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THERMOMETRIC ENZYME LINKED IMMUNOSORBENT ASSAY: TELISA

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Summary

A new method, thermometric enzyme linked immunosorbent assay (TELISA), for the assay of endogenous and exogenous compounds in biological fluids is described. It is based on the previously described enzyme linked immunosorbent assay technique, ELISA, but utilizes enzymic heat formation which is measured in an enzyme thermistor unit.

In the model system studied determination of human serum albumin down to a concentration of 10^{-10} M (5 ng/ml) was achieved, with both normal and catalase labelled human serum albumin competing for the binding sites on the immunosorbent, which was rabbit antihuman serum albumin immobilized onto Sepharose CL-4B.

Introduction

The use of immunosorption for the assay of endogenous and exogenous compounds in biological fluids has received enormous attention during the last decade. First the radioimmunoassay technique (RIA) [1] was developed, which was followed by the enzyme-linked immunosorbent assay technique known as ELISA [2—4] and recently by another enzyme immunoassay technique known as enzyme-multiplied immunoassay, abbreviated EMIT [5,6]. Of these three techniques, assays based on RIA, and at present to a lesser extent on ELISA, are now in wide use in clinical analysis. Normally, in the enzyme-linked immunosorbent assay, an enzyme is covalently linked to a reference sample of the same antigen or antibody to be determined. After complexing to the corresponding matrix-bound antibody or antigen, the non-bound material is separated and an assay for enzymic activity is carried out. The enzymes normally used as marker enzymes include peroxidase and alkaline phosphatase both of which are assayed spectrophotometrically. In the present communication we

wish to report on the use of the ELISA technique in combination with a rather new analytical tool, the enzyme thermistor, which rapidly measures the heat evolved during an enzyme catalyzed reaction [7—9]. As the name implies, it combines the use of a highly general transducer (a thermistor) with a highly specific sensor (an enzyme). By combining the ELISA technique with such a thermometric assay method, for which we suggest the term TELISA (Thermometric Enzyme-Linked Immunosorbent Assay), a much wider choice of enzymes suitable for labelling is available since heat is evolved in most enzymic reactions; further the need for additional separation steps is eliminated. In the model study described here, the concentration of albumin has been determined using the system anti-human serum albumin/human serum albumin and as a marker enzyme catalase.

Materials and Methods

Hydrogen peroxide 30% w/v and glutaraldehyde, were purchased from BDH Ltd. (Poole, U.K.), BrCN from Fluka AG (Buchs, Switzerland) and $Na^{131}I$, 200 mCi/ml, from the Radiochemical Center (Amersham, U.K.). Sepharose CL-4B and Sepharose CL-6B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and porous glass from Pierce Chemicals Co. (Rockford Ill., U.S.A.). Human serum albumin, fraction V, with 15.9% content of nitrogen was obtained from Sigma (St. Louis Mo., U.S.A.) and rabbit anti-human serum albumin (10 mg/ml) from Daco, Denmark. The enzymes used, catalase from beef liver, 19 000 units/mg, and lactoperoxidase, B grade, were obtained from Sigma and Calbiochem (San Diego Calif., U.S.A.), respectively.

^{131}Iodinated human serum albumin

Lactoperoxidase was coupled to Sepharose 4B by the CNBr activation method [10] yielding preparations containing 2.3 mg/ml packed gel. The iodination procedure was carried out at +4°C as described by David [11]: 500 µl human serum albumin solution (5 mg/ml in 0.1 M potassium phosphate buffer, pH 7.0), 0.1 ml (packed) lactoperoxidase gel, 10 µl 5 mM KI and 5 µl $Na^{131}I$ (10 Ci/mg I, 200 mCi/ml). The reaction was started by adding 10 µl of 0.1% (v/v) solution of H_2O_2 in water and was allowed to continue for one hour under gentle stirring. After filtering off the immobilized lactoperoxidase preparation, the mixture was applied to a column of Sephadex G-25 using 0.1 M potassium phosphate buffer, pH 7.0, as running buffer. The protein appeared in the first fractions and the concentration of human serum albumin was estimated from the absorption measured at $A_{280\,nm}$, $E^{1\%}_{280\,nm} = 5.8$.

Apparatus used

Spectrophotometer; LKB Beckman 24, Scintillator; CG 30 Automatic Gamma Spectrometer, Nuclear Chicago. The enzyme thermistor unit was developed at the Chemical Center, Biochemistry 2, University of Lund, the components of which are a—d, (a) Wheatstonebridge; Knauer Temperatur Messgerät, (b) Thermistor; Veco type 41A28, (c) Pump; Varioperpex LKB, (d) Water bath; Hetotherm model 05 PG 623 UO Heto Birkerö.

Preparation of covalently bound proteins

Solid matrix. Anti-human serum albumin was immobilized on Sepharose CL-4B by the CNBr technique [10] yielding 0.5—1.0 mg of protein/ml of packed gel.

Soluble aggregate. Using the bifunctional reagent glutaraldehyde, catalase and human serum albumin were conjugated in the following way: 4.0 mg human serum albumin was dissolved in 0.6 ml 0.5 M potassium phosphate buffer, pH 7.5, 400 μl of catalase suspension (20 mg/ml) was added and the solution cooled on an icebath, 100 μl of 4% (w/v) glutaraldehyde was then added with stirring. The reaction was allowed to proceed with stirring in a refrigerator overnight. Excess glutaraldehyde was allowed to react with 100 μl of fresh, saturated $NaHSO_3$.

Characterization. After separation on Sepharose CL-6B, the human serum albumin and catalase content in the highest molecular weight fraction were determined by both immunological and spectrophotometric methods. Immunoturbidimetric techniques were used to determine the amount of immunoactive antigen in the complex [12]. The catalase catalyzed decomposition of hydrogen peroxide was followed at 240 nm with a spectrophotometer [13].

Assuming that the properties, i.e. immunological and catalytical activity, of the two molecular entities in the complex were unaltered in the coupling step, the two proteins were present in approximately an 1 : 1 ratio.

Analysis procedure

In the enzyme thermistor unit used, the solid matrix containing immobilized

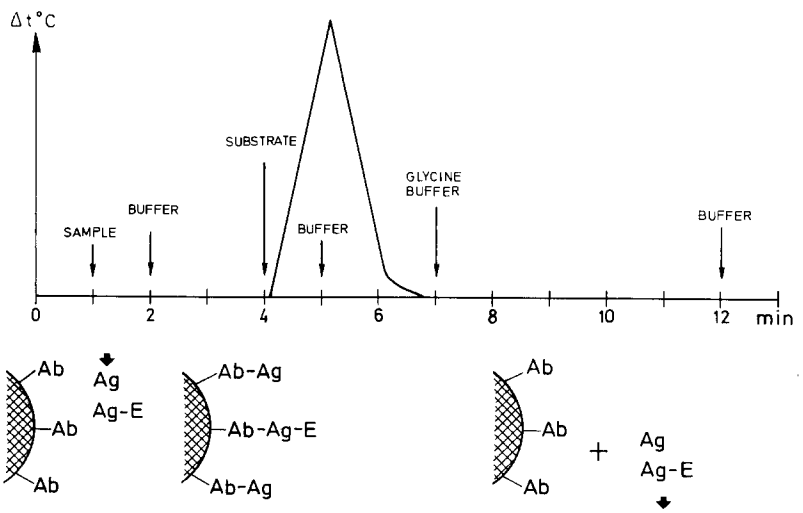

Fig. 1. Schematic presentation of a reaction cycle in the TELISA procedure. The arrows indicate changes in the perfusing medium (flow rate 0.8 ml/min). The cycle starts with potassium phosphate buffer pH 7.0 (0.2 M). At this time the thermistor column contains only immobilized antibodies. At the arrow "sample" a mixture of antigen and catalase-labelled antigen is introduced. The system is then washed with potassium phosphate buffer for two minutes. Now the sites on the antibodies of the column are occupied by antigen as well as by catalase-labelled antigen. The amount of catalase bound is measured by registering the heat produced during a one-minute pulse of 1 mM H_2O_2. After the heat pulse is registered, the system is washed with glycine/HCl (0.2 M, pH 2.2) to split the complex. After five minutes of washing, phosphate buffer is introduced, and the system is ready for another assay.

anti-human serum albumin was packed in either a glass or teflon column. The general scheme of the analytical procedure is outlined in Fig. 1. A known amount of human serum albumin-catalase aggregate was mixed with the sample containing an unknown amount of human serum albumin. This mixture was then pumped through the column. This resulted in a competitive binding of human serum albumin and human serum albumin-catalase to a limited quantity of immobilized anti-human serum albumin. When the substrate, hydrogen peroxide was introduced to the system, the heat of the enzymic reaction was registered by the thermistor and displayed on a strip-chart recorder. The height of the recorder signal obtained was inversely proportional to the amount of human serum albumin in the sample. The sensitivity of the method can be altered by changing the amount of aggregate that is added to the sample as well as the amount of anti-human serum albumin immobilized to the matrix.

A typical example of the assay of albumin in the 0.1—200 μg/ml range is given below: To two ml of the human serum albumin sample, 20 μl of human serum albumin catalase were added. The sample pulse length was one minute and the flow rate 0.8 ml/min. After sample introduction using a three way valve, the system was allowed to equilibrate for 2 min. Then a 1 min pulse of 1 mM H_2O_2 was introduced. After recording the signal, the column was thoroughly washed for five minutes with 0.2 M glycine, pH 2.2.

Results and Discussion

The albumin-catalase aggregates prepared were separated from the reaction mixture by gel filtration on Sepharose CL-6B. The fractions of the first peak obtained, forming the coupled high molecular weight albumin-enzyme aggregate, were collected and used in the subsequent studies. First the presence of albumin and of catalase was established as described earlier using immunoturbidimetric techniques [12] and conventional enzyme assays [13], respectively. In addition electrophoresis was used for further characterization. The different steps involved in the assay procedure are shown schematically in Fig. 1.

Various agents were tested for their ability to dissociate the complex formed between the immobilized antibody and the albumin/albumin-catalase aggregate. Using NaSCN (4 M) [14], a marked decrease in catalase activity on the column as measured with the thermistor unit was observed. However, on introducing phosphate buffer (0.1 M, pH 7.0) a progressive recovery of the catalase activity was observed. This was interpreted as due to refolding of the partially denatured enzyme formed on treatment with the rhodanide solution. When using glycine/HCl buffer, (0.2 M, pH 2.2), as dissociating agent [15], no catalase activity could be observed in the thermistor column and the amount of protein released was identical for a number of consecutive runs. As matrix for the immobilization of the antibody, cross-linked Sepharose (Sepharose CL-4B) was found to be satisfactory as no interference through non-specific adsorption of protein to the support was observed, in contrast to porous glass beads.

In Fig. 2, a standard curve is depicted plotting the heat formed in the enzyme thermistor assays as a function of different quantities of albumin - catalase aggregate added to the flow with a constant concentration of albumin. As can

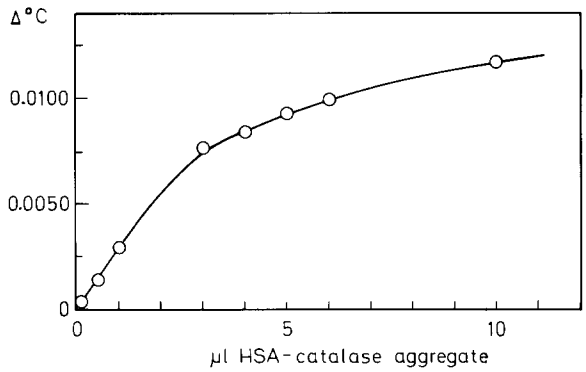

Fig. 2. Standard curve showing temperature change, Δt°C, obtained on introducing a 1 min pulse of 1 mM H_2O_2 to the thermistor column after exposure to varying amounts of human serum albumin (HSA)-catalase aggregate added to 1 ml human serum albumin-solution (1 mg/ml).

be seen, highest and linear response relative to the amount of aggregate added was obtained in the region up to 3 μl (= 0.3 volume per cent) of added aggregate. In Fig. 3a and b standard curves obtained in the subsequent analyses of various amounts of free albumin to which a now constant amount of albumin-catalase aggregate was added are shown. These curves show a similar shape to those obtained with the radioimmunoassay technique in that at the low concentration region the highest sensitivity is found. Furthermore, to obtain increased sensitivity the sample pulse length can be prolonged (Fig. 3).

The coefficient of variation of 0.9% found in these measurements was excellent. To further verify the validity of the results obtained, TELISA experiments were carried out using ^{131}I-labelled albumin (which at the same time correlates the RIA (Radio Immuno Assay) with the ELISA technique). The radioactivity found in the effluent after dissociation of the complex was counted and shown to be in good correlation with the remaining enzyme activity in the column registered by the enzyme thermistor unit. Thus, a decrease of 12% in the column catalase-activity was observed when 1 μg ^{131}I-labelled human serum albu-

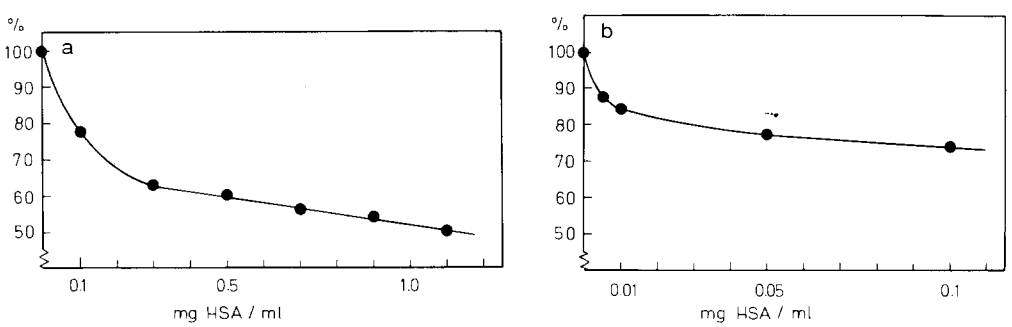

Fig. 3. Standard curve showing the decrease in temperature signal obtained on introducing 1 mM H_2O_2 (1 min pulse) to the antibody-thermistor column after exposure of the bound antibody to a mixture of a fixed amount of aggregate and varying concentrations of free antigen. The temperature change obtained upon exposure to pure antige-catalase complex is set as 100%. (a) 1 min sample pulse. 10 μl aggregate was added to the sample. (b) 4 min sample pulse. 25 μl aggregate was added to the sample.

min/ml was present in the sample to be analyzed. After dissociation of the antibody-antigen complex 850 cpm was found in the effluent. This means that the decrease of 12% in catalase activity is caused by the binding of ^{131}I-labelled human serum albumin to the column instead of enzyme labelled human serum albumin. Likewise 2 and 5 μg/ml of ^{131}I-labelled human serum albumin/ml present in the sample to be analyzed, resulted in a reduction of the catalase produced heat signal by 19 and 31% and a subsequent elution of 1720 and 3620 cpm, respectively.

At present, the total time required for one assay included regeneration time is about 10—11 min after which a new determination can be started. Under the conditions given here the same immobilized antibody preparation was used up to 100 times without showing any decrease in binding capacity towards the antigen (albumin) preparations. However, if decreasing binding capacity of the column, because of undissociated immunocomplexes, becomes apparent, then an intermittent wash with strong dissociating agents should restore the immobilized antibody preparation. The results presented here clearly demonstrate the possibility of assaying metabolites down to at least 10^{-10} M using the TELISA method. Determinations of the serum albumin contents, in serum samples as well as in standard solutions, given as an example of the TELISA-technique in the present communication were in good agreement with both the conventional method based on the use of dyes, e.g. bromocresol green [16] as well as with immunoturbidimetric assay and rocket electrophoresis [17].

In conclusion, we wish to evaluate the procedure described here although it is as yet at an early state of development. Compared to the well-established RIA technique used for assay of hormones and other biological molecules, the sensitivity of the procedures is not at the extremely low level of down to 10^{-17} M reported to have been obtained with the RIA technique. It may also be difficult to reach the same high assay-speed as can be obtained with automated RIA measuring units.

Draw-backs such as short half-life of isotope, expensive equipment and possible health hazards are not encountered however. What has been said above applies of course also to the conventional ELISA technique. Based on earlier results obtained in the analysis of glucose or cholesterol present in the flow, the sensitivity of the enzyme thermistor using either immobilized glucose oxidase or cholesterol oxidase compared well with standard spectrophotometric methods [8].

The advantages of the TELISA technique described here over those of the conventional ELISA method lie, in our opinion, primarily in the fact that (a) it gives a much wider choice of suitable markers as most enzymes can be assayed by heat measurements, thus allowing for instance the use of catalase, characterized by an extremely high turnover number leading to increased sensitivity of the system and (b) that no additional separation step is required.

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