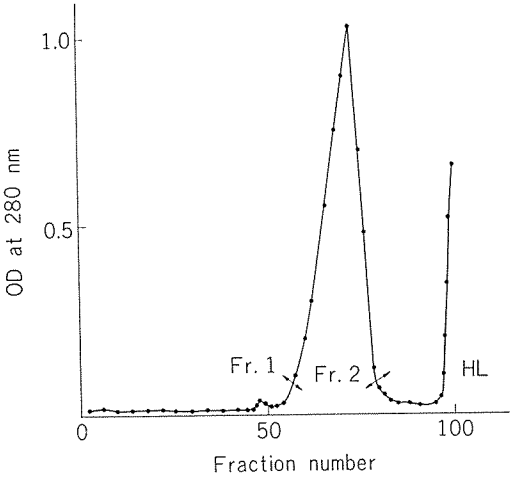

FIGURE 4. *Separation of guinea pig 7 S anti-P_{17} antibody on a Sephadex G-150 column (3×90 cm) in 0.1 N acetic acid at 25 C. 5 ml of HL-antibody (60 mg) mixture were applied and fractions of 5 g were collected. Arrows indicate the fractions pooled.*

HL antibody in the serum. To test for the presence of antibody which reacted with P_{17} but not with HL, after elution 3 times with HL the immunoadsorbent were treated with 2 ml of 1.25×10^{-2} M of P_{17} solution and the eluated was tested by gel filtration on Sephadex G–150 in 0.1 N acetic acid, as described above. However, no antibody protein was detected.

13. *Preparation of normal guinea pig 7 S–γ–globulin (NGG)*

Fifty ml of normal guinea pig serum were dialyzed against 5 liters of 0.05 M sodium phosphate buffer, pH 7.5 at 4 C for 24 hr. The dialyzed serum was applied to a DEAE-cellulose column (6×10 cm) at 4 C and eluted with the same buffer. The fractions in the first peak were pooled and concentrated by pressure dialysis and 5 ml of the concentrated solution (10 mg/ml) were applied to a Sephadex G–150 column (3×90 cm). The 7 S-fractions were pooled and concentrated by pressure dialysis. The solution was extensively dialyzed against PBS, pH 6.0 and used as NGG.

14. *Equilibrium dialysis*

Experiments on the bindings of ^{14}C-acetyl HL and ^{14}C-acetyl P_{17} with purified guinea pig anti-P_{17} antibody were carried out by equilibrium dialysis at 10 C for 60 hr as described previously (Fujio et al., 1971).

The immunological activities of various peptides with structures related to that of P_{17}, were tested by measuring their abilities to inhibit the binding of ^{14}C-acetyl-P_{17} to purified anti-P_{17} antibody by equilibrium dialysis.

15. *Miscellaneous procedures*

After chromatography, peptides were located by their OD at 280 nm and by the ninhydrin reaction. For the latter, 0.3 ml of each fraction was mixed with one ml of ninhydrin reagent (Moore et al., 1954) and boiled for 20 min. After cooling, 4 ml of 50% ethanol were added and the optical density was measured at 570 nm.

The concentrations of peptide, HL and purified antibody were estimated by measuring the OD at 280 nm, using a Zeiss spectrophotometer, Model M4QIII. The extinction coefficient of each protein or peptide was determined, based on its nitrogen content measured by the Kjeldahl-Nessler method (Yokoi and Akashi, 1955).

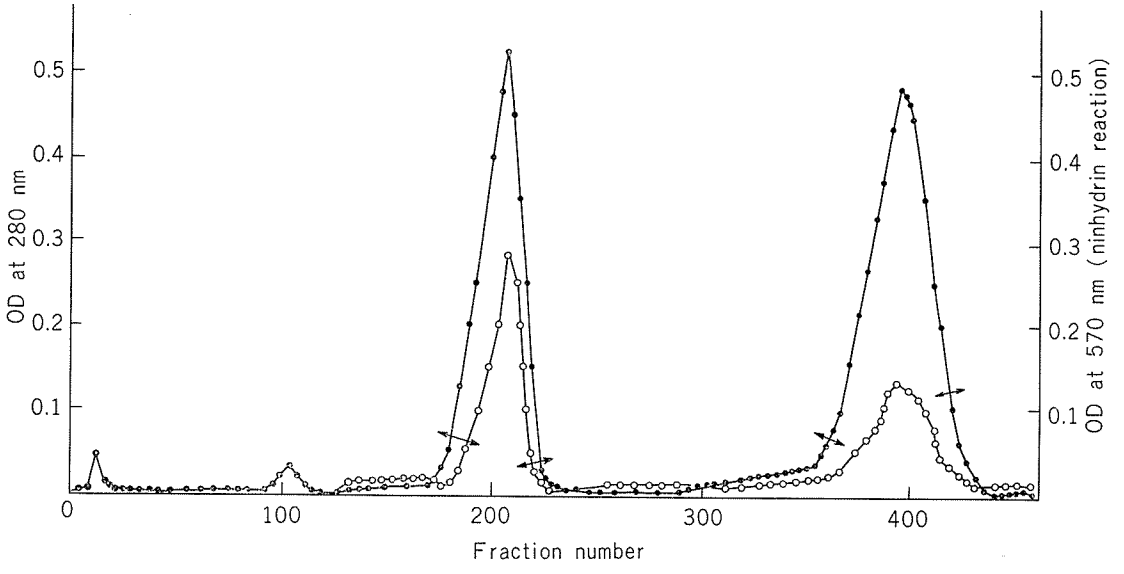

FIGURE 5. *Chromatography of P_{17} after cyanogen bromide treatment on a SE-Sephadex C–25 column (1 × 35 cm). A sample of 98 mg was applied. The column was eluted with a linear gradient formed using 1 liter volumes of 0.2 M sodium acetate buffer, pH 5.5 and 0.3 M sodium acetate buffer, pH 6.0. Fractions of 3.8 ml were collected. Arrows indicate the fractions pooled.* ●——●, *OD at 280 nm;* ○——○, *OD at 570 nm (ninhydrin reaction).*

RESULTS

1. *Cleavage of P_{17} with cyanogen bromide*

P_{17} was treated with cyanogen bromide as described in the Materials and Methods. Then after removal of acetic acid in a rotatory evaporator a sample of 98 mg was applied to a SE-Sephadex C–25 column (1×35 cm). The peptides were separated by elution with a linear gradient formed using 1 litter volumes of 0.2M sodium acetate buffer, pH 5.5 and of 0.3 M sodium acetate buffer, pH 6.0 at 25 C. Fractions of 4 g were collected. The optical density of each fraction, measured at 280 nm, and its ninhydrin reaction are shown in Fig. 5.

Two main peaks were observed. The first gave a strong ninhydrin reaction relative to its optical density at 280 nm, while the second major fraction gave a relatively weak ninhydrin reaction. The two fractions were rechromatographed under the same conditions as in the first chromatography, and then desalted and lyophilized.

The results of amino acid analyses of the two fractions are shown in Table 2.

The results of amino acid analyses show that

TABLE 2. *Amino acid compositions of peptides which are structurally related to P_{17}*

Amino acid[a]	P_{17i} Lys[13]–Asn[27]		P_{17t} Lys[1]–Cys[6]–Homoser[12] | Trp[123]–Cys[127]–Leu[129]		P_{17N} Lys[1]–Asn[27]		P_{17C} Trp[123]–Leu[129]	
	found[a]	calculated[b]	found[a]	calculated[b]	found[a]	calculated[b]	found[a]	calculated[b]
Lys	1.07	1	0.92	1	1.94	2	0.04	0
His	1.08	1			1.04	1		
Arg	2.25	2	2.85	3	3.18	3	1.82	2
Asp	3.13	3			3.05	3	0.05	0
Thr								
Ser	0.75	1			0.65	1	0.08	0
Glu			1.09	1	1.24	1		
Pro								
Gly	3.00	3	2.00	2	4.00	4	1.00	1
Ala			3.02	3	3.34	3	0.08	0
½Cys			1.30	2				
Val			0.95	1	1.07	1		
Met					0.85	1		
Ile			0.84	1			0.89	1
Leu	1.97	2	1.95	2	2.86	3	0.97	1
Tyr	1.72	2			1.69	2		
Phe			0.92	1	1.06	1		
Homoser			0.89	1				
Trp			(0.9)[c]	1			(0.9)[c]	1
CM-Cys					1.08	1	0.92	1

[a] One mg of each peptide was hydrolyzed in 1 ml of constant boiling HCl (5.7 N) for 24 hr. Values are expressed as molar ratios, taking glycine as a standard. Where no numbers are given, the values obtained were less than 0.03 moles per mole of peptide.

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

[c] Trp contents were estimated by the method of Goodwin and Morton (1946).

the first main fraction (P_{17i}) is derived from the internal portion of P_{17} while the second (P_{17t}) is derived from the terminal portion of P_{17}. The minimal molecular weight of the former was calculated as 1,760 and that of the latter as 2,147. The recoveries of P_{17i} and P_{17t} were 82.5% and 52.3% of the theoretical amounts calculated assuming that all the methionyl bonds in P_{17} were split and that the recoveries of the two peptides (P_{17i} and P_{17t}) were both 100%. The relatively low recovery of P_{17t} may be due to aggregation of this peptide and also to heterogeneity in the C-terminal peptide of P_{17}, as described in the next section.

2. *Reduction and alkylation of P_{17}*

P_{17} was reduced and alkylated as described in the Materials and Methods. The two resultant peptides were separated by gel filtration on serial columns of Sephadex G–15 (3× 140 cm) and Bio–Gel P–10 (3×150 cm) in 25% (v/v) acetic acid at 25 C. Fractions of 8 g were collected. The elution pattern is depicted in Fig. 6.

Three peptides fractions were obtained. Fraction 1 had a relatively high ninhydrin value in comparison with its optical density at 280 nm while fraction 3 had a relatively low ninhydrin value in comparison with its optical density at 280 nm. After removal of acetic acid fraction 1 was applied to a CM–23 column (1×35 cm) and eluted with 0.15 M sodium acetate buffer, pH 5.5. Under these conditions intact P_{17} binds to the CM-cellulose column. The results of amino acid analyses of fractions 1 and 3 are listed in Table 2. The results indicate that fraction 1 (P_{17N}) is derived from the N-terminal peptide chain of P_{17} and fraction (P_{17C}) from the C-terminal peptide chain. On amino acid analysis of P_{17N} using 0.3 μmole of peptide no isoleucine could be detected, so contamination of P_{17N} with intact P_{17} or P_{17C} must be less than 1%.

Although in theory the recoveries of fraction 1 (P_{17N}) and fraction 3 (P_{17C}) are 75% and 25% respectively, in practice the recoveries of fractions 1, 2 and 3 were 63%, 5% and 23% respectively. Amino acid analysis of fraction 2 indicated that the peptide is derived from an impurity in the P_{17} preparation. Fraction 3

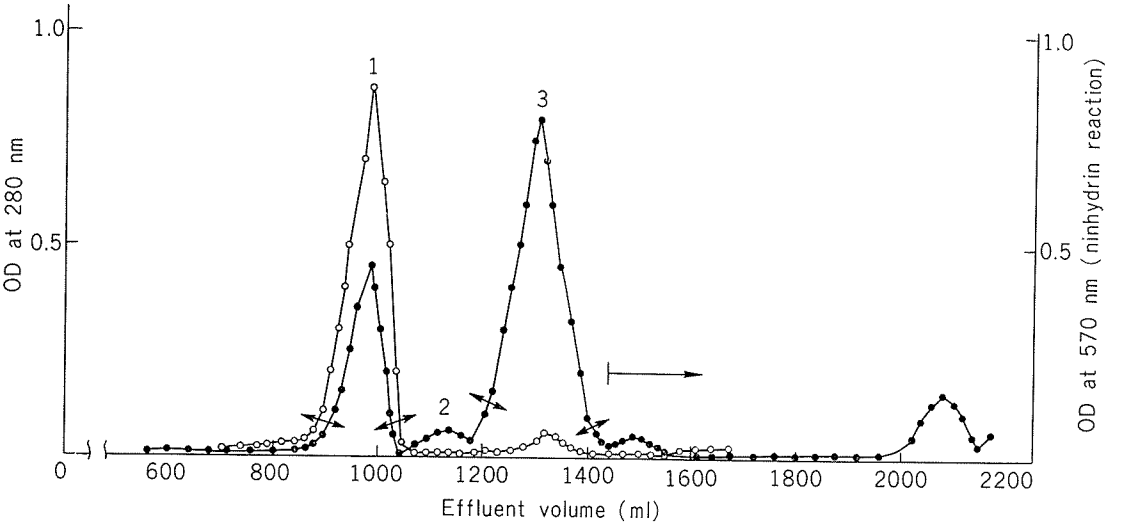

FIGURE 6. *Gel filtration of reduced and alkylated P_{17} on serial columns of Sephadex G–15 (3×140 cm) and Bio–Gel P–10 (3×150 cm) in 25% (v/v) acetic acid at 25 C. 50 mg of reduced and alkylated P_{17} were applied and fractions of 8 g were collected. Numbers on top of peaks indicate fraction numbers and arrows indicate the fractions pooled. The horizontal arrow shows the elution position of the reagents.*

(P_{17C}) also contained about 10% impurity. Since the recovery of total peptides from reduced and alkylated P_{17} in the gel filtration step was 90%, the maximum amount of impurity in the P_{17} preparation must be around 7%.

3. *Immunological activities of various peptide fractions of* P_{17}

The binding of purified guinea pig anti-P_{17} antibody with ^{14}C-acetyl–HL was examined by equilibrium dialysis at 10 C.

Volumes of 50 μliters of various concentrations (6×10^{-6} M to 1.2×10^{-4} M) of ^{14}C-acetyl-HL were dialyzed against 50 μl of purified guinea pig anti-P_{17} antibody (7.0×10^{-6} M) at 10 C for 60 hr. As controls, 50 μliters volumes of various concentrations of ^{14}C-acetyl-HL were dialyzed against the same volume of NGG (7.0×10^{-6} M) under the same conditions. The results are shown as a Scatchard plot of r/c against r, in Fig. 7, where r represents the moles of antigen bound per mole of antibody and c the concentration of free antigen. A very peculiar character of this binding curve is that the experimental points on the Scatchard plot bend sharply at approximately $r=1$. There are two possible explanations for this phenomenon. The first is that when one binding site of guinea pig anti-P_{17} antibody is occupied by HL, the binding of HL to the second binding site is disturbed. The second possibility is that there are two populations of antibody with different affinities for HL, the K_A of one population being 8.9×10^6 (L/M) and that of the other, 4.0×10^5 (L/M). No appreciable binding of ^{14}C-acetyl-HL with

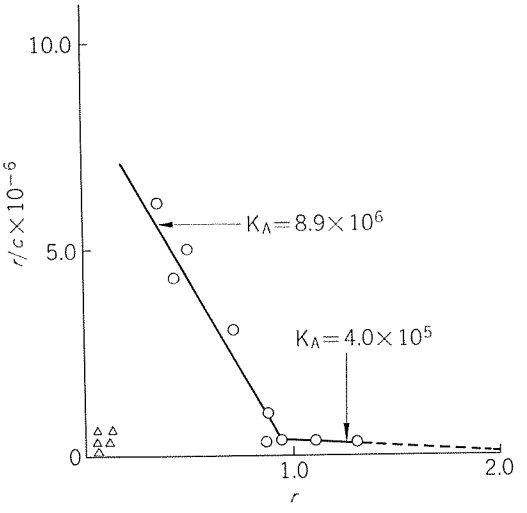

FIGURE 7. *Binding of purified guinea pig anti-P_{17} antibody with ^{14}C-acetyl-HL at 10 C. Values are shown as a Scatchard plot, according to the equation, $r/c=K_An-K_Ar$ where r represents moles of antibody, taking the molecular weight of antibody as 150,000 and that of HL as 14,300; c is concentration of free ^{14}C-acetyl-HL (M); K_A is the association constant for the interaction and n is the maximum number of HL molecules that can be bound per antibody molecule. The dotted line is drawn to intersect the abscissa at $r=2$. ○, binding of ^{14}C-acetyl-HL with guinea pig anti-P_{17} antibody; △, binding of ^{14}C-acetyl-HL with normal guinea pig 7 S-γ-globulin (NGG).*

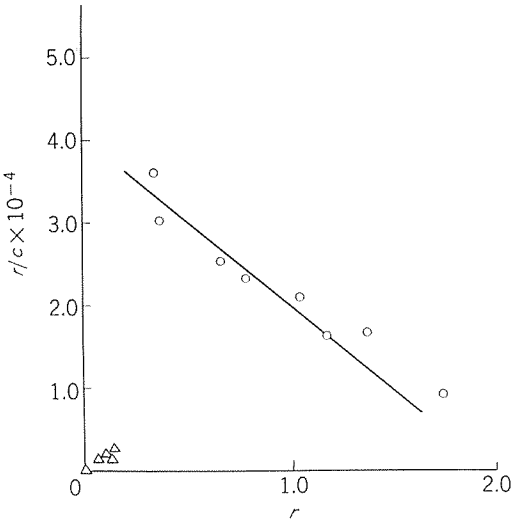

FIGURE 8. *Binding of purified guinea pig anti-P_{17} antibody with ^{14}C-acetyl-P_{17} at 10 C. Values are shown as a Scatchard plot, according to the equation, $r/c=K_An-K_Ar$, where r represents moles of ^{14}C-acetyl-P_{17} per mole of antibody, taking the molecular weight of antibody as 150,000 and that of P_{17} as 3,928; c is the concentration of free ^{14}C-acetyl-P_{17}, K_A is the association constant for the interaction and n is the maximum number of P_{17} molecules that can be bound per antibody molecule. ○, binding of ^{14}C-acetyl-P_{17} with guinea pig anti-P_{17} antibody; △, binding of ^{14}C-acetyl-P_{17} with NGG.*

NGG was observed in the control experiment, as can be seen in Fig. 7.

Next the binding of guinea pig anti-P_{17} antibody with ^{14}C-acetyl P_{17} was examined. Various concentrations (5.87×10^{-5} M to 4.7×10^{-4} M) of ^{14}C-acetyl-P_{17} in PBS were dialyzed against an equal volume of guinea pig anti-P_{17} antibody (2.38×10^{-5} M) or against the same volume of NGG (2.4×10^{-5} M) at 10 C for 60 hr. The results are shown in Fig. 8.

The experimental points on the Scatchard plot show an essentially linear relationship with no bend as in the case of binding with ^{14}C-acetyl-HL. The association constant, K_A, of the binding of ^{14}C-acetyl-P_{17} with the guinea pig anti-P_{17} antibody was calculated as 2.0×10^4 (L/M). The extrapolated value of r is approximately two in this case. These results strongly suggest that the bending of the Scatchard plot for the binding of this antibody with HL is due to steric hindrance, because when the molecular size of the antigen was less, the bending disappeared. However, it is still possible that there are two populations of antibody with similar binding affinities for P_{17} but different affinities for HL.

TABLE 3. *Peptides tested as inhibitors of the binding of ^{14}C-acetyl-P_{17} to guinea pig anti-P_{17} antibody*

Peptide	Sequence	Molecular weight[a]
P_{17}	Lys^1–Cys^6–Asn^{27} | Trp^{123}–Cys^{127}–Leu^{129}	3,928
P_{17i}	Lys^{13}–Asn^{27}	1,760
P_{17t}	Lys^1–Cys^6–$Homoser^{12}$ | Trp^{123}–Cys^{127}–Leu^{129}	2,147
P_{17N}	Lys^1–Asn^{27}	3,086
P_{17C}	Trp^{123}–Leu^{129}	959
P_{17m}	Ala^{11}–Gly^{22}	1,418

[a] The minimum molecular weight of each peptide was calculated from the results of amino acid analyses.

TABLE 4. *Inhibition of binding of ^{14}C-acetyl-P_{17} to guinea pig anti-P_{17} antibody[a] by P_{17} and related peptides*

Inhibitor	Concentration of inhibitor (μM)	Molar ratio of Inhibitor to ^{14}C-acetyl-P_{17}[b]	K_I[c] (L/M)	Mean K_I (L/M)
P_{17}	38.8	0.66	2.2×10^4	2.0×10^4
	77.5	1.32	2.5×10^4	
	155.0	2.64	1.4×10^4	
P_{17t}	171	2.90	9.0×10^3	8.1×10^3
	341	5.83	7.3×10^3	
P_{17i}	2,950	50.3		$<2.0\times10^2$
P_{17N}	2,900	49.4		$<2.0\times10^2$
P_{17C}	3,050	52.0		$<2.0\times10^2$
P_{17m}	2,920	49.7		$<2.0\times10^2$

[a] Antibody concentration, 3.5 mg per ml.

[b] Concentration of ^{14}C-acetyl-P_{17}, 58.7 μmole.

[c] Association constant of inhibitor, $K_I=\frac{(r/r'-1)(1+K_A\cdot c)}{(i)}$; K_A value for the binding of anti-P_{17} antibody with ^{14}C-actyl-P_{17} was 2.1×10^4 (L/M); r represents of antigen bound per mole of antibody in the absence of inhibitor; r' represents moles of antigen bound per mole of antibody in the presence of inhibitor; i is the equilibrium concentration of inhibitor. Dialysis was performed at 10 C for 60 hr.

A maximal binding of 30% P_{17} was observed in this system, so it is unlikely that the observed binding of ^{14}C-acetyl-P_{17} by anti-P_{17} antibody is solely due to impurities in the P_{17} preparation. Again no appreciable binding of ^{14}C-acetyl-P_{17} with NGG was observed in a control experiment.

Next the specificity of guinea pig anti-P_{17} antibody was examined. Various concentrations (3.9×10^{-5} M to 3.05×10^{-3} M) of various peptides, which are components of P_{17}, were mixed with 5.87×10^{-5} M of ^{14}C-acetyl-P_{17}. Then mixtures (50 μliters) were dialyzed against 50 μliters of guinea pig anti-P_{17} antibody (2.38×10^{-5} M) at 10 C for 60 hr. The various peptides used in this experiment are listed in Table 3.

The K_I values of the peptides were calculated by the method of Karush (1956) and are listed in Table 4. The first series of experiments show the inhibition of the binding of labelled P_{17} by various concentrations of cold P_{17}. The mean K_I of P_{17} was 2.0×10^4, which was consistent with the K_A of ^{14}C-acetyl-P_{17} with anti-P_{17} antibody calculated from values in a direct binding experiment. Among the peptides tested, only P_{17t} had inhibitory activity. The K_I of P_{17t} was between half and one third that of P_{17}. Therefore these results strongly suggest that the site of binding of the P_{17} antigenic determinant to antibody is located in the terminal portion of P_{17}, and that the rest of P_{17} may play some role in preserving the conformation of this determinant.

The disulphide bonds of P_{17} also seems to be essential for the antigenic activity of this determinant.

4. *Extent of reactivity of anti-P_{17} antibody with P_{17t}-immunoadsorbent*

Immunoadsorbent seems to be very useful for testing the specificity of an antibody, especially when the affinity of the antibody is low. As shown above the antibody to P_{17} reacts with P_{17t}, but it was uncertain whether all or only part of the anti-P_{17} antibody reacted with P_{17t}. Accordingly P_{17t}-immunoadsorbent was prepared to examine this.

A sample of 12 mg of P_{17t} was coupled to Sepharose 4B as described in the Materials and Methods. One ml of purified anti-P_{17} antibody (4.8 mg) was applied to the top of the P_{17t}-Sepharose column (1×4.5 cm). The column was operated in a cold room and washed with SEDTA. The amount of unbound antibody protein, estimated by measurement of the OD at 280 nm, was 0.23 mg. This corresponds to only 4.8% of the antibody applied. When the column was eluted with 0.1 M citrate buffer, pH 2.2, 4.2 mg of antibody protein was eluted, corresponding to 87.5% of the total antibody protein applied. As a control 1 ml of NGG (5 mg) was applied to the same immunoadsorbent and treated in the same way. More than 90% of the NGG was not adsorbed. From these results, it is concluded that the almost all the antibody molecules which react with P_{17} can also bind to P_{17t}.

DISCUSSION

P_{17} is an intermediate formed during peptic digestion of HL, so it is sometimes difficult to obtain it as a homogeneous preparation. Canfield and Liu (1965) also reported the heterogeneous splitting of peptide bonds in the C-terminal regions of HL, using pepsin to obtain peptides containing disulphide bonds from HL. We reported previously (Fujio et al., 1968) that the C-terminal peptide of P_{17} is composed of Ala^{122}–Leu^{129}, but the P_{17} preparation used in this work contained 3.3 moles of alanine per mole of peptide. The presence of 3 alanine residues in the N-terminal peptide of P_{17} indicates that it does not contain the alanine residue at position 122. The results of amino acid analyses of P_{17t} and P_{17c} also strongly support this assumption that most P_{17} molecules represent the C-terminal peptide Trp^{123}-Leu^{129}. Repeated experiments on peptic digestion of HL also confirmed these results.

The recovery of P_{17} in this work (8 to 10%) was better than in the previous study (3 to 5%) (Fujio et al., 1968). Increase in the ratio of pepsin to HL (>1: 1,000) or prolongation of the digestion time (more than 1 hr) resulted in a lower recovery of P_{17}.

The bending of the Scatchard plot at near $r=1$ was also observed by Sachs et al. (1972) in the reaction of a peptide (99–126) of staphylococcal nuclease with the corresponding antibody in goat antistaphylococcal nuclease antibodies. They reported that when they prepared the univalent fragment of this antibody, experimental points in the Scatchard plot for the binding of the staphylococcal nuclease with the corresponding univalent antibody still showed a sharp bend at half saturation of antibody binding sites. Therefore they concluded unambiguously that there are equal amounts of two antibody populations having different K_A values for staphylococcal nuclease. This is a somewhat striking result, because it must be only rarely that equal amounts of antibodies having different K_A values are formed in a goat.

We also obtained a similar result in a Scatchard plot of the binding of HL with guinea pig anti-P_{17} antibody. However, a plot of the binding of the same antibody preparation with peptide (P_{17}) showed no bend. These results may be interpreted by supposing that the binding of a first HL molecule to the antibody disturbs the further binding of a second HL molecule. However, another possibility is that there are two populations of antibody with similar affinities for P_{17} but different affinities for HL. Further experiments are necessary to clarify this point.

The K_A of the binding of P_{17} to anti-P_{17} antibody was 1/20 to 1/400 of that of the binding of HL to the same antibody preparation. This could be due to partial loss of the structure responsible for binding of the antigenic determinant or to the low effective concentration of peptide with the native conformation of the corresponding amino acid sequence in the native protein. The latter possibility was extensively discussed by Sachs et al. (1972b). They considered that there is an equilibrium between the random form of peptide (P_r) and the native form (P_n), so that the observed K' can be expressed as $K'=K_{conf}K_{assoc}$, where K_{conf} is an constant defined by the conformational equilibrium between [P_r] and [P_n] and K_{assoc} is the true association constant of the binding between P_n and its corresponding antibody. Imanishi (1967) measured the circular dichroism spectrum of P_{17}, in the ultraviolet region but could detect no α-helix in P_{17}, although the same amino acid sequence as P_{17} in native HL should contain approximately 30% of α-helix (Blake et al., 1965). On the other hand, Komatsu et al., (1975) reported that P_{17} is immunogenic in rabbits and that the resultant antibody completely lacks reactivity to native HL. In addition, this antibody preferably reacts with the region of the sequence Ala^{11}–Asn^{27} of P_{17} and the antibody fraction made against native HL reacts with the region of the sequence Lys^{1}–Cys^{6}–Ala^{10}, Trp^{123}–Cys^{127}–Leu^{129} in P_{17}. These findings support the possibility that anti-P_{17} antibody obtained from anti-native HL antiserum recognizes the native conformation in the P_{17} region of HL. The reactivity of P_{17} with the anti-native format antibody must be attained by regaining the native conformation of HL in P_{17}. The mechanism by which peptides acquire the native conformation is not known, but it could be by an "induced fit" mechanism, just as in the enzyme-substrate relationship (Koshland, 1958) or by a shift of equilibrium between P_r and P_n caused by antibody (Sachs et al., 1972b).

Recently, Matthyssens and Kanarek (1974) also reported the antigenicity of the same determinant as P_{17} in HL. They found that P_{17} did not inhibit the precipitin reaction between mononitrolysozyme and goat anti-HL antiserum, and so concluded that tyrosine residues 20 or 23 is determinant in P_{17}. However, in the present study, P_{17t} (sequence Lys^{1}–Cys^{6}–$Homoser^{12}$, Trp^{123}–Cys^{127}–Leu^{129})

reacted with anti-P_{17} antibody while P_{17i} (Lys[13]-Asn[27]), containing both Tyr[20] and Tyr[23] did not. We detected the direct binding of P_{17t} with anti-P_{17} antibody, so the major binding site of P_{17} to the anti-P_{17} antibody seems to be located in the regions of sequence Lys[1]–Cys[6]–Homoser[12], Trp[123]–Cys[127]–Leu[129]. We did not study the roles of Tyr[20] and Tyr[23] in P_{17}, but it seems quite possible that they may be important maintaining the conformation of this determinant or of the subsite for binding to the antibody.

When P_{17} was reduced and alkylated, the resultant two peptides, P_{17N} and P_{17C}, showed no reactivity with anti-P_{17} antibody. These results are consistent with those of Matthyssens and Kanarek (1974), and suggest that a disulphide bond is directly involved in the determinant of P_{17} or that it is necessary for maintaining the native conformation of the determinant in P_{17}. An experiment is now in progress on the reoxidation of an equimolar mixture of reduced peptide (Lys[1]–Cys[6]·H–Asn[27]) and reduced peptide (Trp[123]–Cys[127]·H–Leu[129]). In a preliminary experiment the products were separated by gel filtration on a similar column to that used to separate P_{17N} and P_{17C}. Three fractions were obtained. Amino acid analyses indicated that the first fraction was a dimer of the N-terminal peptide of P_{17}, the second was P_{17} and the third was a dimer of the C-terminal peptide of P_{17}. The recoveries of the three fractions were 30% (fraction 1), 60.5% (fraction 2) and 9.4% (fraction 3), respectively. If the chances of recombination of the two peptides on reoxidation are equal, the recoveries of the three reoxidants should also be equal and should be 33.3%. But the actual recovery of P_{17} was almost twice this value. Therefore, it seems likely that the C-terminal portion and N-terminal portion of P_{17} preferably interact with each other to form a stable conformation. This conformation may be necessary for the antigenicity of P_{17}.

On the other hand, Miyagawa et al. (1974) using cells in a peritoneal exudate from guinea pigs immunized with HL found that P_{17} and P_{17t} can also manifest migration inhibition. In addition, P_{17N} also showed as much migration inhibition activity as P_{17}, although it could not bind circulating antibody to HL.

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