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AN ENZYME IMMUNOSENSOR FOR IgG

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An enzyme immunosensor has been developed for assaying human immunoglobulin G (IgG). The sensor is composed of an oxygen sensoring system and an antibody-binding membrane. The assay procedure involves the competitive immunochemical reaction of the membrane-bound antibody with nonlabeled and catalase-labeled IgG and the electrochemical determination of membrane-bound catalase activity. The analytical result is directly displayed by the output current of the sensor. The sensor exhibited an excellent performance in monitoring specifically human IgG.

INTRODUCTION

Immobilized enzymes have been utilized to yield selective sensors (enzyme electrodes) for many ions and organic substances (1–7). For biological and biomedical sensoring purposes, however, the possibilities for making sensitive and selective sensors for specific protein are an exciting prospect. Assay systems involving use of antigens, haptens, or antibodies labeled with an enzyme have recently been applied to the measurement of substances in biological fluids. These assay systems have been given such names as enzyme- and enzymo-immunoassay (EIA) (8). In the present paper we intend to report the development of an enzyme-immunosensor that is characterized by a unique combination of EIA and electrochemical determination systems. The new sensor depends upon membrane-bound antigen or antibody for its specificity.

Extensive investigations have been made on the development of enzyme-immunoassay (8). Peroxidase, alkaline phosphatase, glucose oxidase, β-galactosidase, and lysozyme have been employed to label antigens

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and antibodies. The EIA involves two tedious steps. One is the separation of antigen–antibody complex from free antigen or antibody, and the other is the photometric determination of enzyme activity with the addition of corresponding substrate and dyes.

To simplify the EIA, the enzyme-immunosensor is provided with an antibody- (or antigen-) binding membrane that is contacted with an oxygen monitoring system, as shown in Fig. 1. In the present paper an enzyme-immunosensor for human immunoglobulin G (IgG) is described as an example. For the purpose of EIA, IgG has been labeled with alkaline phosphatase (9,10), peroxidase (11), glucose oxidase (12), and β-galactosidase (13–15). IgG is labeled with catalase (EC 1.11.1.6) in this investigation. Both nonlabeled and labeled IgG competitively react with membrane-bound antibody. The extent of labeled IgG attached to the membrane can be estimated by the enzymatic assay, in which oxygen generation is directly followed by the oxygen monitoring system.

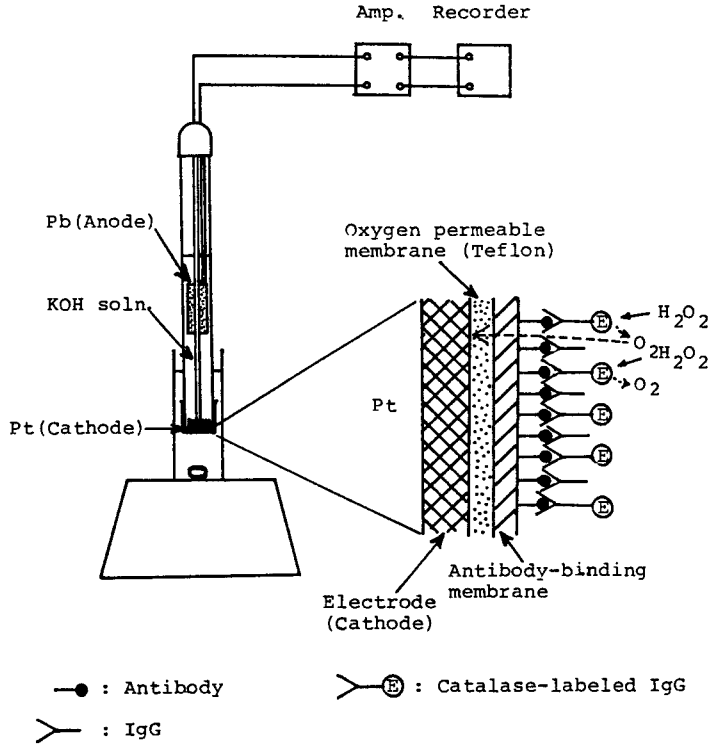

FIG. 1. A schematic representation of the enzyme immunosensor.

MATERIALS AND METHODS

Materials

Human IgG (lyophilized) and antihuman IgG (rabbit) (lyophilized) were obtained from Miles Laboratories (Elkhart, Indiana). Catalase was purchased from Tokyo Kasei Company (Tokyo). All the other reagents were obtained commercially and were used without further purification.

Bromoacetylcellulose was prepared from cellulose powder. Bromoacetic acid (100 g) dissolved in dioxane (30 ml) was added to cellulose powder (10 g), and the suspension was stirred at room temperature for 20 h in a tightly stoppered round-bottomed flask. Bromoacetyl bromide (200 g) was added to the solution. The solution was stirred vigorously for 12 h and then slowly poured into cold deionized water (4°C) with vigorous stirring. The precipitated cellulose was washed exhaustively with water and then with 0.1 M sodium bicarbonate and water. The preparation yielded 22.02% Br, 4.41% H, and 35.22% C.

Preparation of an Antibody-Binding Membrane

Acetylcellulose (150 mg) and bromoacetylcellulose (100 mg) were dissolved in 5 ml of acetone with vigorous stirring at room temperature. The resulting solution was cast on a glass plate (10×16 cm^2). The membrane was dried at reduced pressure. The membrane was peeled off and cut into small pieces (2×2 cm^2 each). Each membrane was immersed in 20 ml of 0.05 M carbonate buffer (pH 9.7) containing 150 mg hexamethylenediamine with moderate stirring overnight. After washing with pH 9.7 carbonate buffer, the membranes were added to 20 ml of 0.05 M carbonate buffer (pH 9.7) containing 100 μl butadienediepoxide. The reaction mixture was allowed to stand at room temperature for 1 h. The membranes were washed with pH 9.0 carbonate buffer and then contacted with 20 ml of 0.05 M phosphate buffer (pH 8.6) containing 5 mg anti-IgG with moderate stirring at 4°C overnight. The preparation was thoroughly washed with water, and then contacted with 0.05 M ethanolamine in pH 8.6 phosphate buffer with stirring at 4°C overnight.

Labeling Human IgG with Catalase

Catalase (10 mg) and human IgG (5 mg) were dissolved in 10 ml of 0.05 M carbonate buffer at pH 9.7. One percent glutaraldehyde (100 μl) was added to the solution. The coupling reaction was continued at room temperature for 60 min. The catalase–IgG composite was separated from

unreacted glutaraldehyde, catalase, and IgG by ultrafiltration. Further purification was conducted by gel filtration of Sepharose 4B and Sephadex G-200.

Assembly of the Enzyme-Immunosensor

Figure 1 gives a schematic representation of the enzyme-immunosensor. The sensor is composed of an oxygen sensoring system and an antibody-binding membrane. The antibody-binding membrane is attached to an oxygen permeable membrane.

Nonlabeled and labeled IgG in a solution may react with the bound antibody at the membrane surface of the sensor. If the sensor is immersed in a hydrogen peroxide solution, the bound catalase decomposes hydrogen peroxide into oxygen and water. The oxygen generated diffuses to the platinum electrode surface through an oxygen permeable membrane, and electrochemically reacts at the electrode with a resulting increase in current.

RESULTS

Enzyme Activity of IgG-Bound Catalase

Lineweaver–Burk plots of the initial rate data yielded straight lines for the two forms of catalase: native and IgG-bound catalase. The measurements were made at 30°C and pH 7.0 using hydrogen peroxide as substrate. The data are shown in Fig. 2. The values of the Michaelis–Menten constant (K_m) for native and IgG-bound catalase were 25 and 87 mM, respectively. The values of K_m were slightly increased by attaching the enzyme to IgG. There was no prominent change in the maximum velocity; $V_{max} = 4.2$ and 4.8 mM/min for native and IgG-bound catalase, respectively. The immobilization of catalase to IgG accompanied no appreciable decrease in the activity.

Immunochemical Reactivity of the Membrane-Bound Antibody

In order to demonstrate the immunochemical reactivity of the membrane-bound antibody, the enzyme-immunosensor was contacted with catalase-labeled IgG. If the membrane-bound antibody retains the immunochemical reactivity, the catalase-labeled IgG can be fixed to the membrane surface through the antigen–antibody complex formation. Since the IgG is labeled with catalase, the antigen–antibody complex formation can be monitored by following the activity of catalase.

Catalase	K_m (mM)	V_{max} (mM/min)
Native	25	4.2
IgG-bound	87	4.8

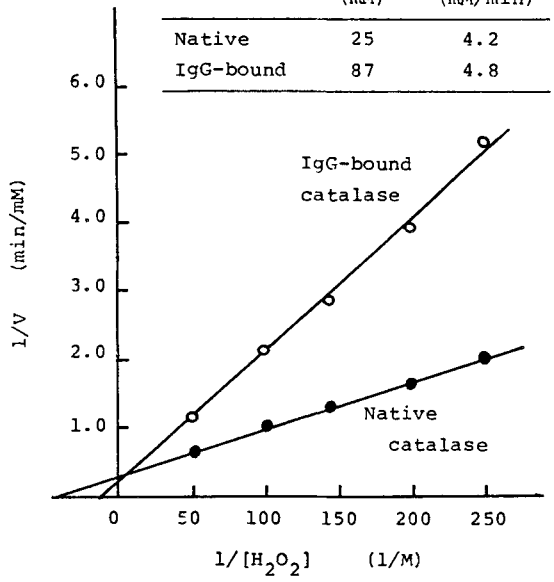

FIG. 2. Lineweaver–Burk plots of the initial rate data for native and IgG-bound catalase at pH 7.0 and 30°C.

The enzyme-immunosensor was incubated in 2 ml of the catalase-labeled IgG solution at 37°C for 30 min. The sensor was thoroughly washed with 0.5 M NaCl solution, and then contacted with 10 ml of 0.1 M phosphate buffer (pH 7.0) that was preliminarily saturated with oxygen at 30°C. The output current of the sensor was continuously recorded. When the sensor gave a constant current (I_0), 50 μl of 3% H_2O_2 was added to the solution. The solution was magnetically stirred during the measurement. The response of the sensor is presented in Fig. 3. The output current increased with time. The increase in the current resulted from the generation of oxygen by catalase fixed at the membrane. A steady-state current (I_s) was obtained in 30 min. On the other hand, the enzyme-immunosensor exhibited no enzyme activity when it was incubated in a free catalase solution under the same conditions. These results indicate that the membrane-bound antibody retained the immunochemical reactivity.

Response of the Sensor

The enzyme-immunoassay based on the new sensor involves two steps, the competitive immunochemical reaction of the membrane-bound

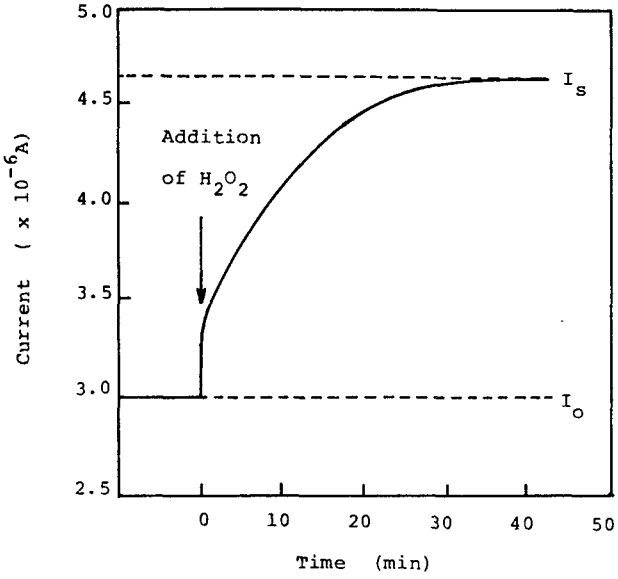

FIG. 3. The response of the sensor. The sensor was contacted with 2 ml of the catalase-labeled IgG solution at 37°C for 30 min. After exhaustive washings, the sensor was immersed in 10 ml of oxygen-saturated buffer at pH 7.0 and 30°C. At time 0, 50 μl of 3% H_2O_2 solution was added.

antibody with nonlabeled and labeled IgG and the electrochemical determination of enzyme activity of the membrane-fixed catalase.

The first step was conducted under the fixed condition of pH 7.0, 37°C, and 30 min. To determine the optimum condition for the second procedure, the output current of the sensor was measured at various H_2O_2 concentrations, pH, and temperature.

The sensor was reacted with catalase-labeled IgG in the absence of nonlabeled IgG at pH 7.0 and 37°C. Since the membrane-bound antibody binds catalase-labeled IgG, the membrane surface of the sensor may be covered with catalase. The sensor depends on the output current for the catalytic action of fixed catalase.

Figure 4 shows the H_2O_2 concentration dependence of the output current. The labeled IgG-binding sensor was contacted with H_2O_2 solution at various concentrations. The pH and temperature were fixed at 7.0 and 30°C. The rate increased with H_2O_2 concentration. As the self-decomposition of H_2O_2 could not be neglected at higher concentration, the concentration of H_2O_2 was fixed at 5 mM in the subsequent measurement.

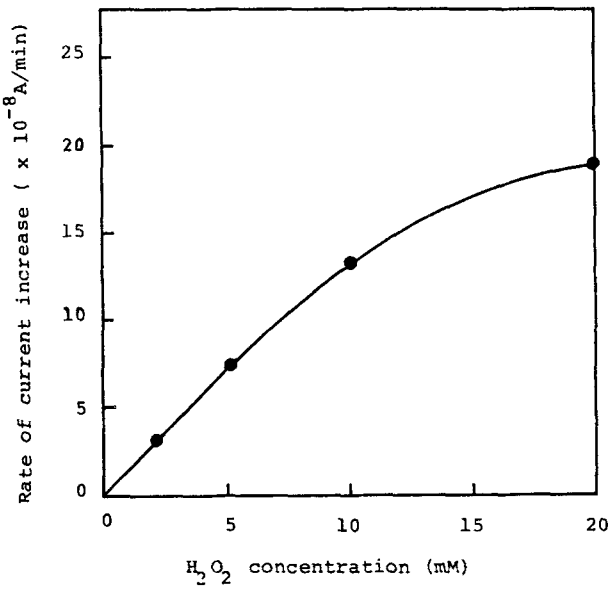

FIG. 4. H_2O_2 concentration dependence of the output current of the sensor. The sensor, which was reacted with catalase-labeled IgG, was contacted with an H_2O_2 solution at various concentrations, pH 7.0, and 30°C.

The effects of pH and temperature on the rate are presented in Figs. 5 and 6. The maximum rate was obtained at pH 7.0. The rate increased with temperature in the range of 20°–40°C. These data show that pH 7.0 and 30°C are suitable conditions for making the electrochemical determination of enzyme activity.

Nonlabeled and labeled IgG competitively react with the membrane-bound antibody. The fractional ratio of labeled to nonlabeled IgG fixed by the membrane might be related to the concentration of nonlabeled IgG in a solution. Therefore, the output current of the sensor is expected to correlate with the concentration of IgG to be determined.

A solution of nonlabeled IgG was made at respective concentration by dissolving human IgG in 0.1 M phosphate buffer (pH 7.0). One milliliter of nonlabeled IgG solution was mixed with 1 ml of catalase-labeled IgG solution. The sensor was contacted with the resulting solution at 37°C for 30 min, and then washed thoroughly with 0.5 M NaCl and 0.1 M phosphate buffer. The electrochemical determination of fixed catalase activity was conducted at an H_2O_2 concentration of 5 mM, 30°C, and pH 7.0. In Fig. 6, the initial rate of current increase was plotted for respective concentration of

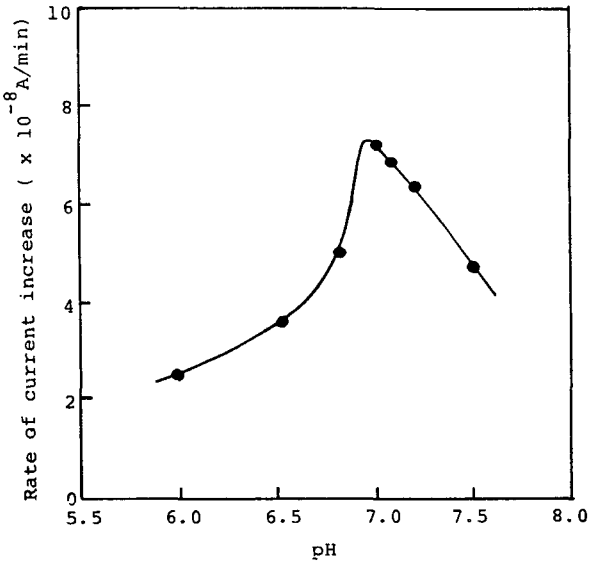

FIG. 5. The pH profile of the sensor in the output current. (1) 2 ml catalase-labeled IgG, pH 7.0, 37°C, 30 min. (2) 50 μl of 3% H_2O_2 at respective pH and 30°C.

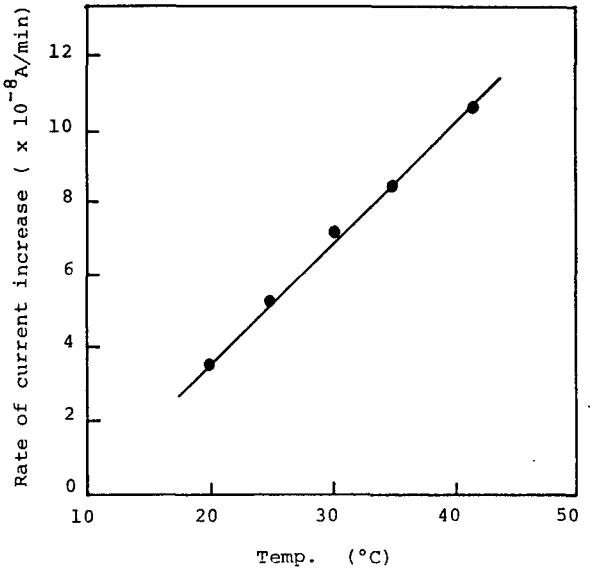

FIG. 6. Temperature dependence of the output current. (1) 2 ml catalase-labeled IgG, pH 7.0, 37°C, 30 min. (2) 50 μl of 3% H_2O_2 at pH 7.0 and respective temperature.

nonlabeled IgG. The total amount of nonlabeled IgG is represented in the abscissa. In the range of 0.1–2.0 mg IgG, the rate of current increase was proportional to the logarithmic scale of IgG.

DISCUSSION

Human IgG-bound catalase retained the activity. Furthermore, the membrane-bound antibody showed an immunochemical reactivity to both nonlabeled and labeled IgG. These results suggest that the immobilization procedures employed are moderate for these biologically active substances.

Catalase was employed as a labeling enzyme because of its high turnover number in oxygen generation, which is easily monitored by the electrochemical method. In the present work the activity was determined by the rate assay. The end-point method should also be applicable.

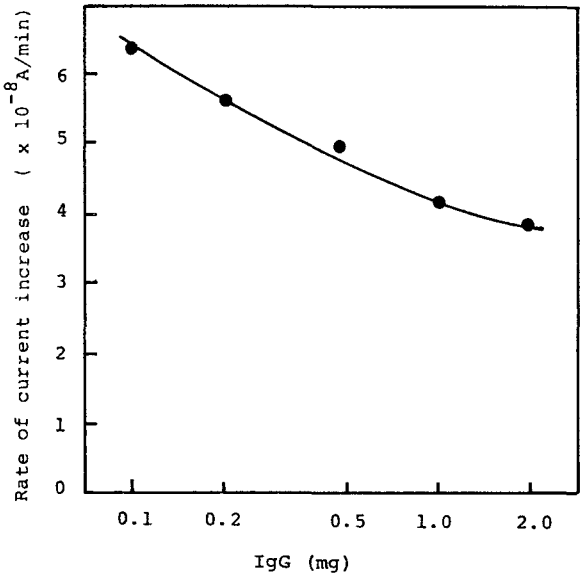

FIG. 7. Calibration curve against the amount of IgG. One milliliter of the catalase-labeled IgG solution was mixed with 1 ml of nonlabeled IgG solution at respective concentrations. The sensor was reacted with the resulting solution at 37°C for 30 min. After thoroughly washing, the sensor was immersed in 10 ml of pH 7.0 phosphate buffer preliminarily saturated with O_2 at 30°C. The enzyme reaction was initiated by the addition of 50 μl of 3% H_2O_2.

The new enzyme-immunoassay requires simple washing of the sensor after the immunochemical reaction. In the enzyme activity assay, only the substrate is required. In addition, the analytical results can be displayed directly in electric signal. Since the sensor has these great advantages, the proposed method is believed to be a significant improvement over conventional EIA.

The calibration curve presented in Fig. 7 covers the concentration range of 0.1–2.0 mg IgG. If a sample solution, such as serum, contains 1000 mg/dl IgG, the new EIA may be operated under the following condition: One milliliter of the catalase-labeled IgG solution, 50 μl of the sample, and 0.05 M phosphate buffer (pH 7.0) are mixed to make 2 ml of testing solution. If the assay is conducted as mentioned above, the sensor is expected to exhibit the current increase rate of 5×10^{-8} A/min.

Since most human serum contains IgG in the concentration range above 800 mg/dl, 50 μl or less of serum is required for making the assay under present conditions.

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