AN ENZYME IMMUNOELECTRODE

Assay of human serum albumin and insulin

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1. Introduction

Convention immunochemical techniques are often very time consuming. Assay of a sample using, e.g., RIA (radio immuno assay) or ELISA (enzyme linked immunosorbent assay) techniques takes in the order of 2–4 h because of long equilibration times for the antibody and antigen to form the specific antbody–antigen complex [1,2]. In recent reports it was demonstrated that when non-equilibrium ELISA was carried out in an enzyme thermistor system assays in a time range of 10–15 min/assay were made possible [3,4].

The present paper deals with efforts to make an enzyme immunoelectrode, thereby combining the advantages of conventional enzyme electrode technique [5–7] with the unique sensitivity and specificity offered in enzyme-linked immunosorbent assays [1].

2. Materials and methods

Human serum albumin (fraction V), insulin, glucose oxidase type V from *Aspergillus niger* and catalase (type C-30 from beef liver) were obtained from Sigma, Mo., USA, rabbit anti-human serum albumin (anti-HSA) was obtained from the University Hospital in Malmö, Sweden and guinea pig anti-porcine insulin from Miles Laboratories, Slough, England. The antibodies were purified by affinity chromatography prior to use. The antiserum was diluted in 0.1 M potassium phosphate buffer, pH 7.0, and was then applied to an antigen–Sepharose column. The column was washed until no absorbance at 280 nm could be detected. By changing the eluting buffer to 0.2 M glycine–HCl, pH 2.2, the antigen–antibody complex was split and the antibody eluted. The fractions containing protein were pooled and rechromatographed on a Sephadex G-25 column equilibrated and eluted with 0.1 M $NaHCO_3$. The fractions thereby eluted were collected and used in further work as purified antibody.

Nylon net, 100 mesh, was obtained from Derma AB, Göteborg, Sweden and dimethylsulphate from Merck, Darmstadt, FRG.

All other chemicals used were of analytical grade.

2.1. *Coupling of antibodies to nylon net*

A previously described coupling procedure for attaching enzymes to nylon tubings [8] was modified. A piece of nylon net (3 × 7 cm) was dipped into dimethylsulphate for 30–45 s and then washed with ice-cold water for 30 s, with ice-cold absolute ethanol for 30 s and with 0.1 M potassium phosphate buffer, pH 7.0, for 1 min. The activated nylon net was put into the coupling solution consisting of 35 ml 0.1 M potassium phosphate buffer, pH 7.40, containing varying amounts of antibodies purified as described above.

Coupling proceeded under slow mixing at +4°C for 15 h. The nylon net was then washed with 0.5 M NaCl for 30 min and with 0.1 M potassium phosphate buffer, pH 7.0. After the washing procedure, the antibody-containing net was, in order to block the still available active groups on the nylon backbone, stored at +4°C for at least 24 h in 0.1 M potassium phosphate buffer, pH 7.0, containing 10 mM ethanolamine. Prior to use the membrane was washed with pure buffer.

2.2. *Preparation of catalase-labelled human serum albumin*

A previously published procedure based on glutaraldehyde aggregation and a subsequent gel-filtration step was used [4].

2.3. *Preparation of glucose oxidase-labelled insulin*

An aggregation procedure based on the activation of the carbohydrate moiety of the glucose oxidase molecule was applied [9].

Glucose oxidase, 6 mg, was dissolved in 3 ml 50 mM acetate buffer, pH 5.60. Potassium periodate, 1.35 mg, was added and the mixture was kept dark under stirring for 4 h at 25°C. Ethylene glycol (250 μl) was then added to destroy unreacted periodate. After 30 min at room temperature the pH was adjusted to 9.0 with 2 M sodium hydroxide and 10 mg of insulin was added. To dissolve the insulin totally 0.1 M $NaHCO_3$ had to be added to a final reaction volume of 8.0 ml. Coupling proceeded overnight at 4°C. Unreacted aldehyde groups were blocked by incubation for 1 h at 25°C after addition of 1 ml of 1 M glycine. The reaction mixture was separated on a Sepharose CL 6B column (15 × 250 mm).

2.4. *Preparation of the electrode*

A Radiometer pO_2-electrode (type E-50460) was used. The signal from the electrode was amplified in a Combi-Analysator U, Eschweiler Co., Kiel, FRG and registered on a chart recorder.

The active surface of the electrode was covered with an antibody-containing nylon net kept in position by a rubber ring. In the continuous-flow experiments the tip of the electrode was mounted in a small flow-through cell (see fig.1).

2.5. *Assay procedure*

The electrode with the antibody containing nylon net was at 25°C first equilibrated against phosphate buffer in equilibrium with atmospheric oxygen. Then a small sample containing varying concentrations of antigen and a known amount of enzyme-labelled antigen was exposed to the antibody–nylon net. Binding of antigen to the antibody–nylon net takes place and because of the existence of two different preparations of antigen (native and enzyme-labelled) competition between the two occurs.

After washing off all unspecifically adsorbed protein, a pulse of substrate (2 mM hydrogen peroxide or alternatively 100 mM glucose in the same buffer) was introduced. The signal from the electrode, reflecting the changed concentration of oxygen because of the enzyme catalyzed reaction, was registered on a chart recorder. After the assay, the bond between antibody and antigen (native as well as labelled) was broken by washing with 0.2 M glycine–HCl buffer, pH 2.2.

3. Results and discussion

In fig.1 is schematically shown the assay procedure used, as well as a generalized picture of the enzyme immunoelectrode device used. The use of a nylon net as support for the antibodies eliminates many of the diffusional restrictions that otherwise will hamper a fast response of the electrode. From the scheme it is thus seen that one assay takes a total of 10 min, including regeneration of the system.

In fig.2 is shown the dependence of the response from the anti-albumin-coated electrode to successive additions of catalase–albumin. A saturation effect is observed. In the following investigation amounts of enzyme-labelled antigen corresponding to the steepest part of the curve were used. On addition of enzyme-labelled antigen to a sample containing native antigen, competition between the two species of antigen will take place for binding to the antibodies on the net covering the electrode. The effect of this competition, expressed as product formation by the bound enzyme, is shown in fig.3A, demonstrating a standard curve obtained after measurements of samples with varying contents of native HSA to which fixed amounts of catalase-labelled HSA had been added. In the system studied here insulin could be determined down to 10^{-6} M and HSA down to at least 10^{-8} M (fig.3).

The antibody-containing membrane was used for several days and showed during this time high operational stability. The sensitivity of the system is still appreciably less than that of, e.g., conventional ELISA and also not as high as that reported for TELISA [3,4], but the system may, after proper optimization, be more sensitive.

A report has appeared in the literature describing electrodes aimed at determining concentrations of antibodies or antigens [10]. None of the electrodes

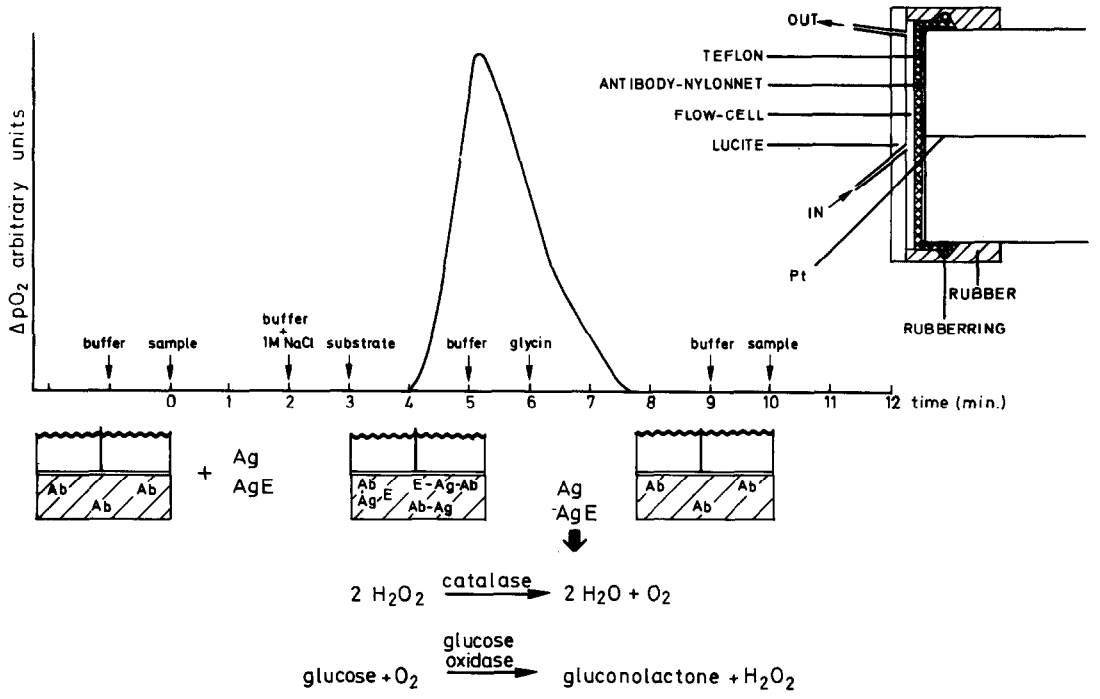


Fig.1. Schematic presentation of a reaction cycle using the enzyme immunoelectrode. The arrows indicate changes in the medium. When the flow device was used flow-rates of 1–2 ml/min were applied, otherwise with the conventional method, the electrode was transferred between the different solutions. In the latter case the time scale given in the figure is over optimistic and an assay cycle takes approximately 30 min. The cycle starts with 0.1 M potassium phosphate buffer, pH 7.0. At the arrow 'sample' a mixture of native and enzyme-labelled antigen moelcules is introduced. After a wash in 0.1 M phosphate buffer, pH 7.0, being 1 M in NaCl for 1 min, the enzyme immunoelectrode is ready for assay. The amount of enzyme trapped in the immunoreaction is assayed by introducing a pulse of a suitable substrate solution followed by buffer until the system is in equilibrium again. A 3 min pulse of 0.2 M glycine–HCl, pH 2.2, is used to split the antigen–antibody complex. After 1 min washing with buffer the system is ready for another assay. In the figure is inserted a generalized picture of the flow-device of the enzyme immunoelectrode.

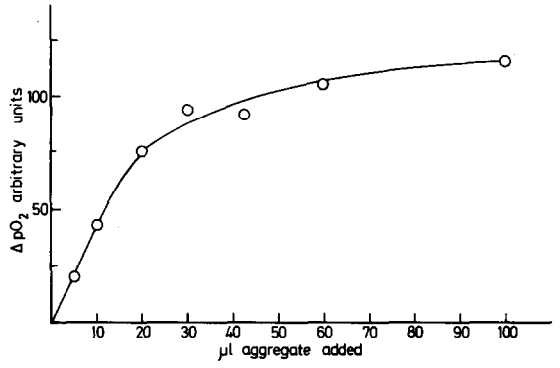
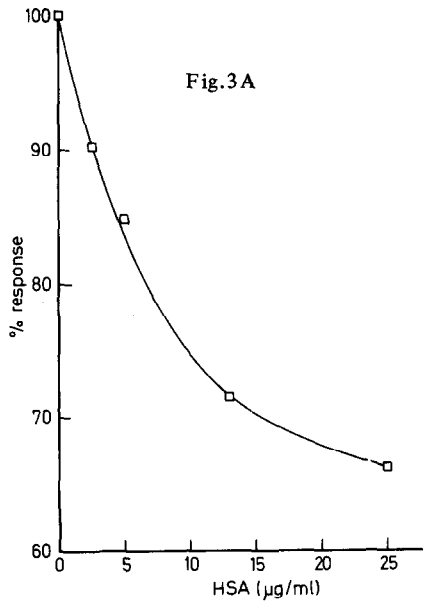


Fig.2. Standard curve showing changes in pO_2 obtained on introducing 2 min pulses of 2 mM hydrogen peroxide to the enzyme immunoelectrode after exposure to varying amounts of HSA–catalase aggregate added to 1 ml HSA-solution (1 mg/ml).

described were based on the ELISA-technique with its high specificity and sensitivity. The use of a platinum electrode coated with a protein-immobilizing membrane was thus described. When, e.g., rabbit anti-human 7 S γ-globulin was immobilized in the membrane, a change in the electric potential was observed when the electrode was exposed to human 7 S γ-globulin.

In earlier reports in the literature the advantages of the ELISA technique over RIA have been discussed [1,4]. The avoidance of radionuclide-labelling eliminates both the health hazards involved in the handling of short-lived isotopes as well as the instability of preparations of the labelled antigen. Besides, the use of radioisotopes involves the use of expensive equipment. In cases where the limits of the sensitivity of



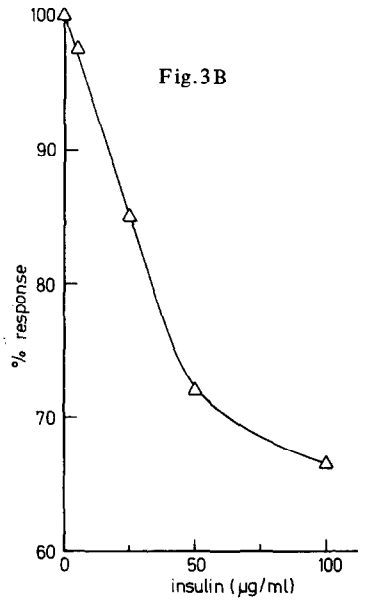


Fig.3. Standard curves showing the changes in pO_2 on introducing 2 min pulses of substrate to the enzyme immunoelectrode afte exposure of the bound antibody to a mixture of a fixed amount of aggregate and varying concentrations of free antigen. The $\Delta p($ obtained upon exposure to pure antigen–enzyme complex is set as 100%. (A) HSA-Assay using an anti-HSA-nylon net covered electrode and catalase labelled HSA. (B) Insulin assay using an anti-insulin-nylon net covered electrode and glucose oxidase labelled insulin.

the RIA-technique are not necessary, enzyme-linked immunoassays may be advantageous.

Conventional ELISA involves separation steps and is laborious and time consuming, whereas the use of immobilized antibodies either in proximity to the transducer, or in flow systems connected to a transducer offers a simple system easy to handle and with great possibilities for automation.

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