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ANTIGENIC STRUCTURES OF HEN EGG-WHITE LYSOZYME III. THE ANTIGENIC SITE CLOSE TO THE CATALYTIC SITE

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SUMMARY The third immunologically active peptide (P_{Ib}) was isolated by limited digestion of hen egg-white lysozyme (HL) with thermolysin. It consisted of two peptides, i.e. sequence 29 to 54 and 109 to 123, and the two peptides were linked together by a single S–S bond (Cys^{30}–Cys^{115}). The peptide specific antibody fraction (anti-P_{Ib}) was separated from specifically purified rabbit anti-HL antibody using peptide-Sepharose immunoadsorbent. The association constant of HL with one batch of anti-P_{Ib} antibody was 1.84×10^5. Two kinds of peptides, sequence 34 to 54 (P_{Ba-1}) and 38 to 57 (P_{Ba-2}) which include one or both residues of the catalytic sites of HL (Glu^{35} and Asp^{52}) were also isolated. An ihnibition test of the binding of ^{125}I–HL with the purified anti-P_{Ib} antibody by these structurally related peptides indicated that the specificity of the anti-P_{Ib} antibody is mainly directed to the region of sequence 38–54.

The efficiency in neutralization of the enzymic activity by anti-P_{Ib} antibody for the two substrates, *M. lysodeikticus* and hexa-*N*-acetyl-chitohexaitol (R-6 mer), was high and was comparable with that in neutralization by the whole population of anti-HL antibodies.

INTRODUCTION

Neutralization of the biological activity of a macromolecule by its antibody is one of the most remarkable properties of an antibody. On the other hand, there are many reports of the presence of neutralizing and non-neutralizing antibodies against one kind of biologically active molecule. To study the mechanism of neutralization by antibody in detail, enzyme has been used as antigen so that the degree of neutralization can be evaluated with high accuracy (Cinader, 1967; Arnon, 1971). Some time ago it was predicted from the results of quantitative precipitin reactions that an enzyme protein might have several antigenic determinants (Kabat and Mayer, 1961), but only recently have the structures responsible for several antigenic sites in a single enzyme protein been examined (Fujio et al., 1968 a, b; Imanishi et al., 1969 a; Omenn et al., 1970 a, b, c). A direct relationship between the specificity of an antibody and its neutralizing ability has also been shown recently (Fujio et al., 1971).

Lysozyme is an enzyme known to be widely distributed in vertebrates. If the structures responsible for the enzymic activity of the enzyme from different sources are exactly the same, it is unlikely that it will be possible to make an antibody which is specific for the active center of enzyme from all these sources. However, Canfield et al. (1971), compared the amino acid sequences of human lysozyme and HL, and found that the amino acid moieties differed at a considerable number of point near the catalytic site. Therefore, it may be possible to make an antibody which is specific for the region close to the catalytic site of enzyme.

Two immunologically active peptides can be obtained by digestion of lysozyme with pepsin (Shinka et al., 1967; Fujio et al., 1968 a, b; Arnon and Sela, 1969.) However, these two kinds of peptide only account for about 60% of the whole precipitable anti-HL antibody (Fujio et al., 1968 a). To find other immunologically active peptides with different specificities from those of the peptic peptides, various proteases including papain, subtilisin, pronase and thermolysin were tested. Among these proteases, thermolysin seemed the most promising to obtain relatively large peptides retaining the immunological activity of the original molecule, because thermolysin has rather restricted substrate specificity (Matsubara, 1970). If thermolysin splits peptide bonds at valine, leucine, isoleucine and phenylalanine residues, HL can be split at 23 loci resulting in 21 fragments. Therefore, limited digestion of HL by thermolysin was chosen to obtain larger peptides. Short-term digestion at elevated temperature with small amounts of enzyme yielded several immunologically active peptides. This paper reports the preparation and characterization of immunologically active peptides obtained by limited digestion of HL with thermolysin. The peptide containing the catalytic site of lysozyme is described in detail.

MATERIALS AND METHODS

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

Six times recrystallized HL was purchased from Seikagaku Fine Biochemicals Co. Ltd. This preparation was digested with thermolysin to prepare immunologically active peptide. Before use for immunization, the preparation was further purified on SE-Sephadex C-25 in 0.2 M sodium phosphate buffer, pH 7.16, and then passed through QAE-Sephadex C-25 in 0.005 M sodium phosphate, pH 8.0.

2. *Thermolysin*

Three times recrystallized thermolysin was purchased from Daiwa-Kasei Co. Ltd. and was used without further purification.

3. *Desalting of peptides*

Peptides were desalted by the method of Dixon (1959). Eluates from a SE-Sephadex column were diluted two fold with deionized water and applied to an IRC-50 colum (H^+-form), using 3 ml of resin (wet vol) per 10 mg of peptide. The IRC-50 column was thoroughly washed with 0.1 N acetic acid and the peptides were eluted with 50% (v/v) acetic acid at room temperature. Acetic acid was removed in a rotary evaporator at a pressure of 15 mm Hg at 37 C. The flasks and apparatus were flushed with nitrogen.

4. *Deionization of urea*

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

5. *High voltage paper electrophoresis*

The homogeneties of the purified peptides were checked by high voltage paper electrophoresis. Pyridine-acetate buffer (pH 3.6 and 6.5) was used as described by Ryle and Sanger (1955). A horizontal type electrophoresis apparatus equipped with a cooling plate (Fuji-Riken Co. Ltd.) was used and 50 v per cm was applied for 2 hr at 2 to 8 C. The peptides were located by staining the paper with 0.2% ninhydrin in 95% ethanol and with stain for peptides (Greig-Leaback, 1960).

6. *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 and 72 hr. The molar ratios of amino acids were calculated taking glycine as a standard.

7. *Production of antisera*

Two mg of highly purified HL with complete Freund's adjuvant were injected into rabbits. Booster injections of 2 mg of HL were given once every five weeks, the last being given after 20 weeks. Blood was taken 7 and 9 days after the last injection and the rabbits were exanguinated 11 days after the last injection. Sera from 12 rabbits were pooled (p # 44). The antibody content of anti-HL antiserum p # 44 was estimated by the quantitative precipitin reaction (Fujio et al., 1971) as 4.00 mg per ml.

8. *Specific purification of rabbit anti-HL antibody*

Rabbit anti-HL antibody was specifically purified as described previously (Fujio et al., 1968a and 1971). Equivalent amounts of HL were added to rabbit anti-HL antiserum p # 44 in the presence of 0.01 M EDTA and the resultant specific precipitates were washed, suspended in 0.1 N acetic acid and incubated at 37 C for 1 hr. The 7S anti-HL antibody fraction was separated on Sephadex G-150 saturated with 0.1 N acetic acid at 25 C. The 7S fraction was dialyzed against 100 volumes of 0.01 N acetate buffer, pH 5.5 at 4 C and then against 100 volumes of 0.002 M sodium phosphate buffer, 0.15 M NaCl, pH 8.0 (DPS, pH 8.0). The yield of specific antibody from rabbit anti-HL antiserum p # 44 was 75% of the total precipitable antibody in the antiserum. This specific antibody preparation was found to consist essentially entirely of 7S IgG when tested by immunoelectrophoresis and gel filtration. More than 92% of the protein in this antibody preparation was precipitable by HL.

The peptide specific antibody fraction was prepared using peptide Sepharose 4B immunoadsorbent (Omenn et al., 1970c; Fujio et al., 1971a).

9. *Turbidimetric assay of peptide-antibody interaction*

The inhibitory activity of peptide on development of turbidity in the HL-anti-HL system was assayed spectrophotometrically as a function of time at 570 mμ, with a Gilford model 2000 automatic recorder equipped with a Beckman DU Spectrophotometer at 25 C, as described by Fuchs et al., (1969).

10. *Equilibrium dialysis*

HL was labelled with ^{125}I by the iodine monochloride method of McFarlane (1958). Conditions were adjusted to attain one atom of iodine per mole of HL. The specific activity of ^{125}I-HL was 10^5 cpm per 10^{-9} mole. Fifty μl of ^{125}I-HL solution of 1×10^{-5} to 4.8×10^{-4} M were put into one compartment of the cell (Fujio et al., 1971) and an equal volume of the purified peptide specific antibody (3–5 mg/ml) was put into the other compartment. Gel-cellophane (Fujio et al., 1968a) was used as a semipermeable membrane and dialysis was continued for at least 45 hr at 10 C. Aliquots of 25 μl were taken from both compartments of the cell and the the concentration of antigen was estimated by counting ^{125}I in a Packard Model 3002, Tricarb Scintillation Spectrometer.

Binding inhibition experiments were carried out as follows. Twenty five μl of various concentrations of inhibitors and 25 μl of ^{125}I-HL were mixed and dialyzed against 50 μl of antibody solution. The association constant of the inhibitor, K_I, was calculated according to the equation (Karush, 1956),

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

where (i) the equilibrium concentration of inhibitor and r and r' are the number of molecules of antigen bound per antibody molecule at an antigen concentration, c, in the absence and prescne of inhibitor, respectively.

11. *Inhibition of enzymic activity by antibodies*

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

Increasing amounts of purified antibody preparations were added to a constant amount of enzyme. The volume was adjusted to 2 ml with 0.02 M sodium phosphate, 0.15 M NaCl, pH 6.0 (PBS, pH 6.0) in the case of *M. lysodeikticus* and to 0.8 ml with 0.025 M sodium diethylbarbiturate-HCl, 0.1 M NaCl, pH 6.0 in the case of hexa-*N*-acetyl-chitohexaitol (R-

6 mer). Two ml of *M. lysodeikticus* suspension (2.4 mg) or 0.2 ml of R-6 mer (300 μg) were added to the enzyme-antibody mixture after preincubation at 37 C for 30 min. The residual activity was measured by incubation at 37 C for 5 min in the case of *M. lysodeikticus* and for one hour in the case of R-6 mer. The decrease in catalytic activity of the enzyme due to antibody is expressed as a percentage of that of the enzyme in the absence of antibody.

12. *Miscellaneous*

Sephadex, Sephadex ion-exchanger and Sepharose 4B were purchased from Pharmacia (Sweden). Bio-Gel P-10 was obtained from Bio-Rad (U.S.A.). All chemicals used were of analytical grade and all except urea and iodoacetic acid were used without further purification.

After chromatography, peptides were located by measuring the optical denisity at 280 mμ and also by the ninhydrin reaction. A sample of 0.2 ml of each fraction was mixed with one ml of 0.2 M citrate buffer, pH 5.0 and one ml of ninhydrin reagent was added to the mixture. Then the procedure of Cocking and Yemm (1954) was followed. The final volume was adjusted to 5 ml with 60% (v/v) ethanol.

Peptides were reduced at 20 C by treatment with 0.1 M 2-mercaptoethanol, 0.005 M EDTA, 0.5 M tris-HCl buffer, pH 8.5 for one hr, keeping the solution at pH 8.3 by adding 2 M tris solution. Ten percent excess (on a molar basis) of recrystallized iodoacetic acid were added to the reduced peptide solution and the pH was kept at 8.3 by adding 2 M tris. Alkylation was continued for one hr at 20 C. The reaction was stopped by passing the mixture through a Sephadex G-25 column equlibrated with 50% (v/v) acetic acid at 25 C.

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

RESULTS

1. *Thermolytic digestion of HL*

Thermolytic digestion of HL was first tested at 37 C at a weight ratio of enzyme to substrate of 1 to 50. Digestion was continued for 24 hr in 0.05 M tris-HCl buffer, 5 mM $CaCl_2$, pH 7.5. The digestion was stopped by adding EDTA at a concentration of 0.01 M. On passing the whole digest of HL through a Sephadex G-50 column, about 70% of the optical density at 280 mμ of the digest was eluted at the same position as intact lysozyme. This fraction could not be distinguished from intact HL on SE-Sephadex C-25 chromatography in 0.2 M sodium phosphate buffer, pH 7.16 at 25 C. Therefore, digestions of HL with thermolysin were next tested at 60 and 70 C. Only the latter temperature was satisfactory to obtain steady proteolysis of HL by thermolysin. Therefore, the following experiments were carried out at 70 C.

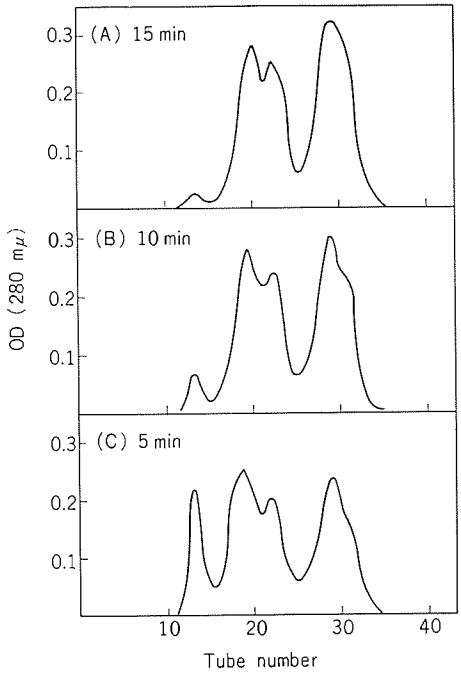


FIGURE 1. *Gel-fitration patterns of thermolytic digests of HL taken at intervals during digestion. The Sephadex G-50 column (1.2×75 cm) was eluted with 5 M guanidine hydrochloride at 25 C. Three g fractions were collected. (A), (B) and (C) represent times of thermolytic digestion.*

Next the effects of the weight ratio of enzyme to substrate and of the digestion time on the size distribution of the digested peptides were examined. The results of preliminary experiments indicated that a ratio of 1: 1,000 gave a reasonable digestion rate at 70 C. Therefore, the time course of thermolytic digestion was examined using an enzyme to substrate ratio 1: 1,000 at 70 C. Figure 1 shows the gel-filtration patterns of digests taken 5, 10 and 15 min after addition of thermolysin to HL at 70 C.

The first peak seems to be intact HL judging from results of SE-Sephadex C-25 chromatography and enzymic assay. During digestion the amount of intact HL decreased, but the sizes of peptides also became smaller. Therefore, in subsequent experiments peptides were prepared from digests obtained after 5 min at 70 C at pH 7.5 using a ratio of thermolysin to HL of 1: 1,000.

2. *Purification and identification of peptides*

Five g of six times crystallized HL were digested as described above. Proteolysis was stopped by adding EDTA. All digests were kept in a frozen state until use. The digest (135 ml, corresponding to 2.5 g of HL) was passed through a Sephadex G-50 column (10 × 90 cm) equilibrated with 20% (v/v) acetic acid

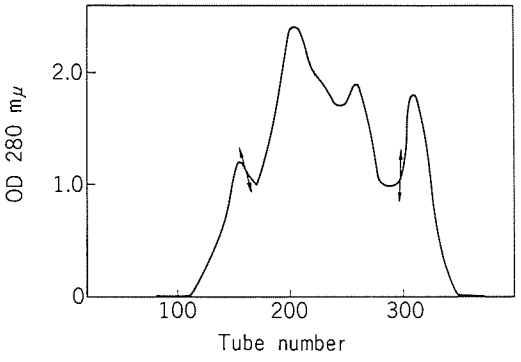


FIGURE 2. *Preparative gel-filtration of a thermolytic digest of HL on a Sephadex G-50 column (10 × 90 cm) in 20% (v/v) acetic acid at 4 C. 20 g fractions were collected.*

at 4 C. Figure 2 depicts the gel-filtration pattern of the whole digest.

Thermolysin and the smaller peptides were removed by this procedure and only the fractions between the arrows in Figure 2 were pooled. The pooled fractions were first concentrated in a rotary evaporator under a pressure of 15 mm Hg at 37 C in a water bath. Evaporation was repeated until almost all the acetic acid had been removed. The preparation was adjusted to a final volume of approximately 11 liter with deionized water and to pH 3.4 with glacial acetic acid. Then it was applied on a SE-Sephadex C-25 column (3 × 40 cm) equilibrated with 0.02 M acetate buffer, pH 3.4. The peptides were separated by elution with two linear gradients. The first gradient was set up using two 3 liter Erlenmeyer flasks, one containing 2.5 liter of 0.2 M sodium acetate buffer, pH 3.4, and the other containing the same volume of 0.17 M sodium acetate buffer, pH 4.5. The second gradient was formed using two 5 liter Erlenmeyer flasks, one containing 3.5 liter of 0.17 M sodium acetate buffer, pH 4.5 and the other the same volume of 0.6 M sodium acetate, pH 8.3. Chloretone (0.02%, w/v) was added as preservative and chromatography was carried out at 25 C. The peptides were eluted at a flow rate of 200 ml per hr using a peristaltic pump and 20 g fractions were collected. Figure 3 shows the elution pattern obtained. The peptides were located by their OD at 280 mμ and by reaction with ninhydrin reagent.

Approximately 16 components were separated in this way. The last peak in the chromatogram seemed to be intact HL from its elution position and enzymic activity against *M. lysodeikticus*. Each peptide except peptide A (P_A) and peptide B (P_B) was desalted by adsorption on and elution from IRC-50 resin, as described in the " Materials and Methods ". P_A and P_B were desalted by gel filtration on Sephadex G-25. Before the peptides were purified further, the ability of each fraction from the SE-Sephadex C-25 column to inhibit the development of turbidity by HL and homo-

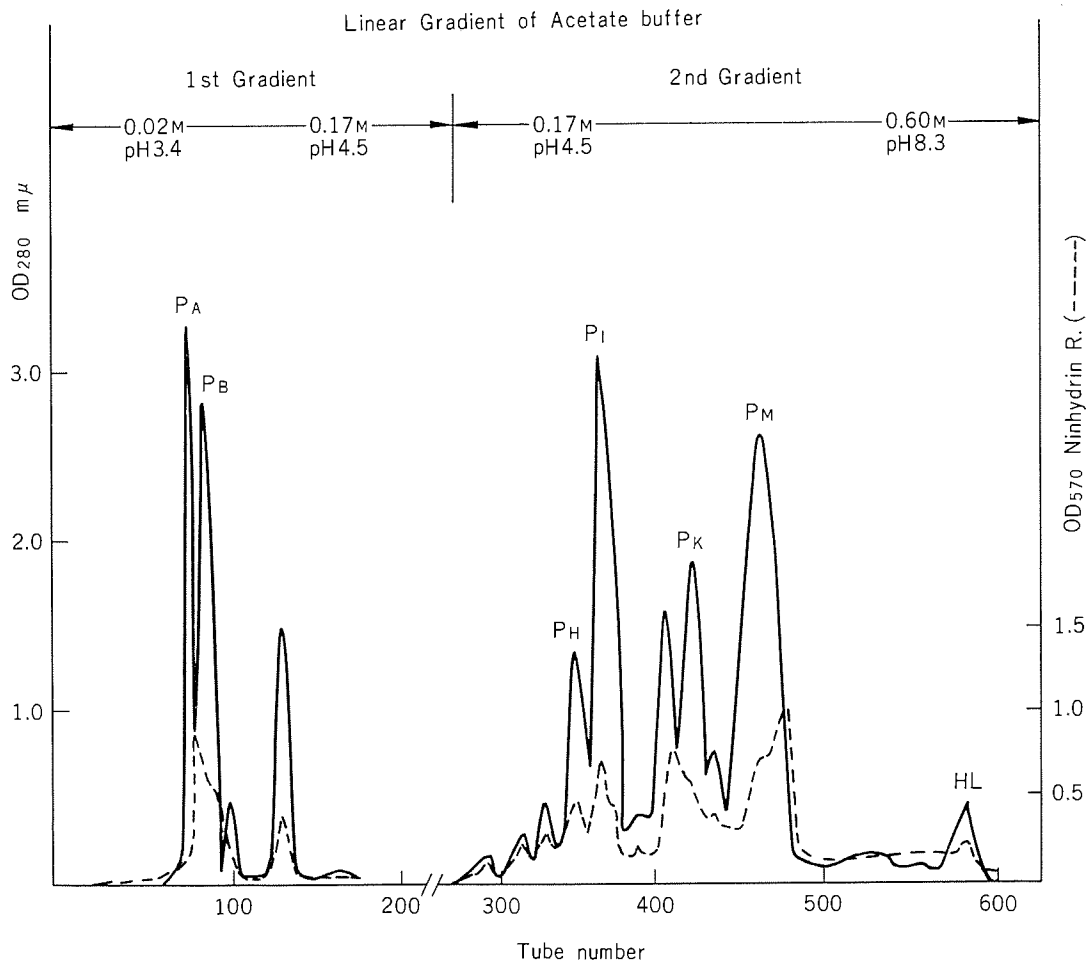


FIGURE 3. *SE-Sephadex C-25 chromatography of a thermolytic digest of HL. An SE-Sephadex C-25 column (3×40 cm) was eluted at a rate of 200 ml per hr and 20 g fractions were collected at 25 C. The solid line represents the optical density at 280 mμ and the dotted line represents that at 570 mμ of the ninhydrin reaction. See text for details.*

logous antibody was tested. Peptides at a weight ratio of 10 to 30 times that of HL were added to purified rabbit anti-HL antibody. Of the 16 peptide fractions tested, P_B, P_I, P_K and P_M were shown to inhibit turbidity formation. These peptides were examined by high voltage paper electrophoresis at pH 3.6 and pH 6.5 and all gave at least two ninhydrin-positive spots. Therefore, they were purified further as follows.

1) Fraction P_B

Samples of fraction PB obtained from two runs of SE-Sephadex C-25 chromatography were pooled and concentrated in a rotary evaporator at 15 mm Hg and 37 C (water bath temperature) to approximately 2 ml. The final volume was adjusted to 5 ml with 50%

(v/v) acetic acid. Fraction P_B was then subjected to gel-filtration on a Sephadex G-25 (fine) column (3×110 cm) equilibrated with 50% (v/v) acetic acid. Chromatography was carried at 25 C and 5 g fractions were collected. Figure 4 shows the result.

The yield of P_{Ba} from 5 g of HL was 140 mg. The homogeneity of P_{Ba} was examined by high voltage paper electrophoresis and, as shown in Figure 5. P_{Ba}, at least three components were separated at pH 6.5. To isolate these components, 10 mg of P_{Ba} were applied to Toyo filter paper 51A (40×60 cm).

The most negatively charged peptide at pH 6.5 was named P_{Ba-1}, the neutral one P_{Ba-2} and the positively charged one P_{Ba-3}. Each peptide was eluted with 50% (v/v) acetic acid and finally purified by gel-filtration on a Bio-Gel P-10 column. The weight ratio of P_{Ba-1}: P_{Ba-2}: P_{Ba-3}: obtained was 4: 10: 1. Both P_{Ba-1} and P_{Ba-2} appeared homogeneous electrophoretically at both pH 6.5 and 3.6. Further characterization of P_{Ba-3} was abandoned because of shortage of material.

The amino acid composition of P_{Ba-1} and P_{Ba-2}, expressed as moles of each amino acid residue per mole of peptide, are shown in Table 1. The minimum molecular weights of P_{Ba-1} and P_{Ba-2} were calculated as 2,235 and 2,217, respectively. The amino acid compositions of the two peptides were compared with the amino acid sequence of HL reported by Canfield et al. (1965). P_{Ba-1} corresponded to the region from Phe^{34} to Gly^{54} and P_{Ba-2} to that from Phe^{38} to Gln^{57} of HL. According to the mechanism of the enzymic action of HL proposed by Philips (1967), Glu^{35} and Asp^{52} are catalytic sites of HL and both were present in P_{Ba-1}. On the other hand, P_{Ba-2} contained only Asp^{52} as a catalytic site of HL.

2) Fraction P_I

Samples of fraction P_I obtained from two runs of SE-Sephadex C-25 chromatography were pooled. The mixture was diluted two fold with deionized water and adjusted to pH 4.5 with acetic acid. The crude peptide solution was applied to a SE-Sephadex C-25 column (3×40 cm). The column was eluted with a gradient formed using 3 liter of 0.17 M sodium acetate buffer, pH 4.5, in the presence of 6 M deionized urea and the same volume of 0.17 M sodium acetate buffer, pH 5.5, in the presence of 6 M deionized urea. Figure 6 shows the elution pattern. Five peaks were obtained and the three major peaks were named P_{Ia},

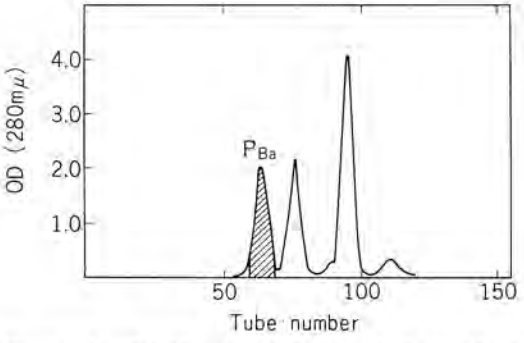


FIGURE 4. *Gel-filtration of P_B on a Sephadex G-25 column (3×110 cm) in 50% (v/v) acetic acid at 25 C. Five g fractions were collected.*

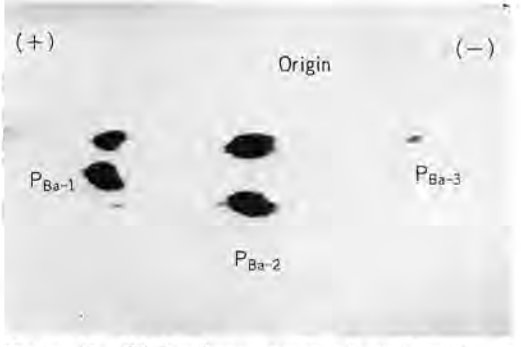
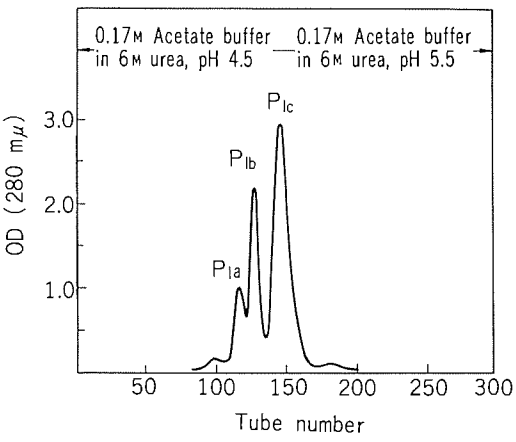


FIGURE 5. *High-voltage paper electrophoresis of P_{Ba}, P_{Ba-1} and P_{Ba-2} in pyridine-acetate buffer at pH 6.5. 180 μg of P_{Ba} and 90 μg each of P_{Ba-1} and P_{Ba-2} were applied to Toyo filter paper (10×60 cm). The run was made at 50 v/cm for 90 min and at 40 v/cm for another 90 min. Peptides were located with peptide-stain (Greig et al., 1960).*

TABLE 1. *Amino acid compositions of P_{Ba-1}, P_{Ba-2} and P_{Ka}*

Amino acid	P_{Ba-1}		P_{Ba-2}		P_{Ka}	
	Found[a]	Phe[34]–Gly[54] [b]	Found[a]	Phe[38]–Gln[57] [b]	Found[a]	Val[29]–S–S–Val[109] / Asn[37] [b] – Trp[123]
Lysine	0	0	0	0	1.63	2
Histidine	0	0	0	0	0	0
Arginine	0.98	1	0.95	1	1.73	2
Aspartic acid	6.05	6	5.01	5	2.56	3
Threonine	3.77	4	3.76	4	0.85	1
Serine	1.70	2	1.06	1	0.80	1
Glutamic acid	2.08	2	2.07	2	1.81	2
Proline	0	0	0	0	0	0
Glycine	2.00	2	2.00	2	1.00	1
Alanine	1.05	1	0.98	1	3.75	4
Half-cystine	0	0	0	0	1.06	2
Valine	0	0	0	0	2.19	3
Methionine	0	0	0	0	0	0
Isoleucine	0	0	0.89	1	0	0
Leucine	0	0	0.88	1	0.09	0
Tyrosine	0.94	1	0.90	1	0	0
Phenylalanine	1.49	2	0.89	1	0.78	1
Tryptophan	Nd	0	Nd	0	Nd	1

a Values are expressed as molar ratios, assuming 2 moles of glycine per mole of P_{Ba-1}, and P_{Ba-2} and one mole of glycine per mole of P_{Ka}.
b The moles of amino acids in the corresponding regions of HL were calculated from the data of Canfield and Liu (1965).



◀ FIGURE 6. *SE-Sephadex C-25 column chromatography (3×40 cm) of P_I at 25 C in the presence of 6 M urea. The gradient was formed using 3.5 l of sodium acetate buffer, 6 M urea, pH 4.5 and the same volume of 0.17 M sodum acetate buffer, 6 M urea, pH 5.5. 15 g fractions were collected. Details are given in the text.*

P_{Ib} and P_{Ic}, respectively. Peaks P_{Ib} and P_{Ic} were desalted with IRC-50 resin and passed through a Bio-Gel P-10 column (5.5×90 cm) to remove impurities derived from urea. The yields of P_{Ib} and P_{Ic} from 5 g of HL were 75 mg and 347 mg, respectively.

Chromatography of P_I on SE-Sephadex C-25

TABLE 2. *Amino acid composition of P_{Ib}*

Amino acid residue	Hydrolysis for		Integer value	Val^{29}—Gly^{54} S S Val^{109}—Trp^{123}
	24 hr	72 hr		
Lysine	1.92	1.92	2	2
Histidine	0	0	0	0
Ammonia	5.42	5.67		
Arginine	3.25	2.88	3	3
Aspartic acid	7.84	7.59	8	8
Threonine	4.34	4.33	4–5	5
Serine	1.85	1.78	2	2
Glutamic acid	3.10	3.05	3	3
Proline	0	0	0	0
Glycine	3.00	3.00	3	3
Alanine	4.73	4.69	5	5
Half-cystine	1.32	1.10	1–2	2
Valine	2.72	2.89	3	3
Methionine	0	0	0	0
Isoleucine	0	0	0	0
Leucine	0	0	0	0
Tyrosine	1.02	1.01	1	1
Phenylalanine	1.78	1.79	2	2
Tryptophan	Nd	Nd		2
Total				41

a Values are expressed as molar ratios, assuming that there are 3 glycines per mole of P_{Ib}.
b Moles of amino acids in the corresponding regions of HL calculated from the data of Canfield and Liu (1965).

in the absence of urea was unsuccessful. The reason for this is unknown, but P_{Ib} may interact with P_{Ic} in the absence of urea. This requires investigation.

The amino acid composition of P_{Ib} is shown in Table 2. The composition is consistent with that of the region from Val^{29} to Gly^{54} and from Val^{109} to Trp^{123} of HL, based on the data of Canfield and Liu (1965). From the results of amino acid analysis the minimum molecular weight of P_{Ib} was calculated as 4,600.

HL was split at Val^{29}, Ile^{55}, Val^{109} and Ile^{124} residues and these points are completely consistent with the reported substrate specificities of thermolysin (Matsubara 1970). But this peptide is obviously an intermediate in thermolytic digestion of HL, because P_{Ib} still contains three susceptible bonds, at Phe^{34}, Phe^{38} and Val^{120}. The yield of P_{Ib} was rather low. Moreover, P_{Ba-1}, P_{Ba-2} and P_{Ka}, which were partly the constituents of P_{Ib}, were obtained from the same thermolytic digest of HL. P_{Ba-1} is the product of P_{Ib} formed on splitting the peptide at Phe^{34}, P_{Ba-2} is produced by splitting HL at the Phe^{38} and Ile^{58} residues and P_{Ka} is the product formed from P_{Ib} by splitting at the Phe^{38} residue. This peptide is described in detail in the next section.

The sequence of P_{Ib} confirmed by amino acid analyses of the two peptides obtained by reduction and alkylation of P_{Ib}. Four mg of P_{Ib} were reduced and alkylated as described in

TABLE 3. *The amino acid compositions of two peptides obtained from reduced and alkylated P_{Ib}.*

Amino acid	P_{Ib-1} [a]	Val^{29}–Gly^{54} [b]	P_{Ib-2} [a]	Val^{109}–Trp^{123} [b]
Lysine	0.93	1	0.85	1
Histidine	0	0	0	0
Ammonia	4.13		1.95	
Arginine	0.89	1	1.74	2
CM-Cysteine	0.88	1	0.96	1
Aspartic acid	5.85	6	2.03	2
Threonine	3.67	4	0.96	1
Serine	1.90	2	0	0
Glutamic acid	2.06	2	0.99	1
Proline	0	0	0	0
Glycine	2.00	2	1.00	1
Alanine	2.85	3	1.76	2
Valine	0.81	1	1.74	2
Methionine	0	0	0	0
Isoleucine	0	0	0	0
Leucine	0	0	0	0
Tyrosine	0.75	1	0	0
Phenylalanine	1.91	2	0	0
Tryptophan	Nd	0	Nd	0
Total		26		15

a Values are expressed as molar ratios, assuming two glycine per mole of P_{Ib-1} and one glycine per mole of P_{Ib-2}.
b Moles of amino acid in the corresponding regions of HL were calculated from the data of Canfield and Liu (1965).

the "Materials and Methods" and separated into two fractions on a Sephadex G-25 column (3×110 cm) eluted with 50% acetic acid. Amino acid analyses of the two peptides showed that the fraction eluted first is P_{Ib-1} and that eluted second is P_{Ib-2}, are shown in Table 3. The amino acid composition of P_{Ib-1} is consistent with that of the larger peptide of P_{Ib} and the amino acid composition of P_{Ib-2} is consistent with that of the smaller peptide of P_{Ib}. Therefore, it is concluded that P_{Ib} is derived from the 29–54 and 109–123 sequences of HL and the two peptides are linked by a single S–S bond (Cys^{30}-Cys^{115}).

Preliminary studies on the amino acids in P_{Ic} indicated that P_{Ic} may be derived from the region from Ile^{58} to Lys^{97} of HL and may have some defect within the loop region (Cys^{80}-Cys^{94}). But the exact localization of this peptide is still under investigation.

The homogeneities of P_{Ib} and P_{Ic} were also tested by high-voltage paper electrophoresis and results are shown in Figure 7. Both peptides gave a single spot.

3) Fraction P_K

Fraction P_K obtained from a SE-Sephadex C-25 column was desalted using IRC-50 resin and lyophilized. The dried material (158 mg) was dissolved into 20 ml of 0.2 M sodium acetate buffer pH 5.0, containing 6 M deionized urea, and applied to a SE-Sephadex C-25 column (1.5×40 cm). The peptide was separated by gradient elution achieved with 1 liter of 0.2 M sodium acetate buffer, pH 5.0, containing 6 M deionized urea, and 1 liter of 0.2 M

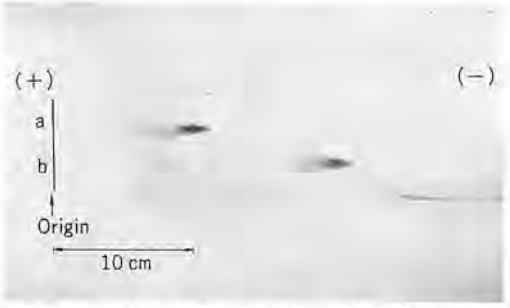


FIGURE 7. *High-voltage paper electrophoresis of P_{Ib} and P_{Ic}. Electrophoresis was carried out in pyridine-acetate buffer, pH 6.5. 200 μg of each peptide were applied to Toyo filter paper 51 A (10 × 60 cm) and 50 v per cm was applied for one hr and 35 v per cm applied for another hr. Peptides were located with ninhydrin stain. a: P_{Ib}; b, P_{Ic}.*

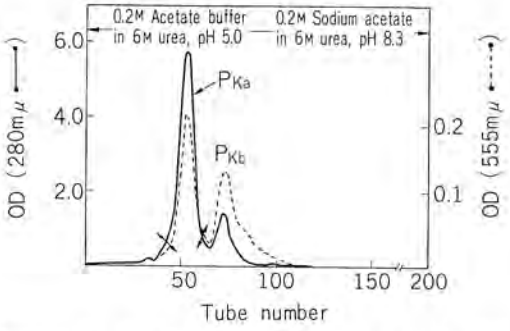


FIGURE 8. *SE-Sephadex C-25 chromatography (1.5 × 40 cm) of P_K in the presence of 6 M urea at 25 C. A gradient was formed using 1 liter of 0.2 M sodium acetate buffer, 6 M urea, pH 5.0 and 1 liter of 0.2 M sodium acetate, 6 M urea, pH 8.3. 10 g fractions were collected. The peptides were located by measuring the optical density at 280 mμ and also by the micro-biuret reaction.*

sodium acetate, pH 8.3, containing 6 M deionized urea. Chromatography was carried out at 25 C and 10 g fractions were collected. The peptides were located by their OD at 280 mμ and also by submitting 200 μl of each fraction to the micro-biuret reaction. The first major peak was named P_{Ka} and the second, P_{Kb}. Figure 8 shows the results. Both fractions were desalted and purified by Bio-Gel P-10 chromatography, like the P_I peptides

FIGURE 9. *High-voltage paper electrophoresis of P_K in pyridine-acetate buffer, pH 6.5. 200 μg of P_{Ka} were applied. Electrophoresis was carried out by applying 50 v/cm for one hr and 35 v/cm for another hr. The peptides were located by the ninhydrin reaction (showed in this figure) and later with peptide stain.*

The yields of P_{Ka} and P_{Kb} from 5 g of HL were. 72 mg and 64 mg, respectively.

The homogeneity of each peptide was checked by high voltage paper electrophoresis at pH 3.6 and at pH 6.5 using pyridine-acetate buffer. The electrophoretic pattern of P_{Ka} is shown in Figure 9. P_{Ka} gave a large main spot and an additional very faint spot with ninhydrin stain.

The amino acid composition of P_{Ka} is given in Table 1. The major fraction in the preparation of P_{Ka} is a peptide derived from regions Val^{29}-Asn^{37} and Val^{109}-Trp^{123}, and the two peptides are connected by a single S-S bond (Cys^{30}-Cys^{115}). In this particular preparation this major fraction constituted approximately 80% of the whole, judging from the results of amino acid analyses and high-voltage electrophoresis. This preparation was used as P_{Ka} in following experiments without further purification.

3. *Immunological activities of thermolytic peptides*

1) Inhibitory activities of peptides in turbidimetric assay

Preliminary studies were made on the immunological activities of the peptide fractions obtained by SE-Sephadex chromatograrphy of the thermolytic digest of HL. Each peptide

in ten to thirty fold excess over HL on a weight basis was put into a micro-cuvette containing 200 μl of the specifically purified rabbit anti-HL antibody (150 μg) and 50 μl of 0.1 M EDTA. The final volume was adjusted to 450 μl with PBS, pH 6.0. As a control, a cuvette was set up with PBS, pH 6.0 in place of peptide solution. Both cuvettes were incubated at 25 C for 30 min. Equal amounts of HL (6.44 μg in 50 μl) were added to these cuvettes and the turbidities of both samples were followed as a function of time at 25 C at 570 mμ for at least one hour.

P_B, P_I, P_K and P_M inhibited turbidity formation by HL and purified rabbit anti-HL antibody.

2) Preparation of P_{Ib} specific antibody

The results of amino acid analyses of thermolytic peptides indicate that P_{Ib} is derived from a completely different region from those of P-8 (Shinka et al., 1967) and P-17 (Fujio et al., 1968 a, b). Moreover P_{Ib} contained the catalytic site of HL (Glu^{35} and Asp^{52}). Therefore, it seemed extraordinarily interesting to see the immunological properties of this antibody fraction which can interact with P_{Ib}. Six mg of P_{Ib} per g of Sepharose were coupled at pH 7.0 (Omenn et al., 1970 and Fujio et al., 1971). Some difficulties arose in the process of the purification of P_{Ib} specific antibody (anti-P_{Ib}) from rabbit anti-HL antibody. When rabbit anti-HL antiserum was passed through the immunoadsorbents and the antibody protein was eluted with 0.1 N acetic acid, the purity of the antibody preparation decreased to approximately 60% of that estimated from the binding of antibody with ^{125}I-HL. Next, specifically purified anti-HL antibody was applied to the immunoadsorbent and the antibody was eluted with 0.1 N acetic acid. But again nonspecific adsorption to the immunoadsorbent was noticed. A minute amount of anti- 8 antibody (the specificity of P-8 is directed to the sequence 57–107) can be detected by the bindng experiment and the antibody preparation gave a precipitin reaction when mixed with HL. Therefore, the antibody preparation in the following experiments was prepared by applying the specifically purified anti-HL antibody and elution of the anti-P_{Ib} antibody was carried out with a solution of P_{Ib} (3.5×10^{-3} M) as described in our previous paper (Fujio et al., 1971). The details were as follows.

P_{Ib} (40 mg) was coupled to 6 g (wet weight) of Sephrose-4B (approximately 6 mg of peptide per g of Sepharose). The immunoadsorbent was poured into a column (1 cm diameter, fitted with a sintered glass filter). Then 400 mg of purified anti-HL antibody in DPS, pH 8.0 were passed through the immunoadsorbent at 4 C and the column was washed with chilled DPS-EDTA. When the optical density of the washing fluid became less than 0.01, the column was placed at 37 C. Then 0.5 ml of P_{Ib} in DPS, pH 8.0 (3.5×10^{-3} M) was placed on the top of the column and the column was eluted with the same buffer at a rate of 5 ml per hr at 37 C. The antibody protein in each fraction was located by the Ouchterlony test using goat anti-rabbit-IgG antiserum. The fractions which gave a positive reaction in the Ouchterlony test were pooled and concentrated by negative pressure dialysis using a Visking tube (8/32).

The concentrated antibody solution was diluted to 100 ml with DPS, pH 8.0 and the last procedure was repeated. More than 90% of the P_{Ib} was removed by this procedure. The residual peptide in the antibody preparation was removed by gel filtration on a Sephadex G-150 column in DPS-EDTA, pH 8.0. Approximately 85% of the total optical density was eluted at the position of 7S-IgG. The fraction of 7S was again concentrated by negative pressure dialysis. The capacity of this immunoadsorbent was calculated as approximately 26 mg of anti-P_{Ib} antibody. The content of anti-P_{Ib} antibody in the rabbit anti-HL antiserum p # 44 was estimated as 200 μg per ml using immunoadsorbent with elution by peptide.

The above method of purification was satisfactory, since the resultant antibody preparation did not give any precipitin reaction when

mixed with HL over a wide range of ratios of HL to antibody and the extrapolated value of "r" was close to two, as reported in the next section. The content of anti-P_{Ib} antibody in rabbit anti-HL antiserum seems to be relatively low and was approximately 5% of the total precipitable antibody in the pooled rabbit anti-HL antiserum (p#44).

3) Binding of rabbit anti-P_{Ib} antibody with ^{125}I-HL and its inhibition by structurally related peptides.

P_{Ib} can interact with some of the population of rabbit anti-HL antibodies, because P_{Ib}-Sepharose immunoadsorbent can adsorb some anti-HL antibodies and this antibody protein can be eluted by P_{Ib}. Equilibrium dialysis of anti-P_{Ib} antibody with ^{125}I-HL was carried out to obtain more exact information on the binding properties of rabbit anti-P_{Ib} antidoby. The results of equilibrium dialysis are shown as a Scatchard plot of r/c against r, in Figure 10 in which r represents the moles of HL bound per mole of antibody and c represents the concentration of free ^{125}I-HL. The extrapolated value of r is approximately two. This suggests that anti-P_{Ib} antibody is more than 90% pure. The experimental points on the Scatchard plot show an essentially linear relationship, suggesting that the affinity of rabbit anti-P_{Ib} antibody is relatively homogeneous. A similar phenomenon was also found with another peptide reactive antibody fraction of the anti-HL antibody population (Fujio et al., 1968 a; Fujio et al., 1971; Sakato et al., 1971). The K_A of the binding of rabbit anti-P_{Ib} antibody was evaluated as 1.84×10^5 M^{-1}.

Next the specificity of rabbit anti-P_{Ib} antibody was studied in more detail. The inhibitory activities of various kinds of peptides, which are or are not structurally related to P_{Ib}, were tested against the bindings of ^{125}I-HL with anti-P_{Ib} antibody. The various peptides of HL used in the inhibition experiment are listed in Table 4. Various concentrations (5×10^{-5} to 7×10^{-4} M: final concentration) of peptides in PBS, pH 6.0 were mixed with 5.4×10^{-5} M (final concentration) of ^{125}I-HL and the

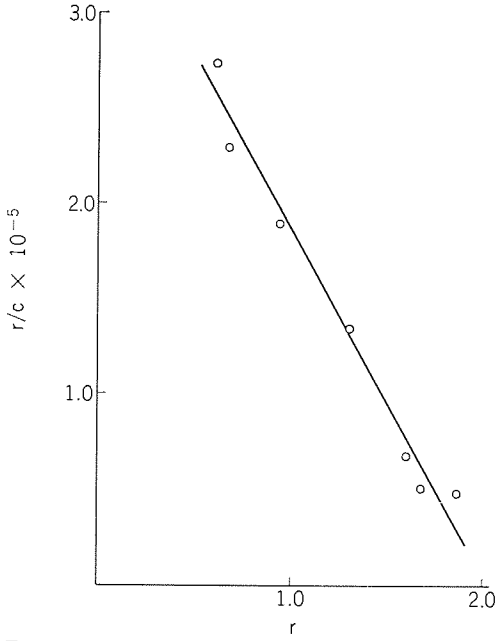


FIGURE 10. *Binding of purified rabbit anti P_{Ib} antibody with ^{125}I-HL at 10 C. The experimental values are shown as a Scatchard plot. Anti-P_{Ib} antibody (2.93 mg/ml) in PBS, pH 6.0 was dialyzed against ^{125}I-HL solution in the same buffer for 65 hr. r: moles of ^{125}I-HL bound per mole of antibody, taking the molecular weight of antibody as 150,000 and that of HL as 14,300, c: concentration of free ^{125}I-HL (M).*

mixtures were dialyzed against purified anti-P_{Ib} antibody (1.96×10^{-5} M) at 10 C. The K_I values were calculated by the method of Karush (1956) and listed in Table 5.

The inhibitory effect of unlabelled HL was also tested to see the effect of labelling on the binding activity with anti-P_{Ib} antibody. The K_I value of unlabelled HL was 1.56×10^5 and was consistent with the K_A value (1.84×10^5) of ^{125}I-HL obtained by direct binding of ^{125}I-HL with anti-P_{Ib} antibody. This indicates that iodine labelling of HL has essentially no effect on the binding activity of HL with anti-P_{Ib} antibody.

The K_I values of P_{Ib}, P_{Ba-1} and P_{Ba-2} are very similar to each other. The K_I value of P_{Ib} was approximately one tenth of that of HL and was very similar with those of P_{Ba-1} and

TABLE 4. *Peptides for binding of ^{125}I-HL with anti-P_{Ib} antibody*

Peptide	Sequence	Molecular weight[a]
P_{Ib}	Val^{29}—Gly^{54} / S / S / Val^{109}—Trp^{123}	4,600
P_{Ba-1}	Phe^{34}—Gly^{54}	2,235
P_{Ba-2}	Phe^{38}—Gln^{57}	2,217
P_{Ka}	Val^{29}—Asn^{37} / S / S / Val^{109}—Trp^{123}	2,552
P_{Ic}	(Ile^{58}—Lys^{97})	—
P_8[b]	Gln^{57}—Ala^{107}	5,437
P_{17}[b]	Lys^{1}—Asn^{27} / S / S / Ala^{122}—Leu^{129}	4,000

a The minimum molecular weight of each peptide was calculated from the results of amino acid analysis.
b Peptides obtained by peptic digestion of HL (Shinka et al., 1967; Fujio et al., 1968a).

P_{Ba-2}. In addition, P_{Ka}, which constitutes a part of P_{Ib} other than P_{Ba-2}, did not cause any detectable inhibition of the binding of ^{125}I-HL with anti-P_{Ib} antibody. These facts suggest that the specificity of anti-P_{Ib} antibody is mainly directed to the region from Phe^{38} to Gly^{54} of HL. The difference in the K_I values of P_{Ib} and HL could be due to a conformational change occurring during isolation of the peptide from the rest of the HL moleculer or coulod be be due to the defect of an additional binding site on the rest of the molecule other than P_{Ib}.

The specificity of the binding activity of anti-P_{Ib} antibody was further proved by its non-reactivity with P_8 (sequence 57–107) and P_{17} (sequence 1–27 and 122–129) which constitute different immunodominant regions of HL from P_{Ib}. P_{Ic}, which seems to be derived

TABLE 5. *Inhibition of the binding of ^{125}I-HL to rabbit anti-P_{Ib} antibody by various peptides*

Inhibitor	K_I[a] (*l/mole*)
HL[b]	1.56×10^5
P_{Ib}	1.10×10^4
P_{Ba-1}	1.41×10^4
P_{Ba-2}	1.41×10^4
P_{Ka}	$<5\times10^2$
P_{Ic}	$<5\times10^2$
P_8	$<5\times10^2$
P_{17}	$<5\times10^2$

a Association constant of inhibitor,

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

K_A of the binding of anti-P_{Ib} antibody with ^{125}I-HL was $1.84\times10^5\ M^{-1}$; r, moles of antigen bound per mole of antibody in the absence of inhibitor; r', moles of antigen bound per mole of antibody in the presence of inhibitor; (i), equilibrium concentration of inhibitor.
b Unlabelled hen egg-white lysozyme.

from a somewhat similar region to P_8, also showed no inhibition at the highest concentration tested. Therefore, it may be concluded that sequence 38 to 54 is the third antigenic site in the HL molecule and its specificity is independent from those of P_8 (sequence 57–107) and P_{17} (sequence 1–27 and 122–129).

4) Neutralization of the enzymic activity of HL by anti-P_{Ib} antibody

The results of equilibrium dialysis indicate that the specificity of anti P_{Ib} antibody is mainly directed to the sequence 38–54 which contains one of the amino acid residue (Asp^{52}) forming the catalytic site of HL and is also located close to the other amino acid residue (Glu^{35}) of the catalytic site. Thus, it seemed interesting to see the efficiency of neutralization of the enzymic activity of HL by this antibody fraction.

Accordingly a constant amount (4.3 μg) of HL was mixed with increasing amounts of purified anti-P_{Ib} antibody and an equal volume of *M. lysodeikticus* was added to the mixture. The solvent was PBS, pH 6.0. Reduction in optical density at 540 mμ was measured at

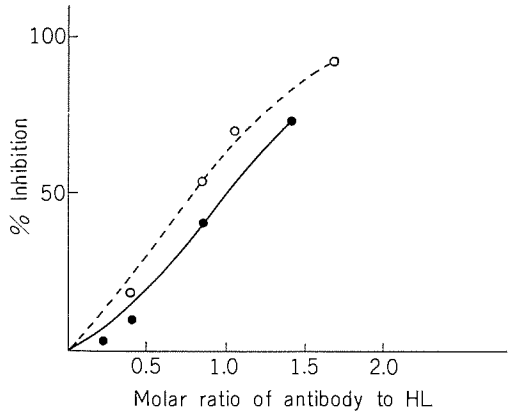


FIGURE 11. *Neutralization of the enzymic activity of HL by increasing amounts of anti-P_{Ib} antibody. M. lysodeikticus was used as substrate. HL solution (4.3 μg) in PBS, pH 6.0 was mixed with various amounts (15 to 100 μg) of anti-P_{Ib} or anti-HL antibody. The percent inhibition of the enzymic activity was plotted against the molar ratios of antibody to HL taking the molecular weight of antibody as 150,000 and that of HL as 14,300.* ●——●, *anti-P_{Ib} antibody;* ○——○, *anti-HL antibody.*

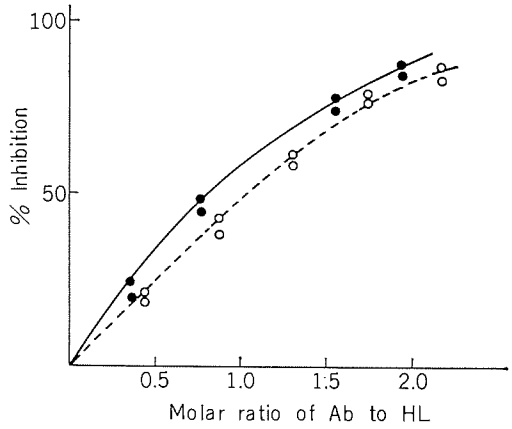


FIGURE 12. *Neutralization of the enzymic activity of HL by increasing amounts of anti-P_{Ib} antibody. Chitohexaitol (Imanishi et al., 1969a, b) was used as substrate. HL solution (4.3 μg) in PBS, pH 6.0 was mixed with various amounts (15 to 100 μg) of anti-P_{Ib} or anti-HL antibody. The per cent inhibition of the enzymic activity was plotted against the molar ratio of antibody to HL taking the molecular weight of antibody as 150,000 and that of HL as 14,300.* ●——●, *anti-P_{Ib} antibody;* ○——○, *anti-HL antibody.*

37 C for 5 min as described previously (Fujio et al., 1968 a). Figure 11 depicts the neutralization of the enzymic activity of HL by increasing amounts of anti-P_{Ib} antibody with *M. lysodeikticus* as substrate. For comparison, the neutralizing activity of purified rabbit anti-HL antibody from which anti-P_{Ib} antibody was prepared was also tested and results are plotted in Figure 11. The efficiency of the neutralizing activity of anti-P_{Ib} antibody was almost the same as that of anti-HL antibody.

Figure 12 shows results on the neutralization of the enzymic activity of HL by increasing amounts of anti-P_{Ib} antibody with chitohexaitol (Imanishi et al., 1969 b) as substrate. Again the efficiency of neutralization of enzymic activity of HL by anti-P_{Ib} antibody was high and was comparable with that by the whole population of anti-HL antibody. As reported in our previous paper (Fujio et al., 1971), a difference in the efficiency of neutralization by antibody fractions with different specificities was always remarkable when a small sized substrate was used. Thus the effect of anti-P_{Ib} antibody in neutralization was in great contrast to that of anti-17 antibody (Fujio et al., 1971) which does not inhibit the enzymic activity of HL with the small sized substrate.

These results are consistent with those of equilibrium dialysis experiments which indicate that the specificity of anti-P_{Ib} antibody may be directed to a region close to the catalytic site of HL.

DISCUSSION

In our previous reports (Imanishi et al., 1969; Fujio et al., 1971), a direct correlation between the efficiency in neutralization of enzymic activity by the antibody of an enzyme and the specificity of each antibody population was emphasized. That is, an antibody with specificity directed to the binding site or the catalytic site of an enzyme may be most important in neutralization of an enzyme, especially when the size of the substrate is small. When consider-

ing the possibility that a rabbit can produce an antibody population with specificity directed to the binding site or catalytic site of HL, the structural similarity or dissimilarity between the active centers of HL and rabbit lysozyme may become a most important factor. From this point of view, a comparison in the primary structures of HL and human leukemia lysozyme (Canfield et al., 1971) is very suggestive. According to Canfield, 77 of the 129 amino acid residues in the two lysozymes are identical, including four disulphide bonds. However, the amino acids in the region close to the catalytic site differ considerably. For example, in the sequence from 34 to 54, 7 of the amino acids in HL differ from those in human lysozyme and there is one insertion. Therefore, it seems very likely that an antibody could be made with specificity directed to the region close to the catalytic site of HL. In any event, our results strongly suggested that the sequence 38–54 is one of the antigenic sites in the HL molecule in rabbits. In addition, the antibody population with specificity directed to sequence 38–54 strongly inhibited the enzymic activity of HL for both large and small sized substrates, as can be expected from its specificity.

The mechanism of neutralization of the enzymic activity of HL by a whole population of anti-HL antibodies must be complex. In addition to anti-P_{Ib} antibody, antibody fractions with specificity directed to the substrate binding sites of HL have been reported (Imanishi et al., 1969 a) and these antibody fractions were shown to be very inhibitory against the enzymic activity of HL with a small sized substrate. On the contrary, anti-17 antibody is known to have no inhibiting activity on the *N*-acetyl-glucosaminidase activity of HL (Fujio et al., 1971). Therefore, to understand the neutralization of enzymic activity by the whole population of anti-HL antibodies it is first necessary to evaluate the contents of each region specific antibody in the total antibody population. Such studies are in progress. At present, only the level of anti-P_{Ib} antibody in the pooled rabbit anti-HL antiserum (P #44) has been evaluatated as approximately 5% of the total precipitable antibody.

Another interesting point about this antigenic site is that the region includes the antiparallel β-structure (sequence 41–54) of HL (Hamaguchi et al., 1964; Hamaguchi, 1964; Phillips, 1967).

Finally it must be mentioned that this work shows that thermolysin is a very good protease to degrade protein into immunologically active peptides. It is especially usefull for rigid proteins with many intramolecular disulphide bonds because it is very stable at elevated temperature.

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