Generation of Colorimetric, Dual Signals from Two Different Enzymes by Using Catalytic pH Shift

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Because enzymes usually require distinct pH conditions for maximum activity, an approach of spontaneous pH shift of solution was devised to derive multiple reactions in a sequence. The two enzymes selected, horseradish peroxidase (HRP) and β-galactosidase (GAL), had dissimilar optimal pH levels, i.e., 5.1 and 7.0, respectively. In a solution, HRP initially reacted at a lower pH range and the GAL reaction was consecutively carried out at a higher range in the presence of a third enzyme, urease, which caused an increase in pH. Under optimal conditions, the multiple system provided comparable performances with those of single reactions. © 1996 John Wiley & Sons, Inc.
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INTRODUCTION

Although immunoassays have been widely used as sensitive means for analyzing complex organic compounds, the analytical mode is exclusively single, i.e., only one analyte is measured in a system, such as in a plastic microwell with coated reagent (Gosling, 1990; Gould and Marks, 1988). In analyzing samples containing more than one analyte, a multi-analyte detection system would provide savings in labor and time. A difficulty in constructing such a system lies in the fact that the generation of signals, each of which is uniquely linked to a different analyte, should generally be carried out under identical conditions. Enzymes used as signal generators usually show their maximum activities under particular chemical conditions, such as the pH of solution (Cleland, 1982; Tipton and Dixon, 1979). Distinct pH requirements for multiple-enzyme reactions cannot be afforded in a single environment. Typical optimal pH values for enzymes that have been extensively used as tracers in immunoassays are 5.1 for HRP, 7.0 for GAL, and 9.8 for alkaline phosphatase (AP) (Paek et al., 1993; Porstmann and Porstmann, 1988; Porstmann and Kiessig, 1992).

Double enzyme immunoassays that might overcome the problem have been performed. Blake et al. (1982) used GAL and AP for dual-signal generation in a microwell. The enzyme reaction of GAL was first carried out and its signal was measured by using colorimetry. After washing the well, the enzyme AP was reacted by adding a substrate solution to produce the other signal. Recently, Macri et al. (1992) and Porstmann et al. (1993) also followed the same procedure using HRP and AP. This type of signal generation may be easy to apply, but it is cumbersome to conduct. Moreover, the second enzyme in solid phase was partially lost by washing after the reaction of the first enzyme, which caused a poor sensitivity of detection.

We have developed a novel method of multiple-signal generation from two different enzymes by shifting the pH of a mixed substrate solution. The pH of the solution was initially maintained at the optimal condition for one enzyme and then spontaneously changed to trigger the other enzyme reaction. These sequential reactions were achieved in the presence of a third enzyme that gave rise to a change in pH. The enzyme urease can increase pH, whereas glucose oxidase and penicillinase can decrease pH (Nilsson et al., 1973). Urease has been selected as the modulator of the dual-signal generation system using the two enzymes HRP and GAL. In this article, the determination of optimal conditions for maximum signal intensities and the evaluation of the dual-signal system are presented.

MATERIALS AND METHODS

Materials

HRP (1000 units/mg protein; EC 1.11.1.7), GAL (600 units/mg protein; EC 3.2.1.23), urease (100 units/mg protein; EC 3.5.1.5), and chlorophenol red-β-galactopyranoside (CPRG) were purchased from Boehringer Mannheim (Germany). 3,3′,5,5′-Tetramethylbenzidine (TMB), *o*-phenylenediamine dihydrochloride (OPD), bromocresol purple, sodium dodecylsulfate (SDS), sodium *N*-lauroylsarcosine (SLS), and heparin sodium salt (grade I-A) were obtained from Sigma (St. Louis, MO). Hydrogen peroxide and Immulon II microtiterwell were purchased from Duksan

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Chemical (Korea) and Dynatech Inc. (Alexandria, VA), respectively. All other reagents used were of analytical grade.

Substrate of urease. The substrate solution for urease was made as described elsewhere (Coulepis et al., 1985) and contained: 8 mg of bromocresol purple; 0.48 mL of 10 m*M* NaOH solution; 100 mg urea; 7.4 mg ethylenediaminetetraacetic acid disodium salt; and 100 mL deionized water. This mixture was adjusted to pH 4.8.

Single substrate of HRP. The substrate solution for HRP was prepared as recommended by the manufacturer. OPD (4 mg) was dissolved in 10 mL of 50 m*M* 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. CPRG (20 mg) was dissolved in 100 m*M* HEPES buffer (pH 7.0) containing 150 m*M* NaCl, 2 m*M* $MgCl_2$, 1% (w/v) bovine serum albumin, and 0.1% (w/v) sodium azide.

Multiple substrates of HRP and GAL. Because the composition of mixed substrate solution for HRP and GAL were varied to obtain high signals from the enzymes, components and their concentration ranges tested is described in each experiment below. The optimal composition of the solution was as follows (composition 1): 0.009% H_2O_2, 200 μg/mL TMB (from 5 mg/mL stock solution in dimethylsulfoxide); 2 mg/mL CPRG; 1 mg/mL urea; 20 m*M* NaCl; 2 m*M* $MgCl_2$, 0.01% (w/v) SDS; and 0.08 m*M* NaH_2PO_4 in 50 m*M* acetate buffer (pH 5.1).

Variation of pH by Using Urease

The enzyme urease was diluted in a range of 1 to 50 pmol/mL with 150 m*M* saline containing 0.1% (w/v) gelatin (Gel-saline) and 10 μL of each diluted solution were then transferred into different microwells. After addition of 100 μL of a multiple substrate solution (composition 2: 0.03% H_2O_2; 100 μg/mL TMB; 1 mg/mL urea; 20 m*M* NaCl; and 2 m*M* $MgCl_2$ in acetate buffer [pH 5.1]) and the equal volume of the substrate for urease into the wells, the color change of bromocresol purple as an indicator of pH shift was detected in a kinetic mode at an absorbance of 570 nm. The enzyme reactions were repeated at various molarities of the acetate buffer (0 to 10 m*M*). All measurements in this article were performed in duplicate and the means were used for plotting.

Stabilization of Oxidized TMB

To prevent the blue signal from fading as pH rose, the following potential stabilizers were tested: 0.1% (w/v) SDS; 0.1% (w/v) SLS; and 50 units/mL heparin. Twenty microliters of the stabilizer was combined with 180 μL of multiple substrate solution (composition 2 with 1.5 m*M* acetate buffer) containing 1 mg/mL CPRG. This mixture was added into a well after 10 μL of 1 pmol/mL HRP and an equal volume of 5 pmol/mL urease diluted with Gel-saline were placed. The blue color produced was measured at 650 nm as a function of time.

Determination of Optimal Buffer Molarity

In the absence of urease, 10 μL of HRP in a concentration range of 0.2 to 1 pmol/mL was located in microwells that already contained the same volume of 0.5 pmol/mL GAL, and 200 μL of substrate solution was added. The substrate solution consisted of 1 mg/mL CPRG and 0.01% SDS in addition to composition 2 with 1 m*M* acetate buffer. After reaction for 1 h, the signals from HRP and GAL were measured at 650 nm and 570 nm (after addition of 50 μL of 100 m*M* Na_2CO_3), respectively. The same procedure was repeated with higher molarity acetate buffers.

Determination of Optimal Substrate Concentrations

Two enzymes, HRP and GAL (10 μL of 0.5 pmol/mL each), were located in separate wells and urease (10 μL of 15 pmol/mL) was placed within the wells. A multiple substrate solution with composition 1 except 0.02 m*M* NaH_2PO_4 and 20 m*M* acetate buffer (pH 5.24), in addition to the variable amounts of H_2O_2 and CPRG, was immediately added. The colors developed in the solutions were monitored as previously described. After 60 min, the reactions were stopped by adding 50 μL of 100 m*M* Na_2CO_3.

Evaluation of Dual-Signal System

Under optimal conditions, the intensities of dual signals produced by using the multiple substrates were compared with those of signals from the single-substrate systems of HRP and GAL. The final assay procedure determined for the dual-signal system was as follows: (1) locate 10 μL of each or both of the enzymes within wells; (2) place 10 μL of urease (300 fmol/well) into the wells; (3) add 200 μL of the multiple substrates of composition 1; (4) perform the enzyme reactions at room temperature (RT) for the first 30 min and at 40°C for the rest of the total 60 min; and (5) measure the absorbances of signals as described. For the single systems, each of the enzymes was located in wells and the respective substrate (see Materials subsection) was added. After incubation for 30 min at RT, the HRP reaction was stopped by adding 50 μL of 1 *M* H_2SO_4 solution and color density was determined at 490 nm. Under identical incubation conditions, the signal resulting from the GAL reaction was detected at 570 nm.

RESULTS AND DISCUSSION

A dual-signal system has been constructed with the following major components that meet suitable criteria:

(1) two enzyme signal generators (HRP and GAL) with high turnover rates; (2) chromogenic substrates (TMB and CPRG) of which the catalytic products can be measured at distinct wavelengths; and (3) a third enzyme (urease) that causes a pH shift within a desired range (Bos et al., 1981; Porstmann and Porstmann, 1988). An enzymatic product of TMB gives a blue color that can be measured at an absorbance of 650 nm (Bos et al., 1981). The substrate, CPRG, in solution has a yellow background color and was catalytically converted to a red-colored product which can be monitored at 570 nm (Porstmann and Porstmann, 1988). Typical optimum pH values for HRP and GAL are 5.1 and 7.0, respectively (Cattaneo and Luong, 1994; Paek et al., 1993; Porstmann and Porstmann, 1988). Across these two values, urease can induce a pH shift by the catalytic action that consumes proton in solution (Moynihan and Wang, 1987; Reithel, 1971).

Signal Generation by Using pH Shift

Signal generation from the two enzymes was initially performed to investigate potential problems under variable conditions of pH toward time. In the presence of urea, the enzyme urease increased pH, which was monitored by the color change of bromocresol purple (Fig. 1). The pattern of catalytic pH shift depended on the urease concentration and the buffer molarity of solution. Under a constant buffer molarity, the time interval before the onset of pH shift from near 5 to higher than 7 was inversely proportional to the urease concentration (Fig. 1, left). Provided 50 fmol of urease was present in a well, the pH of solution was maintained for 20 min at the lower pH range where an enzyme such as HRP could react with maximum activities (Bos et al., 1981). The pH was then abruptly raised by the action of urease, eventually to the higher range, where GAL reaction could take place for the rest of the total time period. The reaction times of each enzyme were also able to be controlled by varying the buffer molarity (Fig. 1, right).

In the generation of colorimetric signals from HRP and GAL, by utilizing the catalytic pH shift, two problems were encountered: instability of the blue signal and influence of one enzyme reaction on another. First, the density of blue color produced by HRP was gradually decreased as pH increased. Second, the intensity of red color resulting from the GAL reaction was higher in the presence of the HRP reaction than in its absence. These effects are analyzed in the next subsection.

Solving Problems

In the first problem, the blue signal represents the color of oxidized TMB as a product of HRP reaction in the presence of TMB and H_2O_2 as substrates (Bos et al., 1981). This oxidized product is a cationic radical (Porstmann and Porstmann, 1988) that could become unstable as pH varies (Cattaneo and Luong, 1994). Since color stability is essential such that the signal can be measured at the end of assays, various potential stabilizers were tested by introducing each into the mixed substrate solution (Fig. 2). Materials selected were two anionic detergents, SDS and SLS, and a negatively charged polysaccharide, heparin. When SDS was added into the solution, more than 95% of the blue color produced during the pH variation was preserved after 60 min, although the maximum signal was approximately 15% lower than that without SDS (control). Unlike SDS, detergent SLS did not protect oxidized TMB. With heparin, the product remained stable even though the level of the enzyme activity was significantly low.

The stabilizing effect of SDS on oxidized TMB may result from either charge interaction between the two molecules or hydrophobic binding. The SDS molecule has a strongly negative charge (Helenius and Simons,

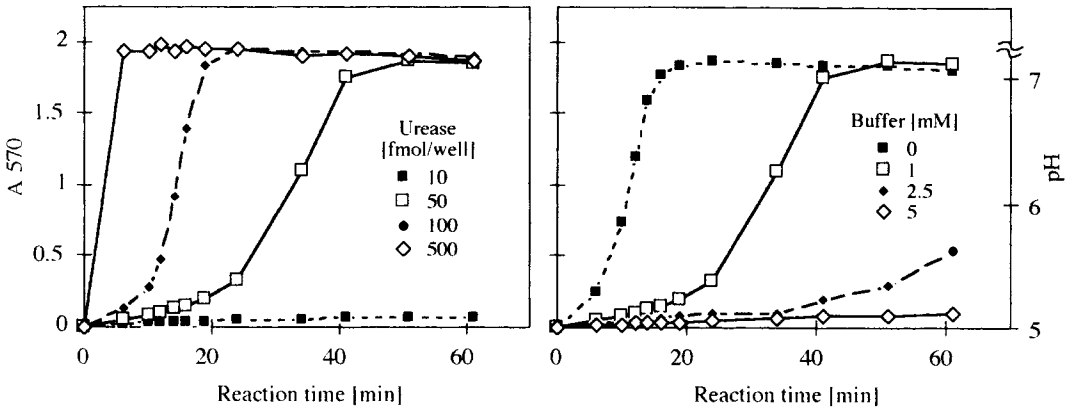

Figure 1. Variation of pH in solution by the action of urease. In the presence of urea, urease increased pH, which was monitored by using a pH indicator (bromocresol purple) at A_{570}. The pH values corresponding to the colors were also determined. Left: At a constant molarity of buffer (final 1 m*M* acetate buffer, pH 5), the concentration of urease was varied. Right: At a constant concentration of urease (50 fmol per well), the buffer molarity was changed. The buffer molarities in the figure indicate final concentrations.

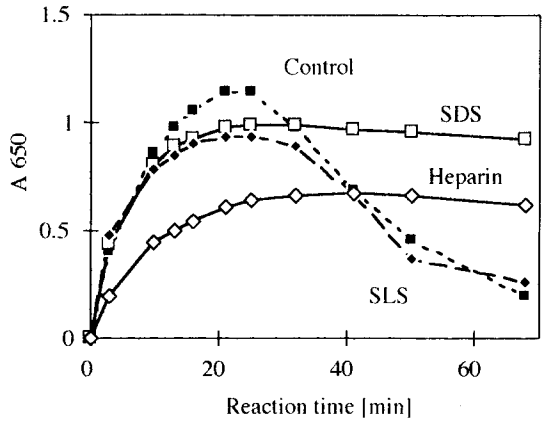

Figure 2. Stabilization of oxidized TMB during pH change. Potential stabilizers tested were SDS, SLS, and heparin. SDS and heparin protected oxidized TMB although inhibitory efffects on HRP were found if compared with control (no stabilizer present).

1975) which may interact with the cationic part of the oxidized TMB. This was supported by the result obtained with heparin which also carries strong anions. SLS detergent was also anionic but only weakly charged as compared with SDS and heparin. Because SLS was ineffective in stabilizing the radical, hydrophobic interaction alone does not seem to be contributive to stabilization. The optimal concentration of SDS was 0.01% in the substrate solution if determined regarding the degree of stabilization and the activities of HRP and GAL.

The second problem confronted was an increased intensity of the GAL signal in the presence of the HRP reaction, as mentioned earlier. We investigated this phenomenon by measuring signals from the two enzymes placed in the same well containing a mixed substrate solution without urease (Fig. 3). Under this condition, the GAL reaction should not take place regardless of the amount of HRP contained since no urease was present.

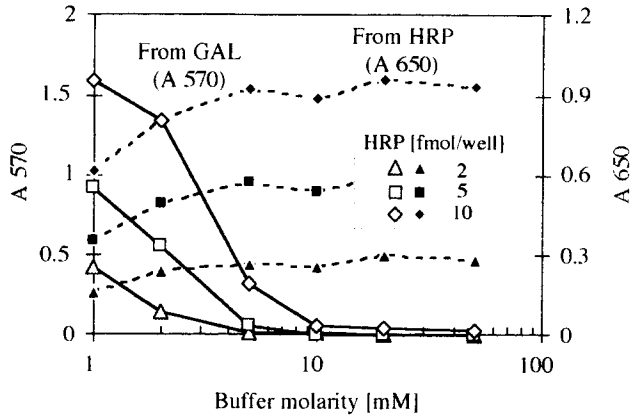

Figure 3. Determination of optimal buffer molarities to minimize the activation effect of the HRP reaction on the generation of signal from GAL. Signals from the two enzymes placed in a well containing a mixed substrate solution were monitored without urease. The GAL reaction (5 fmol/well GAL) was accomplished in the presence of different concentrations of HRP (2, 5, and 10 fmol/well).

However, the red color from GAL was detected in the presence of the HRP reaction and even enhanced in proportion to the HRP concentration if the buffer molarity of solution was lower than 10 m*M*. In this range of molarity, the red signal density decreased as the molarity was increased at a constant amount of HRP. Eventually, no GAL reaction was detectable when the molarity was higher than 10 m*M*. Thus, the red signal produced without urease was due to pH increase caused by the HRP reaction. A minimal influence on the GAL reaction was achieved with either 20 or 50 m*M* buffer solution that was used in next experiments.

Optimization of Variables

To enhance the signal intensities, variables that control the enzyme reaction rates have been optimized. The reaction rate is a function of rate constant and substrate concentration at a constant amount of enzyme (Bailey and Ollis, 1986). The rate constant depends on the temperature used for carrying out the reaction. Reaction times provided for each enzyme are also variables since total amounts of signals accumulated for a certain time period are measured. If the reaction times are kept constant by fixing the urease concentration, the buffer molarity, and total reaction time, major controlling factors remained will be the substrate concentration and the reaction temperature. In the following experiments, the urease concentration was adjusted to initiate the GAL reaction approximately 20 min after the addition of substrates and total reaction time was fixed at 60 min.

Substrate concentrations. Optimal concentrations of substrates, H_2O_2 for HRP and CPRG for GAL, were experimentally determined (Fig. 4). All substrates in desired concentrations were appropriately combined in a solution and added to each enzyme in separate wells. At a constant concentration of CPRG, the blue signal from HRP increased as the concentration of H_2O_2 was decreased in the selected range (Fig. 4, left). The optimal amount of H_2O_2 was approximately 0.009% below which the signal diminished (not shown). This pattern of the H_2O_2 effect was identical to results obtained with conventional systems (Bos et al., 1981; Porstmann and Porstmann, 1988). The GAL activity was also higher at lower concentrations of H_2O_2 although the degree was relatively small. At the optimal amount of H_2O_2, the concentration of CPRG in the solution was varied. The red signal from GAL significantly increased as the CPRG concentration became higher, while the signal from HRP showed a negligible change (Fig. 4, right). The optimum quantity of CPRG was approximately 2 mg/mL in solution as recommended by the manufacturer.

If 0.1 *M* Na_2CO_3 was added to the solution after total reaction time of 60 min, the blue color vanished, while the red signal was maintained approximately constant as shown in Figure 4. The procedure had to be intro-

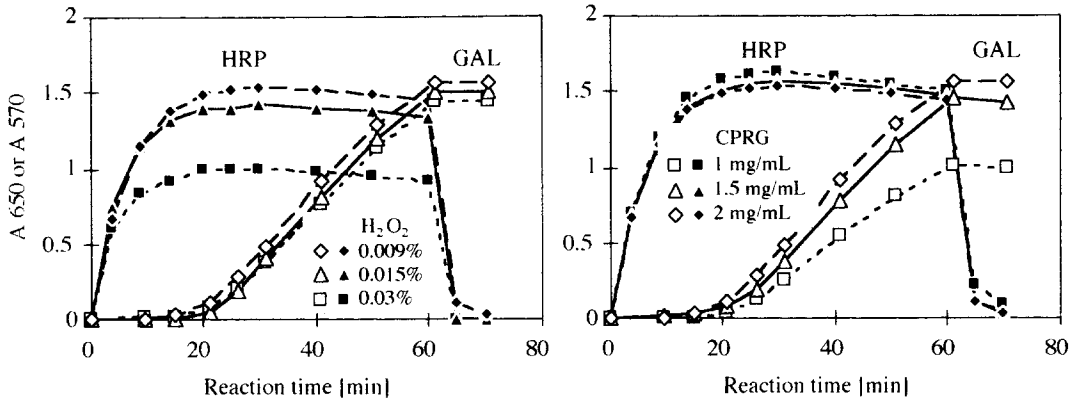

Figure 4. Determination of optimal substrate concentrations toward the signal intensities from HRP and GAL. The two enzymes (5 fmol/well each) were reacted in separate containers for convenience in measuring the two color signals as a function of time. A concentration range of substrate for HRP (H_2O_2) was tested at a constant CPRG amount (left). By using the optimal concentration of H_2O_2 determined, the concentration of substrate for GAL (CPRG) was varied within a selected range (right). Then 0.1 *M* Na_2CO_3 was added at the end of the assays (60 min) to eliminate the blue color.

duced for the detection of the dual signals in multiple assays. In fact, the red signal could not be accurately measured without eliminating the blue color since the absorbance range of the blue color superimposed partly on that of the red signal. The same procedure of signal detection was used in evaluating the dual-signal system (see Evaluation of Dual-Signal System).

Reaction temperature. Preliminary experiments revealed that the signal from GAL ascended at 40°C as compared to that at RT while the signal from HRP descended. The increased GAL signal resulted from acceleration of the GAL reaction at the higher temperature. The descended HRP signal could be due to thermal inactivation of the enzyme (Porstmann and Porstmann, 1988). This problem may be more profound in a reaction with less enzyme. Therefore, the incubation condition providing optimal temperatures for each enzyme, i.e., reaction at RT for the first 30 min and then at 40°C for the rest, was selected as the final protocol.

Evaluation of Dual-Signal System

The performance of the dual-signal system was compared with those of conventional single systems, such as OPD as a substrate for HRP and CPRG for GAL, which have provided the most sensitive signals to date (Porstmann and Porstmann, 1988). The dual-signal generation was carried out under the optimal conditions as determined above, and signals from the conventional systems were developed and detected according to the protocols recommended by manufacturers.

The colors produced from each system were quantified at their maximum absorbances and used to contrast the signal sensitivities (Fig. 5). The plot of HRP signals from the dual system with or without the GAL reaction versus those from the single OPD system showed a linear relationship (correlation coefficients >0.990). The absorbance value from the new system was approximately 30% lower than that from the conventional system under a constant amount of enzyme. The plot also showed that the HRP reaction in the dual system was not affected by the GAL reaction. In the plot for GAL, the two signals from the new system and the single CPRG system also exhibited approximately linear correlation (correlation coefficients >0.985). The magnitudes of signals were 20% to 25% higher in the new system than in the conventional system, which resulted from the incubation of the GAL at a higher temperature. In the dual system, the GAL signal slightly increased in the presence of the HRP reaction as compared to that in the absence. The HRP reaction could accelerate the pH increase and thus resulted in the higher signal as mentioned. However, this will not cause significant

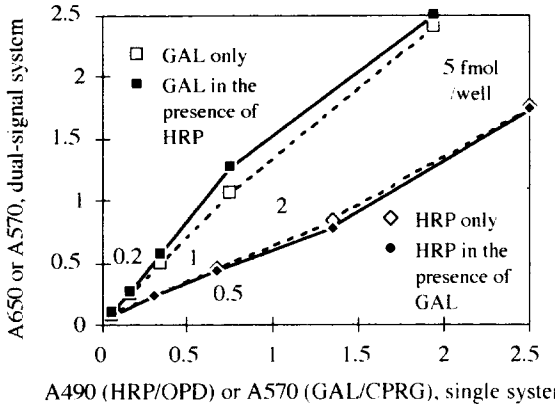

Figure 5. Correlation between the dual-signal system and conventional signal systems. Incubation temperatures used for the dual system were RT for the first 30 minutes and 40°C for the next 30 min. Single systems selected for comparison were OPD as a substrate of HRP and CPRG for GAL. The single enzyme reactions were carried out at RT. In the dual system, signals from one enzyme were measured both in the absence of the other enzyme and in its presence.

problems if the new system is utilized for qualitative analyses such as the detection of infectious diseases. In spite of some differences, all systems had a comparable enzyme detection limit of approximately 0.2 fmol of enzyme per well.

Applications

If the dual-signal generation system is used in multiple immunoassays, the system will introduce an additional variable of managing the third enzyme, urease. This pH modulator can be supplied in a solution to construct the multiple assay in a simple version. After formation of immunologic complexes in the assay, the unbound portion of reagents can be removed with a washing solution containing urea. A drop of this solution remaining after washing may contain a sufficient amount of substrate for urease that can be simply added from a solution. Without complication, the use of the catalytic pH shift described in this article would be a potential approach for the generation of multiple enzyme signals, each of which can uniquely be linked to different analytes.

In conclusion, sequential reactions of two enzymes, HRP and GAL, were derived in a mixed substrate solution by using a third enzyme, urease, which caused a pH shift from one value optimal for HRP to the other for GAL. Colorimetric, dual signals resulting from the enzyme reactions were measured at distinct wavelengths and the detection limits of enzymes under optimal conditions were comparable with those from conventional, single enzyme–substrate systems that were most sensitive.

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