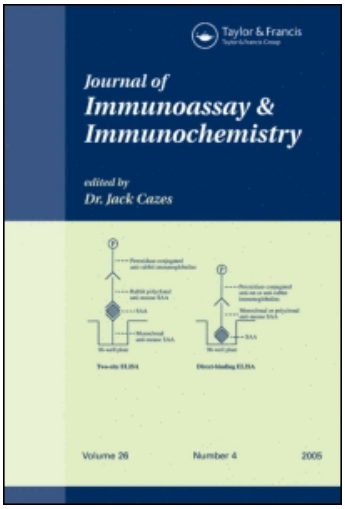

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Binding Kinetics of Monoclonal Antibody Using Antigen-β-Galactosidase Hybrid Protein: Application to Measurement of Peptide Antigenicity

Eun Wie Cho[a]; Myung Kyu Lee[a]; Kil Lyong Kim[a]; Kyung-Soo Hahm[a]
[a] Protein Engineering Research Group, Genetic Engineering Research Institute, Taejon, Korea

To cite this Article Cho, Eun Wie , Lee, Myung Kyu , Kim, Kil Lyong and Hahm, Kyung-Soo(1995) 'Binding Kinetics of Monoclonal Antibody Using Antigen-β-Galactosidase Hybrid Protein: Application to Measurement of Peptide Antigenicity', Journal of Immunoassay and Immunochemistry, 16: 4, 349 — 363
To link to this Article: DOI: 10.1080/15321819508013567
URL: http://dx.doi.org/10.1080/15321819508013567

JOURNAL OF IMMUNOASSAY, 16(4), 349-363 (1995)

BINDING KINETICS OF MONOCLONAL ANTIBODY USING ANTIGEN-β-GALACTOSIDASE HYBRID PROTEIN: APPLICATION TO MEASUREMENT OF PEPTIDE ANTIGENICITY

Eun Wie Cho, Myung Kyu Lee*, Kil Lyong Kim and Kyung-Soo Hahm
Protein Engineering Research Group, Genetic Engineering Research Institute,
KIST, P.O. Box 115, Yusong, Taejon 305-600, Korea

ABSTRACT

A simple method for determination of binding kinetics of a solid-phase antibody using antigen-β-galactosidase hybrid protein was evaluated. To minimize conformational change of the antigen binding site of the antibody when directly binding to a microtiter plate, the microtiter plate was precoated with protein A. The binding and free antigen concentrations were directly obtained from the β-galactosidase activity. This method can be used for analyses of the equilibrium dissociation constant (K_D), and the association (K_{ass}) and dissociation (K_{diss}) rate constants. Peptide antigenicity was also analyzed by competitive ELISA using this method. Since both antigen-β-galactosidase and the peptide used are localized in the fluid-phase, the proper affinity constant (K_A) of the peptide can be estimated from the K_D value of the antigen-β-galactosidase-antibody interaction, and from the IC_{50} value of the peptide.

Key Words: Binding kinetics, monoclonal antibody, antigen-β-galactosidase hybrid protein, competitive ELISA, peptide

INTRODUCTION

The binding kinetics of antigen-antibody interactions are often of interest. Recently, synthetic peptides have been used for study of antigen-antibody interactions (1) and epitope mapping of antigens (2), and these molecules have a

*To whom correspondence should be addressed.

promising future in the field of synthetic vaccines (3). However, some problems exist in binding kinetic studies of peptide-antibody interactions.

The coating efficiency of synthetic peptides on a microtiter plate is dependant on pH and ionic strength (4). Labeling with radioisotopes, fluorescence dyes, or biotinyl groups may induce conformational alteration of peptides, and modify their antibody binding sites in certain cases. Conjugation of a peptide to a carrier, such as bovine serum albumin (BSA) causes difficulty in controling how the peptide is coupled to the carrier (5). A method using a directly captured antibody has also been attempted (6). However antibodies directly bound to microtiter plates sometimes exhibit a decrease in, or complete loss of, their antigen binding capacity (7). A method using biosensor technology has been successfully used for real time binding kinetics of protein and peptide antigens (8), but it requires expensive instrument.

To circumvent some of these limitations, attempts were made to develop a reliable method for the study of antigen-antibody interactions using the antigen(preS2)-β-galactosidase hybrid protein in a fluid-phase and the anti-preS2 monoclonal antibody (H8 mAb) in a solid-phase (9). PreS2 is composed of 55 amino acid residues (10) and is known to be highly immunogenic (11). Hybrid proteins can be easily expressed using a *lac* operon system (12), and can be purified using their β-galactosidase activity (13). Microtiter plates precoated with protein A were used to minimize conformational changes of the antibody. Affinity constants of synthetic peptides were also determined using this method.

MATERIALS AND METHODS

Proteins and peptides

Protein A was purchased from Sigma Chem. Co (USA). H8 mAb was obtained from Dr. H. K. Chung (Seoul National University, Korea). A bacterial strain

producing the preS2-β-galactosidase hybrid protein (14) was provided by Dr. M. H. Yu (Genetic Engineering Research Institute, KIST, Korea).

PreS2-β-galactosidase(MW120KD) was purified by the method of Ullmann (11) and protein concentrations were determined by the method of Lowry (15). The β-galactosidase activity of the hybrid protein was measured by the modified method of Steers *et al.* (16) in 96 well microtiter plates. One hundred μl of reaction buffer (0.05 M Tris-HCl, 0.1 M sodium chloride, and 0.01 M magnesium chloride containing 0.075% o-nitrophenyl-β-galactopyranoside) and 100 μl of stop solution (1 M sodium carbonate) were used.

Peptide synthesis was performed by the solid phase method (17) using Fmoc as N_α-amino protecting group. All peptides were purified by HPLC on a reverse phase C_{18}-column (>90%). Amounts of purified peptides were determined by measuring either their dry weight or UV absorption at 274 nm (tyrosine) or 280 nm (tryptophan). The amino acid sequences of synthetic peptides used in this study are summerized in Table 1.

Determination of the equilibrium dissociation constant (K_D) value

Ninety-six well microtiter plates (Nunc, Denmark) were coated for 2 h at 37 °C with 200 ng of protein A in 0.1 M carbonate buffer, pH 9.6. The remaining sites of the plates were blocked with 20 mM Tris-HCl, 150 mM sodium chloride, pH 7.4 (TBS), containing 3% casein (3% casein-TBS). After washing the wells with TBS-T (TBS containing 0.05% Tween 20), the wells were then incubated for 1 h at 37 °C with 100 ng of H8 mAb in 0.3% casein-TBS. Different concentrations (0~1 μg/well) of preS2-β-galactosidase in 0.3% casein-TBS were then added after washing the wells with TBS-T, then the plates were incubated for 2 h at 37 °C. Concentrations of the free (F) and bound (B) antigen were estimated by measuring β-galactosidase activity in solution, and bound β-galactosidase activity,

TABLE 1

Amino acid sequences of synthetic peptides used in this study.

Peptides	Sequences* 120					125					130					135					140					145
p120-145/adr	M	Q	W	N	S	T	T	F	H	Q	A	L	L	D	P	R	V	R	G	L	Y	F	P	A	G	G
p120-145/ayw	-	-	-	-	-	-	-	-	-	-	T	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	-
p120-145/adw2	-	-	-	-	-	-	A	-	-	-	T	-	Q	-	-	-	-	-	-	-	-	L	-	-	-	-
p123-145/adr				-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
p130-145/adr											-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
p130-145/ayw											T	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	-

Each dashed line (-) indicates the same residues with adr subtype.
*Amino acid sequences of the preS2 peptides of each type were obtained from DNA sequence data of Okamoto *et al.* (18).

respectively. The equilibrium dissociation constant (K_D) of the preS2-β-galactosidase-H8 mAb interaction was obtained from a Scatchard plot (B/F versus B plot) and the nonlinear curve-fitting was done by the modified Scatchard equation (19) using the sigma plot version 4.0 program. For comparison, the K_D value was also determined by the method of Friguet *et al.* (20) as follows: a constant amount of H8 mAb was incubated in solution with various amounts of preS2-β-galactosidase until equilibrium was reached, then the proportion of the antibody which remained unsaturated at each concentration was measured by indirect ELISA.

Determination of the association rate constant (k_{ass}) and dissociation rate constants (k_{diss})

After preparing the wells treated with H8 mAb associated with protein A as above, either 0.5 or 1 μg of preS2-β-galactosidase in 100 μl of 0.3% casein-TBS was added to each well at various time intervals. The wells were then washed and the amount of the bound antigen was determined by the β-galactosidase activity. From a plot of the amount of the bound antigen versus time, the resultant mono-

exponential was analyzed using a linearizing plot, ln (1-B/B_{eq}) versus time, where B_{eq} is the antigen concentration at an equilibrium state. The slope of this linearizing plot is (k_{ass}×F+k_{diss}) (21). In order to determine the rate constants (k_{ass} and k_{diss}), these measurements were made at two different antigen concentrations, and the K_D value was estimated by the equation, K_D=k_{diss}/k_{ass}.

Competitive ELISA for characterization of peptide antigenicity

After preparing the wells treated with H8 mAb associated with protein A as above, 50μl mixtures of different dilutions (1 nM to 300 μM) of the test peptides, and 50 μl of 10 μg/ml of preS2-β-galactosidase in 0.3% casein-TBS were added and incubated for 2 h at 37 °C. After washing, the amount of bound preS2-β-galactosidase was estimated by the β-galactosidase activity. The relationship between the competitor concentration and binding inhibition were plotted and the IC_{50} value, the concentration of the competing peptide when inhibition was 50%, was determined. The affinity constant (K_A) and the inhibition constant (K_i) of the competing peptide were estimated as: K_A=1/K_i=(1+K×F_A)/IC_{50} (21,22), where K is the affinity constant of the preS2-β-galactosidase-H8 mAb interaction (1/K_D), and F_A is the preS2-β-galactosidase concentration corresponding to the IC_{50} value.

RESULTS

Quantification of preS2-β-galatosidase

For determination of binding constants using this method it was necessary to establish a linear relationship between the β-galactosidase activity of preS2-β-galactosidase and its concentration. As illustrated in Fig. 1, β-galactosidase activity corresponded linearly to the antigen concentration. This standard curve was used to determine the amounts of bound and free antigen.

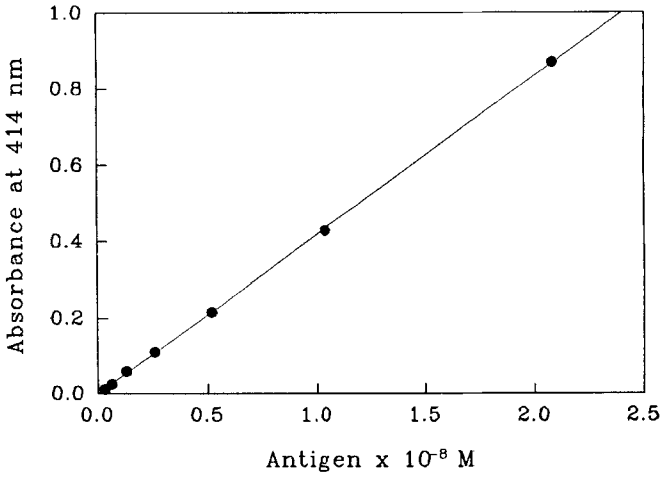

Fig. 1. The relationship between the β-galactosidase activity measured by absorbance at 414nm and the amount of preS2-β-galactosidase.

Determination of the equilibrium dissociation constant (K_D)

To determine the K_D value, increasing concentrations of the antigen were added to wells affixed with H8 mAb associated with protein A. When H8 mAb was directly coated onto the microtiter plate the binding efficiency of preS2-β-galactosidase to the antibody was low (data not shown). This result may be due to a low coating efficiency of the antibody, or to a conformational change of the antibody by passive adsorption (7). Protein A was, therefore, used as a supporter to immobilize the antibody on the microtiter plate.

The binding data obtained were converted to a Scatchard plot (B/F versus B plot) and the K_D value was obtained from a linear regression analysis ($K_D = 3.01 \times 10^{-8}$ M) (Fig. 2A and Table 2). Since the plot looked biphasic (nonlinear concave upwards), analysis of the plot was done by the modified Scatchard equation (19) and two K_D values were obtained from the asymtotes ($K_D = 6.74 \times 10^{-9}$ M and 5.78

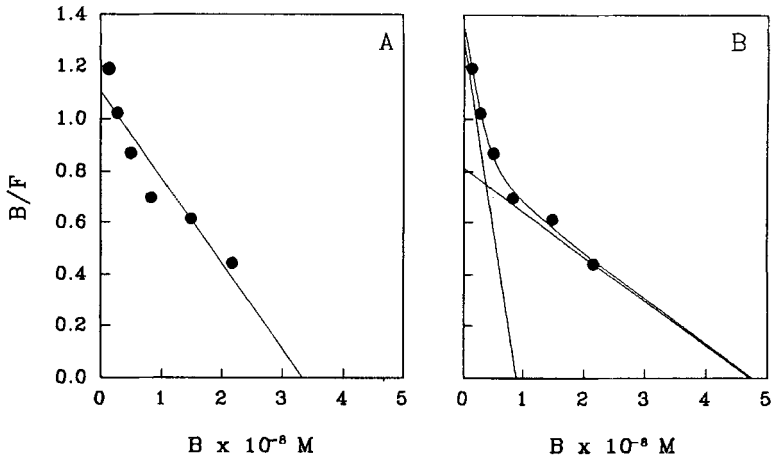

Fig. 2. Scatchard plots of binding data of preS2-β-galactosidase and H8 mAb. The binding between solid-phase H8 mAb and fluid-phase preS2-β-galactosidase was analyzed by simple linear regression (A) and a biphasic plot (B). The resulting K_D values are shown in Table 2.

TABLE 2

Equilibrium dissociation constants of preS2-β-galactosidase-H8 mAb interaction calculated by various methods.

Methods	K_D (M)
Friguet *et al* (20)	4.91×10^{-8}
Scatchard plot:	
linear regration (Fig. 2A)	3.01×10^{-8}
nonlinear curve fitting (Fig. 2B)	6.74×10^{-9}, 5.78×10^{-8} *
k_{diss}/k_{ass} ** (Fig. 3B)	1.72×10^{-8}

* The K_D values of high and low affinity bindings, respectively.

**k_{diss} and k_{ass} were estimated using the slopes of the linearizing plot (Fig. 3B).

$\times 10^{-8}$ M) (Fig. 2B and Table 2). Two populations, one of high (K_D=6.74 $\times 10^{-9}$ M) and one of low affinity (K_D=5.78 $\times 10^{-8}$ M) binding sites, may be caused by the steric hindrance resulting from occupancy of the antigen-binding site by the large antigen preS2-β-galactosidase (MW 120KD). However, low affinity binding still showed a high binding order ($K_D<10^{-7}$). For comparison, the ELISA method of Friguet *et al.* (20) was also performed (Table 2) and the resultant K_D value was 4.91 $\times 10^{-8}$ M, similar to the low affinity binding constant of this method.

Determination of the kinetic rate constants of the antigen-antibody complex

To evaluate the k_{ass} and k_{diss} values of the antigen-antibody complex the time-course binding of preS2-β-galactosidase to H8 mAb was measured. The plot (Fig. 3A) obtained by the bound antigen amount versus time was analyzed using the linearizing plot, ln (1-B/B_{eq}) versus time (Fig. 3B). Using the slope ($k_{ass}\times F+k_{diss}$) of this linearizing plot at two different antigen concentrations (5 μg/ml and 10 μg/ml), the k_{ass} and k_{diss} values were determined to be 1.56×10^{4} $sec^{-1}\cdot M^{-1}$ and 2.68×10^{-4} sec^{-1}, respectively. The K_D value estimated with the rate constants (1.72×10^{-8} M) was similar to the high affinity K_D value determined above (0.67×10^{-8}M) (Table 2).

Competitive ELISA for determination of peptide antigenicity

Previously peptide antigenicity against H8 mAb was determined using an indirect competitive ELISA system (9). Since the coating antigen and the competing peptide used were located in the solid-phase and the fluid-phase in that system, respectively, only the relative peptide antigenicity could be observed. Therefore, attempts were made to identify the true inhibition constants (K_i) of competing peptides in the binding of H8 mAb and preS2-β-galactosidase. Binding of preS2-β-galactosidase to H8 mAb was decreased by increasing amounts of a

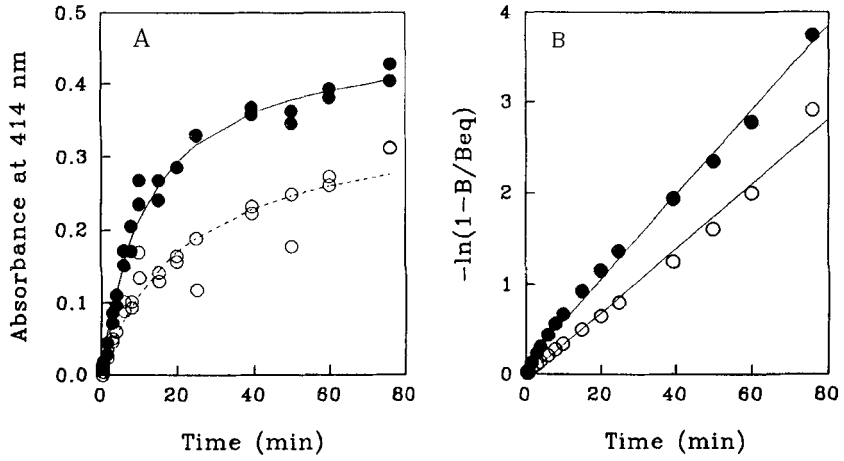

Fig. 3. Time-course binding kinetics between H8 mAb and preS2-β-galactosidase. Panels A and B show a binding versus time plot and a linearizing $\ln(1\text{-}B/B_{eq})$ versus time plot, respectively. The symbols represent the H8 mAb concentrations used; 5 μg/ml (○) and 10 μg/ml (●). Using the slopes ($k_{ass} \times F + k_{diss}$) of the linearizing plot (B) the k_{ass} and k_{diss} values were calculated.

competing peptide (Fig. 4). From inhibition curves, the IC_{50} values of peptides and the affinity constants (K_A) of the competing peptides were estimated (Table 3). The K_A values of long peptides (p120-145/adr, p120-145/ayw and p123-145/adr) were above 10^7, but much lower affinity constants, on the order of 10^5, were observed for truncated peptides (p130-145/adr and p130-145/ayw) excluding the N-terminal residues 120 to 129. On the other hand, p120-145/adw2 showed a loss of almost all antigenicity.

DISCUSSION

The wide use of monoclonal antibodies in a variety of fields has prompted the development of precise, rapid, and convenient methods to characterize the

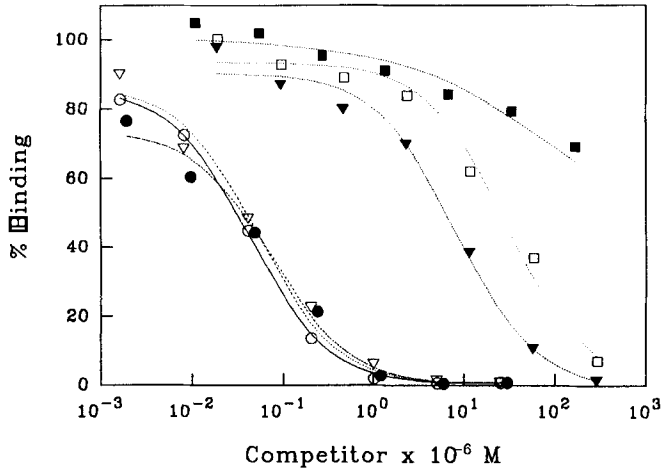

Fig. 4. Inhibition by peptides of the reaction of H8 mAb and preS2(adr)-β-galactosidase. The IC_{50} values were obtained from competitive ELISA data. The symbols represent competing peptides used; p120-145/adr (○), p123-145/adr (●), p120-145/ayw (▽), p120-145/adw2 (■), p130-145/adr (□) and p130-145/ayw(▼).

TABLE 3

The IC_{50} values and affinity constants of various peptides estimated by competitive ELISA.

Peptides	IC_{50} (M)*	K_A (M^{-1})**
p120-145/adr	$3.08x10^{-8}$	$9.05x10^{7}$
p120-145/ayw	$3.91x10^{-8}$	$7.13x10^{7}$
p123-145/adr	$3.66x10^{-8}$	$7.61x10^{7}$
p130-145/adr	$1.12x10^{-5}$	$2.48x10^{5}$
p130-145/ayw	$6.88x10^{-6}$	$4.05x10^{5}$
p120-145/adw2	NA	NA

* The IC_{50} values were calculated from the inhibition curve of competitive ELISA (Fig. 4).

**K_A was estimated by the equation, $K_A=(1+F/K_D)/IC_{50}$. For detail see "MATERIALS AND METHODS".

NA: Not available.

equilibrium and kinetic parameters of antigen-antibody interactions. Since it seems to be difficult to propose the best method, applicable to all antigens, because of the variety of antigens, only appropriate methods have been used for this purpose (6,8,20,23-26).

In the present study the antigen(preS2)-β-galactosidase hybrid protein was used for kinetic studies of the antigen-antibody interaction. Since an antigen molecule is linearly linked to the N-terminus of the β-galactosidase molecule in a hybrid protein, one hybrid protein reacts with one antigen binding site of an antibody without modification of its epitope. Moreover the antigen-antibody binding is directly detectable by its β-galactosidase activity. This method using a solid-phase-antibody and antigen-β-galactosidase makes measurements of the K_D value and rate constants (K_{ass} and K_{diss}) possible.

H8 mAb shows high reactivity with peptides containing the sequence 120/123 to 145 of the preS2 region (9). The K_D value ($0.67\sim5.78\times10^{-8}$ M) of the preS2-β-galactosidase-H8 mAb interaction measured by this method (Fig. 2) is similar to previous results of peptide-antipeptide mAb interactions measured using biosensor technology (8). This indicates that this method is reliable for assay of the antigen-antibody interaction, and the β-galactosidase region in the hybrid protein does not seem to affect the interaction.

In most studies for evaluation of the k_{ass} and k_{diss} values of antigen-antibody interaction, methods based on the use of radioactivity, followed by the seperation of the free and bound molecules, or fluorecence measured in solution have been used. These methods require labelling of either antigen or antibody, but labelling does not always lead to the expected signal and often results in immunological modification of the molecules. Moreover, when fluorecence is used the concentrations of reagents needed for measurments are usually several orders of magnitude higher than the equilibrium dissociation constant, which makes the

experiments difficult (24). To circumvent these problems attempts have been made to measure the dissociation rate constant of antigen-antibody complexes using ELISA methods (24, 25). With our ELISA method the time-course binding of the antigen and antibody can be easily traced. The k_{ass} and k_{diss} values determined by a time-course binding study were 1.56×10^{4} $sec^{-1}\cdot M^{-1}$ and 2.69×10^{-4} sec^{-1}, respectively (Fig. 3). These values are in agreement with rate constant values of peptide-antipeptide mAb interactions measured using biosensor technology (8). These results indicate that ELISA methods can be used for measurement of rate constants.

Competitive ELISA using an indirect ELISA method (9) cannot measure true binding kinetic data since the coating antigen and the competing antigen are located in different phases (solid-phase and fluid-phase, respectively). The method of Friguet *et al.* (20) also has problems, such as a difference between the coating antigen (native protein or peptide conjugated to a carrier) and the competing antigen (peptide). Moreover, peptides with low K_D values dissociate from the antibody and re-equilibrate with the coating antigen (data not shown). In this method, since both antigens used were located in the fluid-phase, the true binding constants can be estimated. The K_A values of long peptides with high antigenicity (9) were approximately 7×10^{7} M^{-1}. These data are similar to the K_A values of peptide-antipeptide mAb interactions (8), indicating that this method can be used for binding kinetic study of peptide-antibody interaction. The K_A values of short peptides with low antigenicity (9) decreased to approximately 1/200 as compared with long peptides, but short peptides completely inhibited the binding of preS2-β-galcatosidase to H8 mAb (Fig. 4). This result agrees with previous results (9) and suggests that antigenicity differences between long and short peptides are caused by comformational differences.

In conclusion, this simple method, which does not require a secondary antibody, can be used for determination of equilibrium and rate constants. Although cloning and expression of an antigen-β-galactosidase hybrid protein are required, it does not cause difficulties because of well established method (12). In the case of a peptide antigen, cDNA encoding the amino acid sequence of the peptide can be easily synthesized by a chemical method. This method is also useful for binding kinetic study of peptides.

ACKNOWLEGEMENT

The authors thank Dr. H. K. Chung and Dr. M. H. Yu for the kind gifts of H8 mAb and the bacterial strain producing preS2-β-galactosidase, respectively. This work was supported by grant N80990 from the Korean Ministry of Science and Technology.

REFERENCES

1. Geysen, H. M. Antigen-antibody interactions at the molecular level: adventures in peptide synthesis. Immunol. Today, 1985; 6: 364.

2. Pancino, G., Chappey, C, Saurin, W. and Sonigo, P. B epitopes and selection pressures in feline immunodeficiency virus envelope glycoproteins. J. Virol., 1993; 67: 664-72.

3. Arnon, R. Synthetic vaccines, vol I and II , Boca Raton, FL, CRC Press, 1987.

4. Geerligs, H. J., Weijer, W. J., Bloemhoff, W., Welling, G. W. and Welling-Wester, S. The influence of pH and ionic strength on the coating of peptides of Herpes simplex virus type 1 in an enzyme-linked immunosorbent assay. J. Immunol. Methods, 1988; 106: 239-44.

5. Briand, J. P., Muller, S. and Van Regenmortel, M. V. H. Synthetic peptides as antigens: Pitfalls of conjugation methods. J. Immunol. Methods, 1985; 78: 59-69.

6. Murray, J. S. and Brown, J. C., Measurement of association constants in ELISA. J. Immunol. Methods, 1990; 127: 25-8.

7. Butler, J. E.. Ni, L., Nessler, R., et al. The physical and functional behavior of capture antibodies adsorbed on polystyrene. J. Immunol. Methods, 1992; 150: 77-90.

8. Zeder-Lutz, G., Altschuh, D., Geysen, H. M., Trifilieff, E., Sommermeyer, G. and Van Regenmortel, M. H. V., Monoclonal antipeptide antibodies: Affinity and kinetic rate constants measured for the peptides and the cognate protein using a biosensor technology. Mol. Immunol., 1993; 30: 145-55.

9. Lee, M. K., Kim, K. L., Hahm, K. S. and Yang, K. H. Structure-antigenicity relationship of peptides from the pre-S2 region of the hepatitis B surface antigen. Biochem. Mol. Biol. Int., 1994; 34: 159-68.

10. Heerman, N., Goldman, U., Schwartz, W., Seyffarth, T., Bangarten, H. and Gelich, W. Large surface proteins of hepatitis B virus containing the pre-S sequence. J. Virol., 1984; 52: 396-402.

11. Milich, D. R. Genetic and molecular basis for T- and B-cell recognition of hepatitis B viral antigens. Immunol. Rev., 1987; 99: 71-103.

12. Casadaban, M. Transposition and fusion of the lac genes to selected promotors in Escherichia coli using bacteriophage lamda and Mu. J. Mol. Biol., 1976; 104: 541-55.

13. Ullman, A. One step purification of hybrid proteins which have β-galactosidase activity. Gene, 1984; 29: 27-31.

14. Kwon, K., Kim, C., Park, J., Han M. and Yu, M., Antigenic determinant mapping in preS2 region of Hepatitis B Surface Antigen. Korean J. Microbiol., 1990; 28: 13-8.

15. Lowry, O. H., Roseberbrough, N. J., Farr, A. L. and Randall, R. J., Protein measurement with the Folin phenol reagent. J. Biol. Chem., 1951; 193: 265-75.

16. Steers, E. Jr., Cuatrecasas, P. and Polland, H. B., The purification of β-galactosidase from *Escherichia coli* by affinity chromatography. J. Biol. Chem., 1971; 256: 196-200.

17. Barany, G. and Merrifield, R. B., Solid-phase peptide synthesis. In: Gross, E. and Meienhofer, J., eds. The peptides vol. 2. New York: Academic Press, 1980: 1-284.

18. Okamoto,H. Imai, M., Shimozoki, M., et al., Nucleotide sequence of a cloned hepatitis B virus genome, subtype ayr: Comparison with genomes of other three types. J. Gen. Virol., 1986; 67: 2305-14.

19. Feldman, H. A. Mathimatical theory of complex ligand-binding systems at equilibrium: Some methods for parameter fitting. Anal. Biochem., 1972; 48: 317-38.

20. Friguet, B., Chaffotte, A. F., Djavadi-Ohaniance, L. and Goldberg, M. E., Measurement of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbant assay. J. Immunol. Methods, 1985; 77: 305-19.

21. Hulme, E. C. and Birdsall, N. J. M., Strategy and tactics in receptor-binding studies. In: Hulme, E. C. ed. Receptor-ligand interactions: A practical approach. New York: IRL Press, 1992: 63-176.

22. Cheng, Y. C.,and Prusoff, W. H., Relationshipbetween the inhibition constant (Ki) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem. Pharmacol., 1973; 22: 3099-108.

23. Hoylaerts, M. F., Bollen, A. and De Bore, M. E. The application of enzyme kinetics to the determination of dissociation constants for antigen-antibody interactions in solution. J. Immunol. Methods, 1990; 126: 253-61.

24. Marie-Pierre, L., Djavadi-Ohaniance, L., Nall, B. and Goldberg, M. E. Measurement of the dissociation rate constant of antigen/antibody complexes in solution by enzyme-linked immunosorbant assay. J. Immunol. Methods, 1994; 170: 167-75.

25. Raman, C. S., Jemmerson, R., Nall, B. T. and Allen, M. J. Diffusion-rates for monoclonal antibody binding to cytochrom c. Biochemistry, 1992; 31: 10370-9.

26. Azimzadeh, A. and Van Regenmortel, M. H. V. Measurement of affinity of viral monoclonal antibodies by ELISA titration of free antibody in equilibrium mixtures. J. Immunol. Methods, 1991; 141: 199-208.