Simultaneous Dual-Enzyme Immunoassays in a Solid Phase

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Received August 30, 1996

A method of dual-signal generation from two different enzymes was developed and utilized to simultaneously perform dual immunoassays in a single microwell. Two enzymes selected as tracers were horseradish peroxidase (HRP) and β-galactosidase (GAL). 3,3',5,5'-Tetramethylbenzidine (TMB) and chlorophenolred-β-galactopyranoside (CPRG) as chromogenic substrates for the respective enzyme were used. Although the two enzymes showed their maximum activities at distinct pH conditions (pH 5.1 for HRP and 7.5 for GAL), the enzyme reactions were able to be concurrently carried out at pH 5.75 in a dual-substrate solution without signal loss. This performance was achieved by increasing TMB concentration two-fold, introducing potassium salt as activator of GAL reaction, and extending total reaction time 50%. The signal generation method was then used for dual-enzyme immunoassays to detect antibodies with co-immobilized Hepatitis C virus antigens (core and NS5) and a Hepatitis B virus antigen (PreS(2)) in a microwell. Dose-response curves of the assays revealed cooperativity between different antigen-antibody complex formation, which suggested that dual immunoassays can only be used for qualitative screening tests unless the antigens immobilized were spatially separated.

Introduction

A difficulty in performing dual-enzyme immunoassays, by which two different analytes can be simultaneously measured, is to carry out the enzyme reactions in a single solution for signal generation.[1,2] An enzyme reacts with a specific substrate and may need certain chemicals such as cofactor(s) and stabilizer(s) to maintain its activity. The activity is also critically affected by pH of solution.[3,4] For enzymes to react within a system, the required conditions except pH can be provided by simply adding each chemical into a solution. However, because enzymes usually show their maximum activities at distinct pH conditions,[5] enzyme reactions carried out at a single pH other than the optima may cause a significant decrease in the amount of signals produced and, thus, in the sensitivity of immunoassays.[1]

Conventional method of signal generation from two different enzymes present in a solid phase was a sequential addition of substrates for accomplishing the enzyme reactions in an individual mode. Blake *et al.*[1] used β-galactosidase (GAL) and alkaline phosphatase (AP) for dual-signal generation in a microwell. The GAL reaction was first performed and the signal produced was measured by using colorimetry. The well was washed, and AP was reacted by adding a substrate to generate the other signal. Marci *et al.*[6] and Porstman *et al.*[7] also followed the same procedure with horseradish peroxidase (HRP) and AP. However, the washing step required between the two reactions partially eliminated the bound enzyme for the second reaction and resulted in a poor sensitivity of immunoassay as a consequence.

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To circumvent such problem, we previously reported a method of dual-signal generation in a multiple substrate solution by using catalytic pH shift.[2] The pH of the solution was initially maintained at the optimal condition for one enzyme and then changed by the action of a third enzyme (*e. g.*, urease) introduced to trigger the other reaction. This method can be applied in principle to any multiple enzyme reactions by selecting appropriate pH modulating enzyme and buffer as medium. An alternative of dual-signal generation, which may be limited to a pair of enzymes chosen, can be devised by selecting a pH mid-way between the two optimal values for the enzymes. Although this approach requires modification of conditions of enzyme reactions to eliminate signal loss, the procedure of signal generation would become relatively simple.

In this investigation, we have selected two enzymes, HRP and GAL, and two substrates of each enzyme, 3,3',5,5'-tetramethylbenzidine (TMB) and chlorophenolred-β-galactopyranoside (CPRG), for dual-signal generation at a single pH. To this end, optimal physical and chemical conditions for the both of enzyme reactions were determined. Utilization of the method developed was demonstrated by applying it to the detection of antibodies with immobilized antigens on a solid surface.

Materials and Methods

Materials. HRP (1,000 units/mg protein; EC 1.11.1.7), GAL (600 units/mg protein; EC 3.2.1.23), CPRG, anti-human goat polyclonal antibody-GAL conjugate (anti-hAb-GAL), and anti-rabbit goat polyclonal antibody-HRP conjugate (anti-rAb-HRP) were purchased from Boehringer Mannheim (Germany). TMB, o-phenylenediamine dihydrochloride (OPD), and poly-L-lysine hydrobromide (MW 567,200; PLL) were obtained from Sigma (St. Louis, MO, U.S.A.). Hydrogen peroxide, Immulon II microwell, and 1-

ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC) were purchased from Duksan Chemical (Korea), Dynatech Inc. (Alexandria, VA, U.S.A.), and Pierce (Rockford, IL, U.S.A.), respectively. All other reagents used were of analytical grades.

Core and a nonstructural region (NS5) of Hepatitis C virus (HCV) were produced as recombinant antigens from *E. coli* and purified as described elsewhere.[8,9] Human sera positive to HCV was generously provided by Korean Red Cross. Normal human sera from healthy blood donors negative for HCV were pooled and used as negative control. A Hepatitis B virus (HBV) antigen, PreS(2), was synthesized according to the manual for solid phase peptide synthesis (Applied Biosystems, Model 431A, U.S.A.). Antiserum to PreS(2) was derived by immunizing rabbits with purified PreS(2) according to a standard method.[10] All sera were stored as frozen in aliquots at − 20 °C.

Single substrate of HRP. The substrate solution for HRP was prepared as recommended by the manufacturer. Four miligrams of OPD were dissolved in 10 mL of 50 mM phosphate-citrate buffer, pH 5.0, and 4 μL of 30% hydrogen peroxide was added to this solution.

Single substrate of GAL. The substrate solution for GAL was made according to the protocol prepared by the manufacturer. Twenty miligrams of CPRG were dissolved in 100 mM Hepes buffer, pH 7.0, containing 150 mM NaCl, 2 mM $MgCl_2$, 1% (w/v) bovine serum albumin, and 0.1% (w/v) sodium azide.

Dual substrates of HRP and GAL. Since the composition of mixed substrate solution for HRP and GAL was variable to obtain high signals from the enzymes, components and their concentration ranges tested will be described in each experiment below. An optimal composition of the solution was as follows: 0.006% H_2O_2, 200 μg/mL TMB, 2 mg/mL CPRG, 20 mM NaCl, 2 mM $MgCl_2$, and 50 mM KCl in 50 mM acetate buffer, pH 5.1 (Acetate Buffer), of which mixture was finally adjusted to pH 5.75 with 0.2 M Na_2HPO_4 (Composition 1).

Enzyme reactions at different pH and temperature. The effect of pH and temperature on the GAL and HRP reactions in a multiple substrate solution was tested. The substrate solution used in this experiment was 0.006% H_2O_2, 100 μg/mL TMB, 2 mg/mL CPRG, 20 mM NaCl, and 2 mM $MgCl_2$ in Acetate Buffer (Composition 2). Each 10 μL of 0.4 pmol/mL GAL and 0.25 pmol/mL HRP diluted with 140 mM NaCl containing 0.1% gelatin (Gel-Saline) was located within microwells, 200 μL of the substrate solution was added, and the reactions were performed at RT or 37 °C for 30 min. Colorimetric signals produced from GAL and HRP were measured at the absorbances of 570 nm and 650 nm, respectively. The identical procedure was followed with substrate solutions adjusted to different pH values with 0.2 M Na_2HPO_4.

Optimization of dual-substrate composition. The chemical composition of the multiple substrates in Composition 2 was modified to enhance the enzymatic signals. The concentration of substrate, TMB, was increased two-fold by transferring the equivalently higher volume of a stock solution (10 mg/mL TMB in dimethyl sulfoxide). Ten microliters of 0.4 pmol/mL GAL or 0.25 pmol/mL HRP were placed within microwells, and the enzymes were reacted at 37 °C for 30 min after adding 200 μL of the substrate solution. Colorimetric signals produced were measured and compared with the control, *i.e.*, 100 μg/mL TMB. To activate the GAL reaction, 50 mM KCl was supplemented to the solution in Composition 2 with two-fold higher TMB. The same experimental protocol as in the test of TMB was followed, and the result was compared with that in the absence of KCl.

Immobilization of antigens. HCV antigens, core and NS5, and a HBV antigen, PreS(2), were separately conjugated to PLL by using a heterobifunctional cross-linker, EDC,[11] and then immobilized on the inner surface of microwells by physical adsorption. Each antigen-PLL conjugate was diluted to 5 μg/mL based on PLL with 10 mM carbonate buffer, pH 9.6, and 200 μL of each solution was incubated in different microwells at 37 °C for 1 h. After washing with de-ionized water, 250 μL of 10 mM phosphate buffer, pH 7.4, containing 140 mM NaCl (PBS) and 0.5% (w/v) casein (Casein-PBS) was added and incubated at 37 °C for 1 h. Such prepared wells were washed and used for the binding of antibody below. For co-immobilization of the two antigens, each conjugate was diluted to 20 μg/mL PLL, and an equal volume of the conjugates (100 μL each) was added into a microwell. The same procedure as for the immobilization of single antigen was then followed.

Determination of enzyme reaction time. An optimal reaction time for signal generation was determined by measuring signals from the enzymes present in a solid phase. A human serum positive to HCV and a rabbit antiserum raised against PreS(2) diluted with Casein-PBS containing 0.1% Tween-20 (Casein-PBS-TW) were incubated within the wells with immobilized HCV antigens or PreS(2). After washing, each second antibody labeled with GAL or HRP were added for incubation. Then, the multiple substrates in Composition 1 were transferred into the wells, and the colorimetric signals were generated at 37 °C and measured against time. These signals were compared with the signals generated in the respective single substrate of the enzymes (see Materials) at 37 °C (GAL) or RT (HRP) for 30 min. The HRP signal in the OPD substrate was measured at 490 nm after adding 50 μL of 1 M H_2SO_4. Incubation for antigen-antibody binding reactions was performed at 37 °C for 1 h.

Different antigen-antibody complex formation. Different binding complexes were simultaneously formed within the wells with co-immobilized antigens as prepared above. To examine the variation of the binding of antibodies to the HCV antigens, 20 μL of human serum (HCV positive or negative) was combined with 180 μL of Casein-PBS-TW with or without rabbit antiserum to PreS(2) (1/100 dilution), and added into the wells. After washing, anti-hAb-GAL alone or in the combination with anti-rAb-HRP was incubated to detect single or dual complexes formed. The amount of complexes formed with the HCV antigen was measured by using the single substrate of GAL. For monitoring the change of the antibody binding to PreS (2), 180 μL of diluted antiserum to PreS(2) was mixed with 20 μL of Casein-PBS-TW containing a positive antiserum to HCV or none, and this solution was incubated within the wells. The dual complexes and the single were detected by

the enzyme conjugate mixture and the single HRP conjugate, respectively. The enzymatic signal from HRP was produced in the single substrate (OPD) and measured as mentioned. Non-specific binding of the enzyme conjugates was tested by using only the buffer in place of antiserum.

Dose-response curves of dual-enzyme immunoassays. By utilizing the wells with co-immobilized antigens and the multiple substrates, simultaneous dual-enzyme immunoassays were performed to obtain dose-response curves. Antisera diluted with Casein-PBS-TW were used as positive samples, and a normal serum and the buffer as negative samples. Twenty microliters of HCV antiserum in a concentration were combined with 180 μL of PreS(2) antiserum in different concentrations, and incubated in the wells with the immobilized antigens. After the wells were washed, anti-hAb-GAL and anti-rAb-HRP were mixed in Casein-PBS-TW, and 200 μL of this mixture was incubated in the wells. After washing, the multiple substrates in Composition 1 were added and reacted at 37 °C for 45 min. The HRP signal was measured at 650 nm, and the GAL signal was detected at 570 nm after adding 50 μL of 0.1 M Na_2CO_3. The same procedure was repeated with diluted HCV antiserum in different ratios.

Results and Discussion

Major components of a model system of dual-enzyme immunoassays (Figure 1) are antigens immobilized on a solid matrix such as microwell, analytes (different antibodies), and second antibody-enzyme conjugates as signal generators. Antigens are immobilized by co-incubating them within the inner surface of microwell. If a sample containing analytes is added to the antigens, specific antigen-antibody binding reactions take place and antibodies are captured on the solid surface. To detect the binding complexes formed, enzyme-labeled second antibodies are bound to the complexes, and substrates of the enzyme labels are then added to generate colorimetric signals which are uniquely related to each analyte.

We selected two enzymes, GAL and HRP, to be covalently coupled to each second antibody and chromogenic substrates for the respective enzyme, CPRG and TMB, that provide most sensitive colorimetric signals to date.[5] CPRG has a yellow background color and turns to red as a result of the enzyme reaction of GAL. This color change can be monitored at the absorbance of 570 nm.[5] The other chromogen, TMB, is colorless but produces a blue color resulting from the catalytic conversion of HRP in the presence of hydrogen peroxide. The blue color is measured at the absorbance of 650 nm.[12]

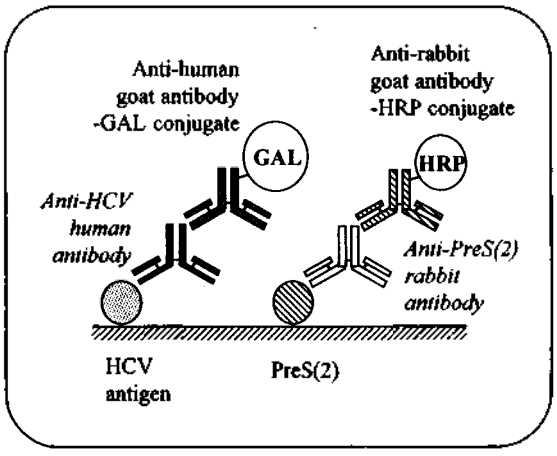

Figure 1. Model system of dual-enzyme immunoassays.

Because each enzyme, in general, requires particular chemical and physical conditions for its maximum activity, we determined optimal conditions of a dual-substrate solution such that the both of GAL and HRP can react at the same time and generate colorimetric signals in a single environment. This signal generation method was then applied to detect antigen-antibody complexes formed with antigens immobilized on solid surface.

Generation of Dual Signals

Enzyme reactions at different pH. The effect of pH on the enzyme activities was examined by accomplishing the reactions of GAL and HRP in a dual-substrate solution adjusted to variable pH (Figure 2; see data obtained at a constant temperature). The HRP activity, measured by the reaction with TMB, showed the maximum at pH 5.1 and proportionally decreased as pH rose. Almost no activity was detected at pH 7.0 or higher. On the other hand, GAL showed a maximum activity at pH 7.0 in the range used. The activity decreased at lower pH values and eventually vanished at pH 5.1. These results illustrate that the optimal pH condition for one enzyme cannot be used for the other.

These unique pH requirements for signal generation have been a limitation in performing dual-enzyme immunoassays in a single environment. This problem, however, may be resolved by selecting a pH between different optimal values for the respective enzyme. Although the use of pH other than the optima resulted in decreases of the enzyme activities, this lowered performance of signal generation has been overcome by 1) adjusting the concentration of substrate, 2) introducing an activator, and 3) elongating total

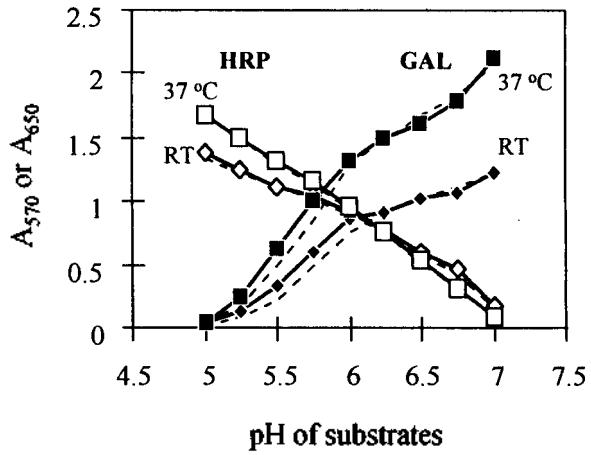

Figure 2. Signal generation from GAL and HRP incubated at different pH and temperatures. The enzyme reactions were performed either in a dual-substrate solution (solid curves) or in single substrates of each enzyme (dashed curves).

reaction time as shown below. Prior to these tests, a temperature optimal for the enzyme reactions was determined.

Determination of optimal temperature. The colorimetric signal from GAL in a constant concentration was 51 to 89% higher at 37 °C than at RT between pH 5.5 and 6.25 (Figure 2). The use of the higher temperature for the HRP reaction increased its signal in a lower pH range (pH≤ 6.0), but a little decreased in a higher pH. These results suggest that the both enzyme reactions can be carried out at the higher temperature without significant loss of signals.

The dual-substrate solution used was formulated by including the substrates of the two enzymes into a acetate buffer, pH 5.1, and the pH was adjusted by adding appropriate amount of Na_2HPO_4. Because chemical compounds required by one enzyme might inhibit the activity of the other enzyme, the results obtained with the multiple substrates (solid curves with markers in Figure 2) were compared with those with each single substrate (dashed curves). The enzyme signals produced in the dual and single substrates were not significantly different.

Optimization of chemical composition. The chemical composition of the multiple substrate solution was optimized to enhance the signals in a middle pH range (Figure 3). As mentioned, substrates included in the dual-substrate system are TMB and H_2O_2 for HRP, and CPRG for GAL. The optimal concentration of H_2O_2 is 0.006%, and, in both of the lower and higher concentrations, the HRP signal diminishes.[12] On the other hand, the GAL signal increases in proportion to the concentration of CPRG up to 2 mg/mL above which the signal becomes approximately constant.[2] The remaining substrate, TMB, is usually used in a concentration of 100 μg/mL (from 10 mg/mL stock in dimethyl sulfoxide).[12,13] However, we found that the TMB concentration can be increased two-fold without precipitation, and, at this concentration, the HRP signal was intensified (Figure 3, top). The degree of intensification was 25 to 38% and the maximum was attained in a pH range of 5.75 to 6.0. The use of the more TMB, however, caused a little decrease in the GAL signal although the effect was relatively negligible.

An enhancement of the GAL signal was also achieved by using potassium salts (Figure 3, bottom) among potential activators tested. The addition of KCl to the substrate solution resulted in 23 to 36% increase of the GAL signal as compared to the control, *i.e.*, no KCl added. The compound slightly increased the HRP signal as well. Similar effects were obtained with 0.4% K_2SO_4.

So far, optimal physical and chemical conditions of the multiple substrates except pH have been determined. For the pH of the solution, we have selected a value, *i.e.*, pH 5.75, which was favorable to the HRP reaction. As compared to the maxima, the signals from HRP and GAL under the selected pH condition decreased 30% and 53%, respectively (see the pH dependence at 37 °C in Figure 2). The decrease in the HRP signal can be completely restored by increasing the TMB concentration (Figure 3, top). However, the reduced GAL signal can only be partially recovered by using the activator, potassium salt (Figure 3, bottom), and the signal is still approximately 30% lower than that obtained at the optimal pH. To eliminate this signal loss, we used an approach of elongation of total reaction time as

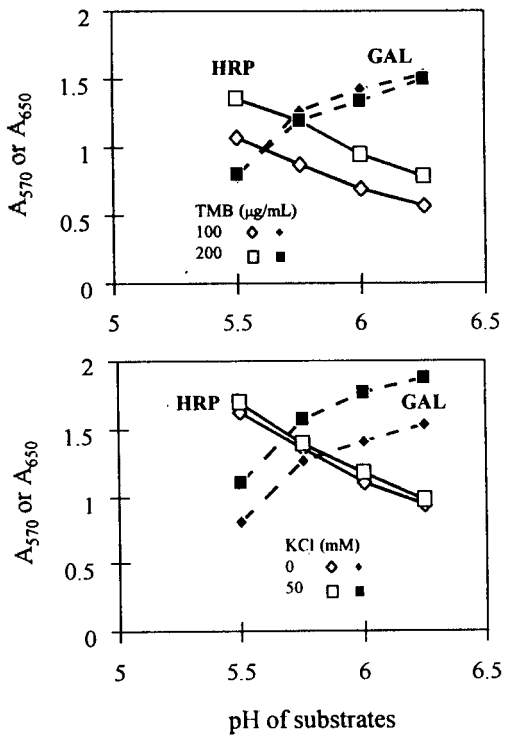

Figure 3. Enhanced signals from GAL and HRP under optimal concentrations of substrate and activator. The effect of concentrations of TMB as a substrate of HRP (top panel) and KCl as an activator of GAL reaction (bottom panel) were tested.

shown in the next subsection.

Determination of total reaction time. Because the dual-signal generation method will be applied for solid-phase immunoassays, total reaction time was determined by measuring signals from the enzymes present on solid surfaces (Figure 4). To this end, an enzyme-labeled second antibody was bound to the antibody analyte that was already reacted with the antigen immobilized on the inner surface of microwell. Under this condition, the intensity of the GAL signal produced for 30 min in the dual-substrate solution (pH 5.75) was approximately 35% lower than that obtained after the same time period in a single substrate (control; CPRG in Hepes buffer, pH 7.0) as expected (Figure 4, left panel). The signal, however, linearly increased as the reaction time extended (left panel, shaded bars) and, after 45 minute reaction, almost reached the level of the control signal. Differently from this pattern of signal generation, the HRP signal approached the maximum within 30 min and, thereafter, the signal increase was minimized (right panel, shaded bars). The maximum signal measured was comparable to a HRP control signal produced from another substrate for HRP, OPD, that has been widely used in immunoassays.[5] Based on these results, we set total reaction time for 45 minute to eliminate the GAL signal loss that occurred under the pH condition selected.

Construction of Model System

To demonstrate the utilization of the signal generation method developed, a model system of dual-enzyme immunoassays has been constructed (Figure 1). Antigens used

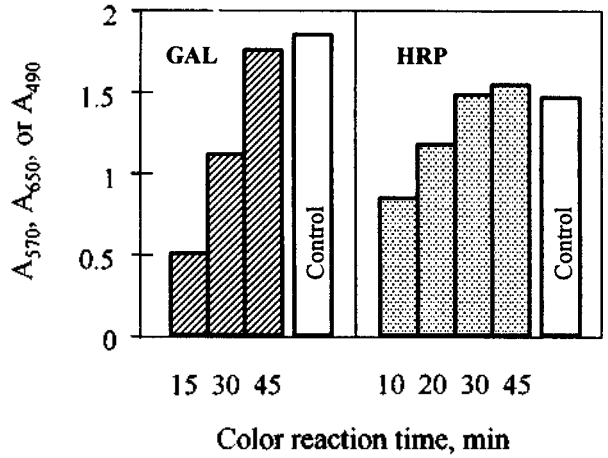

Figure 4. Determination of total enzyme reaction time under optimal conditions of a dual-substrate solution. The signals produced against time were compared with control (CPRG for GAL or OPD for HRP).

to capture analytes (antibodies specific to the antigens) were two recombinant proteins of HCV, core and NS5, and a synthetic peptide of PreS(2) present on HBV. Samples containing the analytes, *i.e.*, anti-HCV and anti-PreS(2) antibodies, were human sera drawn from donors who have been infected by HCV and a rabbit antiserum raised against PreS(2). Finally, anti-human goat polyclonal antibody conjugated to GAL and anti-rabbit goat antibody labeled with HRP were used for the generation of signals related to the respective analyte.

Different complex formation with co-immobilized antigens. By using the model system devised, we first studied on the different binding complex formation with the antigens immobilized on the same solid surface (Figure 5). In this experiment, single substrates (CPRG for GAL or OPD for HRP) for each enzyme were used to reduce complexities in interpreting the experimental results. To measure the binding complex formed with the HCV antigens (Figure 5, top), test samples used were two positive human sera containing anti-HCV antibodies (p74 and p102), a negative serum (p53), and a control (casein, no serum contained). Each sample was incubated with the immobilized antigens in the presence of anti-PreS(2) antibody (dual) or in the absence (single). Non-specific binding (NSB) of the enzyme conjugate was measured without analyte. If the signal of 'dual' was compared with that of 'single', the amount of binding complex formed with the HCV antigens was significantly reduced by the presence of the other complex with PreS(2). Non-specific binding of the enzyme conjugate and the analyte was negligible although a serum effect slightly appeared (compare the results with p53 and casein).

The other binding complex formed with PreS(2) was monitored by using a HRP substrate, OPD (Figure 5, bottom). A rabbit antiserum as sample containing anti-PreS(2) antibody was diluted to a range between 1/100 and 1/500, and incubated with the antigens in the presence (dual) of a positive antiserum to HCV (p102) or in the absence (single). Contrary to the results in the above, the amount of complex with PreS(2) increased in the presence of the other complex with the HCV antigens, and this effect was gradually reduced as the complex density of PreS(2) approached the maximum (saturation). In these measurements, non-specific binding of any components was also insignificant.

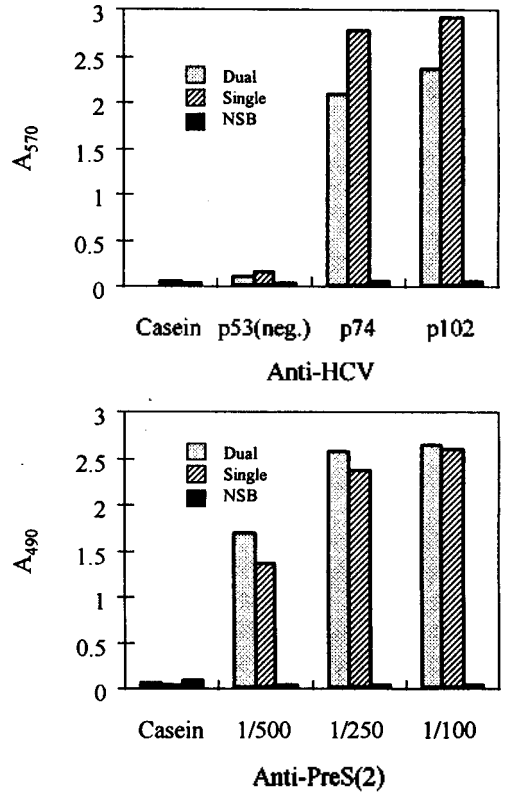

Figure 5. Different complex formation of antibodies with co-immobilized HCV antigens and PreS(2).

Inhibition or activation of a binding by the other can be caused by several factors. First, since the antigens co-immobilized on the same surface forms a patch (locally, high density),[14,15] a simultaneous binding of antibodies to adjacent antigen molecules is difficult to occur due to the large molecular size of immunoglobulin. This can result in a competition between different antigen-antibody reactions, which may cause an inhibition of a binding by the other with a higher avidity. Second, the complex formation can also be affected by the molecular size of the second antibody-enzyme conjugate. The enzyme, GAL (M_r=465,000), is much larger than HRP (M_r=40,000), and the antibody labeled with the larger enzyme would have a lower accessibility to the immobilized antigens (see Figure 5, top).[15,16] Finally, a binding reaction can be enhanced by the other, which might result from molecular interaction. Protein molecules, in general, can interact each other via weak forces such as van der Waals force and electrostatic force. This interaction may create a binding of molecule induced by other molecules that are already bound on solid surface (see Figure 5, bottom).[7,13]

Dose-response curves. To demonstrate a simultaneous detection of multiple analytes in a single microwell, the model system of immunoassay was combined with the dual-signal generation method, and dose-response curves for each analyte were obtained (Figure 6). A human serum containing anti-HCV antibody, p102, was selected and diluted

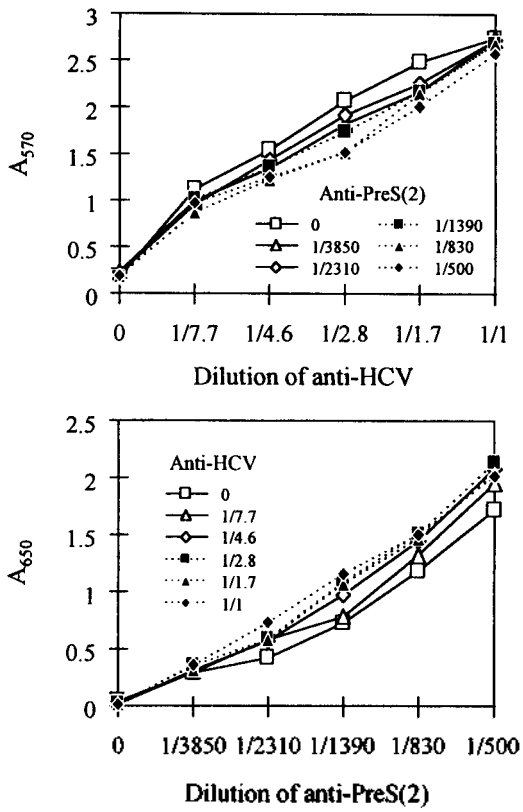

Figure 6. Dose-response curves of dual-enzyme immunoassays.

to a range. Each diluted sample was mixed with different concentrations of anti-PreS(2) antibody. After these mixtures were incubated with the co-immobilized antigens, binding complexes formed were subsequently reacted with the second antibodies labeled with distinct enzymes, GAL and HRP. Signal generation from these enzymes were performed in the dual substrates under the optimal conditions as determined above. If anti-HCV antibody was solely present in the combined samples, a red color from GAL, which was related to this analyte, was produced and measured at 570 nm (open square in Figure 6, top). If anti-PreS(2) was the only analyte in the samples, a blue signal from HRP was generated and quantified at 650 nm (open square in Figure 6, bottom). The intensities of these two signals were directly proportional to the analyte concentrations.

In case of the presence of the both analytes, the two colorimetric signals were produced at the same time. The red color under this condition cannot be accurately measured since the absorbance range of the blue color superimposed partly on that of the red signal. For this reason, after measuring the blue signal 0.1 M Na_2CO_3 was added to the solution. The blue color was then vanished while the red signal was remained constant against time. The same procedure of dual-signal detection was used throughout experiments for obtaining dose response curves.

Although the pattern of dose-response curves for an analyte was similar as shown in Figure 6, the curve location changed in response to the concentration of the other analyte. The curves for the anti-HCV antibody (Figure 6, top) was shifted down as the concentration of anti-PreS(2) antibody increased, which represented an inhibition of the formation of a binding complex by that of the other complex. To the contrary, the curves for the anti-PreS(2) antibody moved upward in proportion to the concentration of anti-HCV antibody (Figure 6, bottom). This standed for an activation effect of a complex formation on the other. These results were consistent with those shown in Figure 5. The presence of the cooperativity between two different complex formation suggested that the dual-enzyme immunoassay can only be utilized for qualitative screening tests such as the detection of infectious diseases in blood banks.

Quantitative analyses would also be possible provided the cooperativity is minimized. This condition may be obtained by immobilizing each antigen on spatially separated areas of the solid surface. One antigen is coated on the half of total surface by transferring an equivalent volume into microwell, and residual sites are then blocked with an inert protein (*e.g.*, casein). The next half surface can be coated with the other antigen by adding a liquid volume that covers the total surface. Such prepared microwell could be used for quantitative assays according to the protocol presented in this investigation.

In conclusions, simultaneous reactions of two different enzymes, GAL and HRP, as signal generators were carried out at a midpoint between the two optimal pH values for each enzyme without impairing the signal yields. This was achieved by optimizing the chemical composition of a multiple substrate solution, the reaction temperature, and total reaction time. The utilization of the signal generation method developed was demonstrated in dual-enzyme immunoassays for antibodies with co-immobilized antigens on the same solid surface. The model system generated dose-response curves for each analyte in an identical pattern but revealed cooperativity between different complex formation.

References

1. Blake, C.; Al-Bassam, M. N.; Gould, B. J.; Marks, V.; Bridges, J. W.; Riley, C. *Clin. Chem.* **1982**, *28*, 1469.
2. Paek, S. H.; Kim, J. H. *Biotech. Bioeng.* **1996**, *51*, 591.
3. Cleland, W. W. *Meth. Enzymol.* **1982**, *87*, 390.
4. Tipton, K. F.; Dixon, H. B. F. *Meth. Enzymol.* **1979**, *63*, 183.
5. Porstmann, B.; Porstmann, T. In *Nonisotopic Immunoassay*; Ngo, T. T., Ed.; Plenum Publishing Corp.: New York, U. S. A., 1988; p 57.
6. Marci, J. N.; Spencer, K.; Anderson, R. *Ann. Clin. Biochem.* **1992**, *29*, 390.
7. Postmann, T.; Nugel, E.; Henklein, P.; Dopel, H.; Ronspeck, W.; Pas, P.; von Baehr, R. *J. Immunol. Meth.* **1993**, *158*, 95.
8. Cho, J. M.; Lim, K. J. Korea Patent Application 91-13596.
9. Cho, J. M.; Choi, D. Y. Korea Patent Application 93-07232.
10. Schetters, H. In *Methods of Immunological Analysis*; Masseyeff, R. F., Ed.; VCH: Weinheim, Germany, 1993; Vol. 2, p 176.
11. Bauminger, S.; Wilchek, M. *Meth. Enzymol.* **1980**, *70*, 151.
12. Bos, E. S.; van der Doelen, A. A.; van Rooy, N.; Schuurs, A. H. W. M. *J. Immunoassay* **1981**, *2*, 187.

13. Paek, S. H.; Bachas, L. G.; Schramm, W. *Anal. Biochem.* **1993**, *210*, 145.
14. Kricka, L. J. *Ligand-Binder Assays*; Dekker: New York, U. S. A., 1985; p 76.
15. Schramm, W.; Paek, S. H. *Anal. Biochem.* **1992**, *205*, 47.
16. Matson, R. S.; Little, M. C. *J. Chromatogr.* **1988**, *458*, 67.

Conformation and Biological Activity of Mastoparan B and Its Analogs I

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Received August 30, 1996

The mode of action of mastoparan B, an antimicrobial cationic tetradecapeptide amide isolated from the hornet *Vespa basalis*, toward phospholipid bilayers was studied with synthetic mastoparan B and its analogs with individual Ala instead of hydrophobic amino acids (1-Ile, 3-Leu, 6-Leu, 7-Val, 9-Trp, 13-Val, 14-Leu) in mastoparan B. Mastoparan B and its analogs were synthesized by the solid-phase method. Circular dichroism spectra showed that mastoparan B and its analogs adopted an unordered structure in buffer solution. In the presence of neutral and acidic liposomes, most of the peptides took an α-helical structure. The calcein leakage experiment indicated that mastoparan B interacted strongly with neutral and acidic lipid bilayers than its analogs. Mastoparan B also showed a more or less highly antimicrobial activity and hemolytic activity for human erythrocytes than its analogs. These results indicate that the hydrophobic face in the amphipathic α-helix of mastoparan B critically affect biological activity and helical contents.

Introduction

Mastoparan (MP), an antimicrobial cationic tetradecapeptide amide isolated from the venom of wasp (*Vespula lewisii*), is an amphiphilic α-helical peptide and its primary structure is Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2.[1] MP toxin possesses a variety of biological activities such as activation of mast cell degradation histamine release,[2,3] phospholipase A_2[3,4] and C,[4,5] erythrocyte lysis and binding to calmodulin.[6] MP is also turned out to enhance the permeability of artificial membranes and biomembranes[7] and activate GTP-binding regulatory proteins (G-proteins) in a manner similar to that of G-protein-coupled receptors *in vitro*.[8] Structure-activity relationship studies with the use of various natural and synthetic compounds have shown that the amphiphilic α-helical structure with cationic amino acid residues on one side and hydrophobic residues on the other side is crucial to show such biological activity.[2,9] However, more recent study[10] has reported that such structural feature is necessary but not itself sufficient to stimulate GTPase of G-protein.

A similar peptide, namely mastoparan B (MP-B), was isolated from the venom of the hornet *Vespa basalis*[11] (Figure 1). This peptide not only caused liberation of histamine from rat peritoneal mast cells, but also possesses an more potent hemolytic activity than MP. MP-B has been shown to elicit cardiovascular depressor[12] and to inhibit the growth of Gram-positive and -negative bacteria, at a minimum concentration of 19.5 μg/mL.[13] Based on this relation, we have reported that MP-B has antimicrobial activity against both bacteria and leakage ability.[14] This peptide revealed the amphiphilic property as shown in the helical wheels.[14] NMR studies of MP-B in trifluoroethanol (TFE)-containing aqueous solution have indicated that residues 3-14 adopt an amphiphilic α-helical structure in which the residues with hydrophilic side chains (*i.e.* Lys-4, Ser-5, Ser-8, Lys-11, Lys-12) are located on one side and the residues with hydrophobic side chains (*i.e.* Leu-3, Ile-6, Trp-9, Ala-10, Val-13, Leu-14) located on the other side of the molecule.[15] The previous CD studies have shown that MP and MP-B take a random structure in buffer solution and α-helical structure in the presence of phospholipid bilayers,[9] but MP also adopts α-helical structure at high ionic strength (more than 1 M NaCl) in aqueous solution.[16] MP-B has more hydrophilic amino acid residues on the hydrophilic side of the amphiphilic structure (1-Leu, 5,8-Ser, 2,4,12-Lys, 9-Trp) as compared with those of MP (1-Ile, 2-Asn, 5,8-Ala, 4,12-Lys, 9-Leu), although both peptides have almost same residues on the hydrophobic side. Such more hydrophilic surface on the molecule might lead to the change in its interaction with membranes, resulting in the alternation in its biological activity. The interaction of MP-B and its analogs with phospholipid bilayers have not reported as yet. Thus in order to attain further information of the relationship between the hy-