Immunoaffinity layering of enzymes

Stabilization and use in flow injection analysis of glucose and hydrogen peroxide

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Abstract A general procedure for the high yield immobilization of enzymes with the help of specific anti-enzyme antibodies is described. Polyclonal antibodies were raised against Aspergillus niger glucose oxidase and horseradish peroxidase in rabbits and the gamma globulin (IgG) fraction from the immune sera isolated by ammonium sulphate fractionation followed by ion-exchange chromatography. Immobilization of glucose oxidase and horseradish peroxidase was achieved by initially binding the enzymes to a Sepharose matrix coupled with IgG isolated from anti-(glucose oxidase) and anti-(horseradish peroxidase) sera, respectively. This was followed by alternate incubation with the IgG and the enzyme to assemble layers of enzyme and antibody on the support. The immunoaffinity-layered preparations obtained thus were highly active and, after six binding cycles, the amount of enzyme immobilized could be raised about 25 times over that bound initially. It was also possible to assemble layers of glucose oxidase using unfractionated antiserum in place of the IgG. The bioaffinity-layered preparations of glucose oxidase and horseradish peroxidase exhibited good enzyme activities and improved resistance to heat-induced inactivation. The sensitivity of a flow injection analysis system for measuring glucose and hydrogen peroxide could be remarkably improved using immunoaffinity-layered glucose oxidase and horseradish peroxidase. For the detection of glucose, a Clark-type oxygen electrode, constructed as a small flow-through cell integrated with a cartridge bearing immunoaffinity-layered glucose oxidase was employed. The hydrogen peroxide concentration was analysed spectrophotometrically using a flow-through cell and the layered horseradish peroxidase packed into a cartridge. The immunoaffinity-layered enzymes could be conveniently solubilized at acid pH and fresh enzyme loaded onto the support. Immunoaffinity-layered glucose oxidase was successfully used for the on-line monitoring of the glucose concentration during the cultivation of Streptomyces cerevisiae.

Introduction

Procedures that result in the binding of large quantities of enzyme on supports are advantageous in nearly all applications requiring immobilized enzymes. Such strategies are particularly helpful in the construction of enzyme-based biosensors that necessitate the fixation of large amounts of enzymes on a relatively small area for maximum sensitivity (Vandenberg et al. 1994). We have recently demonstrated that quantities of glycoenzyme immobilized on insoluble supports can be raised remarkably by bioaffinity layering that involves building of alternate layers of the lectin concanavalin A (Con A) and glycoenzymes (Farooqui et al. 1997). In addition to the high accessibility of the layered enzymes for their substrates, a layer-by-layer increase in the stability against inactivation was exhibited by the immobilized glycoenzyme preparations. The sensitivity of a flow-through glucose-monitoring cartridge integrated into a flow injection analysis system (FIA) was enhanced very significantly by increasing the amount of glucose oxidase immobilized by affinity layering (Farooqui et al. 1997). The bioaffinity-layering procedure described, however, is restricted only to glycoenzymes with high affinity towards Con A. In view of the observation that the bioaffinity layering of the glycoenzyme was possible at pH values where Con A exists predominantly as dimers, it was envisaged that enzyme layering should also be possible with bivalent anti-enzyme antibodies. A preliminary report on the assembly of layers of glucose oxidase on a glassy carbon electrode surface using mouse IgG, anti-mouse IgG–glucose-oxidase conjugate.
and monoclonal antibodies to glucose oxidase produced in mouse is available (Bourdillon et al. 1994). Work described in this article relates to the use of polyclonal anti-(glucose oxidase) and anti-(horseradish peroxidase) sera in the assembly of glucose oxidase and horseradish peroxidase layers, respectively, on a Sepharose support. A flow-through cartridge containing the immunoaffinity-layered glucose oxidase was integrated into a FIA system equipped with a Clark oxygen electrode for use in the analysis of glucose. The hydrogen peroxide concentration was measured spectrophotometrically with the help of a cartridge bearing the layered horseradish peroxidase connected to a flow-through cell. Immunoaffinity layering markedly improved the detection limits of the sensors and the former could be conveniently used for on-line monitoring of the glucose concentration in the culture medium during the cultivation of S. cerevisiae.

Materials and methods

Glucose oxidase (EC1.1.3.4, type VIIS from A. niger, 230 units/mg protein), peroxidase (EC1.11.1.17, type II, 150 units/mg solid from horseradish), CNBr-activated Sepharose 4B Fast Flow (Pharmacia, Sweden), 2-2′-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS). All other fine chemicals were purchased from Sigma (St. Louis, USA).

Immunization of animals and isolation of IgG

Glucose oxidase and horseradish peroxidase were injected into healthy albino rabbits for the production of polyclonal antibodies. Animals received subcutaneously 300 μg enzymes dissolved in 0.5 ml of 20 mM sodium phosphate, pH 7.2, mixed with equal volumes of Freund’s complete adjuvant as the first injection. Boosters with 150 μg glucose oxidase or horseradish peroxidase in Freund’s incomplete adjuvant were administered every week after resting the animal for 15 days. After each booster, blood was collected through an ear vein and was allowed to clot at room temperature for 3 h. Serum was collected after centrifugation and stored in small aliquots at −20 °C. The Ouchterlony double-diffusion technique (Ouchterlony 1949) was used to detect the presence of antibodies. For the isolation of the IgG fraction, the precipitate obtained at 20–40% ammonium sulphate saturation of the antiserum was collected and dialysed against 20 mM sodium phosphate, pH 7.0, in a total volume of 3 ml for 12 h at 20 °C. The matrices were then washed thoroughly with the buffer to remove the unbound enzyme. The IgG support with bound enzyme was further incubated alternately with fresh IgG and enzyme for the formulation of successive layers. For each successive binding, the amount of enzyme and IgG incubated with the support was raised 1.5–2.5 times. After each incubation, the amount of matrix-bound IgG or enzyme was determined by subtracting the amount of the unbound protein from that added either by measuring the protein (Lowry et al. 1951) or the enzyme activity (Iqbal and Saleemuddin 1983).

For the preparation of immunoaffinity-layered glucose oxidase using unfractionated antiserum, CNBr-Sepharose with coupled IgG (5 mg/ml gel) was used. After the first incubation with the enzyme at the concentration described above, the matrix was washed thoroughly to remove the unbound enzyme and incubated with unfractionated antiserum at a protein concentration 10-times higher than that used for layering with isolated IgG. After appropriate binding cycles, the matrix was rinsed and allowed to remove all the non-specifically bound proteins. The alternate incubations of the support with unfractionated antiserum and enzyme were continued the required number of times.

Measurements with FIA systems

A cartridge filled with 1 ml Sepharose support with immunoaffinity-layered glucose oxidase or horseradish peroxidase was integrated in a FIA system connected to a transducer. The signals generated by the action of glucose oxidase and horseradish peroxidase were determined using an oxygen electrode or spectrophotometer, respectively. The FIA used was EVA-Line (Eppendorf, Netheler and Hinz, Hamburg, Germany) controlled by a 486 personal computer. The controlling and evaluating software was CAFCA (Computer Assisted Flow Control and Analysis, Analyscon, Hannover, Germany).

For the continuous analysis of glucose, a Clark-type oxygen electrode, constructed as a small flow-through cell (Analyscon, Hannover, Germany) was used (potential +600 mV). Oxygen consumption during enzymatic reactions was measured and the signal amplified. The flow rate of the glucose dissolved in 20 mM potassium phosphate, pH 7.0, was 1.0 ml/min and the sample volume was 30 μl.

For the quantification of hydrogen peroxide, a spectrophotometer was used as a transducer and the oxidation of ABTS was measured at 425 nm (Kleinhammer and Mattesberger 1986). The photometer used a flow through cell and was of the Skalar 6010 type. The stopped-flow technique was used during the analysis, in which the buffer flow (1 ml/min) was stopped for 40 s to 1 min, once the sample reached the cartridge.

Elution and reloading of enzyme

For the solubilization of affinity-bound glucose oxidase on IgG coupled to VA epoxy carrier, O.2 M glycine/THI, 0.15 M NaCl, pH 2.5, or 3.0 M KSCN, pH 7.6, was pumped through the cartridge containing the immobilized preparation at a flow rate of 0.25 ml/min for 15 min. The cartridges were washed with the buffer, analysed for possible residual signal and reloaded with glucose oxidase as described earlier.

Cultivation of S. cerevisiae

S. cerevisiae was cultivated on a semi-synthetic medium containing 30 g/l glucose (Schatzmann 1975). The cultivation was carried out in a 2.0-l bioreactor (Biotest B, Braun Melsungen, Melsungen, Germany), with aeration of 1vvm (2.0 l/min), pH 4.0, temperature 32 °C and stirred at a speed of 600 rpm Analysis of the cultivation fluid for glucose was made using an in situ sampling device (Scheper et al. 1996) on-line with the cartridge bearing an affinity-layered glucose oxidase support. Off-line glucose analysis in the medium was performed using a YSI analyser.