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Immobilization of β galactosidase from *Aspergillus oryzae* via immunoaffinity support

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ABSTRACT

Polyclonal antibody bound cellulose support has been exploited for the immobilization and stabilization of β galactosidase from *Aspergillus oryzae*. Immunoaffinity bound β galactosidase retained 96.5% of the initial activity on the support. Immobilized β galactosidase showed broad-spectrum pH optima, pH 4.6–5.5 and temperature at 50–60 °C whereas the soluble enzyme exhibited activity peak at pH 4.6 and 50 °C. Immunoaffinity bound enzyme preparation was quite stable to thermal denaturation and it retained 72% activity after 4 h incubation at 60 °C whereas under these conditions the soluble β galactosidase lost almost its full activity. IgG-cellulose immobilized β galactosidase was quite stable against the inactivation caused by proteolytic enzymes; trypsin and pepsin. Moreover, this immobilized enzyme preparation retained 64%, 71% and 65% activity in the presence of 4.0 M urea, 5% $CaCl_2$ and 5% galactose. IgG-cellulose bound β galactosidase exhibited 80% of its original activity after 2 months storage at 4 °C while the soluble enzyme showed only 35% of the initial activity. After 10th repeated use immobilized β galactosidase retained 46% activity.

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1. Introduction

Bioaffinity based methods have several advantages over the other known methods used for the immobilization of enzymes [1]. This method provides oriented immobilization of enzymes that facilitates good expression of activity and the possibility of direct immobilization [2,3]. Among the bioaffinity pairs, the antigen and antibody pair is highly specific and this pair could be exploited for the immobilization of all kinds of enzymes. Immobilized antibodies have become powerful tools in biosensor technology, diagnostics and therapeutics [4,5]. In the last few years, purification of antibodies has grown to become the largest class of proteins in clinical-phase development intended for the therapeutic and diagnostic applications [6,7]. Numerous sorbents have been designed for their separation, employing different adsorption–desorption principles [8]. Stabilization of enzymes against the inactivation induced by different types of denaturants has been accomplished using a multitude of immobilization strategies including immunoaffinity, covalent coupling, adsorption, microencapsulation, polymer entrapment and chemical aggregation [9].

Enzyme immobilization can be economically advantageous when the immobilization promotes a significant increase in the stability of enzyme and the support can be reused [10,11]. Procedures, which utilize the affinities of biomolecules and ligands for the immobilization of enzymes are gaining wider acceptance in the construction of enzyme based analytical devices as well as for other applications [12,13].

β Galactosidases from different sources are currently being used in the production of lactose free milk products. Hydrolysis of lactose improves product sweetness, makes milk consumption more easier for the people who suffer from lactose intolerance, increases product quality and process efficiency in the dairy industry [14]. Lactose is the main carbohydrate present in milk (4–5%) and dairy products [15,16]. Its concentration in food products is indicative of the amount of lactose present in these products. Lactose is determined in dairy products in the milk industry in order to provide process efficiency and product quality control [17]. There are several methods to detect lactose concentration such as spectrophotometry, polarimetry, infrared spectroscopy, titrimetry and chromatography. However these methods are expensive, tedious and time-consuming due to long sample preparation. These disadvantages have forced the development of bioaffinity-based methods for the detection of lactose [18].

Abbreviations: DEAE, diethyl aminoethyl; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; ELISA, enzyme linked immunosorbent assay; LB, Langmuir–Blodgett; ONPG, *o*-nitrophenyl β-D-galactopyranoside; TBS, Tris buffer saline.

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Here an effort has been made to immobilize β galactosidase from *Aspergillus oryzae* on an immunoaffinity support, IgG-cellulose. The stability of immunoaffinity immobilized β galactosidase has been investigated against denaturants such as pH, heat, urea, proteolytic enzymes (pepsin, trypsin), $CaCl_2$ and galactose. Immunoaffinity support immobilized β galactosidase was also investigated for its storage stability and reusability.

2. Materials and methods

2.1. Materials

A. oryzae β galactosidase (3.2.1.23), *o*-nitrophenyl β-D-galactopyranoside (ONPG), cyanogen bromide (CNBr), galactose and glucose were obtained from Sigma Chem. Co. (St. Louis, MO, USA). Cellulose and DEAE-cellulose, chemicals and reagents used in electrophoresis and immunodiffusion were obtained from SRL Chemicals (Mumbai, India). All the other chemicals and reagents used were of analytical grade.

2.2. Immunization

Commercially available purified β galactosidase was injected into healthy male albino rabbits weighing 2–3 kg for the production of anti-β galactosidase polyclonal antibodies. The animals received subcutaneously 300 μg of β galactosidase 0.5 mL mixed and emulsified with equal volume of Freund's complete adjuvant as first dose [19]. Booster doses of 150 μg of β galactosidase mixed and emulsified with Freund's incomplete adjuvant were administered weekly after resting the animal for 15 days. After each booster dose blood was collected from the ear vein of the animal and allowed to clot at room temperature for 3 h. Serum was collected by centrifugation at 1600 × *g* for 20 min at 4 °C. After adding sodium azide (0.2%) serum was stored at −20 °C.

2.3. Purification and characterization of polyclonal antibodies

The antiserum was fractionated with 20–40% ammonium sulphate. The sample was kept overnight with constant stirring at 4 °C to precipitate proteins. The precipitated proteins were collected by centrifugation at 1600 × *g* for 20 min at 4 °C. The pellet obtained was re-dissolved in a minimum volume of 0.02 M sodium phosphate buffer, pH 7.2 and was subjected to extensive dialysis against the same buffer to remove the traces of ammonium sulphate.

Further antibodies raised against β galactosidase were purified by ion exchange chromatography. The dialyzed protein sample from ammonium sulphate precipitated antiserum was passed through DEAE-cellulose column (1.20 cm × 10.0 cm) and the fractions containing purified anti-β galactosidase antibodies were pooled for further use [9].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gel under denaturing conditions and native PAGE on 7.5% gel were run to separate proteins present in DEAE-cellulose purified anti-β galactosidase antibodies [20]. The staining and de-staining of the gel was also performed by the same procedure. Molecular weight marker proteins (myosin, 205 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; soybean trypsin inhibitor, 20 kDa and lysozyme, 14.3 kDa) were also run in one lane adjacent to the purified IgG.

2.4. Immunodiffusion

Ouchterlony double immunodiffusion was used to prove the presence of antibodies against β galactosidase. Immunodiffusion was performed in 1.0% (w/v) agarose prepared in normal saline [3]. The purified anti-β galactosidase antibodies were employed for preparing immunoaffinity support.

2.5. Direct binding ELISA

Polystyrene (96 well) microtitre plate was coated with 100 μL of antigen (β galactosidase) at a concentration of 10 μg/mL prepared in antigen coating buffer (0.05 M bicarbonate buffer, pH 9.6) and then incubated for 2 h at 37 °C followed by overnight storage at 4 °C. The wells were then washed three times with TBS-T buffer. The unoccupied sites were blocked with 2% fat milk in TBS (150 μL, each well) followed by incubation for 5–6 h at room temperature. The wells were then washed twice with TBS-T. The test and control wells were then diluted with 100 μL of serially diluted serum. Each dilution was in TBS buffer. Serially diluted blanks corresponding to each dilution were also present. The plate was then incubated for 2 h at room temperature and overnight at 4 °C. The plate was washed again with TBS-T buffer (five times). Bound antibodies were assayed with an appropriate conjugate of anti-rabbit IgG alkaline phosphatase, 100 μL of it was put in each well and kept at room temperature for 2 h. Washing of the plate with TBS-T (five times) and with distilled water (two times) was followed by addition of *p*-nitrophenyl phosphate (50 μg/100 μL) in each well and incubation at 37 °C for 30–45 min. The absorbance of each well was monitored at 405 nm on a Lab system ELISA Reader.

2.6. Preparation of immunoaffinity support

Cellulose powder (5.0 g) was activated by cyanogen bromide (CNBr) as described by Porath et al. [21]. Cellulose was washed thoroughly with distilled water in a sintered glass funnel. The gel was sucked dry and suspended in 10.0 mL of 1.0 M Na_2CO_3 and stirred slowly by placing on a magnetic stirrer at 4 °C for 30 min. CNBr (1.0 g) dissolved in 1.0 mL of acetonitrile was added to the beaker containing cellulose and was again stirred for 10 min in cold. The whole mass was transferred immediately to a sintered funnel and washed thoroughly with sufficient volume of 0.1 M bicarbonate buffer, pH 8.5, distilled water, and again with same buffer. Washed activated cellulose was dried and re-suspended in 5.0 mL of 0.1 M bicarbonate buffer, pH 8.5. Purified antibodies (60 mg) was mixed with activated cellulose (5.0 g) and stirred overnight in cold. Cellulose unbound antibodies were removed by centrifugation at 1600 × *g* for 20 min at 4 °C. Antibody bound matrix was extensively washed with 0.1 M bicarbonate buffer, pH 8.5 containing 1.0 M NaCl. The washed suspension was treated with 7.0 mL of 0.1 M glycine for 2 h at 4 °C. Antibody bound cellulose was successively washed with 0.1 M sodium bicarbonate buffer, pH 8.5, containing 1.0 M NaCl, distilled water and finally with 0.05 M sodium phosphate buffer, pH 7.0. The quantity of bound antibody was calculated by subtracting the unbound protein in the washings from that of total added protein.

2.7. Immobilization of β galactosidase on IgG-cellulose

β Galactosidase (5200 U) from *A. oryzae* was incubated with 5.0 g of IgG-cellulose in 0.1 M sodium phosphate buffer, pH 6.2 overnight at 4 °C. IgG-cellulose bound enzyme was then thoroughly washed with 0.05 M sodium phosphate buffer, pH 6.2, to remove unbound enzyme. Finally, the immobilized enzyme was stored in sodium acetate buffer, pH 4.6 for its further use.

2.8. Effectiveness factor (η)

The effectiveness factor (η) value of the immobilized preparation represents the ratio of actual and theoretical activity of the

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