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Further Studies on the Specificity of the N- and C-terminal Antigenic Determinant of Hen Egg-white Lysozyme

Youn-mun Ha

Microbiology, Medical School, Kyung Hee University

=國文抄錄=

鷄卵白 Lysozyme의 N-末端과 C-末端 抗原決定基에 대한 硏究

慶熙大學校醫科大學 微生物學敎室

夏 潤 文

鷄卵白 Lysozyme N-와 C末端 抗原決定基(P_{17}: sequence Lys^{1}-cys-^{6}-Asn^{27}, Trp^{12}_{3}-Cys^{127}-Leu^{129})의 特異性에 對하여 硏究했다. ^{14}C-acetyl Lysozyme과 精製한 guinea pig 抗—P_{17} 抗體와의 結合을 Scatchard plot上에서 나타낸 結果 그 實驗値가 겨의 $r=1$였다. 이것은 Lysozyme에 對하여 各各 다른 親和性을 가진 2個의 抗體群의 存在나 그렇잖으면 第1의 抗體結合部位에 最初의 Lysozyme分子가 結合함으로 因하여 抗體分子의 第2의 結合部位에 다른 Lysozyme 分子의 結合을 방해하는 즉 steric hindrance에 의한 可能性을 示唆한다. 여러가지 peptides의 抗原活性을 ^{14}C-acetyl-P_{17}과 抗—P_{17} 抗體와의 結合阻害시험에서 測定했다. 그 結果 단지 P_{17}과 $P_{17}t$(sequence Lys^{1}-cys^{6}-Homoser^{12}, Trp^{123}-cys^{127}-Leu^{128})만이 억제되었고 그 K_i値가 各各 2.0×10^{4}과 8.1×10^{3}이였다. 이를 結果를 綜合하면 P_{17}의 抗—P_{17} 抗體와의 직접적 結合部位는 P_{17}의 末端部位에 屬在해 있는 것을 암시해 준다. 한편 P_{17}의 나머지 部分은 이 抗原決定基 構造를 維持하는데 重要한 役割을 할 것으로 생각되며 또한 이 決定基內의 한개의 disulphide 結合은 免疫學的 活性을 나타내는데 必須的인 것으로 믿어진다.

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

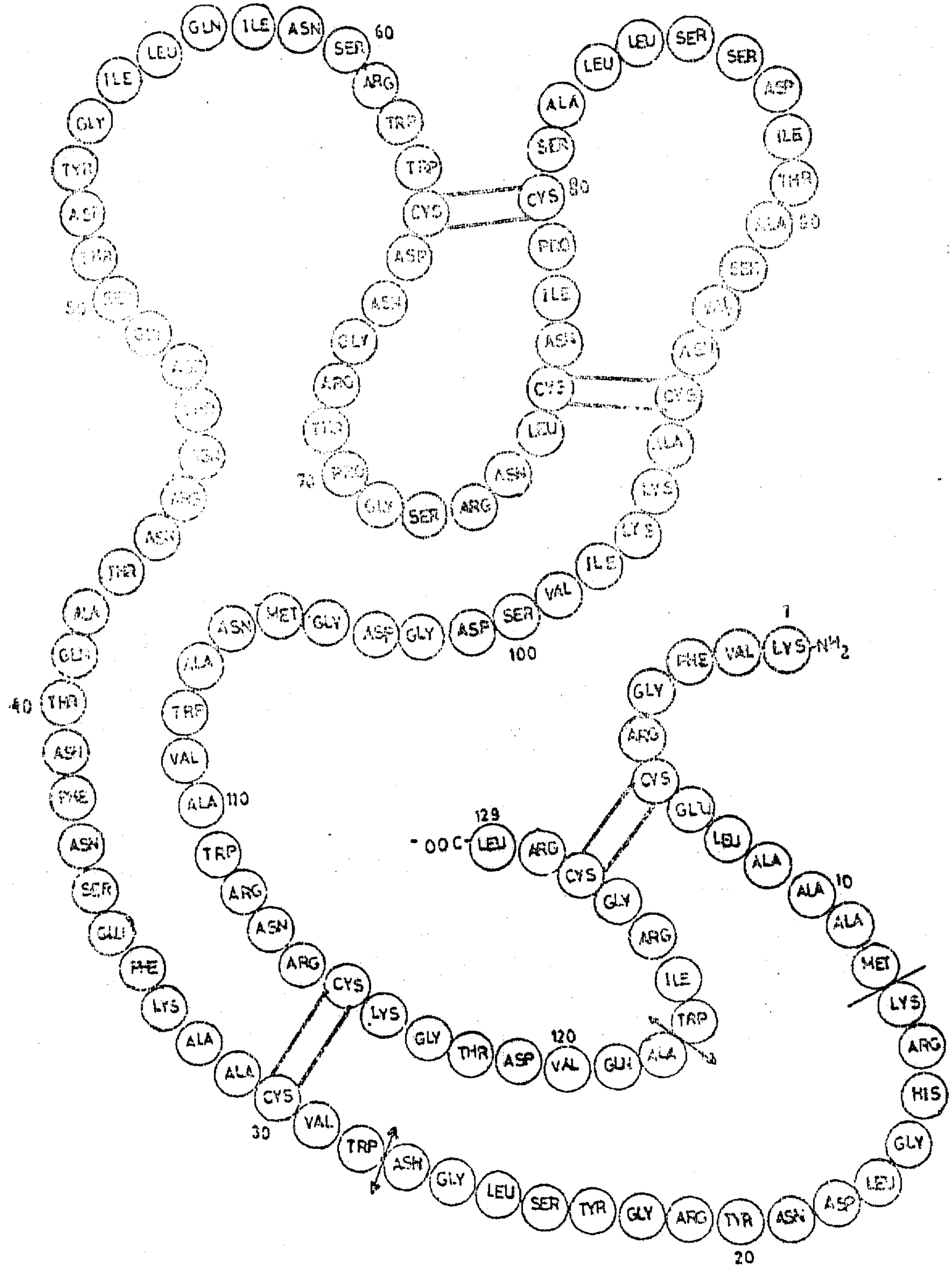

Fig. 1. Primary structure of hen egg-white lysozyme.

1,000. 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 determinant.

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. Finall, 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 peptids (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 0.2M sodium phosphate buffer, pH 7.16 and then through QAE-Sephadex C-25 in 0.005M phosphate butter, 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 (Sakao 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 4N 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 (3×40cm) at 25 C and P_{17} was eluted with a linear gradient formed using 3 liters each of 0.15M NaCl, 0.05M sodium acetate buffer, pH 3.88 and 0.12M sodium phosphate buffer, pH 8.0. For further purification 500mg of crude P_{17} were applied to a CM-23 column (3×40cm) at 25C and eluted with a linear gradient formed using 3 liters each of 0.12M sodium phosphate buffer, pH 6.0 and 0.18M sodium phosphate buffer, pH 6.0. All chromatographic procedures were carried out at 25C and 0.05% chloretone was

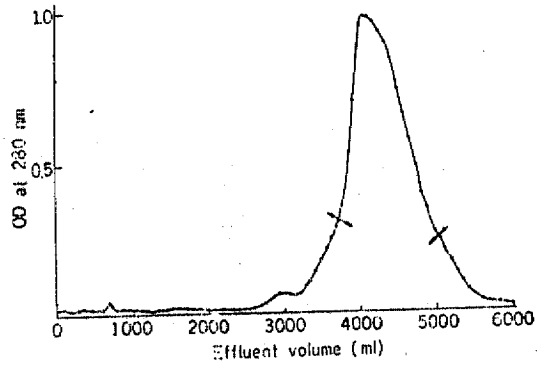

Fig. 2. Rechromatography of P_{17} on a CM-23 column (3×10cm) at 25C. 500mg. of crude P_{17} were applied and the peptide was eluted with a linear gradient formed using 3 liters volumes of 0.12M sodium phosphate buffer, pH 6.0 and of 0.18M sodium phosphate buffer, pH 6.0. Arrows indicate the fractions collected.

used a preservative.

The elution pattern obtained on rechromatography is shown in Fig. 2.

The main fractions of eluate were pooled and desalted. For further purification 400mg of P_{17} in 20ml of 1.0N acetic acid were applied to a BioGel P-10 (100-200 mesh) column (5.5×90cm) equilibrated with the same solvent and fractions of 20g were collected. Usually a single peak was obtained. 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 (2×140cm) 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} prepararions always contained less than 0.01% of intact HL.

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

The material in the peptic digest of HL eluted from the CM-23 column with 0.15M NaCl, 0.05M

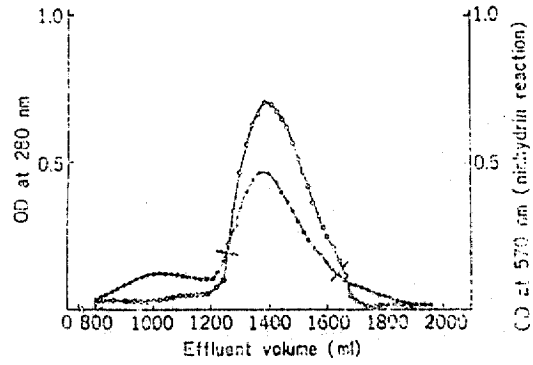

Fig. 3. Rechromatogrphy of P_{17m} on a SE-Sephadex C-25 column (1×35cm) at 25C. 300mg of crude P_{17m} were applied and the peptide was eluted with a linear gradient formed using 1 liter volumes of 0.14M sodium acetate buffer, pH 5.5 and 0.16M sodium acettate buffer, pH 5.5. Arrows indicate the fractions collected. ●——●, OD at 280 nm; ○——○, OD at 570nm (ninhydrin reaction).

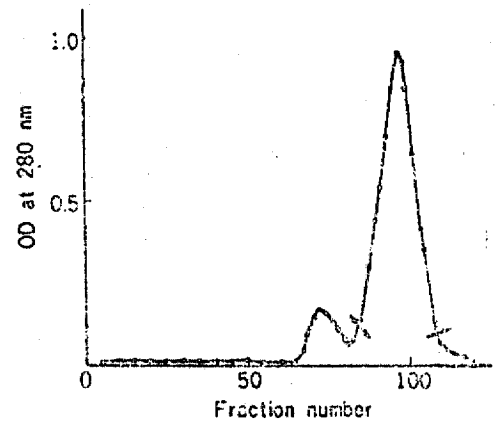

Fig. 4. Purification of P_{17m} by gel filtration through a Bio-Gel P-10 column (3×155cm) in 25% v/v) acetic acid at 25C. 130mg of P_{17m} obtained by rechromatography on SE-Sephadex C-25 were applied. Fractions of 8g were collected. Arrows indicate the fractions collected.

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 (1×35cm) equilibrated with 0.05M sodium acetate buffer, pH 3.88. All chromatographic and gel filtration procedures were carried out at 25C with 0.05% chloretone added to the buffers as preservative. A linear gradient, formed using 1 liter of 0.14M sodium accetate buffer, pH5.5, was used. Material eluted in a volume of 1,200ml to 1,620ml was collected. This fraction was rechromatographed on a SE-Sephadex C-25 column (1.35cm) using the same gradient as in the first chromatography. Fig. 3 shows the elution pattern of the peptide on rechromatography.

The peptide was desalted and purified by gel fitration on a Bio-Gel P-10 (100-200 mesh) column (3×155cm) in 25% (v/v) acetic acid. The gel filtration pattern is shown in Fig. 4. The second peak was collected and acid was removed by repeated evaporation in a rotatory evaporator under reduced pressure. The peptide was finally lyophilized. Re-

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 acide residues found at concentrations of more than 0.03 mole per mole of peptide are listed.

[b] The peptide was hydrolyzed for 24hr in constant boiling HCl (5.7N). 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).

sults of amino acid analysis of this peptide are shown in Table 1.

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 100mg of P_{17} in 50ml of 70% formic acid was mixed with 500mg of cyanogen bromide. The reaction mixture was stirred with a magnetic stirrer at 40C for 20hr. Then it was concentrated in a rotatory evaporator under reduced pressure and subjected to gel filtration on a Bio-Gel P-10 column (3×145cm) in 25% (v/v) acetic acid to remove cyanogen bromide.

6. Reduction and alkylation of P_{17}

Dithiothreitol was added to at final concentration of 0.01M to a solution of 50mg of P_{17} in 5ml of 0.4M Tris-HCl buffer, pH 8.5 containing 0.004M EDTA and the reaction mixture was stirred at 25C for 1 hr. Then recrystallized iodoacetic acid in NaOH was added to a final concentration of 0.02M and the mixture was kept at pH 8.3 by addition of Tris for 1 hr at 25C. 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 5.7N HCl at 105±1C 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 300g, were obtained from a single commercial farm. A does of 500μg of highly purified HL in 0.4ml of complete Freund's adjuvant was injected into all four foot pads of the guinea pigs. A booster injection of 500μg 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 pooled.

9. Quantitative precipitin reaction

Volumes of 100μ liters 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 0.01M EDTA, as described previously (Fujio et al., 1971). The antibody content of pooled guinea pig anti-HL antiserum

was estimated as 6.00mg 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 6ml of cyanogen bromide solution (50mg/ml). The suspension was adjusted to pH 11.0±0.5 at 20C for 8 min and then thoroughly washed first with chilled deionized water and then with 60ml of chilled 0.1M sodium phosphate buffer, pH 6.5. The activated Sepharose 4B cake was suspended in 10ml of peptide solution (10mg/ml) and the suspension was gently stirred at 4C for 22hr. The immunoadsorbent was then washed once with 20ml of 0.1M sodium bicarbonate and 3 times with 20ml volumes of 1.0N acetic acid and finally treated with 0.05M monoethanolamine at pH 8.0. By this method, approximately 40mg of P_{17} were coupled to 6ml of Sepharose and 12mg of P_{17} were coupled to 3ml of Sepharose.

11 Labelling of HL and P_{17}

One-^{14}C acetic anhydride (12.9mCi/mmole) was purchased from Daiichi Pure Chemicals Co. Ltd.

After purification by chromatography, HL (2.1μ mole) was dissolved in 3ml of dimethylsulfoxide and 60μ liters of 1-^{14}C-acetic anhydrde (3.1μ mole) were added. The mixture was stood at room temperature for 2hr with occasonal shaking (once every 15min). Then it was subjected to gel filtration on a Sephadex G-25 fine (Pharmacia, Sweden) column (3×77cm) equilibrated with 0.15 NaCl, 0.02M 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} (7.5μ mole) in 3ml of dimethylsulfoxide was mixed with 220μ liters of 1-^{14}C-acetic anhydride (11.4μ mole) for 2hr. The reaction mixture was then subjected to gel filtration on Sephadex G-15 (3×140cm) 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 120ml of pooled guinea pig anti-HL antiserum was mixed with 12ml of 0.1M EDTA, cooled in an ice bath and applied to the P_{17}-Sepharose (40mg of P_{17} coupled) column (2×2cm) as described in section 9 of the Materials and Methods. The immunoadsorbent was kept at 4C and the antiserum was applied at a rate of 12ml per hr. Then the column was washed with 120ml of 0.14SM NaCl, 0.01M trisodium EDTA, pH 7.5 (SEDTA). The unbound fraction of antiserum (120ml) and the washing fluid (120ml) were pooled and concentrated to the original volume (120ml) 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 300ml) until the OD of the washing fluid at 280nm was less than 0.01. The P_{17} specific antibody (anti P_{17} antibody) was recovered by elution with 2ml of 3×10^{-3}M HL in SEDAT at 4C and then with 48ml of SEDTA. These elution procedures were repeated 3 times and all the eluates were pooled. The eluates were concentrated by pressure dialysis to approximately 5ml and applied to a Sephadex G-150 column was kept at 25C and fractions of 5g were collected. The gel filtration pattern is shown in Fig.5.

The first fraction seems to be so called 19S 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 immedi-

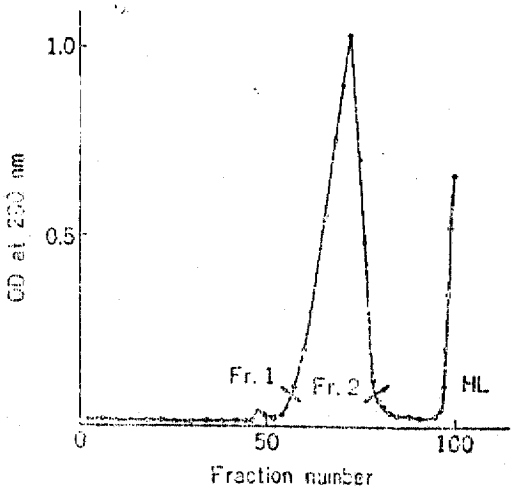

Fig. 5. Separation of guinea pig 7S anti-P_{17} antibody on a Sephapex G-150 column (3×90 cm) in 0.1N acetic acid at 25C. 5ml of HL-antibody (60mg) mixture were applied and fractions of 5g were collected. Arrows indicate the fractions pooled.

ately neutralized with NaOH. The antibody protein was concentrated to 5mg per ml and extensively dialyzed against PBS. This antibody fraction was found to be so-called 7S 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 7S antibody eluted was combined with the first fraction and used as anti-P_{17} antibody in the following experiments. Approximately 80mg of anti-P_{17} antibody were obtained from 120ml of guinea pig anti-HL antiserum. This represents 11.1% of the total precipitable anti-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 2ml of 1.25×10^{-2}M of P_{17} solution and the eluated was tested by gel filtration on Sephadex G-150 in 0.1N acetic acid, as described above. However, no antibody protein detected.

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

Fifty ml of normal guinea pig serum were dialyzed against 5 liters of 0.05M sodium phosohate buffer, pH 7.5 at 4C for 24hr. The dialyzed serum was applied to a DEAE-cellulose column (6×10cm) at 4C and eluted with the same buffer. The fractions in the first peak were pooled and concentrated by pressure dialysis and 5ml of the concentrated solution (10mg/ml) were applied to a Sephadex G-150 column (3×90cm). The 7S-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 10C for 60hr 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 280nm and by the ninhydrin reaction. For the latter, 0.3ml of each fraction was mixed with one ml of ninhydrin reagent (Moore et al., 1954) and boiled for 20min. After cooling, 4ml of 50% ethanol were added and the optical density was measured at 570nm.

The concentrations of peptide, HL and purified antibody were estimated by measuring the OD at 280nm, 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).

RESULTS

1. Cleavage of P_{17} with cyanogen bromide

P_{17} was treated with cyanogen bromide as des-

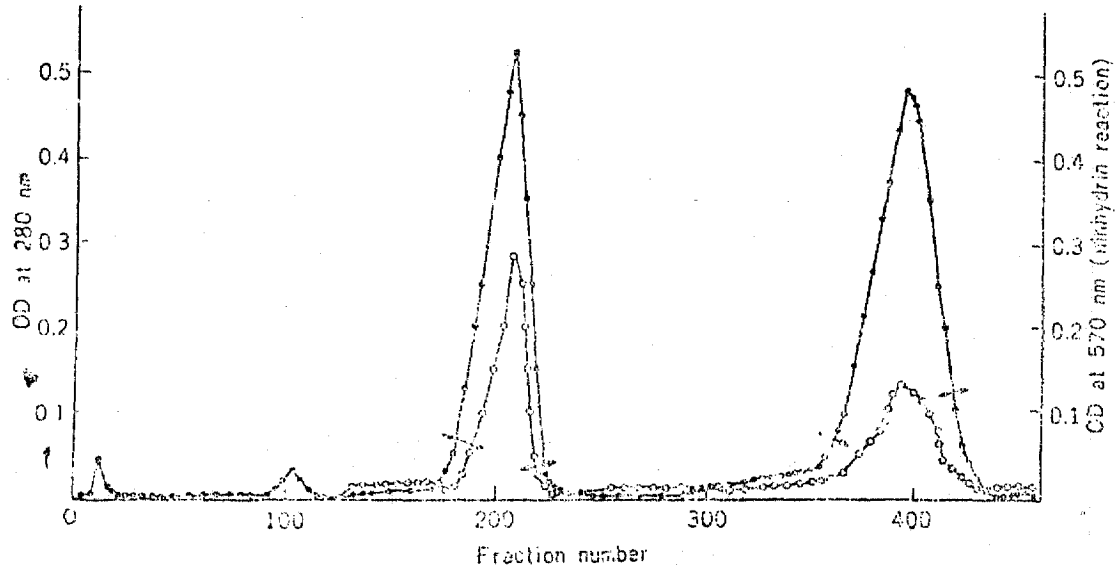

Fig. 6. Chromatography of P_{17} after cyanogen bromide treatment on a SE-Sephadex C-25 column (1×35cm). A sample of 98mg mas applied. The column was eluted with a linear gradient formed using 1 liter volumes of 0.2M sodium acetate buffer, pH 5.5 and 0.3M sodium acetate, buffer, pH 6.0. Fractions of 3.8ml were collected. Arrows indicate the fractions pooled. ●——●, OD at 280nm; ○——○, OD at 570nm (ninhydrin reaction).

cribed in the Materials and Methods. Then after removal of acetic acid in a rotatory evaporator a sample of 98mg was applied to a SE-Sephadex C-25 column (1×35cm). 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 0.3M sodium acetate buffer, pH 6.0 at 25C. Fractions of 4g were collected. The optical density of each fraction, measured at 280nm, and its ninhydrin reaction are shown in Fig. 6.

Two main peaks were observed. The first gave a strong ninhydrin reaction relative to its optical density at 280nm, 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 fracitons are shown in Table 2.

The results of amino acid analyses show that the first main fraction (P_{17I}) is derived from the internal portion of P_{17} while the second (P_{17II}) 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_{17II} 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_{17II}) were both 100%. The relatively low recovery of P_{17II} 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 column of Sephadex G-15 (3×140cm) and Bio-Gel P-10 (3×150cm) in 25%(v/v) acetic acid at 25C. Fractions of 8g were collected. The elution pattern is depicted in Fig. 7.

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

Amino acid[a]	P_{17I} Lys[12]–Asn[27]		P_{17t} Lys[1]–Cys[6]–Homoser[12] | Trp[123]–Cys[127]–Leu[128]		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 (5.7N) 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).

Three peptides fractions were obtained. Fraction 1 had a relatively high ninhydrin value in comparison with ts optical density at 280nm while fraction 3 had a relatively low ninhydrin value in comparison with its optical density 280nm. After removal of acetic acid fraction 1 was applied to a CM-23 column (1×35cm) and eluted with 0.15M 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 0.3μmole 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

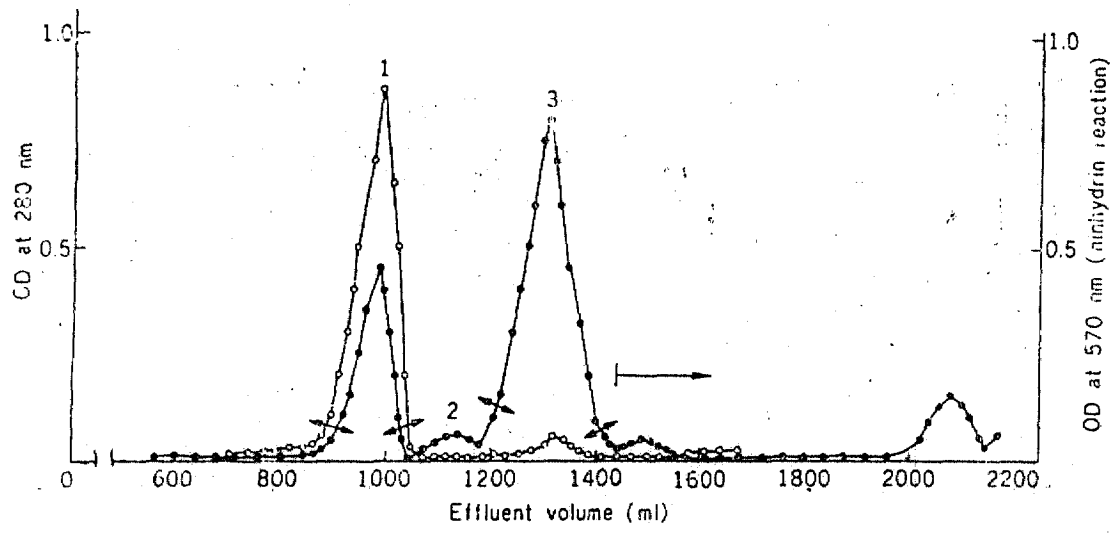

Fig. 7. Gel filtration of reduced and alkylated P_{17} on serial columns of Sephadex G-15 (3×140cm) and Bio-Gel P-10 (3×150cm) in 25% (v/v) acetic acid at 25C. 50mg of reduced and alkylated P_{17} were applied and fractions of 8g were collected. Numbers on top of peaks indicate fraction numbers and arrows indicate the fractions pooled. The horisontal arrow shows the elution position of the reagents.

is derived from an impurity in the P_{17} preparation. Fraction 3(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 10C.

Volumes of 50μ liters of various concentrations (6×10^{-6}M to 1.2×10^{-4}M) of ^{14}C-acetyl-HL were dialyzed against 50μl of purified guinea pig anti-P_{17} antibody (7.0×10^{-5}M) at 10C for 60hr. As controls, 50μ liters 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. 8, 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^{5} (L/M) and that of the other, 4.0×10^{5} (L/M). No appreciable binding of ^{14}C-acetyl-HL with NGG was observed in the control experiment, as can be seen in Fig. 8.

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 10C for 60hr. The results are shown in Fig. 9.

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

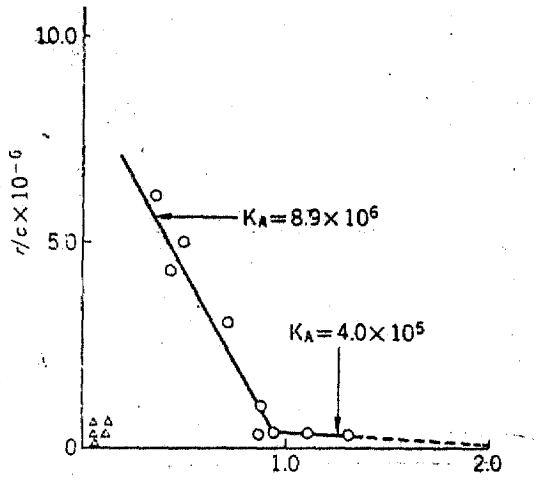

Fig. 8. Binding of purified guinea pig anti-P_{17} antibody with ^{14}C-acetyl-HL at 10C. Values are shown as a Scatchard plot, according to the equation, $r/c=K_An-K_Ar$ where r represents moles of antibody, taking the molecular weight of antibody as 150,000 and that of HL as 14,300. c is concentration 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 7S-γ-globulin (NGG).

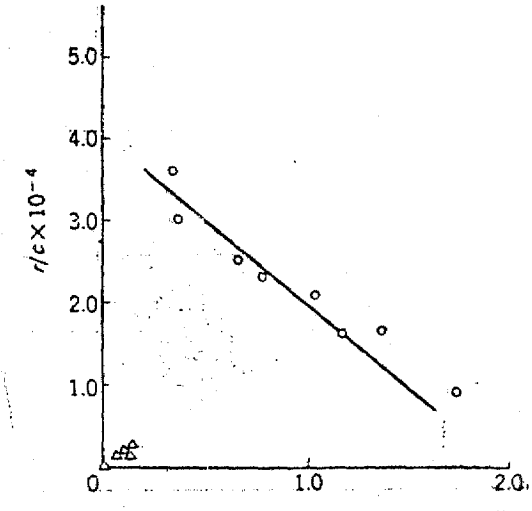

Fig. 9. Binding of puirified guinea pig anti-P_{17} antibody with ^{14}C-acetyl-P_{17} at 10C. Values are shown as a Scatchard plot, according to the equation, $r/c=K_An-K_Ar$, where r represents moles of ^{14}C-acety-P_{17} per mole of antibody, taking the molecular weight of antibody as 150,000 and that of P_{17} as 3,928; 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.

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.

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

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}–Len^{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_{17t}	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, 3.5mg 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 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 cocentration of inhibitor. Dialysis was performed at 10C for 60 hr.

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 (50μ liters) were dialyzed against 50μ liters of guinea pig anti-P_{17} antibody (2.38×10^{-5}M) at 10C for 60hr. 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 experments 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 antibody reacted with P_{17t}. Accordingly P_{17t}-immunoadsorbent was prepared to examine this.

A sample of 12mg of P_{17t} was coupled to Sepharose 4B as described in the Materials and Methods. One ml of purified anti-P_{17} antibody (4.8mg) was applied to the top of the P_{17t}-Sepharose column (1×4.5cm). 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 280nm, was 0.23mg. This corresponds to only 4.8%

of the antibody applied. When the column was eluted with 0.1M citrate buffer, pH2.2, 4.2mg of antibody protein was eluted, corresponding to 87.5% of the total antibody protein applied. As a control 1ml of NGG (5mg) 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 $P_{17'}$.

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 aear $r=1$. This suggests 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 antibody. Only P_{17} and $P_{17'}$ (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.

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