A GENERAL ENZYME THERMISTOR BASED ON SPECIFIC REVERSIBLE IMMOBILIZATION USING THE ANTIGEN–ANTIBODY INTERACTION

Assay of hydrogen peroxide, penicillin, sucrose, glucose, phenol and tyrosine

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1. Introduction

Enthalpimetric enzyme assay has appeared to be very general since the transducer registers any heat produced in an enzyme catalyzed reaction [1–3].

The combination of immobilized enzymes and enthalpimetric assays – the enzyme thermistor technique – has shown to be a general and versatile analysis procedure. Here an insulated micro-column filled with porous glass onto which enzyme is immobilized is used. The sample is pumped through the column and the heat produced by the enzyme catalyzed reaction during the passage of the substrate pulse, is registered by means of a thermistor immersed in the top of the bed.

Enthalpimetric assays have also been shown to be applicable to enzyme immunoassays, TELISA (thermometric enzyme immunosorbent assay) [4,5] Thus, it was possible to determine antigens in the concentration range 10^{-8}–10^{-10} M using immobilized antibodies in the thermistor unit and enzyme labeled antigen mixed with free antigen in the solution to be analyzed.

However, one drawback in using the conventional enzyme thermistor assay has been the time-consuming and laborious exchange between different analyses.

The present report deals with the application of a reversible specific immobilization technique which creates conditions for change from one enzyme assay to another within a few minutes.

2. Materials and methods

Rabbit anti-human serum albumin (anti-HSA) was obtained from the University Hospital in Malmö, Sweden. The antiserum was purified further by an affinity chromatography step [6].

Human serum albumin (HSA), Fraction V, catalase type C-30 from beef liver 10 000 U/mg, tyrosinase (*o*-diphenol oxygen oxidoreductase EC 1.10.3.1) from mushrooms, glucose oxidase (EC 1.1.3.4) Type V from *Aspergillus niger* (200 U/mg) and invertase (β-D-fructofuranoside fructohydrolase EC 3.2.1.26) from Baker's yeast (170 U/mg) were all purchased from Sigma, St. Louis, Mo. Penicillinase from *Bacillus cereus* was obtained from Riker Laboratories, Loughborough, England. Sepharose CL 4B was bought from Pharmacia Fine Chemicals, Uppsala, Sweden. All other reagents used were of analytical grade.

2.1. *Apparatus*

The enzyme thermistor unit was the same as described earlier [2,7]. The antibody-containing Sepharose CL 4B preparation was packed in an insulated glass-column with the flow upwards, and with a thermistor immersed in the top of the bed. The heat signals caused by enzymic conversion of pulses of substrate were registered by the thermistor and then amplified and registered on a chart recorder.

2.2. *Preparation of anti-HSA–Sepharose CL 4B*

The purified anti-HSA from 1 ml antiserum (approx. 2.5 mg) was added to 5 ml 0.1 M $NaHCO_3$ containing 3 g (wet wt) BrCN-activated and washed Sepharose CL 4B [8]. Coupling proceeded at + 4°C for 15 h. The gel

was then washed with 0.5 M NaCl and 0.1 M potassium phosphate buffer, pH 7.0.

2.3. *Preparation of enzyme–albumin conjugates*

In all the couplings excess of albumin in relation to enzyme was used. In a typical coupling of glucose oxidase to HSA, 10 mg glucose oxidase and 30 mg HSA were mixed in 2 ml 0.1 M potassium phosphate buffer, pH 7.0, 100–200 μl 4% glutaraldehyde was added, and the reaction proceeded at + 4°C for 17 h. The coupling process was stopped by the addition of 2 ml of either 1 M ethanolamine or 1 M glycine, both solutions with pH 7.0. The reaction mixture was then dialyzed against 3 liters of potassium phosphate buffer, pH 7.0.

When tyrosinase–albumin conjugates were to be prepared the coupling solution was 0.1 M in benzoic acid to protect the enzyme during the coupling procedure, and the dialysis buffer was 35 mM in benzoic acid.

After dialysis, the conjugates were chromatographed on Sepharose CL 6B (30 × 1.5 cm) to separate the high molecular weight fraction from unreacted protein. At a later stage this chromatographic step was omitted from the procedure.

2.4. *Assay procedure*

The enzyme thermistor unit was equilibrated by continuously pumping buffer through it (0.74 ml/min). The enzyme–albumin conjugate was diluted in the same buffer and introduced into the flow by means of a three-way valve. One minute after the enzyme pulse, a pulse of 0.5 M KCl in 0.1 potassium phosphate was passed through in order to wash out any protein not specifically bound by the antibody. Then the enzyme thermistor was ready for use, and substrate pulses could be introduced. When the enzyme activity declines or another enzyme activity is needed, the column is washed with a 2 min pulse of 0.2 M glycine–HCl, pH 2.2. By this wash the antibody–antigen interaction is split and the antibody-column is, after equilibration with a proper buffer, ready for reloading with new enzyme.

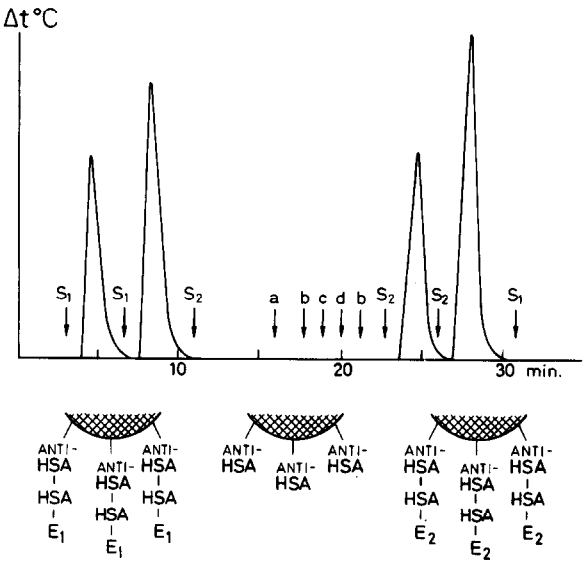

Fig.1. Schematic presentation of an assay-cycle. The arrows indicate changes in the perfusion medium, normally 0.1 M potassium phosphate buffer, pH 7.0 (flow rate 0.74 ml/min). The cycle starts with an enzyme–antigen (E_1–HSA) bound to the antibody–(anti-HSA)-containing support material. At the arrows marked, S_1 substrate for enzyme E_1 is introduced as pulses, and at the arrows marked, S_2 substrate for enzyme E_2 is introduced. The heat signals obtained upon substrate pulses are represented by the peaks. At the arrow '*a*', a 2 min pulse of 0.2 M glycine–HCl, pH 2.2, is introduced in order to split the complex and to wash the system. After a pulse of potassium phosphate buffer ('*b*'), new enzyme–antigen complex (E_2–HSA) is introduced ('*c*'), followed by a 1 min pulse of 0.1 M potassium phosphate 0.5 M in KCl ('*d*') and buffer ('*b*'). The system is then ready for new assays.

3. Results and discussion

In fig.1 is shown the general principle behind the procedure described here. It can be seen that the exchange between two different enzyme assays takes a total of 8–10 min, provided the same buffer can be used, otherwise a few additional minutes are needed.

In fig.2A a standard curve for assays of hydrogen-peroxide using albumin-labeled catalase immunologically bound to the HSA–Sepharose in the thermistor column is depicted. After a change of the enzyme to, e.g., albumin-conjugated penicillinase, penicillin G could be assayed (fig.2B).

In table 1 some of the metabolites assayed using this reversible binding technique are shown.

It has been shown earlier that the immobilized antibodies (rabbit anti-HSA) are stable for a long time (up to a month at 27°C, with continuous buffer or sample flow) and also stable for many association–dissociation steps between antigen and antibody [4–6]. In the cases referred to, the immunological function of

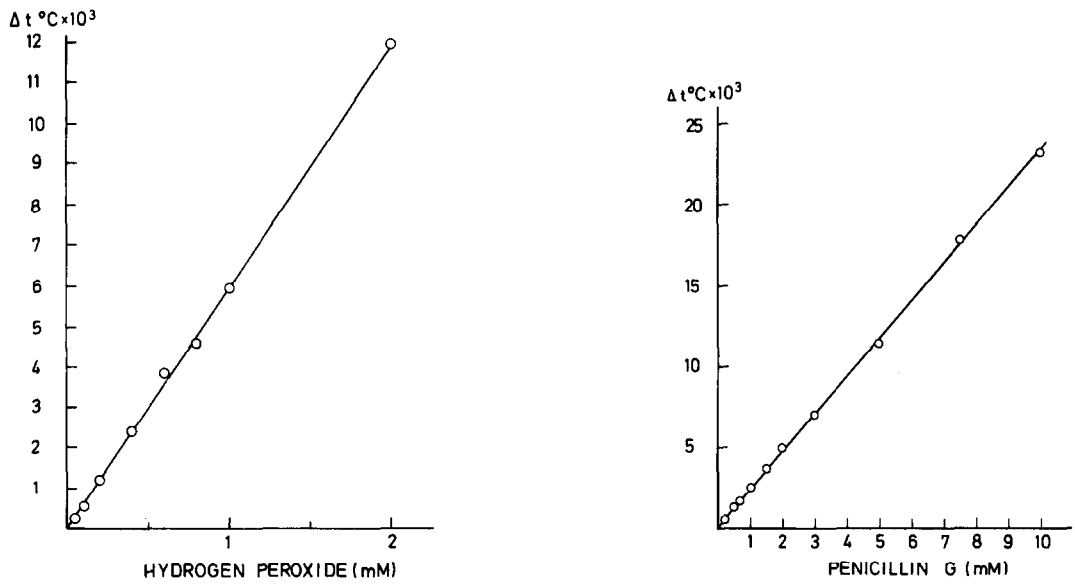

Fig.2. (A) Measured peak heights (Δt°C) obtained from a catalase–HSA–anti-HSA–Sepharose column as a function of the concentration of hydrogen peroxide dissolved in 0.1 M potassium phosphate buffer, pH 7.0 (1 min pulse). Fig.2. (B) Measured peak height (Δt°C) obtained from a penicillinase–HSA–anti-HSA–Sepharose column as a function of the concentration of penicillin G dissolved in 0.1 M potassium phosphate buffer, pH 7.0 (1 min pulse).

the immobilized antibodies have been of vital interest since they have been used in direct enzyme-immunoassays and in spite of that no decrease in response could be observed [5]. However, in the system reported here, a slight decrease in capacity to bind antigen-conjugated enzyme to the column should not markedly influence the operational capacity of the system.

To ensure that the antibodies were saturated with enzyme-conjugated albumin, successive additions of enzyme conjugates were carried out until constant response upon substrate addition was obtained. In all subsequent measurements saturating amounts of enzyme conjugates were used.

One great advantage with most analytical devices based on immobilized enzymes has always been their high operational stability. This character has been obtained by using great excess of enzyme so that initially only a small fraction of the potentially active enzyme molecules have been exposed to substrate, but as the activity of the preparation declines more and more of the earlier latent enzyme molecules become active [9]. In the present system no such overloading

Table 1

Metabolite	Enzyme	Concentration range (M)
Hydrogen peroxide	Catalase	5×10^{-5}–10^{-2}
Penicillin G	Penicillinase	10^{-4}–10^{-2}
Phenol	Tyrosinase	10^{-4}–10^{-3}
Tyrosine	Tyrosinase	10^{-4}–10^{-3}
Sucrose	Invertase	10^{-3}– 5–10^{-2}
Glucose	Glucose oxidase	5–10^{-5}– 5–10^{-4}

is necessary since the system can always be fresh, simply by replacing an old enzyme preparation.

The use of only a small amount of enzyme makes this system, in most cases studied, almost as sensitive as the conventional system in lower concentration regions. However, the conventional systems containing great excess of enzyme, also show, at least during the first period of their operational lives, a linear response even up in higher concentration regions [2,7]. An advantage of using column materials with only low degrees of substitution is that systems more sensitive to inhibitor analysis are developed [9,10].

The analysis of phenol and sucrose were in the present device markedly less sensitive than comparable results using glass-immobilized enzymes. Glass-bound invertase gave a linear response curve towards sucrose in the concentration range 10^{-5}–10^{-1} M and for phenol, using glass-bound tyrosinase linearity was obtained in the region 10^{-5}–10^{-2} M. These differences may probably be ascribed to a lower specific activity of the albumin-conjugated proteins even though a marked increase in tyrosinase-activity was noticed after protection with benzoic acid during the coupling procedure.

The process of cross-linking enzymes and human serum albumin has been shown to be mild and in most cases to give high yields, provided proper coupling conditions are used. The addition of substrates, inhibitors or combinations of both to protect regions of the enzyme vital to the catalytic process may be used [11, 12]. Thus malate dehydrogenase is reported to lose most of its activity upon glutaraldehyde treatment [13], but when protection is carried out by an abortive ternary complex, very high yields of active enzyme were obtained [14].

In the preparation of the complexes used here, excess of albumin was used to increase the chances of producing the bifunctional aggregate desired at a minimum of enzyme cost.

During the last years different reports on reversible immobilization of enzymes have appeared in literature [15,16]. Those methods may be easier to handle than the method here reported, but on the other hand, they have been general methods with no specificity. When working with analytical devices designed to be used in, e.g., crude solutions containing proteins and even active enzymes as well as a mixture of substrates, it is of vital interest to have a specific reversible coupling procedure so that the enzyme can be replaced either by fresh enzyme of the same species or of enzymes from other sources for quite different analytical demands. As shown in another report, the use of enzyme-immuno-systems may also be used in combination with other transducers, e.g., electrodes [17].

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