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Concanavalin A layered calcium alginate–starch beads immobilized β galactosidase as a therapeutic agent for lactose intolerant patients

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ABSTRACT

A novel therapeutic agent in the form of β galactosidase immobilized on the surface of concanavalin A layered calcium alginate–starch beads has been developed. Immobilized β galactosidase exhibited significantly very high stability against conditions of digestive system such as pH, salivary amylase, pepsin and trypsin. Soluble and immobilized β galactosidase exhibited same pH-optima. However, the immobilized enzyme retained greater fraction of catalytic activity at higher and lower pH to pH-optima as compared to soluble enzyme. Immobilized enzyme preparation was quite stable under conditions present in mouth, stomach and intestine. Immobilized β galactosidase retained 65% activity even after its sixth repeated use.

1. Introduction

β galactosidases are abundant among micro-organisms, animals and plants (Haider and Husain, 2007a). These enzymes are increasingly employed in the dairy industry. Their capacity as biocatalysts is to hydrolyze lactose in milk and whey which helps in the production of low-lactose dairy products for people intolerant to this sugar and also serves to recycle whey which can be used as an additive for human or cattle feed (Zhou et al., 2003; Phadtare et al., 2004). Lactase is the common name for lactase-phlorizin hydrolase (LPH), an enzyme located in the brush border of small intestinal enterocytes that is necessary for the digestion of lactose. Human LPH is encoded by the lactase gene located on the long arm of chromosome 2 (Kerber et al., 2007). Lactose intolerance occurs in children shortly after weaning, when production of the lactose-digesting enzyme, lactase is down-regulated in the gut (Stefano et al., 2001).

The symptoms of lactose intolerance tend to be primarily gastrointestinal in origin (Wilson, 2005). When lactase is absent or deficient, hydrolysis of lactose is incomplete as it is osmotically active, the undigested sugar will pull fluid into the intestine. Thus these lactose intolerant people are discouraged from consuming milk and may lose a major source of calcium and high quality protein from their diets (Suarez et al., 1995; Kim et al., 1999; Heyman, 2006). Milk is the most important source of energy during the first year of a human's life, providing almost half the total energy requirement of infants (Vesa et al., 2000). In order to prevent lactose intolerance, the need for lactose-free milk and its products arises.

Immobilized enzymes are generally more stable and there are many potential applications that range from chemical synthesis to biotechnology and medicine (Liang et al., 2000). Immobilization of β galactosidase in liposomes may be useful in order to overcome the shortcoming of lactose hydrolysis. β galactosidase microencapsulation in lipid vesicles has been delivered for treating lactose intolerance but there was a problem of contact between the enzyme and substrate (Walde and Ichikawa, 2001; Monnard, 2003; Nogales and Lopez, 2006). Very few other immobilized β galactosidase preparations have shown their potential in targeting lactose present in small intestine. Based on applications, there are many different types of polymers used for enzyme immobilization (Propkop et al., 1998). Chitosan is biocompatible and has been used in many applications including drug delivery systems. The disadvantage of chitosan is its limited solubility in water and the low pH of chitosan solution tends to denature most proteins and cells (Taqieddin et al., 2002). Starch is a high molecular weight polymer and is used as the coating polymer which afforded the probiotic strain survives in the adverse environmental conditions (O'Riordan et al., 2001; Lanthong et al., 2006). Now need has arisen to develop such a preparation where enzyme would be immobilized on large

Abbreviations: Con A, concanavalin A; ONPG, *o*-nitrophenyl β-D-galactopyranoside; SβG, soluble β galactosidase; IβG, immobilized β galactosidase.

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surface area of the support and it could easily hydrolyze lactose deposited in the lumen of the small intestine.

Here an attempt has been made to immobilize β galactosidase on the large surface of concanavalin A (Con A) layered calcium alginate–starch beads. In order to examine the suitability of this immobilized β galactosidase as oral therapeutic agent for the treatment of patients suffering from lactose intolerance, we have studied the stability of immobilized β galactosidase against the conditions of alimentary canal/digestive system, such as varying pH, trypsin, pepsin and salivary α amylase. The reusability of immobilized enzyme in the buffer of varying pH and in the assay buffer was also investigated.

2. Materials and methods

2.1. Materials

Aspergillus oryzae β galactosidase (3.2.1.23), galactose and glucose were obtained from Sigma Chem. Co. (St. Louis, MO) USA. *o*-Nitrophenyl β-D-galactopyranoside (ONPG) and starch were obtained from SRL, Chemicals, Mumbai, India. Sodium alginate was the product of Koch-Light Lab (Colnbrook, UK). Jack bean meal was procured from DIFCO, Detroit, USA. All other chemicals and reagents used were of an analytical grade.

2.2. Preparation of calcium alginate–starch beads

An aqueous mixture of sodium alginate (2.5%) and starch (2.5%) was slowly extruded as droplets through a 5.0-mL syringe with attached gauge needle No. 20 into 0.2 M calcium chloride solution. The formation of calcium alginate–starch beads was instantaneous and the beads were further gently stirred in calcium chloride solution for 2 h (Matto and Husain, 2006). The beads were washed and stored in 0.1 M acetate buffer, pH 4.6 at 4 °C, until further use.

2.3. Binding of Con A on the surface of calcium alginate–starch beads

Jack bean extract (10%, w/v) was prepared by adding 5.0 g of jack bean meal to 50 mL of 0.1 M Tris–HCl buffer, pH 6.2 with slight modification from the earlier used method (Haider and Husain, 2007b). Calcium alginate–starch beads were incubated overnight with jack bean extract (25 mL) containing Con A, at 30 °C with mild stirring. β galactosidase (2100 U) was incubated overnight with Con A layered calcium alginate–starch beads at room temperature (30 °C) with slight stirring. The bound enzyme was separated from the unbound enzyme by repeatedly washing with 0.1 M sodium acetate buffer, pH 4.6.

2.4. Crosslinking of immobilized β galactosidase

Con A layered calcium alginate–starch beads immobilized β galactosidase was crosslinked by 0.5% (v/v) glutaraldehyde 2 h at 4 °C. Ethanolamine was added to a final concentration of 0.01% (v/v) to stop the crosslinking. Crosslinked beads were allowed to stand with ethanolamine for 90 min at 30 °C. The integrity of crosslinking was examined by incubating beads in 1.0 M methyl-α-D-glucopyranoside for 2 h. No enzyme activity was released from the beads; it indicated complete crosslinking of the immobilized enzyme.

2.5. Effect of pH on the activity of soluble and immobilized β galactosidase

The activity of β galactosidase (2.0 U) was measured in buffers of various pH values. The buffers used were glycine–HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0–6.0) and Tris–HCl (pH 7.0–10.0). The molarity of each buffer was 0.1 M. The activity at pH 4.6 was taken as control (100%) for the calculation of percent activity.

2.6. Stability of soluble and immobilized β galactosidase at pH 2.0, 4.6 and 7.0

The stability of soluble and immobilized β galactosidase was monitored by incubating the enzyme in the buffers of different pH (2.0, 4.6 and 7.4) for various time intervals at 37 °C. After incubation in varying buffers, beads were washed with assay buffer and their activity was determined by ONPG.

2.7. Reusability of immobilized β galactosidase in the buffers of varying pH

The activity of immobilized β galactosidase was initially checked in 0.1 M sodium acetate buffer, pH 4.6. β galactosidase bound beads were taken out from the assay tubes and washed and incubated in 0.1 M glycine–HCl buffer, pH 2 for 30 min for assaying the activity with ONPG. These beads were further washed with 0.1 M sodium acetate buffer pH 4.6 and the same beads were taken for determining the activity of β galactosidase in Tris–HCl, pH 7.4. The same procedure was repeated after 1 and 2 h.

2.8. Effect of salivary amylase on the activity of soluble and immobilized β galactosidase

Con A layered calcium alginate–starch beads surface immobilized β galactosidase (2.0 U) was incubated with increasing concentrations of salivary α amylase (20–200 U) in 0.1 M sodium acetate buffer, pH 4.6 for 4 h at 37 °C. The activity of the enzyme without salivary amylase treatment was considered as control (100%) for the calculation of remaining activity.

2.9. Effect of trypsin/pepsin on the activity of soluble and immobilized β galactosidase

Soluble and immobilized preparations of β galactosidase (2.0 U) were incubated with increasing concentrations of trypsin/pepsin (0.025–0.150 mg mL^{-1}) at 37 °C for 1 h. After incubation period the β galactosidase activity was determined as described in the assay procedure.

2.10. Reusability of immobilized β galactosidase

Calcium alginate–starch beads were taken in triplicates and were assayed for the activity of β galactosidase. After each assay, beads were taken out and stored in 0.1 M sodium acetate buffer, pH 4.6 for 6 h. This procedure was repeated for six successive cycles. The activity determined for the first time was considered as control (100%) for the calculation of remaining percent activity after each use.

2.11. Assay of β galactosidase

β galactosidase was determined by measuring the release of *o*-nitrophenol from *o*-nitrophenyl β-D-galactopyranoside at 405 nm (Haider and Husain, 2008).

One unit (1.0 U) of β galactosidase activity is defined as the amount of enzyme that liberates 1.0 μmole of *o*-nitrophenol (ε_m = 4500 L mol^{-1} cm^{-1}) per minute under standard assay conditions.