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HIGHLY INHIBITORY ANTIBODY FRACTION AGAINST ENZYMIC ACTIVITY OF EGG WHITE LYSOZYME ON A SMALL SUBSTRATE

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SUMMARY A highly inhibitory antibody fraction against the enzymic activity of hen egg white lysozyme (HL) was isolated by dissociation of the HL-anti HL complex with tri-N-acetylglucosamine. Recovery of this antibody was approximately 7 to 8% of the total precipitable antibody in HL antiserum. It completely inhibited the enzymic activity of HL on a small substrate (reduced penta-, and hexa-N-acetylglucosamine) when added at a molar ratio of antibody to enzyme of less than 2 : 1 in the presence of about 700 molar excess of substrate over enzyme. Kinetic studies of the inhibition suggest that this antibody fraction contained an antibody which behaves as a competitive inhibitor of the enzymic actions of HL and duck egg white lysozyme (DL) on small substrates.

DL did not give any precipitin reaction with this antibody fraction. The specificity of the highly inhibitory antibody fraction was related to neither peptide 7a (Glu^{57}–Ala^{107}) nor peptide 17 (the peptide located at the N- and C-terminal region of HL). The significance of these findings is discussed.

INTRODUCTION

The mode of inhibition of an enzyme by its antibody may be very complex, because an enzyme protein can generally combine with two or more antibody molecules with different specificities. On the other hand, studies on the structures and functions of enzymes suggest that relatively restricted areas of enzyme molecules are responsible for their functions (Phillips, 1967 and Hille et al., 1967). Therefore, the role of an antibody molecule in inhibition of the catalytic activity of an enzyme may depend largely on the specificity of the antibody molecule.

Two immunologically active peptides were isolated from hen egg white lysozyme (HL) and were shoun to be two distinct antigenic determinants of HL (Shinka et al., 1967, Fujio et al., 1968a and Fujio et al., 1968b). Two specific antibody fractions were isolated using immunoadsorbent and each could only react with one of the active peptides described above. These two antibody fractions differed in their inhibitions of the catalytic activity of HL (Imanishi et al., 1968). This again emphasizes the significance of antibody specificity in neutralization of the catalytic activity of an enzyme

by the corresponding antibody.

Recently, Rupley established methods for isolating chitin oliogosaccharides for use as small sized substrates for lysozyme (1964). Phillips (1967) applied X-ray crystallographic analysis to the HL-chitin oligosaccharide complex and suggested that a relatively restricted area of the HL molecule is responsible for its enzymic activity. Therefore, if there are any antibodies with specificities directed to the binding site of substrate, to the catalytic site of the enzyme molecule and/or to areas close to these, these antibodies would be dissociated from the enzyme-antibody complex by the substrate itself or by a competitive inhibitor. According to Dahlquist et al. (1966), among the chitin oligosaccharides tested tri-N-acetyl glucosamine (3 NAG) showed the highest association constant to HL and its complex was only degraded very slowly. Therefore, an attempt was made to select a restricted population of antibodies with specificity for the active center of HL. One way to do this was to dissociate the specific precipitates of HL and its rabbit antibodies with 3NAG. Although the degree of inhibition of catalytic activity of an enzyme by its antibody generally tends to be more complete when a substrate of larger size is used (Cinader, 1967), the antibody fraction dissociated by 3NAG strongly inhibits the activity of HL on substrates of low molecular weight. Moreover, inhibition by this antibody fraction seems to be competitive.

MATERIALS AND METHODS

1. *Hen egg white lysozyme (HL)*

Six times recrystalized HL (Lot No. 4112) was purchased from Seikagaku-Kogyo Co. Ltd. HL was purified by SE-Sephadex C-50 (2.3 meq per g., Pharmacia) chromatography in 0.2 M sodium phosphate buffer, pH 7.16 at 25 C. Fractions containing the main peak were pooled and rechromatographed in the same way.

2. *Duck egg white lysozyme (DL)*

DL was purified by SE-Sephadex C-50 column chromatography in 0.2 M sodium phosphate buffer, pH 6.5 at 25 C as described in our previous reports. (Fujio et al., 1968a and Imanishi et al., 1969).

3. *HL antisera*

Rabbits were immunized with HL purified as described in 1, using complete Freund's adjuvant as described previously (Fujio et al., 1968a). Each rabbit received an initial dose of 2 mg of antigen followed by 3 booster injections at 5 weeks intervals. Blood was taken 8, 10 and 12 days after the last injection. The sera from more than 8 rabbits were pooled.

4. *Tri-N-acetyl glucosamine (3NAG)*

Chitin was purchased from Eastman Organic Chemicals Co. 3NAG was prepared from a partial hydrolyzate of chitin with 12 N HCl by charcoal-celite column chromatography and gel filtration on a Sephadex G-25 superfine column as described previously (Rupley, 1964 and Imanishi et al., 1969).

5. *Enzyme assay*

Two kinds of substrate of quite different sizes were used for enzyme assay.

1) *Micrococcus lysodeikticus* as substrate

Micrococcus lysodeikticus was treated with UV-light and lyophilized. Dried cells were kept at —20 C until use. Then 2 ml of 1.2 mg per ml of cell suspension in 0.02 M sodium phosphate, 0.15 M NaCl, pH 6.0 (PBS) were mixed with equal volumes of enzyme solution or enzyme-antibody mixture in PBS at 37 C. The reduction of OD at 540 mμ in the first 5 minutes of incubation was measured as described in our previous report (Shinka et al., 1962). Lytic activities of HL ranging from 0.3 μg to 3 μg per ml can be measured with an error of less than 5%.

2) Reduced penta-, and hexa-N-acetylglucosamine as substrates

Chitin oligosaccharides were prepared by our modification (Imanishi et al., 1969) of the method of Rupley (1964). The crude penta-, and hexa-N-acetylglucosamines, which were eluted from a charcoal-celite column, were reduced with sodium borohydride and purified on a Sephadex G-25 column (superfine). As mentioned in our previous report (Imanishi et al., 1969), if penta-, or hexa-N-acetylglucosamine is used as substrate of lysozyme, the error in the assay may be almost 20%, because there is a high blank due to reducing groups in the substrate itself. Therefore, the reducing ends

of penta- and hexa-N-acetylglucosamines were converted to N-acetylglucosaminitols by reduction (R-5 mer and R-6 mer).

R-5 mer or R-6 mer (300–500 μg) in 0.2 ml of 0.025 M sodium diethylbarbiturate-HCl, 0.1 M NaCl. pH 6.0 (VBS) was mixed with 0.8 ml of enzyme solution or enzyme antibody mixture in the same solvent at 37 C. The increments in reducing power of the reaction mixture were measured by Park and Johnson's method (1949) with appropriate controls.

Using this method 0.5 μg to 6.0 μg of HL activity with a reaction time of 30 minutes with R-6 mer, and 4 hours with R-5 mer as substrate can be measured with an error of less than 5%.

6. *Inhibition of enzymic activity by antibody*

The inhibitory effects of various antibody fractions with either *M. lysodeikticus* or reduced chitin oligosaccharides as substrate were measured.

Increasing amounts of antibody fraction were added to a constant amount of enzyme and the volume was adjusted as described in the section on "enzyme assay." A constant amount of substrate was added to the enzyme-antibody mixture after incubation at 37 C for 30 minutes. The residual activity was measured at 37 C for 5 minutes in the case of *M. lysodeikticus*, for 30 minutes in the case of R-6 mer and for 4 hours in the case of R-5 mer. The decrease in catalytic activity of the enzyme due to the antibody was expressed as a percentage of the catalytic activity of the enzyme in the absence of antibody.

Kinetic studies on the three components system were carried out, adding the enzyme solution after preincubation of antibody with substrate. In the Lineweaver Burk plot and also in the method of Hunter and Downs, each point is the mean from triplicate experiments and is plotted by the method of least squares.

7. *Preparation of ^{125}I labelled hen egg white lysozyme (^{125}I-HL)*

HL was iodinated with triiodide solution (0.046 M I_3 in 0.14 M KI) by the method of Covelli et al. (1966). Iodination was chosen to avoid the formation of diiodo-tyrosine. Triiodide solution was titrated with standardized $Na_2S_2O_3$ solution using soluble starch as an indicator.

Cold triiodide solution ($I_2 = 2.1 \times 10^{-6}$ moles) was mixed with 500 μc of Na ^{125}I free from reducing agents. The next morning, the mixture was added to HL solution (29.8 mg in 3 ml of 0.2 M sodium phosphate buffer, pH 8.0). The molar ratio of I_2 to HL was kept at 1 : 1. The reaction mixture was stirred at 0 C for 60 minutes. The labelled HL was dialyzed against deionized water for 2 days in a cold room changing the deionized water twice a day. Finally the trace of free ^{125}I present was removed by passage through Sephadex G-25 column. The mean value for I atoms per mole of HL was approximately 0.2.

8. *Equilibrium dialysis using [1–^{14}C] acetyl-peptide*

Two kinds of immunologically active peptide, one corresponding to the region of Glu^{57}-Ala^{107} (peptide 7a) and the other to that of Lys^{1}-Asn^{27} linked to Ala^{122}-Leu^{129} by Cys^{6}-Cys^{127} (peptide 17) of HL, were prepared from the peptic digest of HL as described in our previous reports (Shinka et al., 1967 and Fujio et al., 1968 a). Both peptides were acetylated with [1-^{14}C] acetic anhydride. The specific activity of the [1-^{14}C] acetylated peptide 7a was 265 count/min per μg and that of peptide 17 was 466 count/min per μg. Equilibrium dialysis was performed at 10 C for 36 hours in PBS (pH 6.0). The details of the procedure were described in our previous reports (Fujio et al., 1968a and 1968b).

9. *Immunoadsorbent*

Bromoacetylcellulose was prepared according to the method of Robbins et al. (1967). Approximately 90 mg of peptide 17 were coupled to 400 mg of bromoacetylcellulose. The resulting immunoadsorbent could remove 30 mg of anti-peptide 17 antibody.

10. *Analytical ultracentrifugation*

The sedimentation velocity of the antibody prepreation was examined with a Hitachi analytical ultracentrifuge at 47,000 rev/min, and 20 C. The solvent was PBS, pH 6.0.

RESULTS

1. *Dissociation of an antibody fraction from the HL-anti-HL complex by 3 NAG.*

An equivalent amount of ^{125}I-HL (11.7 mg), calculated from the results of a preliminary quantitative precipitin reaction, was added to 90 ml of HL antiserum #P32. The reaction

mixture was incubated at 37 C with gentle shaking for 1 hour and then kept at 4 C for 24 hours. The resulting specific precipitate was separated by centrifugation at 3,000 rev/min for 30 minutes at 4 C. This precipitate was washed 3 times with 40 ml volume of chilled PBS and further washed by stirring it with 40 ml of PBS at 37 C for one hour and then centrifuging the mixture at 25 C. The latter treatment was repeated 2 or 3 times. The loss of antibody protein during washing at 37 C was usually less than 2.5% of the total precipitable antibody and the total antibody protein in the final washing fluid was less than 1% of this.

Then 40 ml of 3 NAG solution (1.7×10^{-2} M) in PBS were added to the thoroughly washed specific precipitate. The suspension was stirred at 37 C for one hour and centrifuged at 3,000 rev/min for 30 minutes at room temperature. This procedure was repeated twice more and the supernatants were pooled. In this way 18.5 mg of antibody protein and 0.47 mg of ^{125}I-HL were dissociated into the supernatant, as calculated from measurement of the OD at 280 mμ and the radioactivity of ^{125}I. The supernatant fluid was concentrated by ultrafiltration, during which almost all the 3 NAG added dialyzed out. Acetic acid was added to the concentrated antibody solution to a concentration of 0.1 N. The antibody fraction was separated from ^{125}I-HL and from 3 NAG by passage through a Sephadex G-150 column (3×75 cm) saturated with 0.1 N acetic acid. The gel filtration pattern is shown in Fig. 1.

The antibody fraction dissociated by 3 NAG (diss. Ab-A) represented 7 to 8% of the total precipitable antibody in the original serum. Measurement of sedimentation velocity showed that diss. Ab-A is a so called 7S-class γ-globulin.

The residual specific precipitate, which was not dissociated by 3 NAG, was dissolved in 0.1 N acetic acid and the antibody fraction was separated on a Sephadex G-150 column as described previously (Fujio et al., 1968a). The 7S fraction (resd. Ab-A) was collected and extensively dialyzed against PBS, pH 6.0.

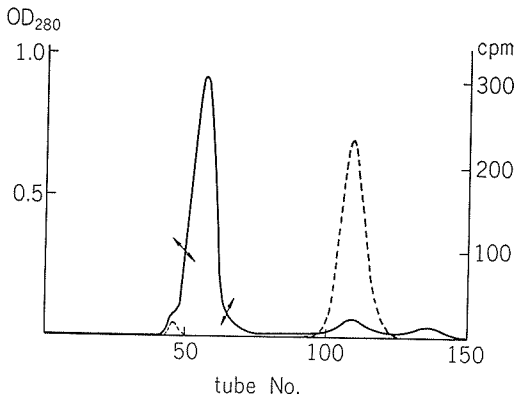

FIGURE 1. *Dissociation of antibody protein from antigen on a Sephadex G-150 column.*

A mixture of ^{125}I-HL (0.47 mg) and antibody (18.5 mg) in 10 ml of 0.1 N acetic acid was applied to a Sephadex G-150 column (3 × 75 cm) saturated with 0.1N acetic acid and the column was operated at 25C. The column was eluted with the same solvent and 4 gr fractions were collected.

——— *OD at 280 mμ*

---------- *Radioactivity*

2. *Inhibitions of HL activity by the two fractions of antibody*

The efficiencies of inhibition of the catalytic activity of HL by the two fractions of antibody were measured using substrates of both high and low molecular weight.

Fig. 2 shows the inhibitions by diss.Ab-A and resd.Ab-A of HL activity using *M. lysodeikticus* as substrate. The two antibody fractions inhibited HL activity equally. Complete inhibition was obsereved with a molar ratio of antibody to HL of less than 2 : 1.

On the other hand, there was a great difference in the inhibitions by the two antibody fractions with R-5 mer as substrate.

Fig. 3 shows that diss.Ab-A caused 100% inhibition at a molar ratio of antibody to HL of less than 2 : 1, while at the same molar ratio and with even more antibody resd. Ab-A caused only 50% inhibition.

A similar experiment with R-6 mer as substrate gave essentially the same results.

Two immunologically active peptides were

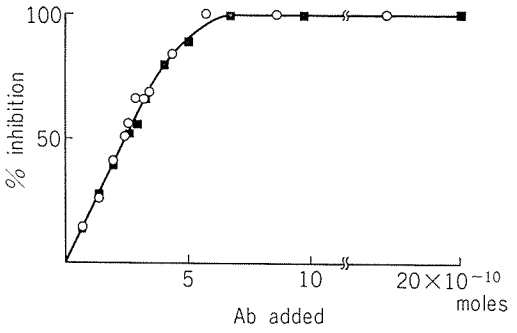

FIGURE 2. *Inhibition of HL activity on M. lysodeikticus by:* (○———○) *and resd.Ab-A* (■———■). *A constant amount of HL* (3.5×10^{-10} *moles) was mixed with various amounts of antibodies.*

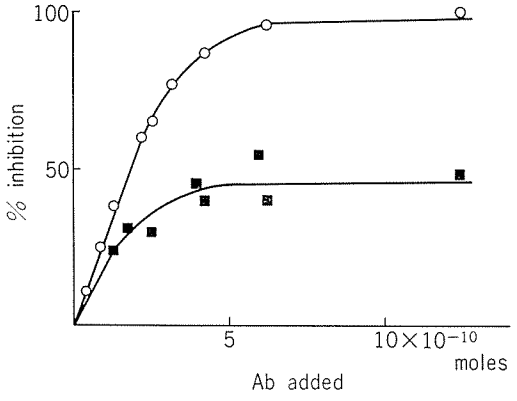

FIGURE 3. *Inhibition of HL activity on a small substrate* (2.4×10^{-7} *moles of R-5 mer) by: diss.Ab-A* (○———○) *and resd.Ab-A* (■———■). *A constant amount of HL* (3.5×10^{-10} *moles) was mixed with various amounts of antibodies.*

isolated from HL (Shinka et al., 1967 and Fujio et al., 1968a), so it was interesting to study the specificity of diss.Ab-A with respect to its reactivity with them. The bindings of [1−^{14}C] acetyl-peptide 7a and -peptide 17 by diss. Ab-A were measured by equilibrium dialysis as described earlier. Approximately 1.0 mg of diss.Ab-A per cell was used and dialysis was continued for 36 hours at 10 C. The [1−^{14}C] acetyl-peptide 7a did not manifest any detectable binding with diss.Ab-A, but the [1−^{14}C] acetyl-peptide 17 bound a fraction of antibody in diss. Ab-A. The content of anti-peptide 17 antibody in diss.Ab-A was evaluated as 10 to 15% from the maximal amounts of bound peptide 17, taking the valency of antibody as two. The results imply that diss.Ab-A consists of at least two kinds of antibody differing in specificity. This is also consistent with the fact that diss.Ab-A still manifests a precipitin reaction on addition of HL. But a maximum of only 60% of antibody protein can be precipitated by HL.

3. *Removal of anti-peptide 17 antibody from the highly inhibitory antibody fraction*

It seems unlikely that the strong inhibition of the catalytic activity of HL by diss.Ab-A is due to the presence of anti-peptide 17 antibody in it, because peptide 17 does not contain any structures responsible for binding or catalytic activity of HL on a substrate of low molecular weight (Johnson et al., 1965, Phillips, 1966, Blake et al., 1967 and Phillips, 1967). Therefore, antipeptide 17 antibody was removed by treating HL antiserum with peptide 17-immunoadsorbent. The amount of antibody in the original HL antiserum precipitated by HL was 3.27 mg/ml, and after adsorption it was 1.96 mg/ml. Specific precipitates were made by adding HL to the adsorbed antiserum. The antibody fraction (diss.Ab-B) dissociated by 3 NAG was prepared as described in a previous section. The residual antibody fraction (resd. Ab-B) was also prepared from the residual specific precipitate after treatment with 3 NAG. Fig. 4 illustrates the inhibition of diss.Ab-B on HL and DL activities with *M. lysodeikticus* as substrate.

The results of similar experiment using R-6 mer as substrate are shown in Fig. 5.

The efficiencies of inhibition of HL activity with substrates of high (*M. lysodeikticus*), and low (R-6mer) molecular weight by diss.Ab-B were quite similar to that of diss.Ab-A Thus, the inhibitory effect of an antibody fraction dissociated by 3 NAG is not altered by removal of anti-peptide 17 antibody. From these results

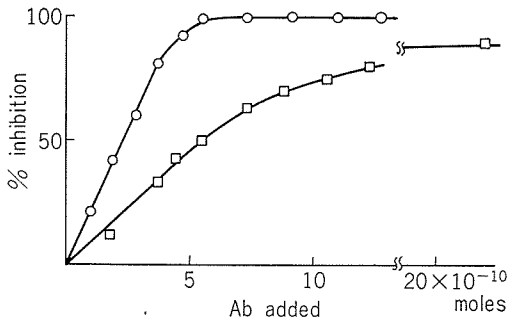

FIGURE 4. *Inhibition of HL* (○———○) *and DL* (□———□) *activities on M. lysodeikticus by diss. Ab-B (prepared after absorption by peptide 17). The amount of enzymes in the system was* 3.5×10^{-10} *moles.*

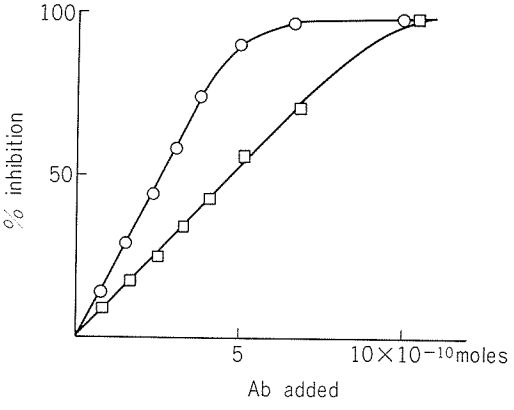

FIGURE 5. *Inhibition of HL* (○———○) *and DL* (□———□) *activities on a small substrate (*3.64×10^{-7} *moles of R-6 mer) by diss.Ab-B (prepared after absorption by peptide 17). The amount of enzymes in the system was* 3.5×10^{-10} *moles.*

and also from those on binding by peptide, it is clear that the specificity of the highly inhibitory antibody fraction was correlated neither with peptide 7a nor peptide 17.

Figs. 4 and 5 also show the cross reactivity of diss.Ab-B with DL using substrates of high and low molecular weight.

The precipitations of diss.Ab-B by HL and DL were tested by the Ouchterlony technique. Diss. Ab-B (1.31 mg/ml) solution gave a precipitin line with HL solution (103 μg/ml) but not with DL at a wide range of concentrations. Thus diss. Ab-B still shows specificities of more than one antibody. But the specificity of diss. Ab-B is clearly much more limited than that of untreated HL antiserum or of the residual antibody fraction, because the former, unlike the latter two fractions, gave no precipitin line with DL. The inhibition of HL activity by resd.Ab-B was similar to that by resd.Ab-A, that is, at a molar ratio of antibody to enzyme of 2 : 1 it caused almost 100% inhibition with *M. lysodeikticus* as substrate but less than 50% inhibition with a small substrate.

4. *Effect of substrate concentration (R-6mer) on inhibitions of enzyme by various antibody preparations*

To elucidate the mechanisms of inhibitions of enzymes by various antibody preparations, the effect of substrate concentration on the inhibition was tested.

Constant amounts of HL or DL mixed with constant amounts of antibody (the molar ratios of antibody to enzyme were 1 : 1 or less) were incubated at 37 C for 30 minutes. After incubation, various concentrations of R-6mer were added to enzyme-antibody mixtures and the residual enzymic activities were measured as described earlier. The results are shown in Fig. 6.

The dependency of the degree of inhibition on the substrate concentration is great in the DL-diss. Ab-B system and slight in the HL-diss.Ab-B system. These results imply that some competitive mechanisms are involved in the inhibitions of the two enzymes by diss.Ab-B. On the contrary, no appreciable substrate concentration dependency can be detected in the inhibition of HL activity by resd.Ab-B. Therefore, its mechanism of inhibition is non-competitive.

5. *Kinetic studies using a small substrate*

The modes of inhibition of the two enzymes by various antibody preparations were further investgated by kinetic analysis of enzyme-substrate (low molecular weight) interaction in the

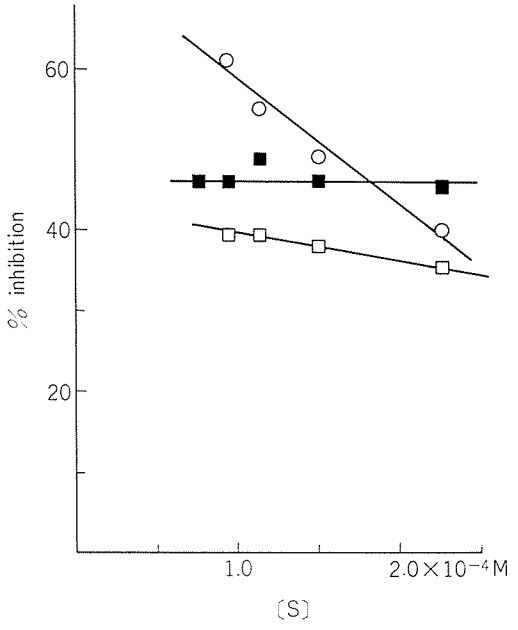

FIGURE 6. *Effect of substrate concentration on inhibitions of the activities of HL and DL by two fraction of anti-HL antibodies. Percentage inhibition of the activity of DL (2.24×10^{-7} M) by diss.Ab-B (1.83×10^{-7} M) in the presence of various concentrations of R-6 mer* (○———○). *Percentage inhibition of the activitity of HL (2.17×10^{-7} M) by diss.Ab-B (1.75×10^{-7} M) in the presence of various concentrations of R-6 mer* (□———□). *Percentage inhibition of the activity of HL (2.17×10^{-7} M) by resd.Ab-B (2.26×10^{-7} M) in the presence of various concentrations of R-6 mer* (■———■).

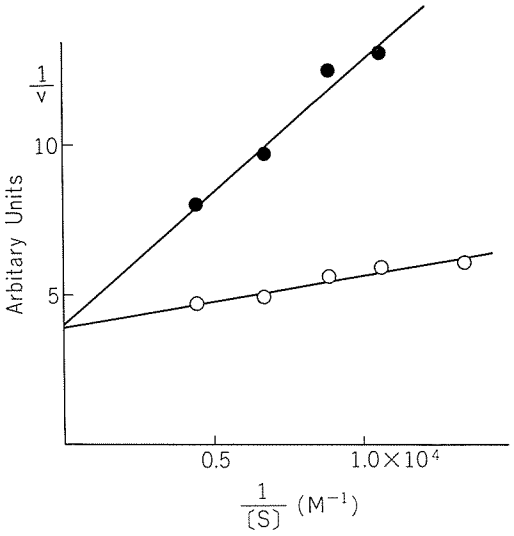

FIGURE 7. *Competitive type inhibition of DL activity by diss.Ab-B (Lineweaver-Burk plot). The DL concentration was 2.24×10^{-7} M. The substrate was R-6 mer. DL alone* (○———○). *DL in the presence of 1.83×10^{-7} M of diss.Ab-B* (●———●).

presence of various antibody preparations. The initial velocity (v) of hydrolysis of various concentrations of R-6mr was measured in the presence of a fixed amount of antibody.

1) Lineweaver Burk plot

The inhibition of DL activity by diss. Ab-B in the presence of various concentrations of R-6 mer is illustrated in Fig. 7 as a Lineweaver-Burkplot (1934). The molecular weights of antibody, HL or DL and R-6 mer are taken as 150,000, 14,300 and 1,320, resectively.

Since the exact concentration of the antibody fraction reacting with DL is not known, it was estimated from the inhibition data shown in Fig. 4 as 42% of diss. Ab-B assuming that DL activity was inhibited by the corresponding antibody with the same efficiency as the HL activity was inhibited by diss. Ab-B. The Lineweaver Burk plot indicates that the mode of inhibition of DL activity by a fraction of antibody in diss.Ab-B is a fully competitive type. The maximal velocity (V) of DL activity was not influenced by the presence of antibody, but the apparent Km value of DL-R-6-mer interaction changed from 4.46×10^{-5} M to 2.18×10^{-4} M in the presence of antibody.

Similar experiments were also made using the HL- diss. Ab-B system and the HL-resd. Ab-B system and Lineweaver Burk plots of these experiments are shown in Fig. 8.

In the HL-diss. Ab-B system, the V value in the presence of antibody was 67% of that in the absence of antibody and the apparent Km value in the presence of antibody was also larger than that of HL alone. This indicates that the mode of inhibition of HL activity by diss. Ab-B is so-called " mixed type." On the other hand,

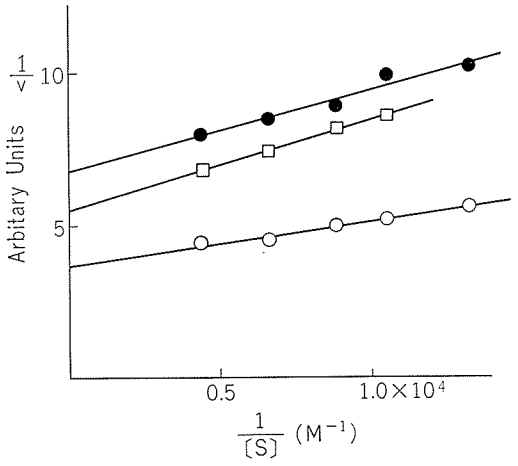

FIGURE 8. *Analysis of the type of inhibition of HL activity by the two antibody fractions (Lineweaver-Burk plot). The concentration of HL in this experiment was* 2.17×10^{-7} M. *Molar concentration of R-6 mer: [S]. HL alone* (○———○). *HL in the presence of* 2.26×10^{-7} M *of resd.Ab-B* (●———●). *HL in the presence of* 1.75×10^{-7} M *of diss.Ab-B* (□———□).

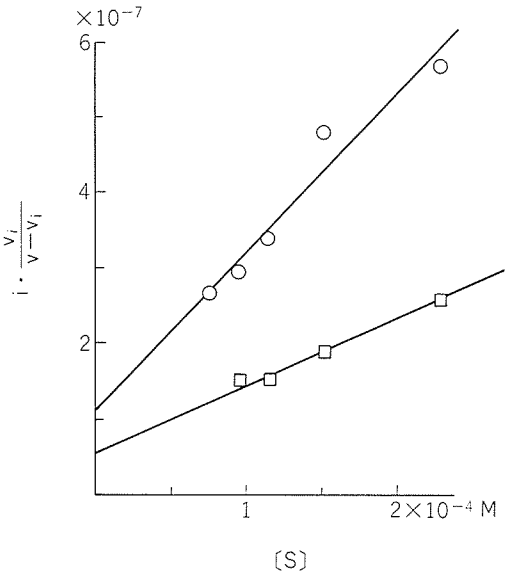

FIGURE 9. *Graphical evaluation of* K_i *value from the interaction of DL and diss.Ab-B. The concentration of DL was* 2.24×10^{-7} M. *Molar concentration of R-6 mer: [S].* 7.4×10^{-8} M *of diss.Ab-B* (○———○). 1.83×10^{-5} M *of diss.Ab-B* (□———□).

in the HL-resd.Ab-B system, the V value in the presence of antibody was 55% of that of HL alone but the apparent Km values of HL in the absence (3.90×10^{-5} M) and presence (3.92×10^{-5} M) of antibody were quite similar. This implies that the inhibition of HL activity by resd. Ab-B is non-competitive. But, in these two systems, more than one antibody may inhibit HL activity. If so the meanings of these analyses would not be simple and hence attempts are in progress to fractionate these antibodies further.

2) Determination of inhibitor constants

To measure the affinities of these antibodies, which behaved as inhibitors, to the enzyme the method of Hunter and Downs (1945) was applied to evaluate the K_s value (the dissociation constant of the enzyme-inhibitor complex). Data obtained in the previous experiments were recalculated and plotted as follows. In the DL-diss. Ab-B system the mode of inhibition fully competitive, as shown in the previous section, and the equation is

$$i\frac{v_i}{v-v_i}=K_i+\frac{K_i}{K_m}S$$

where i is the concentration of inhibitor (antibody), v is the velocity when the substrate concentration is S, v_i is the velocity in the presence of inhibitor and K_m is the Michaelis constant. If then $i\frac{v_i}{v-v_i}$ is plotted against S, a straight line will be obtained which cuts the ordinate at K_i and has a slope of K_i/K_m. Fig. 9 shows this plot, calculated from the data on the inhibition of DL activity by diss. Ab-B in the presence of two concentrations of antibody. The K_i value obtained from this plot is 1.12×10^{-7} M when tne concentration of antibody is 7.4×10^{-8} M and is 0.548×10^{-7} M when the concentration of antibody is 1.83×10^{-7} M. Therefore the association constants (Ka) of diss. Ab-B to DL calculated from the two K_i values are 8.93×10^{2} L/M and 1.82

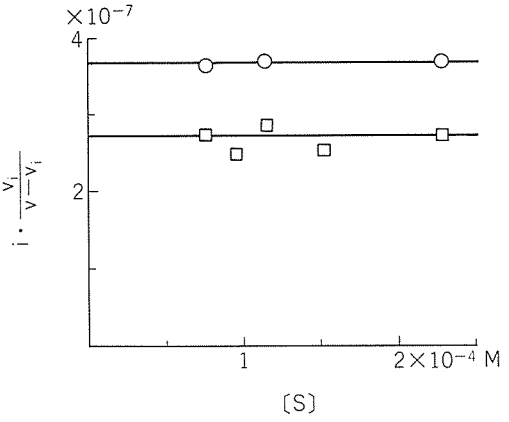

FIGURE 10. *Graphical evaluation of K_i value from the interaction of HL and resd.Ab-B. The concentration of HL was 2.17×10^{-7} M. Molar concentration of R-6 mer: [S]. 1.70×10^{-7} M of resd. Ab-B* (○———○). *2.26×10^{-7} M of resd.Ab-B* (□———□).

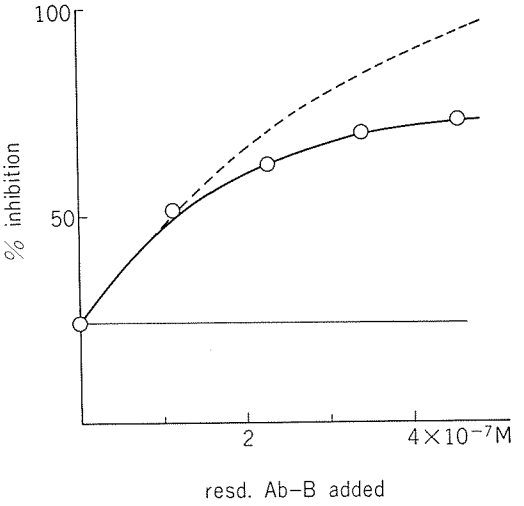

FIGURE 11. *Effect of mixing two antibody fractions on the degree of inhibition of HL activity on a small substrate. HL solution (2.17×10^{-10} moles in 0.3 ml) was added to mitures containing constant amounts of diss.Ab-B (0.87×10^{-10} moles) and various amounts of resd.Ab-B (1.1×10^{-10} moles to 4.55×10^{-10} moles). HL alone, a mixture of HL and diss.Ab-B, and mixtures of HL and various amounts of resd.Ab-B were also prepared as controls. The final volume of all samples was adjusted to 0.8 ml with VBS, pH 6.0 and mixtures were incubated at 37 C for 30 minutes. R-6 mer (2.28×10^{-6} moles in 0.2 ml of VBS, pH 6.0) solution was then added and the enzymic activity of each mixture was measured as described earlier.*

---------- *Percentage inhibition calculated as the sum of the inhibitions of HL activity by diss.Ab-B and by resd.Ab-B separately.*

——————— *Percentage inhibition of HL activity by a mixture of diss.Ab-B and resd.Ab-B in different molar ratios.*

$\times10^6$ L/M. That is the K_a value in this system is in the order of 1×10^6 L/M. The facts that this plot gives a straight line and that the line has a certain slope to the abscissa, imply that the inhibition involved in this system is competitive.

The same kind of plot taken from data on inhibition of HL activity by resd. Ab-B is shown in Fig. 10. The K_i value is calculated as 3.66×10^{-7} M when the concentration of antibody is 1.70×10^{-7} M and 2.72×10^{-7} M when the concentration of antibody is 2.26×10^{-7} M. This K_a values calculated from the two K_i values are 2.74×10^6 L/M and 3.68×10^6 L/M, respectively. As seen from Fig. 10, the value of $i\dfrac{v_i}{v-v_i}$ is independent of the concentration of substrate and equal to K_i. This suggests again that the mode of inhibition in this system is purely non-competitive.

6. *Mutual effect of antibody fractions*

In studies on the inhibition of HL activity by HL antibody, it was noticed that the efficiency of inhibition of HL activity by the whole antibody population is generally low using a small substrate, although the presence of a highly inhibitory antibody fraction against HL activity on the small substrate has been proven. Therefore the possibility that one population of antibody influenced the degree of inhibition of HL activity by the other population of antibody was tested. Increasing amounts of resd. Ab-B (1.14×10^{-7} to 4.55×10^{-7} M) were added to a constant amount of diss. Ab-B (0.87×10^{-7} M) and the mixtures were incubated with HL (2.17×10^{-7} M) for 30 minutes at 37 C. A constant amount of R-6 mer (2.28×10^{-6} M)

was added to each mixture and the catalytic activity was measured as described earlier. The degrees of inhibition by the two antibody preparations were also measured separately. Fig. 11 shows that the degree of inhibition by the mixture of the two antibody fractions was clearly lower than the sum of their separate inhibitions. The mechanism involved is not clear at the moment, but it seems that the inhibitory activity of one kind of antibody against HL can be disturbed by the presence of another kind of antibody.

DISCUSSION

The significance of the role of antibody in neutralization or inhibition of biological activities has long been noticed as one of the most important defense mechanisms of living matter. It was also found that all the antibodies induced by a biologically active material are not equally active in neutralizing its function. For instance, the avidity or affinity of antibodies to a given antigen was emphasized in neutralization of toxin by antitoxin (Raynaud et al., 1959) and also in virus neutralization (Jerne et al., 1956). Actually some conditions were found to increase the affinity to a given antigen (Eisen et al., 1964 and Fujio et al., 1966) using antihapten antibody as a model.

On the other hand, with enzyme as a model of biologically active material, the degree of neutralization or inhibition of enzyme activity by its antibody seems to be largely related to the size of the substrate on which the enzyme acts (B. Cinader, 1967). In general, partial or no inhibition of enzymic activity by its antibody is often observed when substrates of low molecular weight are used. In such cases, the specificity of the antibody may play a most important role in the inhibition of a catalytic activity of an enzyme. In practice, an antibody with specificity directed to the binding site of a substrate or the catalytic site of an enzyme, would play a most important role in neutralization of the enzyme, when the size of the substrate is small. An approach to selection of such an antibody fraction from a whole population of antibody to an enzyme was made by Kaplan et al. (1964) by removing the cross reacting antibody from anti-tryptophan-synthetase antibodies by the mutant antigen. Arnon et al. (1967) and Shapira et al. (1967) also succeeded in isolating an efficient inhibitor type of antibody from anti-papain antibodies using the cross-reacting enzyme, chymopapain.

In this work, a more direct approach to selection of such a type of antibody from the whole population of anti-HL antibodies was made utilizing the dissociation of the HL-anti-HL antibody complex by a competitive inhibitor to HL, 3 NAG. The antibody fraction dissociated by 3 NAG actually has a high efficiency in inhibition of the enzymic activity of HL on a low molecular weight substrate. In addition, the mode of inhibition of HL by this antibody fraction seems to be competitive in nature and the results of kinetic studies infer that the inhibition of DL activity by the dissociated antibody is fully competitive. But the dissociated antibody fraction can still give a precipitin reaction with HL and this implies that the dissociated antibody fraction has the specificities of more than one kind of antibody. The reason for this is not clear at the moment but there seem to be at least two explanations for it.

1) There is more than one kind of antibody and the binding site of each antibody only partly overlaps the binding site of the substrate in a different area.

2) Tri-N-acetylglucosamine not only dissociates the antibody from the binding site of the substrate, but also causes a relatively wide spread conformational change in addition to changing the structure of the binding site for substrate. Hence the antibody could be released from an area other than the binding site for the substrate. These possibility must be tested further by fractionating the dissociated antibody with respect to its specificity. Nevertheless, the specificity of the antibody in the highly inhibitory antibody fraction seems to be very restricted compared with that of other

fractions of antibody tested, because it gave no precipitin reaction with DL, although the residual antibody fraction and the original HL antiserum showed clearly precipitin reactions with DL.

Fellenberg and Levine (1967) also reported that complement fixation by the HL-anti HL reaction is partially inhibited by a high concentration of 3 NAG. Arnon (1968) suggested that this inhibition of the lysozyme-antilysozyme system might be due to steric hindrance. However we suggest that there is a distinct antibody population in rabbit HL antiserum with high efficiency for neutralization of HL activity even with substrate of low molecular weight.

Another interesting phenomenon observed was that when the dissociated antibody fraction was mixed with the rest of the antibody fraction, the degree of inhibition of the mixture was less than the sum of the inhibitions by each fraction separately. A similar phenomenon was observed with a ribonuclease system by Cinader and Lafferty (1963). Again there are two possible explanations of this: one is that the antigenic determinants in HL may partly overlap and the other is that a conformational change is induced by the binding of one type of antibody which prevents the binding of another type of antibody. An attempt to estimate the amount of so called highly inhibitory antibody fraction in the residual antibody fraction met with the difficulty that the presence of one antibody fraction affected the inhibitory capacity of the other antibody fraction. At any rate, for a full understanding of the mechanism of neutralization of HL activity by antibody, it is essential to elucidate the specificities of each member of the antibody population formed in response to the various parts of the structure of the HL molecule. Studies along these lines are under way (Fujio et al., 1962; Shinka et al., 1962; Shinka et al., 1967; Fujio et al., 1968a and 1968b and Imanishi et al., 1968).

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