BIKEN JOURNAL Vol. 14, 395–404, 1971

THE IMMUNOLOGICAL PROPERTIES OF REGION SPECIFIC ANTIBODIES DIRECTED TO HEN EGG-WHITE LYSOZYME[1]

HAJIME FUJIO, NOBUO SAKATO and TSUNEHISA AMANO

Department of Immunochemistry, Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka

(Received July 27, 1971)

SUMMARY Two immunologically active peptides, peptide-8 (sequence 57–107) and peptide-17 (sequences 1–27 and 122–129), were prepared from hen egg white lysozyme (HL). Consequently, two region specific antibody fractions, anti-8 and anti-17 antibodies, were isolated from rabbit anti-HL antisera using the two peptide immunoadsorbent. Equilibrium dialysis showed that the K_A of the binding of anti-8 antibody by ^{125}I-HL was 9.8×10^4 (M^{-1}) and that of the anti-17 antibody was 1.5×10^5 (M^{-1}). The heterogeneity index of a Sips plot of anti-8 antibody was 1.02 ± 0.05 and that of the anti-17 antibody was 0.97 ± 0.02.

The anti-17 antibody neutralized the enzymic activity of HL with comparable efficiency to that of the whole population of anti-HL antibodies using *M. lysodeikticus* was used as substrate, but it did not influence the enzymic activity of HL using hexa-N-acetyl-chitohexaitol (R-6 mer) as substrate. On the other hand, anti-8 antibody neutralized the enzymic activity of HL for both substrates. The relationship between antibody specificity and neutralization is discussed.

INTRODUCTION

Enzyme proteins such as lysozyme (Shinka et al., 1967; Fujio et al., 1968a, b) and Staphylococcal nuclease (Omenn et al., 1970a, b, c) have been shown to have several antigenic determinants. Thus, antibodies provoked against a highly purified enzyme molecule consisted of a variety of antibodies with different specificities. Antibodies usually inhibit enzymes, though in some cases they cause activation (Pollock, 1964; Suzuki et al., 1969). In addition, their efficiency of neutralization of enzymic activities varies depending on their specificities (Imanishi et al., 1969a). Since different parts of a protein molecule play different roles in maintaining the function and conformation of the protein, the most probable explanation for the diverse functions of antibodies is that they differ in antibody specificity.

A direct approach to this problem is to separate and characterize each determinant group in an enzyme protein and antibody populations with specificities directed to different restricted areas of the enzyme molecule. This approach

1 Parts of this work were reported at the Symposium on Immunochemistry in Japan (Imanishi et al. 1968, Fujio et al. 1969 and 1970) and at the 8th International Congress of Biochemistry (Amano et al. 1970).

was first attempted in our laboratory (Fujio et al., 1959; Fujio et al., 1962; Shinka et al., 1962) and now detailed information on the antigenicity of lysozyme has been obtained from many laboratories (Arnon and Sela, 1969; Maron et al., 1971; Pecht et al., 1971; Habeeb, 1969; Strosberg and Kanarek, 1970; Bonavida et al., 1969; Young et al., 1970).

Two immunologically active peptides were isolated from different regions of the lysozyme molecule and so two kinds of region specific antibody fractions could be prepared using these two peptide-immunoadsorbents. This paper reports the properties of the two region specific antibodies, and especially their behaviors in neutralization of lysozyme activity.

MATERIALS AND METHODS

1. *Preparation of active peptides 8 and 17*

Six times crystallized HL was obstained from Seikagaku-Kogyo Co. Ltd. HL was digested with pepsin for one hour and active peptide-8 and peptide-17 were isolated by CM-cellulose chromatography and by gelfiltration on Sephadex G-50 as described before (Fujio et al., 1968a). Peptide 8 was highly purified by repeated chromatography on CM-cellulose to remove a trace of intact lysozyme.

2. *Production of antisera*

Six times crystallized HL was further purified for immunization. HL was chromatographed on SE-Sephadex C-25 in 0.2 M sodium phosphate buffer, pH 7.16 at 25 C. The main peak obtained from the first chromatograph was passed through QAE-Sephadex A-25 in 0.005 M sodium phosphate buffer, pH 8.0 to remove a trace of acidic components of egg-white. Two mg of a highly purified HL preparation was injected with complete Freund's adjuvant into a rabbit. Booster doses of 2 mg HL were given every five weeks, the last injection being given after 20 weeks. Blood was taken 7 and 9 days after the last injection and animals were exsanguinated 11 days after the injection. All rabbits produced lysozyme antibody and antisera contained 3 to 5 mg of antibody per ml estimated by the precipitin reaction. The antisera of 25 rabbits were pooled and the lipid component was removed by ultracentrifugation before further purification.

3. *Quantitative precipitin reaction*

The procedures used were essentially those of Heidelberger and Kendall (1935) except that the reaction was carried out in the presence of 0.01 M EDTA, instead of heating the antisera, to avoid complement fixation.

4. *Immunoadsorbents*

Bromoacetylcellulose (BAC-cellulose) was prepared by the methods of Robbins et al. (1967).

Approximately 200 mg of peptide-8 were coupled to 900 mg of BAC-cellulose at pH 4.6 and 50 mg of antibody directed to peptide-8 (anti-8) could be eluted with 0.1 N acetic acid at 37 C. Two hundred and eighteen mg of peptide-17 coupled to 1.4 g of BAC-cellulose at pH 8. This amount of immunoadsorbent can also bind approximately 40 mg of antibody directed to the region of peptide-17 (anti-17).

Sepharose-immunoadsorbents were also prepared by the methods of Omenn, Ontjes and Anfinsen (1970c). Fifty mg of peptide-8 coupled to 15 ml of Sepharose 4B (wet volume) in 0.1 M sodium phosphate buffer, pH 7.0 and 40 mg of anti-8 could be eluted with 0.1 N acetic acid at 37 C. Sixty mg of peptide 17 coupled to 15 ml (wet volume) of Sepharose 4B in 0.1 M sodium phosphate buffer, pH 6.5 and 57 mg of anti-17 could be eluted with 0.1 N acetic acid at 37 C. Antibodies were allowed to react with immunoadsorbents in the presence of 0.01 M EDTA at 4 C. Then they were washed with 2 mM phosphate, 0.15 M NaCl, 0.01 M EDTA, pH 8.0 (EDTA-PBS) till the optical density (λ= 280 mμ) of the fluid became less than 0.01. In the case of BAC-cellulose, antibody was eluted by incubating the adsorbent in 0.1 N acetic acid at 37 C for 1 hr with stirring. Sepharose-immunoadsorbent was used in a column and antibody was eluted by pumping 0.1 N acetic acid through the column (10 ml per hr with columns of 2 cm diameter) at 37 C. Eluate with an optical density (λ=280 mμ) of more than 0.01 was collected.

5. *Inhibition of enzymic activity by antibody*

Inhibitions of the enzymic activity of lysozyme by various antibody fractions were measured with either *M. lysodeikticus* or hexa-N-acetylchitohexaitol as substrate as described in our previous reports (Fujio et al., 1968a; Imanishi et al., 1969a, b).

Increasing amounts of antibody fraction were added to a constant amount of enzyme and 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 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 30 min in the case of R-6 mer. The decrease in catalytic activity of the enzyme due to antibody is expressed as a percentage of the catalytic activity of the enzyme in the absence of antibody. Purified anti-HL antibody was also prepared as described previously (Fujio et al., 1968a). HL was added to rabbit HL antiserum in the ratio of equivalence in the presence of 0.01 M EDTA. The resulting specific precipitate was thoroughly washed with EDTA-PBS and dissociated by treatment with 0.1 N acetic acid. The anti-HL antibody was separated from HL on a Sephadex G-150 column saturated with 0.1 N acetic acid.

Normal rabbit γ-globulin (NRγ) was also prepared for a control experiment. Normal rabbit serum was adjusted to half saturation of ammonium sulfate at 0 C. The precipitate was dissolved in deionized water and fractionated on a column of Sephadex G-150 saturated with EDTA-PBS. The 7-S fraction was collected and used as NRγ.

6. *Radioactive labelling of peptides and of HL*

Peptide-8 and peptide-17 were labelled with a 2 or 3 molar excess of ^{14}C-acetic anhydride (Daiichi Chemicals Co. Ltd., 45.5 mc/m mole) in dimethylsulfoxide at 20 C for 1 hr and purified by passage through a Sephadex G-25 column (3×150 cm) in 10% acetic acid (v/v). Conditions were adjusted to provide about one acetyl group per mole of petide.

Radioactivity of ^{14}C-acetylpeptides was measured by adding 100 μl aliquots of samples to 4 ml of scintillator fluid (10 ml of Bio-solve BBS-3, a product of Beckmann, mixed with 90 ml of toluene containing 4 g of PPO) using a Beckman LS-200B Liquid Scintillation Counter.

HL was labelled with ^{125}I by the iodine monochloride method of McFarlane (1958). The conditions were adjusted to attain one atom of iodine per mole of HL. The concentration of ^{125}I-HL was measured using 25 μl aliquots in a Packard Model 3002, Tricarb Scintillation Spectrometer.

7. *Equilibrium dialysis*

A micro-dialysis cell like that described by Eisen (1971) was used. Fifty μl of 1×10^{-5} to 4.8×10^{-4} M ^{125}I-HL solution was put into one compartment of the cell and an equal volume of purified anti-8 or anti-17 was put into the other compartment. Three to 5 mg per ml of purified antibody solution were used and the molar concentration was calculated taking the molecular weight of antibody as 150,000. The solvent was PBS, pH 6.0.

Gel-cellophane, used as a semipermeable membrane, was obtained from Dai-Seru Co. Ltd. (Fujio et al., 1968a). The cell was rotated at 5 rev/min in a water bath at 10 C for 24 hr and equlibrium of ^{125}I-HL was attained within 24 hr at 10 C. Twenty-five μl aliquots were taken from both compartments of the cell and the concentration of antigen was estimated by counting ^{125}I. Values for binding were calculated from the equation.

$$r/c = nK_A - rK_A$$

where n is the valence of the antibody, r the moles of antigen per mole of antibody, c the concentration of free antigen and K_A is the association constant (Karush and Karush, 1971).

The heterogeneity index, a, of K_A was also calculated using Sips equation:

$$\log [r/(n-r)] = a \log c + a \log Ko$$

where Ko is the mean association constant.

8. *Miscellaneous methods*

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

RESULTS

1. *Preparations of region specific antibodies*

Initially, bromoacetylcellulose was used for the preparations of region specific antibody fractions. As described in Materials and Methods, peptide-Sepharose immunoadsorbents can bind 4 to 5 times more antibody than peptide-cellulose adsorbents at a given level of the bound peptide. Therefore in later ex-

periments, peptide-Sepharose immunoadsorbents were mainly used. Some experiments were carried out with both adsorbents and essentially the same results were obtained.

First the effect of acid treatment on the binding activities of anti-8 and of anti-17 was studied, because acetic acid was used throughout eluting antibodies from immunoadsorbents. Anti-8 and anti-17 were first eluted with the respective peptide solutions, as in the hapten-antibody system. Then, these antibody fraction were treated with various concentrations of acetic acid, the binding capacities of the acid treated antibodies were compared with that of antibodies eluted with peptide.

In a typical experiment, 100 ml of rabbit HL antiserum containing 3.0 mg/ml of precipitable antibody were passed through a peptide-8-Sepharose column consisting of 109 mg of peptide-8 coupled to 30 ml (wet volume) of Sepharose. The column was thoroughly washed with EDTA-PBS at 4 C. Then the column was warmed to 37 C and the anti-8 antibody was eluted with 6.5 ml of a 1.4×10^{-3} M solution of ^{14}C-acetyl peptide-8 at 37 C. The column was further washed with EDTA-PBS. A similar experiment was carried out using peptide-17-Sepharose. The amount of antibody protein in each eluate was measured with goat anti-rabbit γG antiserum. The eluates were pooled and concentrated by pressure dialysis. The concentrated antibody solutions were diluted with EDTA-PBS and pressure dialysis were repeated three times. Finally, each antibody preparation was passed through a Bio-Gel P-200 column (3×90 cm) in EDTA-PBS at 25 C to remove the peptide more completely.

Only 0.072 mole of peptide-8 and 0.10 mole of peptide-17 per mole of antibody were detected by measurement of radioactivity. The contents of anti-8 and anti-17 were calculated by the Kjeldahl-Nessler method and by measurement of radioactivity after the second pressure dialysis and found to be 0.324 mg and 0.326 mg per ml of serum, respectively. No UV adsorbing materials could be detected in the eluate from either peptides-Sepharose column on further elution with 1.0 N acetic acid.

The anti-8 and anti-17 antibody preparations, which were isolated under the very mild conditions described above, were each divided into three portions. The concentration of acetic acid in the first portion of each antibody was adjusted to 0.1 N and that in the second was adjusted to 1.0 N. The protein concentration of each antibody preparation was 0.5 mg/ml. These preparations were incubated at 37 C for 1 hr. During purification of the antibody by immunoadsorbents, it was noticed that loss of antibody protein due to acid denaturation can be minimized if the antibody protein is first dialyzed against buffer of low ionic strength and then against buffered physiological saline. Björk and Tanford (1971) reported similar conditions for renaturation of the heavy chain of rabbit γG. Therefore, the antibody protein eluted with acid was first dialyzed against 100 volumes of 0.01 N acetate buffer, pH 5.5 for three days with two changes of buffer and then against PBS, pH 6.0, by the method of Björk and Tanford (1971). The third portion of each antibody preparation was dialyzed directly against PBS, pH 6.0.

The 6 preparations of antibody were concentrated to approximately 3 mg per ml by pressure dialysis. Then binding experiments with ^{125}I-HL were carried out by equlibrium dialysis. As seen in Table 1, 0.1 N acetic

TABLE 1. *The effect of acid treatment on the binding properties of rabbit anti-HL antibody fractions*

Fraction of anti-HL antibody	K_A[a] (M^{-1}) after treated with		
	(Control)	0.1 N Acetic acid	1.0 N Acetic acid
Anti-8	2.4×10^5	1.5×10^5	0.92×10^5
Anti-17	1.5×10^5	1.1×10^5	0.32×10^5

a Binding of various antibody preparations by ^{125}I-HL was examined at 10 C in PBS, pH 6.0. For details see text.

acid did not influence the binding properties of either antibody. The K_A of anti-8 antibody which had been treated with 0.1 N acetic acid appeared slightly lower than that of the control, but it was within the range of error of the method used. However, treatment with 1.0 N acetic acid definitively decreases the K_A values of both antibody fractions. Fortunately almost all the anti-8 and anti-17 antibodies were eluted with 0.1 N acetic acid and usually no protein could be eluted in the eluate with 1.0 N acetic acid after elution with 0.1 N acetic acid. Therefore, all the antibody fractions used in the following experiments were prepared by elution with 0.1 N acetic acid.

2. *Bindings of anti-8 and anti-17 antibodies by* ^{125}*I-HL*

Two kinds of region specific antibodies were separated using peptide-Sepharose immunoadsorbents as described above. These antibody fractions did not give any measurable precipitates when mixed with HL. Therefore, the antibody activity of these preparation was first tested by direct binding experiments using equilibrium dialysis.

Fifty μl of anti-8 antibody (4.5 mg/ml) or anti-17 antibody (3.6 mg/ml) were put into one compartment of a dialysis cell and equal volumes of various concentrations (1.0×10^{-5} to 4.8×10^{-4} M) of ^{125}I-HL were added to the other compartment of cells. Both antigen and antibody were dissolved in PBS, pH 6.0. The cells were rotated at 10 C for 24 hr. The specific activity of the ^{125}I-HL used in this experiment was approximately 10^4 count/min per 10^{-9} mole of HL. Alquots were taken from each compartment of the cells and counts were made in a Packard auto-gamma counter. At least 1,000 counts were recorded for each Sample. The amounts of bound HL was calculated from the difference in the counts in the two compartments.

Results are shown in Fig. 1. Scatchard plots of the two antibody preparations both gave straight lines. The association constant of the anti-8 antibody was calculated as 9.8×10^4 (M^{-1}) and that of anti-17 antibody was 1.5×10^5 (M^{-1}). Sips plots of these binding data were also made by the method of Karush (1962) and are shown in Fig. 2. The heterogeneity index, a, of the association constant of anti-8 antibody was 1.02 ± 0.05 and that of anti-17 antibody was 0.97 ± 0.02.

Most of the antibodies produced against protein antigens are believed to recognize the conformations of intact preteins (Crumpton et al., 1966; Sela 1967). Therefore, it seemed interesting to see the differences between the binding of these region specific antibodies by the corresponding peptides and by intact HL.

Volumes of 25 μl of various concentrations of peptide-8 or peptide-17 and 25 μl anti-8 or anti-17 antibody were put into one compartment of a dialysis cell and 50 μl of ^{125}I-HL (Ca. 3×10^{-4} M) were added to the other compartment. Cold HL was also mixed with the respective antibody solution and dialyzed against ^{125}I-HL solution. Aliquots of 25 μl were taken from both compartments for counting.

The association constant of the inhibitor, K_I, was calculated from the equation,

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

where i is the concentration of inhibitor, r the moles of antigen bound per mole of antibody in the absence of inhibitor, and r′ is the moles of antigen bound per mole of antibody in the presence of inhibitor (Karush, 1956). Table 2 shows that the K_I of peptide-8 is approximately one fifth of that of intact HL and then K_I of peptide-17 is about one tenth of that of intact HL.

These differences between the K_I values of the peptides and intact HL may be due to a conformational difference between the isolated peptide and the corresponding portion of the intact molecule or to the defect of the additional portion of the HL molecule other than the isolated portion. The K_I of unlabelled HL is similar to the K_A of ^{125}I-HL. This suggests that the values of K_A calculated using iodinated

HL are close to the value given on reaction with intact HL.

On the other hand, peptide 17 did not inhibit the binding of ^{125}I-HL by anti-8 antibody, and peptide-8 did not influence the binding of ^{125}I-HL by anti-17 antibody. This confirms that the specificity of the antigenic determinant involved in the peptide-8 region is independent of that of the peptide-17 region, as reported previously (1968b). This finding also proves that the purification procedure used to separate the region specific antibody fractions in this work is satisfactory.

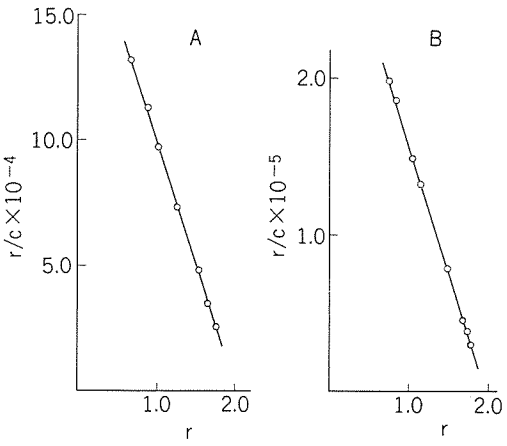

FIGURE 1. *Binding of purified region specific antibodies by ^{125}I-HL at 10 C: anti-8 antibody (4.5 mg/ml), B: anti-17 antibody (3.6 mg/ml), r: moles of ^{125}I-HL bound per mole of antibody, c: concentration of free ^{125}I-HL (M).*

TABLE 2. *Inhibition of the bindings of ^{125}I-HL to region specific antibodies by two active peptides*

Antibody	K_I[a]		
	Peptide-8	Peptide-17	HL[b]
Anti-8 ($K_A=9.8\times10^4$)	1.6×10^4	$<1\times10^2$	8.4×10^4
Anti-17 ($K_A=1.5\times10^5$)	0	1.7×10^4	1.4×10^5

a for calculation, see text.
b unlabelled hen egg-white lysozyme.

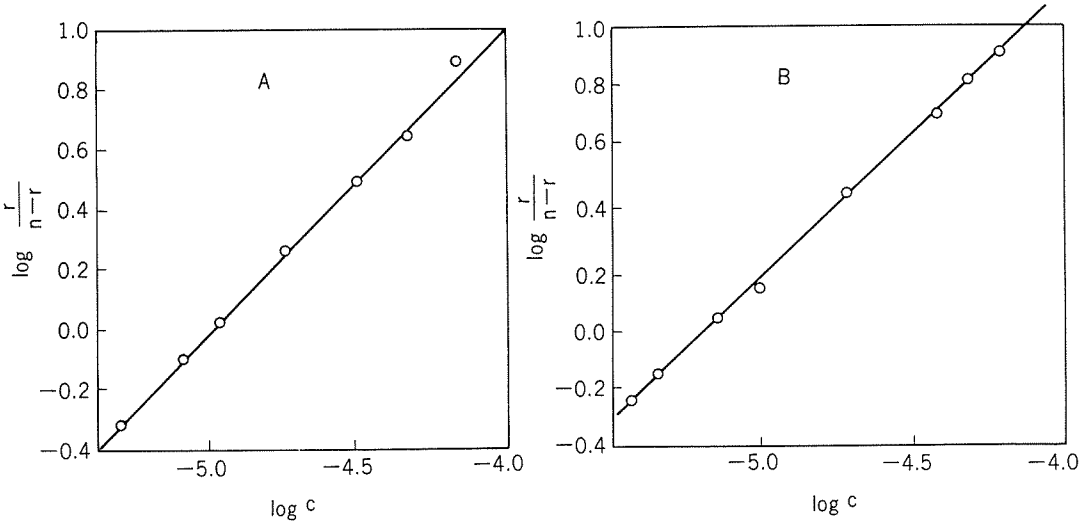

FIGURE 2. *Sips plots for the bindings of ^{125}I-HL by region specific antibody fractions.*
A: Binding of ^{125}I-HL by anti-8 antibody, B: binding of anti-17 antibody, n: numbers of binding sites per mole of antibody, r: moles of antigen bound per mole of antibody, c: concentration of free antigen (M) at equilibrium.

3. *Neutralization of the enzymic activity of HL by the region specific antibody fractions*

The specificity of one of the antibody populations in the rabbit HL antiserum is directed to the region of 57 to 107 (peptide-8) and that of the other is directed to the region of 1 to 27 and 122 to 129 (peptide-17) of the amino acid sequence of HL. Thus it seemed interesting to compare the behavior of the two distinct populations of antibodies in neutralization of lysozyme activity. The same preparations of anti-8 and anti-17 antibodies were used as in binding experiment, and their effects on the enzymic activity of HL were examined. Fig. 3 shows the inhibitory effects on the lytic activity of HL by anti-8 and anti-17 antibodies with *M. lysodeikticus* as substrate. For comparison, purified anti-HL antibody, which supposedly contains the whole population of antibodies directed to HL, was also tested. The enzymic activity of HL was neutralized 90% by addition of an equimolar amount of anti-17 antibody. This is comparable with the inhibition caused by the whole population of HL antibodies.

Fig. 4 shows the inhibition of β-1, 4 N-acetyl-glucosaminidase activity of HL by the two antibody fractions with hexa-N-acetyl-chitohexaitol as substrate. Anti-8 antibody inhibited the N-acetyl-glucosaminidase activity of HL, but the anti-17 antibody did not influence the enzymic activity at all when the small sized substrate was used, although the same preparation of anti-17 antibody inhibited the lytic activity of HL with high efficiency.

These results are consistent with the picture obtained by X-ray crystallographic analysis of the complex of HL and chitin oligosaccharide (Phillips 1967). According to the proposed mechanism, peptide-8 is located closed to the area of the binding site for a small substrate, whereas the region corresponding to the area of peptide-17 is not related to the binding site for small substrates or the catalytic site of HL. Normal rabbit γ-globulin was also used as a control. NRγ did not influence the enzymic activity of HL for either substrate, as shown Figs. 3 and 4.

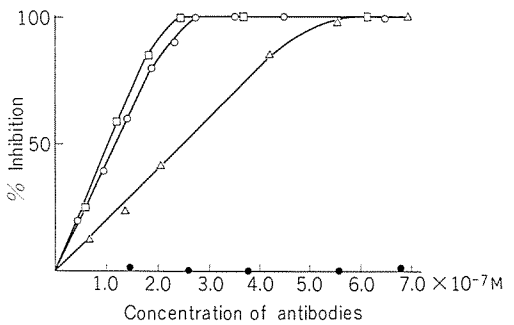

FIGURE 3. *Neutralization of the enzymic activity of HL by various antibody species.*
M. lysodeikticus was used as substrate.
The concentration of HL in the reaction mixture was 1.92×10^{-7} *M.*
○——*Anti-HL antibody;* △——*Anti-8 antibody;* □——*Anti-17 antibody;* ●——*NR*γ.

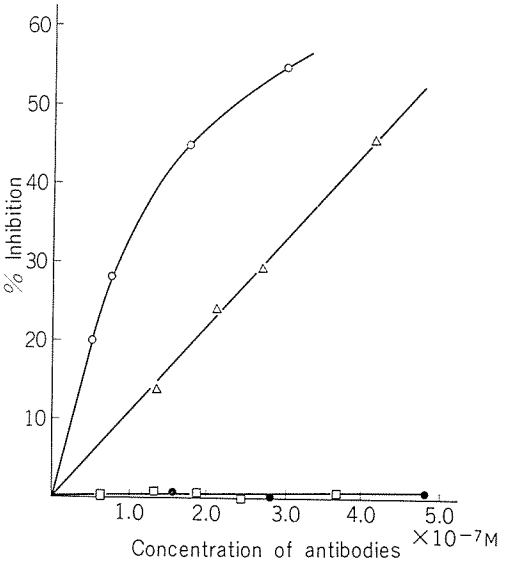

FIGURE 4. *Neutralization of the enzymic activity of HL by various antibody species.*
Hexa-N-acetyl-chitohexaitol was used as substrate.
The concentration of HL in the reaction mixture was 1.92×10^{-7} *M.* ○——*Anti-HL antibody;* △——*Anti-8 antibody;* □——*Anti-17 antibody;* ●——*NR*γ.

DISCUSSION

In discussing the efficiency of neutralization of biological activity of macromolecules by a humoral antibody, the significance of the specificity and the affinity of the antibody are always mentioned. Recently, some evidence was obtained suggesting the significance of the specificity of the antibody in neutralization (Arnon et al., 1967; Imanishi et al., 1969a). But it was not possible to obtain direct evidence of a correlation between specificity of the antibody and its effect in neutralization, because of the complexity of the specificities of antibodies provoked against a biologically active macromolecule. For the same reason, there is little information on the affinity of different antibody populations directed to specific regions of a macromolecule.

Difficulties may arise in isolation of immunologically active peptides, if the function of the determinant group in the protein is dependent only on the conformation. Neverthless, active fragments have been isolated from many proteins (Crumpton and Wilkinson, 1965, Atassi et al 1968 and 1970; Omenn et al., 1970a, b, c).

In our studies differences between the K_I values of each antibody with the peptide and with the intact molecule may reflect conformational changes occurring on isolation of the peptides from the whole molecule.

Sela and his colleagues also succeeded in isolating an active peptides which corresponded to the region of 60 to 83 of HL (Sela and Arnon, 1969; Maron et al., 1971). This unique peptide forms the loop and its conformation seems to play a role in immunological activity (Pecht et al., 1971). The binding characteristics of the loop peptide were also reported recently. The homogeneity of the binding of peptide-17 was reported in our previous paper (Fujio et al., 1968a), and the association constants of the bindings of HL by both anti-8 and anti-17 antibodies were also reported (Fujio et al., 1969). Isagholian and Brown also pointed out the highly restricted heterogeneity of the K_A of the active peptide of oxidized pancreatic ribonuclease with the corresponding antibody (1970). The narrow range of K_A for the binding of angiotensin by the corresponding antibody, which was provoked by the injection of angiotensin-polylysine conjugate, was also reported by Haber et al., (1967). Therefore, the homogeneity of the association constants of the antibodies directed to restricted region of protein antigen seems to be a fairly general phenomenon. Neverthless, the electrophoretic heterogeneities of such antibody preparations are still apparent, as will be reported later (Sakato et al., 1971). It is uncertain whether this kind of heterogenity is related to the structure of the active region of the antibody simply reflects the heterogeneity of a region other than active site. The close similarity in the binding characteristics suggests that the latter possibility is more likely.

The mechanism of neutralization of enzymic activity by an antibody was thoroughly discussed by Cinader (1967) and more recently by Arnon (1971). Arnon et al., (1968) also reported the roles of antibody fractions in anti-HL antibodies in neutralization of the catalytic activity of HL. In our studies, when a large sized substrate was used, both region specific antibodies neutralized the enzymic activity, and one of the antibody fractions inhibited the enzymic activity as much as the whole population of anti-HL antibodies. This suggests that aggregate formation is not a prerequisite for efficiency in neutralization of an enzyme by its antibody. The significance of the specificity of antibodies in neutralization was clearer using a small sized substrate. Anti-8 antibody, the specificity of which is supposedly directed to the region of the binding sites for substrate, neutralized the enzymic activity. On the other hand, anti-17 antibody, the specificity of which is supposedly directed to a region which is not essential for enzymic activity, did not neutralize the enzymic activity.

In addition, we must emphasize that even with a small sized substrate, the special fraction of anti-HL antibodies, which was isolated

by dissociation of specific precipitates with a competitive inhibitor to enzyme, has even higher neutralization activity than the whole population of anti-HL antibodies (Imanishi et al., 1969a).

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