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Use of Soybean Peroxidase in Chemiluminescent Enzyme-Linked Immunosorbent Assay

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A procedure for the production of conjugates of soybean peroxidase (SbP) oxidized by sodium periodate and anti-mouse IgG antibody (Ab) was optimized. A sandwich chemiluminescent enzyme-linked immunosorbent assay (ELISA) for determination of mouse IgG using SbP and specific Ab was developed, and SbP-catalyzed oxidation of luminol was carried out in the absence of any enhancer. Comparison of conjugates produced by labeling Ab by soybean and horseradish peroxidases in the chemiluminescent ELISA showed that in the case of SbP a rate of emission decay formed through luminol oxidation was significantly lower. Application of the soya enzyme allowed the development of the immunoassay with improved sensitivity and a wider linear range.

KEYWORDS: Peroxidase; soya; ELISA; chemiluminescence; luminol; hydrogen peroxide; enhancement

INTRODUCTION

Peroxidase (EC 1.1.11.7) is one of the most widely distributed plant enzymes. Peroxidases were isolated from various plant sources (*1*−*5*), but presently, only the cationic isoenzyme *c* of the peroxidase purified from horseradish roots (HRP-C) is widely used in practice. This enzyme is applied for removal of aromatic amines and phenols from industrial waters, in organic synthesis, for bleaching of industrial dyestuffs, etc. (*6*−*10*). However, the widest application of the peroxidase is found in enzyme-linked immunosorbent assays (ELISA) as an enzyme label of immunochemical reagents (*11*).

Multiple methods for the detection of enzyme activity of peroxidase-labeled immunoreagents have been developed including colorimetry, fluorimetry, and luminescence (*12*). Luminescence detection is markedly more sensitive than other methods (*13*). Luminol is the peroxidase substrate commonly used in luminescence assays, but its oxidation catalyzed by HRP-C shows low efficiency (*14*). Attempts to use enhancers to increase the reaction efficiency in chemiluminescence immunoassays (*15*, *16*) gave some improvement. Presently, the most used enhancer is *p*-iodophenol. This allowed the development of a number of immunokits for determination of various compounds.

Kinetic studies of the enhanced chemiluminescence reaction catalyzed by HRP-C demonstrated that the chemiluminescence intensity increased for the first few minutes, reached a maximum, and then decreased sharply (*17*, *18*). The emission decay is related to HRP-C inactivation as a result of the interaction of the enzyme and radical products of substrate oxidation. Hence, luminol oxidation catalyzed by HRP-C results in an unstable luminescence signal.

Previously, we showed that the luminol oxidation by hydrogen peroxide was efficiently catalyzed by anionic soybean (SbP) and palm tree peroxidases in the absence of any enhancer (*19*, *20*). Another feature of these peroxidases is the production of a long-term signal of chemiluminescence through luminol oxidation. It should also be noted that the stability of the anionic peroxidases is significantly higher than that of HRP-C (*21*−*24*) that is an important parameter for storage of the enzyme-containing immunoreagents. On the basis of these facts, we suggest that the anionic peroxidases are promising enzymes for immunoassays with chemiluminescence detection. Herein, we describe some advantages of using commercially available SbP in chemiluminescent ELISA.

MATERIALS AND METHODS

Peroxidases of soya beans *Glycine max* (RZ 1.5) and horseradish roots (RZ 3.0) were purchased from Enzymol International (United States) and Biozyme (United Kingdom) and used without further purification. Luminol-HCl, *p*-iodophenol, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), Triton X-100, and Tris were obtained from Sigma (St. Louis, MO), and H_2O_2 (30%) was from Merck (Darmstadt, Germany). All other reagents were of analytical grade. The concentration of H_2O_2 was estimated by measuring the absorbance using $\epsilon_{240} = 43.6$. All solutions were prepared using milliQ grade water.

Immunoreagents. The mouse IgG was produced from serum by consecutive combination of salt precipitation, ion exchange chromatography, and affinity chromatography on protein A (*25*). Rabbit anti-mouse IgG antibodies were purified from immune sera by affinity chromatography using mouse IgG immobilized on BrCN-Sepharose

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4B (*25*). Conjugates of rabbit anti-mouse IgG antibody with SbP and HRP-C were produced using the periodate method (*26*) with some modifications: 125 μL of a solution of $NaIO_4$ was added to 1 mL of an aqueous solution of SbP or HRP-C with a concentration of 10 mg/mL. The sodium periodate concentration in feed varied from eight to 40 mM. For HRP-C conjugate production, the $NaIO_4$ concentration was 17 mM. The reaction was carried out at constant agitation for 20 min at room temperature in the dark. The oxidized SbP was purified by dialysis against the 10 mM acetate buffer, pH 4.4, overnight at 4 °C. Then, 600 μL of solution of rabbit anti-mouse IgG antibody (10 mg/mL in 0.1 M bicarbonate buffer, pH 9.5) was added to the obtained SbP solution. After agitation of the mixture for 2 h at room temperature in the dark, the solution was mixed with 100 μL of aqueous solution of $NaBH_4$ (4 mg/mL) and stirred for 1 h in the dark. The conjugate was purified by gel filtration on a Superose 12 HR column (1.6 cm × 70 cm) in 50 mM phosphate buffer, pH 7.0, using a Pharmacia FPLC apparatus at a flow rate of 30 mL/min and room temperature or by dialysis against the 10 mM phosphate buffer, pH 7.0, overnight at 4 °C. The conjugates were stored in 50% glycerol at −20 °C.

Enzyme Assay. The peroxidase activity was usually determined spectrophotometrically as follows: 10 μL of enzyme solution was added to 2 mL of 10 mM citrate-phosphate buffer, pH 3.0, containing 50 μM ABTS and 0.2 mM H_2O_2 as substrates, and the absorbance change at 414 nm was measured at 25 °C by UV-2401 PC spectrophotometer (Shimadzu, Japan).

Immunoassays. *Colorimetric ELISA.* Ninety-six well transparent polystyrene microtiter plates ("high binding" grade, Costar, United States) were coated by adding into each well 100 μL of rabbit anti-mouse IgG antibodies (5 μg/mL) dissolved in 50 mM carbonate buffer, pH 9.5, and incubated at 4 °C for 20 h. The plates were washed three times with washing solution (10 mM sodium phosphate, 137 mM NaCl, 0.05% Triton × 100, pH 7.4 (PBST). Then, IgG mouse solution with concentration that was varied in the interval from 0 to 20 μg/mL was added to the wells (100 μL per well) and incubated for 1 h at 37 °C. The plates were washed again as described above. Conjugates of rabbit anti-mouse IgG antibody with SbP or HRP-C (dilution of 1:1000) were added to the wells (100 μL per well). The plates were incubated for 1 h at 37 °C and then washed with PBST three times.

The substrate solution for colorimetric detection was prepared by adding 1 mL of ABTS solution (14.6 mM in 50 mM citrate-phosphate buffer, pH 4.0) and 7 μL of concentrated hydrogen peroxide solution (8.8 M) to 9 mL of 50 mM citrate-phosphate buffer, pH 4.0. Then, 100 μL of freshly prepared substrate solution was added into each well, and the plates were left at room temperature for 15 min. Finally, the plates were stirred and read using a plate reader (Molecular Devices, United States) at a wavelength of 405 nm.

Chemiluminescent ELISA. Chemiluminescent ELISA was carried out similarly to the colorimetric ELISA using 96 well white nontransparent polystyrene microtiter plates (Dynatech, United States) instead of Costar plates. The substrate solution for chemiluminescence detection of SbP activity optimized previously (*20*) was prepared by adding 400 μL of 160 mM luminol dissolved in 2 M NaOH and 100 μL of 0.88 M hydrogen peroxide solution to 11.5 mL of 100 mM Tris-HCl buffer, pH 8.5. For HRP-C, the substrate solution was prepared by mixing 72 μL of 160 mM luminol, 120 μL of 50 mM *p*-iodophenol dissolved in DMSO, 14 μL of 0.88 M hydrogen peroxide solution, and 11.8 mL of 100 mM Tris-HCl buffer, pH 8.5. After the performance of immunological steps of the assay, 100 μL of freshly prepared substrate solution was added to each well and stirred. Finally, the chemiluminescence intensity was monitored vs time for each well by an Amerlite luminometer (Amersham, United Kingdom).

RESULTS AND DISCUSSION

SbP was first purified from soya beans by Sessa and Anderson (*27*). Like other secretory peroxidases, SbP is a glycoprotein; therefore, carbohydrate chains of this enzyme can be oxidized by $NaIO_4$ to form aldehyde groups. This reaction previously developed by Nakane and Kawaoi (*28*) is widely used for the production of conjugates of HRP-C and antibody/antigen molecules where aldehyde groups obtained at oxidation of peroxidase react with amino groups of antibody/antigen. Because the degrees of glycosylation of SbP and HRP-C are different (*1*, *29*) and the activity and stability of plant peroxidases depend on the degree of their deglycosylation (*30*), at the first stage of the work, the conditions for SbP oxidation should be optimized.

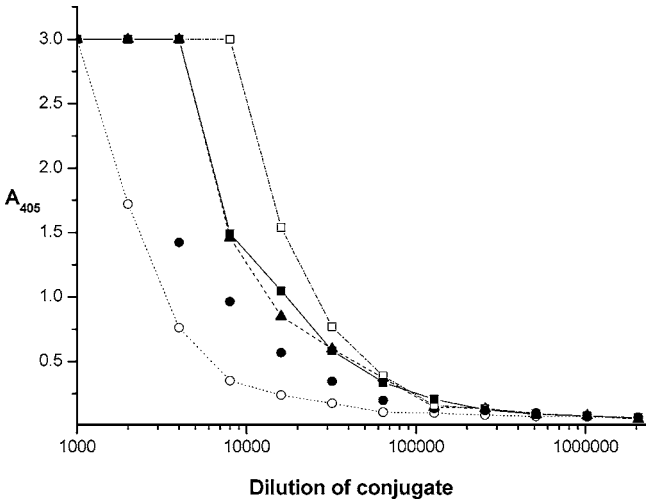

Figure 1. Comparison of conjugates of SbP and rabbit anti-mouse IgG antibody produced in the presence of 8 (open circles), 17 (closed circles), 25.5 (closed triangles), 32 (open squares), and 40 mM $NaIO_4$ (closed squares) in colorimetric ELISA. The enzyme activity was measured using ABTS as the substrate.

At the optimization, the concentrations of $NaIO_4$ in SbP solution varied in the interval 8−40 mM; then, the oxidized SbP was bound with purified anti-mouse IgG antibody. The obtained conjugates were compared in colorimetric ELISA. **Figure 1** shows that the most active conjugate was synthesized using 32 mM $NaIO_4$ solution. Interestingly, in the case of SbP, the favorable $NaIO_4$ concentration is significantly higher than that used in HRP-C oxidation (17 mM). At the concentrations of the oxidant other than 32 mM, less active conjugates of SbP and anti-mouse IgG antibody were produced.

To find the reason for this phenomenon, the gel filtration of the obtained conjugates was carried out on Superose 12. Comparison of chromatograms for the conjugates synthesized at various concentrations of $NaIO_4$ demonstrated that increasing the oxidant concentration resulted in a decrease of the protein peak area characteristic for intact SbP (**Figure 2A**). This means that the yield of the conjugate synthesis directly depends on the oxidant concentration.

In parallel, we observed an appearance of protein peaks characteristic for SbP conjugates. The observation of some high molecular weight peaks means that in the course of the synthesis a set of the conjugates with different ratios between their components (enzyme/antibody ratio) is produced. It should also be noted that at low concentrations of the oxidant (8 mM $NaIO_4$) the major peak corresponded to the conjugate with the equivalent ratio of SbP/antibody (the retention time 135 min), whereas at high $NaIO_4$ concentrations (32 and 40 mM) the major peak corresponded to a higher molecular weight conjugate with the retention time of 80 min (**Figure 2B**).

On the basis of the obtained results, further work was carried out with SbP conjugate synthesized in the presence of 32 mM $NaIO_4$. In chemiluminescent ELISA, this conjugate was compared with that of HRP-C bound with the same antibody by the traditional procedure. For this, we used an immunological reaction between mouse IgG and rabbit anti-mouse IgG antibody

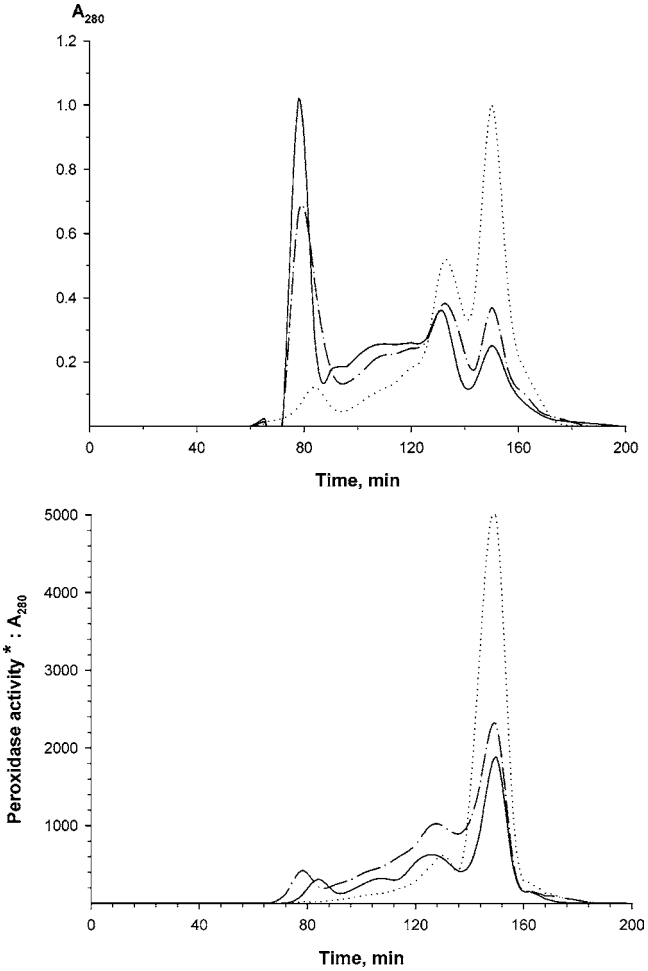

Figure 2. Gel filtration of conjugates of SbP and rabbit anti-mouse IgG antibody produced in the presence of 8 (dotted line), 32 (dashed line), and 40 mM $NaIO_4$ (solid line) on a Superose 12 HR column (1.6 cm × 70 cm) equilibrated with 50 mM phosphate buffer, pH 7.0, at the rate of 30 mL/h. The conjugates detected by measuring the optical density at 280 nm (**A**) and peroxidase activity/optical density at 280 nm ratio (**B**).

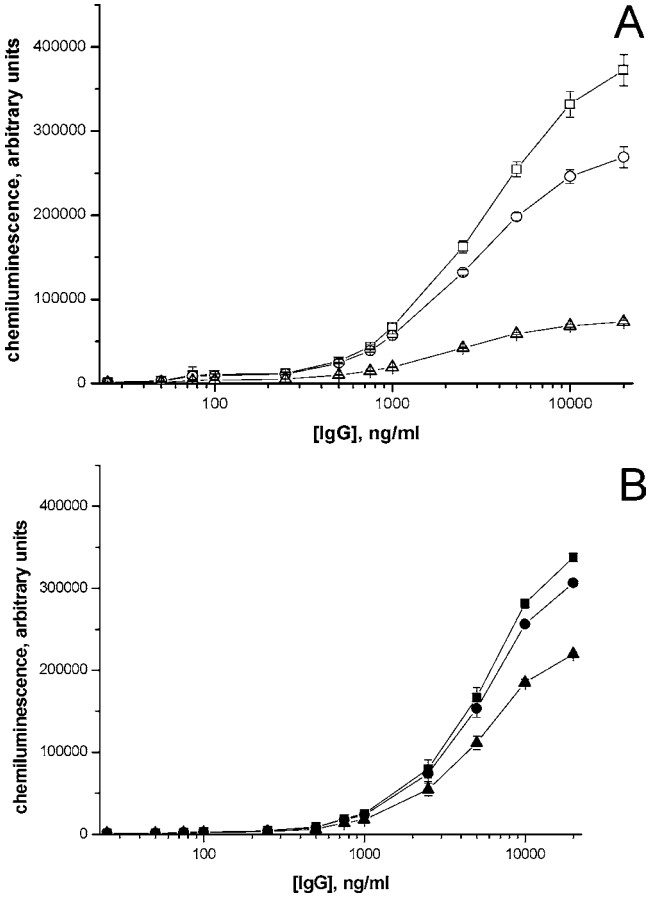

Figure 3. Calibration curves for determination of mouse IgG by chemiluminescent ELISA using rabbit anti-mouse IgG antibody labeled by HRP-C (**A**) and SbP (**B**). Each point represents the mean ± SD of eight measurements. Chemiluminescence was measured 2 (squares), 4 (circles), and 12 min (triangles) after the initiation of peroxidase-catalyzed oxidation of luminol.

selected as a model reaction because of availability of the immunoreagents. However, the assay for determination of mouse IgG also has a practical value, since presently a lot of immunokits include murine monoclonal antibodies as their active components and, therefore, there is a need for their quantitative measurement.

Using HRP-C conjugate in the sandwich chemiluminescent ELISA, the standard calibration curves for mouse IgG were obtained (**Figure 3A**). The kinetic study showed that the chemiluminescence intensity for HRP-C conjugate was sharply decreased. At the same time, the emission decay did not affect major parameters of the assay. Despite the measurement time, the linear range (250−2500 ng/mL, $R^2 = 0.995$), the lower detection limit (signal-to-noise ratio of 3) (25 ng/mL), and the RSD (11.1−11.3%) of the assay were identical.

As reported previously (*20*), SbP is able to catalyze luminol oxidation by hydrogen peroxide. Moreover, the presence of enhancer in the reaction medium poorly affects the SbP catalysis. By this reason, for immunoassay using SbP conjugate, we prepared the substrate solution without the addition of *p*-iodophenol. The results of the sandwich chemiluminescent ELISA for determination of mouse IgG using the conjugate of SbP and anti-mouse IgG antibody are presented in **Figure 3B**. Contrary to the HRP-C conjugate, for SbP conjugate, the chemiluminescence intensity decayed with a significantly lower rate. Interestingly, the initial intensity, for instance in 2 min, for both conjugates was similar. Comparison of parameters of the immunoassays showed that with SbP conjugate the assay was more sensitive and its linear range was a broader, e.g., 100−10000 ng of mouse IgG/mL at similar detection limits and RSDs. The observation of the wider range for determination of mouse IgG using SbP conjugate is explained by the higher stability of the enzyme to treatment by radical products of luminol oxidation that was shown in a separate experiment (data not shown). Previously, the similar stabilizing effect was found for other anionic peroxidases isolated from palm tree leaves (*19*). The obtained higher sensitivity of the SbP immunoassay is likely related to a higher sensitivity of SbP in the reaction of luminol oxidation as compared to HRP-C, which we recently reported (*20*).

Thus, this work clearly demonstrated that the replacement of horseradish peroxidase with SbP in the synthesis of immunoconjugates allows the development of the improved chemiluminescent ELISA.

ABBREVIATIONS USED

SbP, soybean peroxidase; HRP-C, isozyme *c* of horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay.

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