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Preparation and Properties of Glucose Isomerase Immobilized on Indion 48-R

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ABSTRACT

Partially purified glucose isomerase from *Streptomyces thermonitrificans* when coupled to glutaraldehyde-activated Indion 48-R, retained 30–40% activity of the soluble enzyme. However, an approximately twofold increase in the activity could be achieved by binding the enzyme in the presence of glucose. Binding the enzyme to matrices presaturated with either glucose or fructose and influence of lysine modification on the activity of the soluble enzyme revealed that the comparatively low activity observed in case of the enzyme bound in the absence of substrate is the result of the nonspecific binding of either substrate or product to the matrix. Immobilization did not affect the pH and temperature optima of the enzyme, but it lowered the temperature stability. Immobilization resulted in a marginal increase in the K_m and a threefold decrease in the V_{max}. Substrate concentrations as high as 36% glucose could be converted to 18.5% fructose in 5 h, at pH 7.0 and 70°C. The bound enzyme, however, showed inferior stability to repeated use and lost approx 40% of its initial activity after five cycles of use. Indion 48-R bound glucose isomerase could be stored, in wet state, for 30 d without any apparent loss in its initial activity.

Index Entries: Glucose isomerase from *Streptomyces thermonitrificans*; immobilization on Indion 48-R; temperature stability; lysine modification.

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INTRODUCTION

Glucose isomerase (E.C. 5.3.1.5), which catalyzes the reversible isomerization of glucose to fructose, is a commercially important enzyme used for the industrial production of high fructose syrup (HFS) from starch. The enzyme itself is considered to be a major cost center, and hence, considerable attempts have been made to develop suitable procedures to immobilize the enzyme, since immobilization offers advantages like reusability, greater stability, and better process control. A thermophilic strain of *Streptomyces thermonitrificans* (*1*) was found to produce high levels of intracellular glucose isomerase. The enzyme exhibited good stability at high temperatures and, therefore, was selected for immobilization studies. If an immobilized enzyme is to be used for the production of food or pharmaceuticals, the matrix and the reagents used for immobilization should be nontoxic. In view of this, Indion 48-R (a crosslinked macroporous anion-exchange resin) was selected because polystyrene resins show good chemical and mechanical stability, are resistant to microbial contamination, and can be easily derivatized. Furthermore, binding the enzyme via glutaraldehyde was preferred since this method is simple, nontoxic, and can be carried out over a wide pH range. Hence, in the present studies, attempts were made to bind glucose isomerase to glutaraldehyde activated Indion 48-R, so as to obtain a highly active and stable immobilized system, suitable for commercial purposes.

MATERIALS AND METHODS

D-glucose (Sarabhai M. Chemicals, India); D-fructose (Loba Chemie Indoaustranal Co., India); glutaraldehyde (50% v/v, Fluka AG, Switzerland); bovine serum albumin and maleic anhydride (Sigma Chemical Co., USA); sodium borohydride (BDH, India); formaldehyde (S.D. Fine Chemicals Pvt. Ltd., India) and malt extract, yeast extract, casamino acids, and peptone (Difco Products, USA) were used. Indion 48-R (a crosslinked macroporous polystyrene anion-exchange resin) was from Ion Exchange India Ltd, Bombay, India. All other chemicals used were of analytical grade.

The thermophilic strain of *Streptomyces thermonitrificans* (NCIM 2007) was routinely maintained on MGYP slants (g/L: 3, malt extract; 3, yeast extract; 5, peptone; 10, glucose; and 20, agar; adjusted to pH 7.0 with NaOH) at 50°C.

Enzyme Production

The inoculum was prepared by inoculating 50 mL Callens' (*2*) medium (g/L: 20, sorbitol; 10, casamino acids; 5, yeast extract; 2.7, KH_2PO_4; 5.2, K_2HPO_4; 2.0, $MgSO_4 \cdot 7H_2O$; 3, NH_4NO_3; and 10, xylose; adjusted to pH 7.0 with NaOH) with a 4-d-old, well-sporulated Streptomyces culture,

followed by incubation on a New Brunswick thermostated shaker (200 rpm) at 50°C for 24 h. Enzyme production was carried out using 10% inoculum in 250-mL conical flasks containing 50 mL of Callens' medium followed by incubation at 50°C for 16 h, as described above. The cells were harvested by centrifugation (9226*g* for 20 min), washed twice with distilled water, and stored frozen.

Partial Purification of the Enzyme

Unless otherwise stated, all the operations were carried out at 4°C. One gram (wet wt) of the cells was suspended in 5 mL of 50 m*M* potassium phosphate buffer, pH 7.0, containing 1 m*M* Co^{2+} and 5 m*M* Mg^{2+}, and sonicated using a Ralsonic sonicator, at 10,000 kc for 6 min. The cell debris was removed by centrifugation (9226*g* for 20 min), and the supernatant was subjected to heat treatment at 65°C for 20 min followed by ammonium sulfate precipitation (0.7 saturation). The precipitated protein was collected by centrifugation (9226*g* for 20 min), dissolved in minimum volume of 50 m*M* potassium phosphate buffer, pH 7.0 (containing 1 m*M* Co^{2+} and 5 m*M* Mg^{2+}), and dialyzed extensively against the same buffer to remove the ammonium sulfate. The precipitate obtained after dialysis, if any, was removed by centrifugation, and the clear supernatant was used as the source of enzyme. The specific activity of the partially purified enzyme, which ranged from 0.9–1.0, was used for immobilization studies.

Enzyme Assays

Glucose isomerase activity determination was carried out at pH 7.0 and 70°C, essentially according to the method of Chen et al. (*3*). The fructose formed was estimated spectrophotometrically, according to Dische and Borenfreund (*4*) and modified by Marshall and Kooi (*5*).

The immobilized enzyme was assayed in a similar manner, by incubating 50 mg (wet wt) of the matrix with 2 mL of the standard reaction mixture, at pH 7.0 and 70°C, in a thermostated shaker water bath (75–100 rpm) for 10 min, followed by estimating the fructose formed. One unit of the enzyme is defined as the amount of enzyme required to liberate 1 μmol of fructose/min under the assay conditions.

Determination of Protein

Protein was estimated according to the method of Lowry et al. (*6*) using bovine serum albumin as standard.

Immobilization Technique

Indion 48-R was regenerated by alternatively washing with 0.5*M* NaOH and 0.5*M* HCl. The regenerated resin (1 g wet wt) was activated by incubating with 5 mL of 1% (v/v) glutaraldehyde in 50 m*M* sodium bicarbonate buffer, pH 9.0, at 10°C for 5 h, under mild agitation. The resin

was then washed free of excess glutaraldehyde and used immediately for coupling the enzyme.

In a typical experiment, 15–20 U of partially purified glucose isomerase in 5 mL of 50 m*M* potassium phosphate buffer, pH 7.0, containing 1 m*M* Co^{2+}, 5 m*M* Mg^{2+}, and 100 m*M* glucose were incubated with 1 g (wet wt) of 1% (v/v) glutaraldehyde-activated resin, at 10°C for 16 h, under mild agitation. The unbound enzyme and protein were removed by decantation, and the matrix was washed successively with the coupling buffer, 1*M* NaCl in the coupling buffer, and finally with the assay buffer (potassium phosphate buffer, pH 7.0) until the washings showed no enzyme activity. The amount of enzyme and protein bound to the matrix was determined by estimating the difference in the enzyme activity and protein before loading on the matrix and after coupling.

Determination of Efficiency

Efficiency of the immobilized enzyme was determined by assaying 50 mg (wet wt) of the immobilized preparation, under standard assay conditions, followed by calculating the ratio of measured activity to bound activity. Specific activity of the immobilized preparation is defined as measured activity/mg protein/g matrix.

Chemical Modification Studies

For chemical modification studies, the enzyme was purified to homogeneity by preparative PAGE, using 7.5% polyacrylamide gel, at pH 8.3 (Deshmukh and Shankar, unpublished data). The amino groups of glucose isomerase were reversibly blocked by maleic anhydride according to Butler et al. (*7*). Maleic anhydride was dissolved in acetonitrile, and the concentration of the reagent was 500 m*M*. Acetonitrile at this concentration did not have any adverse effect on the activity and stability of the enzyme, during the incubation period. Purified glucose isomerase (100 μg) in 2 mL of 50 m*M* potassium phosphate buffer, pH 9.0, containing 1 m*M* Co^{2+} and 5 m*M* Mg^{2+}, was treated at room temperature with a total of 40 μL of maleic anhydride. The reagent was added in four installments (10 μL each), and the pH of the reaction mixture was maintained at 9.0 by the addition of 0.1*M* NaOH. After every addition, an aliquot was removed and assayed for the enzyme activity. Enzyme sample incubated in the absence of maleic anhydride served as control.

Reductive methylation of the purified enzyme was carried out according to Means and Feeney (*8*). To 1 mL of the purified enzyme (100 μg) in 200 m*M* sodium borate buffer, pH 8.0, containing 1 m*M* Co^{2+} and 5 m*M* Mg^{2+}, at 0°C, 0.1 mL of sodium borohydride (0.5 mg/mL) was added, followed by six aliquots (5 μL each) of 3.5% (v/v) formaldehyde, at an interval of 10 min. At the end of the reaction, the residual activity of the modified enzyme was determined under standard assay conditions. Enzyme samples incubated in the absence of formaldehyde served as control.

Production of Fructose Using Immobilized Glucose Isomerase

Isomerization was carried out in shake flasks by incubating 1 g (wet wt) of the immobilized enzyme (5.7 U, measured activity) with 2 mL of 36% (w/v) glucose, in a thermostated shaker water bath at 70°C, pH 7.0, and following the conversion by estimating fructose at an interval of 1 h up to 6 h.

RESULTS AND DISCUSSION

In a typical experiment, when 15.6 U of partially purified glucose isomerase were reacted with 1 g (wet wt) of 1% (v/v) glutaraldehyde-activated Indion 48-R at pH 7.0 and 10°C for 16 h, 6.6 U were bound. The effectiveness factor (η) of the immobilized preparation was approx 0.39, indicating the efficiency of the immobilized system to be 39%. However, when the binding was carried out in the presence of glucose (i.e., freezing the enzyme in its native conformation), a significant increase (1.5–2.0-fold) in the efficiency occurred. The increase in the efficiency of the enzyme, bound in the presence of glucose, may be owing to the protection of the active site region of the enzyme during coupling. When an enzyme is bound to an insoluble matrix via glutaraldehyde, primarily ϵ-amino groups of lysine are involved in the binding. The pure enzyme was therefore subjected to maleation and reductive methylation, to evaluate the role of lysine in the catalytic activity of *S. thermonitrificans* enzyme. Modification of lysine residues, however, did not have any adverse effect on the enzyme activity, suggesting that lysine may not have a role in the catalytic activity of the enzyme. This observation also indicates that the increase in the efficiency of the enzyme, bound in the presence of substrate (glucose), cannot be correlated to the protection of catalytically active lysine residues during coupling.

In an immobilized system, the expressed activity depends on the rate not only of diffusion of the substrate, but also of the product. In other words, apart from the accessibility of the substrate, the release of the product can also influence the activity of the bound enzyme. Koizumi and Okada (*9*), while studying the transformation of sugars using anion-exchange resins, noted that glucose binds to the matrix. Since Indion 48-R, too, is an anion-exchange resin, the nonspecific binding of both glucose and fructose was checked by incubating 1 g (wet wt) of Indion 48-R, with 100 m*M* of either glucose or fructose, at room temperature, for 3 h. The results showed a significant nonspecific binding of both the sugars (approx 10–12 mg/g wet wt resin) to the matrix. This observation suggests that the low activity observed in case of the enzyme bound in the absence of glucose could be the result of decreased availability of the substrate (i.e., glucose or low levels of the product, i.e., fructose, as a result of nonspecific

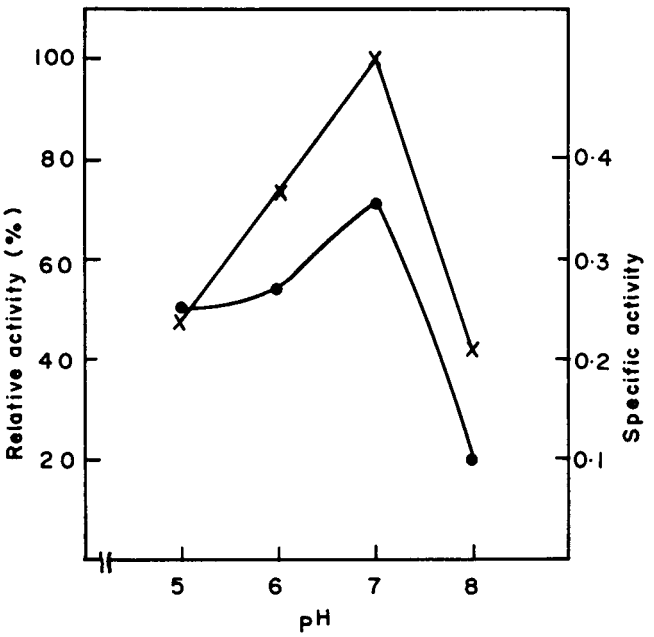

Fig. 1. Effect of coupling pH on the activity of Indion 48-R bound glucose isomerase. Relative activity (X) and specific activity (●). 1 g (wet wt) of 1% (v/v) glutaraldehyde-activated matrix was incubated with 12.5 U of partially purified glucose isomerase at different pH (5.0–8.0) and 10°C for 16 h, and efficiency of the immobilized enzyme was determined as described under Materials and Methods.

binding of these sugars on to the matrix. To verify this possibility, the activated matrix was preincubated with 100 m*M* of either glucose or fructose, and after removing the unbound sugars, was used for coupling the enzyme. When the enzyme was bound under the above conditions, a significant (1.5-fold) increase in the efficiency was observed. Comparable results were obtained when the enzyme was bound in presence of fructose. The above results substantiate our view that the low activity observed in case of the enzyme bound in the absence of glucose can be correlated to decreased availability of the substrate, as a result of nonspecific binding of glucose to the matrix. However, the increased activity observed in the case of the enzyme bound to the matrix presaturated with either glucose or fructose or when the coupling was carried out in the presence of either of these sugars can be the result of a decrease in the nonspecific binding of either substrate or product, resulting in higher concentration of fructose in the reaction mixture.

Optimization of coupling pH on the efficiency of the immobilized preparation showed that the most active preparations were obtained when the coupling was carried out at pH 7.0. The decrease in the efficiency of the enzyme bound at higher pH (>7.0) was owing to the overcrowding of the enzyme on the matrix, as there was a decrease in the specific activity of the bound enzyme (Fig. 1). Influence of glutaraldehyde concentration

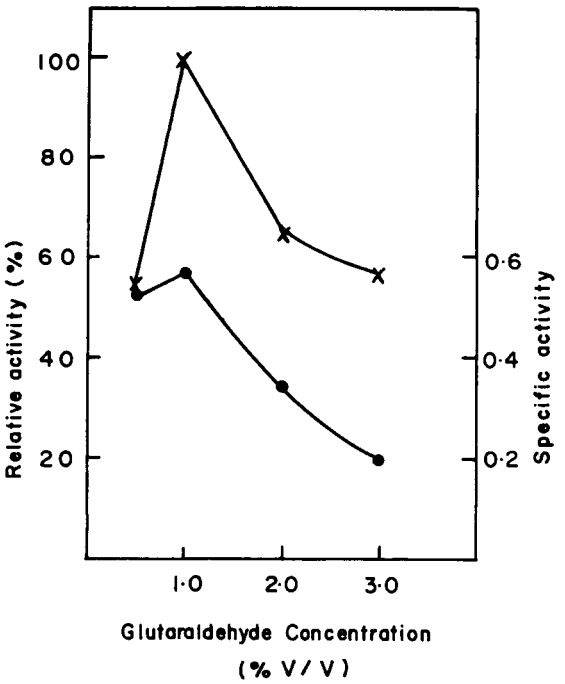

Fig. 2. Effect of glutaraldehyde concentration on the activity of Indion 48-R bound glucose isomerase. Relative activity (X) and specific activity (●). 1 g (wet wt) of the matrix was activated with varying concentrations of glutaraldehyde (0.5–3% v/v) at pH 9.0 for 5 h. Coupling of glucose isomerase to the activated matrix was carried out by incubating 16 U of the enzyme with the activated matrix at pH 7.0, followed by determining the efficiency of the bound enzyme under standard assay conditions.

showed the enzyme bound to 1% (v/v) activated Indion 48-R to be most active (Fig. 2). Effect of matrix-to-enzyme ratio on the activity of the bound enzyme revealed that after an initial increase, the efficiency decreased with increase in the enzyme load, and most active preparations were obtained when 15–20 U of partially purified enzyme was reacted with 1 g (wet wt) of 1% v/v glutaraldehyde activated Indion 48-R at pH 7.0. The decrease in the efficiency at higher enzyme load (> 20 U) can be attributed to the overcrowding of the enzyme on the matrix, since there was a decrease in the specific activity of the bound enzyme (Fig. 3). The results of a typical procedure for the immobilization of partially purified glucose isomerase from *S. thermonitrificans* on Indion 48-R, under optimized conditions, are given in Table 1.

When 17.4 U of partially purified glucose isomerase was reacted with 1 g (wet wt) of 1% (v/v) glutaraldehyde activated Indion 48-R, in the presence of 100 m*M* glucose, at pH 7.0 and 10°C for 16 h, 8.3 U were bound. The effectiveness factor (η) of the immobilized preparation was approx 0.69, indicating the efficiency of the immobilized preparation to be 69%.

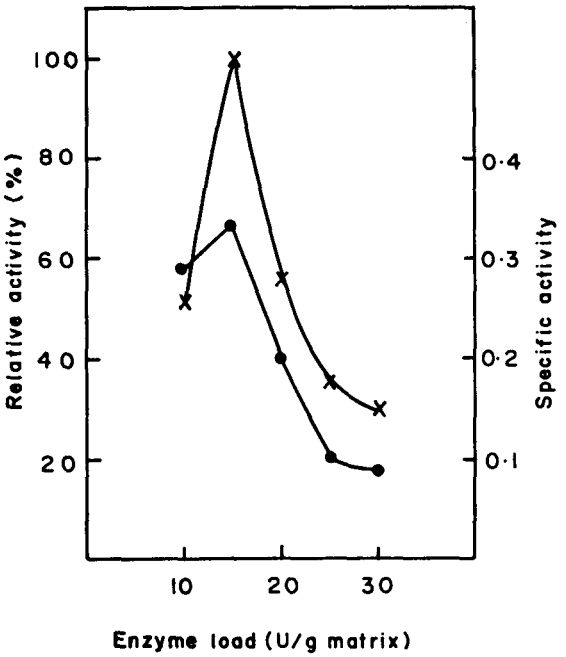

Fig. 3. Effect of enzyme load on the activity of Indion 48-R bound glucose isomerase. Relative activity (X) and specific activity (●). 1 g (wet wt) of 1% (v/v) of glutaraldehyde-activated matrix was incubated with varying amounts of the enzyme (10–30 U) at pH 7.0 and 10°C for 16 h, and efficiency of the immobilized system was determined as described under Materials and Methods.

Table 1
Immobilization of Glucose Isomerase on Glutaraldehyde-Activated Indion 48-R

Enzyme loaded		Enzyme bound		Activity of the complex, U expressed	Efficiency,* %
U	Protein, mg	U	Protein, mg		
17.4	18.1	8.3	8.9	5.7	68.7

*Efficiency = (Activity of the complex/Bound activity) × 100

Comparison of the pH activity profiles of soluble and immobilized enzyme showed no change in the optimum pH (7.0), suggesting the absence of partitioning effects in the microenvironment of the immobilized enzyme. Similarly, no change was observed in the optimum temperature of the enzyme (80°C) as a result of immobilization. Evaluation of kinetic parameters showed a marginal increase in the K_m of the bound enzyme (330 m*M*) compared to that of the soluble enzyme (266 m*M*). However, the V_{max} of the enzyme (200 μmol/min/mg) showed a threefold decrease (66.6 μmol/min/mg) as a result of immobilization. The marginal increase in the

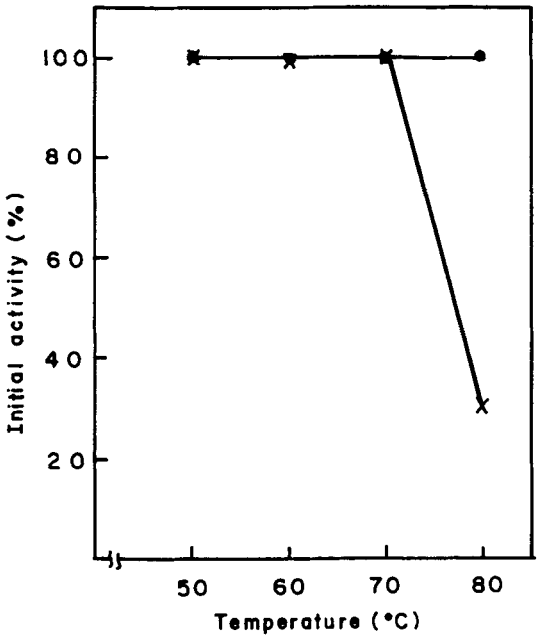

Fig. 4. Temperature stability of soluble (●) and immobilized (X) glucose isomerase. Soluble and immobilized enzymes (0.2 U) were preincubated at different temperatures (50–80°C) for 1 h in 50 m*M* potassium phosphate buffer, pH 7.0, containing 1 m*M* Co^{2+} and 5 m*M* Mg^{2+}, and their residual activities were determined as described under Materials and Methods.

K_m points toward the absence of significant diffusional barriers, whereas the decrease in the V_{max} can be correlated to blocking or masking of some of the active sites during coupling.

In batch operations, Indion 48-R bound enzyme could convert high concentrations of glucose (36%) to 18.5% fructose, in 5 h at pH 7.0 and 70°C, with a conversion efficiency of 51%. The bound enzyme, however, showed inferior temperature stability compared to its soluble counterpart, as indicated by the inactivation pattern. Although the soluble enzyme completely retained its activity at 80, the bound enzyme lost more than 70% of its initial activity (Fig. 4). The inferior temperature stability of the immobilized glucose isomerase indicates the lack of rigidity of the enzyme structure in the bound form. The bound enzyme also showed less stability to repeated use and lost approx 40% of its initial activity after five cycles of use. In the absence of any detectable leaching (as evidenced by the absence of enzyme activity in the supernatant), the decrease in the activity, after every use, can be correlated to slight inactivation of the enzyme. The bound enzyme, however, showed good storage stability and could be stored in 50 m*M* potassium phosphate buffer, pH 7.0, containing 1 m*M* Co^{2+} and 5 m*M* Mg^{2+} for 30 d, without any apparent loss in its initial activity.

With commercial application in mind, we have attempted to develop a simple protocol for the preparation of a highly active and stable immobilized glucose isomerase, suitable for large-scale operations. Our observations have commercial significance because the immobilized system shows very high retention of activity and can isomerize high concentrations of glucose to fructose. The polystyrene matrix used in the present studies is stable, can afford good flow rates, and is nonbiodegradable. The immobilized system, however, showed poor operational stability. Hence, efforts in this direction are required if Indion 48-R bound glucose isomerase is to find successful industrial application.

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