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ENZYME IMMUNOSENSOR USING HAPTEN-BOUND MEMBRANE AND Fab'-β-D-GALACTOSIDASE COMPLEXES

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An enzyme immunosensor using a hapten-bound membrane and Fab'-β-D-galactosidase complexes has been developed to improve the reproducibility and the capability of multi-cyclic operation. The proposed method may be useful for repeated uses of the enzyme immunosensor without exchanging the membranes.

Previously, we have reported the enzyme immunosensor systems for insulin[1] and theophylline.[2] In the enzyme immunosensor system, which utilizes antibody-bound membranes, sensitivity and selectivity are considerably influenced by the characteristics of the antibody-bound membrane used. In order to enable the repeated uses of the same antibody-bound membrane, it is necessary to dissociate antigen-antibody complexes. This procedure causes denaturation of the antibodies, which results in the necessity of exchanging the membrane. In this communication we report a new type of enzyme immunosensor using the theophylline hapten-bound membrane (polypropyrene) and Fab'-β-D-galactosidase complexes. Theophylline, which is used in the treatment of acute and chronic respiratory deseases, has also recently been suggested for the prevention of apnea in infants. Plasma level monitoring has been recomended for this drug because of the relatively narrow therapeutic range.

As an oxygen-permeable membrane of the oxygen electrode, polypropyrene was selected because of its nonspecific adsorbability of proteins. The theophylline hapten was bound covalently on the membrane according to the method of Ngo et al.[3] Briefly, the surface carboxylic groups were generated by acidic oxidation of the polypropyrene membrane and were reacted with excess ethylenediamine via carbodiimide. The theophylline hapten was then coupled on the amine substituted polypropyrene membrane using carbodiimide. The anti-theophylline IgG was purified from anti-

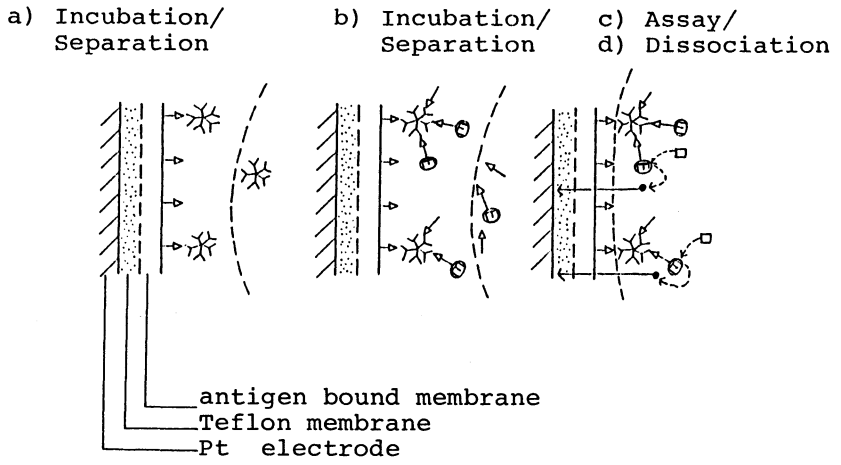

Fig. 1. Principle of the enzyme immunosensor using antigen bound membrane and Fab'-β-D-galactosidase complexes.

: Antigen, : Antibody, E : Enzyme, ● : Product, □ : Substrate.

theophylline antiserum which was prepared as described before.[4] FITC (fluorescein isothiocyanate)-labeled IgG was prepared according to Goldman.[5] Fab' fragment (antigen binding fragment of antibody) was prepared by pepsin digestion of IgG. Fab'-β-D-galactosidase complex was prepared by conjugating dimaleimide-treated Fab' fragments of anti-theophylline IgG with β-D-galactosidase according to Kato et al.[6] The reason for the use of this enzyme is that a number of sulfhydryl groups in the enzyme molecule are independent of the enzyme activity.[7] The theophylline hapten-catalase complex was prepared using glutaraldehyde.

The principle of the sensor is shown in Fig. 1. The theophylline was assayed by an enzyme immunosensor as follows. (a) The enzyme immunosensor was first incubated in Fab'-β-D-galactosidase solution at 30 °C for 20 min. Then it was washed with 0.2 mol dm^{-3} glycine-HCl buffer (pH 2.8) for 10 min to separate free and non-specifically bound Fab'-β-D-galactosidase. (b) It was further incubated in the sample solution containing unknown amount of theophylline and known amount of enzyme-labeled theophylline at 20 min. To separate free and nonspecifically adsorbed enzyme-labeled theophylline, it was washed with 0.2 mol dm^{-3} glycine-HCl buffer (pH 2.8). (c) The sensor was immersed in 3 ml of 0.05 mol dm^{-3} phophate buffer (pH 7.0) and equilibrated. Then 400 μl of 3% H_2O_2 was introduced. The generation of oxygen was detected by a Clark-type oxygen electrode and amperometric current was measured. From the rate of initial current increase (di/dt), the concentration of theophylline was determined. After the assay, the sensor was immersed in 0.2 mol dm^{-3} glycine-HCl buffer (pH 2.2) to dissociate the antigen-antibody complex.

From the result of radio immunoassay of polypropyrene membrane using ^{14}C-theophylline, the number of theophylline hapten bound to the membrane was estimated to be about $0.7 \times 10^{12}/cm^2$. The number of Fab' molecules conjugated per β-D-galactosidase molecule was about 6, which was calculated both from the fluorescence measurement of Fab' fragments and the enzymatic activity of β-D-galactosidase. This value was relatively high in comparison with the data of Yoshitake et al.[8] The number of theophylline hapten molecules conjugated per catalase molecule was determined by differential spectrophotometry[9] at 280 nm and 629 nm. The number of molecules of theophylline hapten per molecule of catalase was 6.2. To determine the optimal assay conditions, the effects of incubation time and pH on the output current in each step were studied. Throughout the experiment, the incubation temperature was kept at 30 °C according to our previous report.[1] In the first step (a) the rate of current increase (di/dt) was saturated at about 15 min and showed a maximum at pH 7.0 (data not shown). In the second step (b) the rate of current increase was also saturated at about 15 min and the maximum rate of current increase was obtained at pH 7.0. These results suggested that pH 7.0 would be favorable for the antigen-antibody reaction and that the antigen-antibody reaction took place within 15 min. From the results described above, the optimal assay condi-

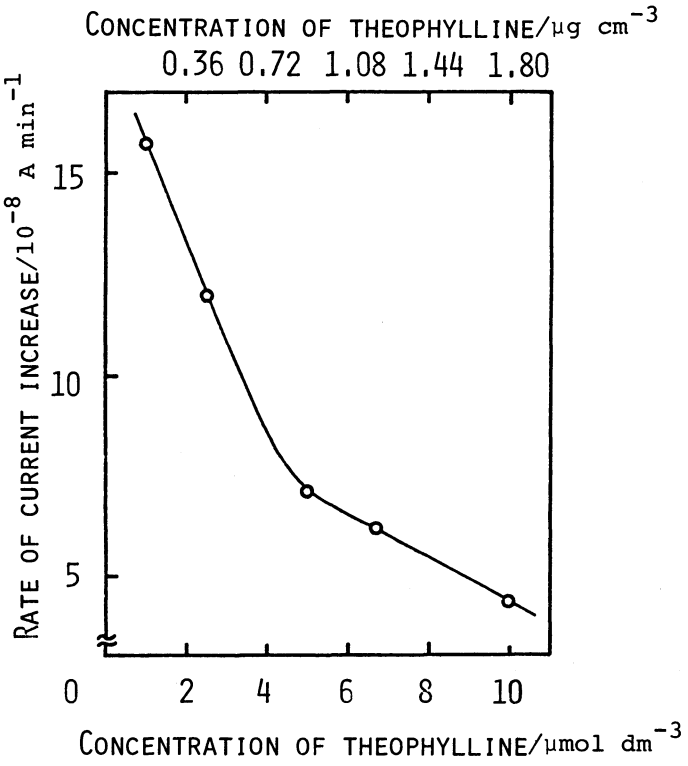

Fig. 2. Calibration curve for theophylline.
(conditions as in the text)

tions were determined as follows : step (a); incubation at pH 7.0, 30 °C for 20 min (concentration of Fab' was 2.90 nmol dm^{-3}), step (b) : incubation at pH 7.0, 30 °C for 20 min (concentration of catalase labeled theophylline was 4.75 μmol dm^{-3}). The correlation between the initial rate of current increase and the concentration of theophylline is shown in Fig. 2. Each point represents the average value of two experiments. The rate of current increase decreased with increasing concentration of theophylline and a linear relationship was obtained for 1 - 5 μmol dm^{-3} . The reproducibility of the sensor at the concentration of 2.5 μmol dm^{-3} was also examined. The standard deviation and the coefficient of variation of ten trials were 0.44 and 5.8%, respectively. Over ten trials the reproducibility of the sensor was markedly reduced. A possible explanation of this behavior is the elimination of theophylline hapten, which was bound on the polypropyrene membrane, during the dissociating procedure. In the case of the sensor using an anti-theophylline IgG bound membrane and theophylline hapten-catalase complexes, less than five assays were possible because of denaturation of the antibodies (di/dt at sixth run was about 40% of the initial value).

The feasibility of the enzyme immunosensor using a hapten-bound membrane and Fab'-β-D-galactosidase complexes was evaluated. The described immunosensor was less susceptible to denatuation of the antibodies than the sensor using an antibody-bound membrane, and it had a longer cyclic lifetime. The proposed method may be useful for repeated uses of the enzyme immunosenosr without exchanging the membrane.

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