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Immobilization of invertase attached to a granular dimer acid-co-alkyl polyamine

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Abstract

Invertase was immobilized onto the granular dimer acid-co-alkyl polyamine after activation with carbodiimide. The K_m value for immobilized enzyme (53.6 mM) was much greater than that of the free enzyme (20.6 mM). V_{max} values were 6.44×10^{-5} mol dm^{-3} min^{-1} and 5.45×10^{-5} mol dm^{-3} min^{-1} for free and bound, respectively. The optimal pH values for free and covalently bonded enzymes were 4.56 and 5.50, respectively. The optimum temperature for both free and covalent invertase was 55°C. The enzyme activities, after storage for 1 month, were found to be 21.0 and 99.0% of the initial activity values for free and covalently bonded, respectively. The immobilized enzyme that was used 50 times in 5 days in repeated batch experiments showed 100% of its original activity.

Keywords: Invertase; Immobilization; Covalent binding; Carbodiimide

1. Introduction

Many methods have been described for the attachment of enzymes to supporting structures. It has been shown that the supporting material can have a modifying effect on the kinetics of the attached enzyme. The covalent attachment of an enzyme to a water-insoluble support offers numerous advantages in enzyme immobilization. The coupling of the enzyme to a functionalized support is experimentally easy to perform and consists of adding the enzyme to a suspension of the polymer, allowing the coupling to proceed and removing any non-covalently bound enzyme by suitable washing. The activity of a covalently bonded enzyme derivative depends on a number of factors which can be summarized as size and shape of the carrier material, the nature of the coupling method, the composition of the carrier material and the specific conditions during coupling. So far, enzymes have been insolubilized by their attachment to either beads or fiber and in this form used in either packed beds or stirred tank reactors, or else they have been insolubilized by their attachment to porous sheets (Filippusson & Hornby, 1970). Immobilization is often accompanied by changes in the enzymatic activity, optimum pH, affinity to the substrate and stability. The extent of these changes depends on the enzyme and carrier support and on the immobilization conditions.

There are many reactions of industrial interest when immobilized enzymes are used as catalysts. Most biochemical catalysts are used for conversion of biological materials, but there is growing awareness of their potential applications in chemical conversions. The choice between use of the soluble free and immobilized enzymes depends on the cost of the enzyme, the nature of the conversion process, and the relative operational stabilities of the two forms. By their nature, some food processes, such as meat tenderization and baking, involve the addition of the enzymes at the final processing stage, making reuse impossible. Sometimes the ability to remove the immobilized enzyme from the product stream, ensuring minimal contamination by protein, may influence the choice, but the main factor is the operational stability of the enzyme. As long as the enzyme can be stabilized by modification or

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immobilization, reuse may be worthwhile (Kennedy & Cabral, 1987).

Invertase, from *Candida utilis*, has been immobilized on porous cellulose beads by an ionic-guanidino bonding. The immobilized invertase showed optimum activity between pH 4.0 and 5.4, while the free enzyme had a sharp optimum at pH 4.1. Above 55°C, the immobilized enzyme was more stable than the free enzyme (Dickensheets, Chen & Tsao, 1977). Invertase has been immobilized on diazotized 4-aminobenzoylcellulose. The optimum coupling conditions, namely enzyme concentrations, time and pH have been determined (Simionescu, Popa & Dumitru, 1987).

The method of preparation and the physical properties of porous (75–85% porosity) cellulose beads have been studied (Tsao & Chen, 1977). The invertase has been covalently bonded to the porous cellulose beads and chemical procedures have been employed for immobilizing enzymes on ordinary cellulose applied to the porous cellulose beads. More enzyme loads onto these beads than ordinary cellulose (Chen & Tsao, 1977).

Invertase has been ionically bonded to the poly-(ethylene-vinylalcohol) membrane surface-modified with two aminoacetals with different molecular length, 2-dimethyl-aminoacetoaldehyde and 3-(*N*,*N*-dimethyl-amino-*n*-propane-diamine) propionaldehyde dimethyl-acetal (Imai, Shiami, Uchida & Miya, 1986).

Invertase immobilized onto corn grits has been utilized in the hydrolysis of highly concentrated sucrose solutions, producing liquid sugar solutions containing glucose and fructose (Monsan & Combes, 1984).

Invertase has been immobilizied with nitrated copolymers of methacrylic acid and methacrylic acid-*m*-fluoroanilide in the presence of divinylbenzene as a cross-linking agent (Maneche & Singer, 1960). Invertase bound to diazotized polystyrene showed changed inhibitor behavior toward aniline and tris buffer (Filippusson & Hornby, 1970). Invertase was immobilized into photographic gelatin by chemical cross-linking with formaldehyde and chromium (III) acetate using polyester film (Akbulut, Sungur & Pekyardımcı, 1993). Invertase was immobilized into polyacrylonitrile (PAN) and carboxyl-modified polyacrylonitrile (PAN-AA) ultrafiltration membranes (Mathias & Alexandra, 1997).

Specific immunoaffinity supports for the immobilization of invertase were constructed by coupling the affinity-purified anti-neoglycoprotein-I or anti-neoglycoprotein-II antibodies to cyanogen bromide-activated Sepharose 4B. Both the affinity adsorbants were effective in binding and improving the thermal stability of invertase (Farahdiba & Saleemuddin, 1997).

In this study, invertase was immobilized onto a new support material which could be a good candidate for continuous flow columns. Activation of acid groups proves a certain number of available binding sites for the enzyme. Covalent binding of invertase to organic polymeric supports is more advantageous than an entrapment process since diffusional restriction of soluble sucrose is decreased considerably. On the other hand, hydrophilic carriers confer more stablity to the immobilized invertase. Effects of the immobilization process on enzyme activity, kinetic parameters, storage and reuse capability of the enzyme were investigated. Twelve mg enzyme were bonded for 1 g polymeric support. In a previous study, these values were found to be 3.5 and 2.8 mg for 1 g carrier (Imai et al., 1985).

2. Materials and methods

2.1. Materials

Absorption spectra and absorbances were recorded using a Unicam UV 2-100, Double Beam UV–Visible spectrophotometer. The pH values were measured with a Hanna HI 8521 pH meter.

2.2. Reagent

Invertase (β-fructofuranosidase, E.C. 3.2.1.26) was obtained from Sigma. Sucrose was a product of Sigma and used as the substrate. Dimer acid-co-alkyl polyamine was supplied from Aldrich. Chemicals used in the preparation of buffers were supplied from Merck and used without further purification. 1-Cyclohexyl-3-(2-(morpholino-ethyl) carbodiimide metho-*p*-toluene sulfonate) was obtained from Dow Chemical Company (USA). Glucose of Biochemica-grade was from Fluka. All other chemicals were of reagent grade and used as received (Merck).

2.3. Activation with carbodiimide

Granular dimer acid-co-alkyl polyamine (0.1 g) was added to a carbodiimide solution (0.05 g in 10 ml phosphate buffer), kept in a shaking water bath for 4 h at 30°C and left at that temperature overnight. The activated copolymer was separated and washed with phosphate buffer (15 ml).

2.4. Enzyme immobilization

The activated copolymer were added to the enzyme solution (20 ml containing 0.4 mg enzyme per ml phosphate buffer) and the immobilization reaction was carried out at 30°C in a shaking water bath for 4 h. Copolymer was separated and the unbound enzyme was removed by washing with phosphate buffer (15 ml). The immobilized enzymes were used freshly or stored at 4°C. The reactions for activation of polymeric support and enzyme binding are shown in Fig. 1.

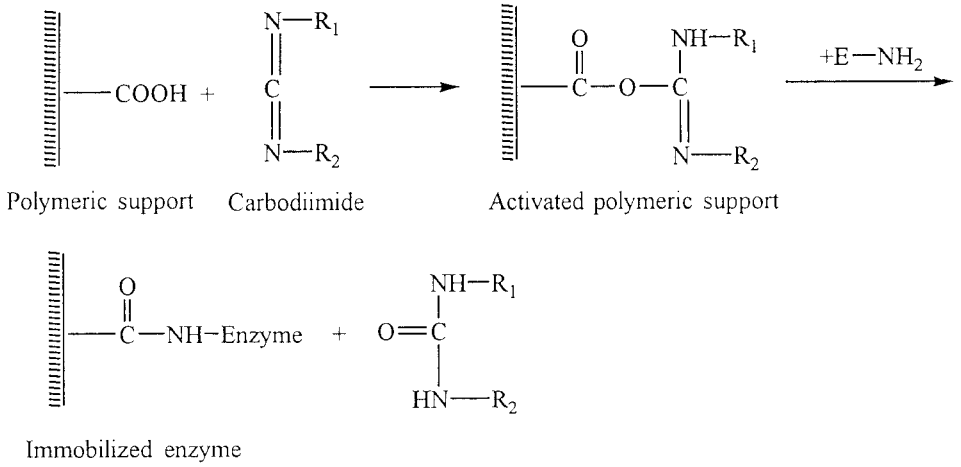

Fig. 1. The reaction for activation of polymeric support and enzyme binding.

2.5. Enzyme assay

The immobilized and free invertase activities were determined by Folin–Wu assay; solution containing free or immobilized enzyme in phosphate buffer (0.2 mg enzyme or 0.1 g copolymer in 1.0 ml phosphate buffer) was placed in a test tube. Substrate solution (1% sucrose) was added to the tubes and incubation was for exactly 15 min. At the end of 15 min the tubes were removed from the water bath (30°C) and 1 ml of alkaline copper sulphate solution was added to terminate the reaction. Then the tube was placed in the boiling water bath until the brown colour was obtained, then cooled in water at room temperature. Then 1 ml of fosfomolybdic acid reagent was added to the tube and mixed thoroughly (by vortex). Finally, 10.0 cm^3 of phosphate buffer was added to the tube. The absorbance was read photocolorimetrically at 640 nm versus a blank solution, which was prepared in the same manner, but lacking the enzyme. The amount of glucose was obtained from the calibration curve and used in the calculation of enzyme activity. The activity of invertase (mols of glucose + fructose formed/mg protein/min) was calculated (1 U of enzyme activity is defined as that amount of enzyme which hydrolyses 1 μmol sucrose/min under the present assay conditions).

2.6. Enzyme binding efficiency

After the enzyme immobilization process and separation of granular dimer acid-co-alkyl polyamine, the supernatant and washing solutions were collected and UV absorbance of solutions was measured at 280 nm by using a UV-spectrophotometer (Unicam UV/Vis spectrometer UV2). The amount of unbound enzyme was calculated from the enzyme calibration curve which was obtained at the same wavelength.

3. Results and discussion

3.1. Parameters affecting enzyme activity

The activities of free and immobilized enzyme were calculated by measuring the absorbance of the solutions at 640 nm when the reactions were carried out at variable pH, temperature, substrate concentration, reuse and storage time. The measured absorbance values were plotted versus time and the initial slopes of these curves were used to calculate the activities of the enzymes with the help of the glucose calibration curve.

3.2. Effect of pH

The effect of pH was studied in the pH range 3.0–8.0 at 30°C. The maximum activity for free enzyme was at pH 4.56. For CDI-activated. samples the optimum pH was found to be 5.50 (Fig. 2). In previous studies, the highest activity was observed between pH 4.1 and 4.6 (Monsan & Combes, 1984). The immobilized invertase showed optimum activity between pH 4.0 and 5.4, while the free enzyme had a sharp optimum at 4.1 (Paul et al., 1977). Free and immobilized invertase showed an optimum pH of 4.0 (Imai et al., 1986). Free and immobilized invertase showed an optimum pH of 4.5 (Simionescu et al., 1987).

3.3. Effect of temperature

The effects of temperature on the activity of free and immobilized invertase are given in Fig. 3. Free and immobilized enzymes had optimum temperatures of approximately 55°C. Both temperature profiles were fairly similar up to 55°C. However, above this temperature the immobilized enzyme was more stable than the free enzyme (Monsan & Combes, 1984; Paul et al.,

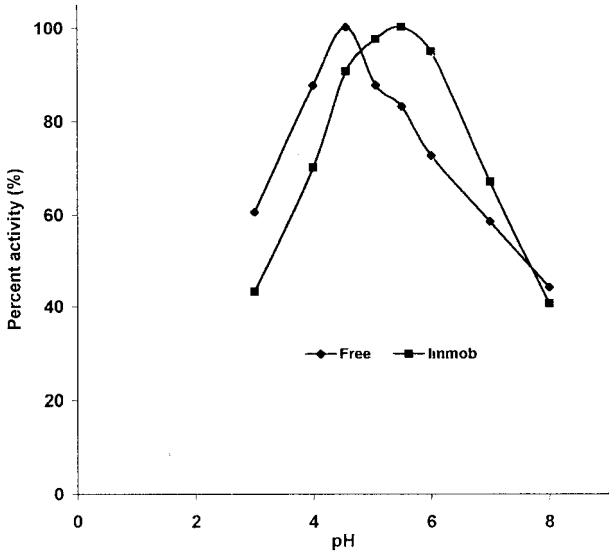

Fig. 2. Effect of pH on the activity of free and immobilized invertase.

1977). Free and immobilized invertase reported had an optimum temperature of 50°C (Simioneseu et al., 1987). However, some reports are of 55 and 60°C, respectively (Imai et al., 1986) and 50 and 60°C, respectively (Farahdiba & Saleemuddin, 1997).

3.4. Kinetic parameters

The activities of free and immobilized enzymes for various substrate concentrations are plotted in a Lineweaver–Burk graph, from which V_{max} and K_m values are calculated in Fig. 4. V_{max} defines the highest possible velocity when all the enzyme is saturated with substrate. Therefore, this parameter reflects the intrinsic characteristics of the immobilized enzyme, but this may be affected by diffusional constraints. K_m is defined as the substrate concentration that gives a reaction velocity of 1/2 V_{max}. This parameter reflects the effective characteristics of the enzyme and depends upon both partitioning and diffusional effects.

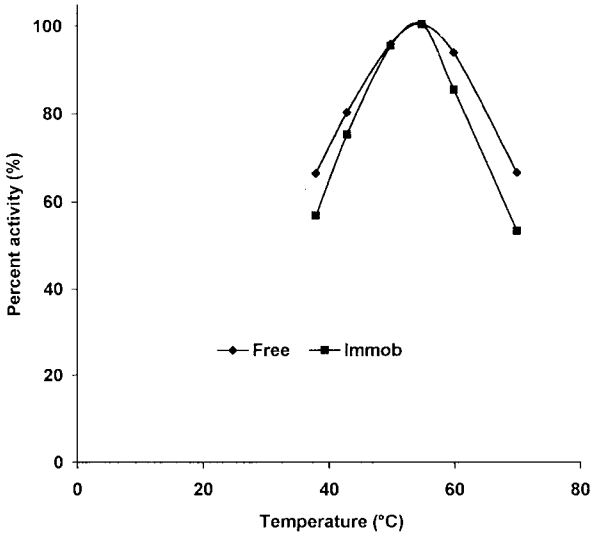

Fig. 3. Effect of temperature on the activity of free and immobilized invertase.

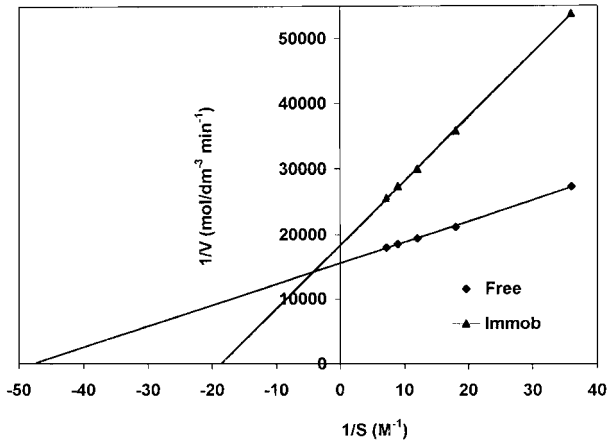

Fig. 4. Lineweaver–Burk plots for free and immobilized invertase.

The Michaelis constant, K_m, of free invertase and the apparent K_i of the immobilized enzyme on cellulose beads were previously determined to be 46.9 and 130 mM, respectively (Dickensheets et al., 1977). The Michaelis constants, K_m, of free and immobilized invertase were determined to be 1.3×10^{-2}, 7.6×10^{-2} and 3.6×10^{-2} M, respectively (Imai et al., 1986). The K_m values for the immobilized and free enzymes were reportedly 40 and 17–20 mM, respectively (Filippusson & Hornby, 1970) and 84 and 30 mM, respectively (Simionescu et al., 1987). The Michaelis–Menten constants K_m with the small substrate maltose were reportedly 3.5 mM for free and 3.2 mM for covalently bound AG, whereas V_{max} values with maltose and starch dropped to 32 and 22% respectively, for the immobilized versus free enzyme (Mathias & Alexandra, 1997). In this study, for free and immobilized invertase, V_{max} values were estimated as 6.44×10^{-5} g/dm^3 min and 5.45×10^{-5} g/dm^3 min, respectively. The Michaelis constants, K_m, of free and immobilized invertase were determined to be 20.6 and 53.6 mM, respectively (Fig. 4). This indicates that the formation of the enzyme–substrate-complex is more difficult with the immobilized invertase.

3.5. Storage stability

Enzymes are not stable during storage in solutions and their activities decrease gradually over time. Immobilization definitely puts the enzyme into a more stable position in comparison to free enzyme. After storage for 1 month, the enzyme activities were found to be 21 and 100% of the initial activity values for free and

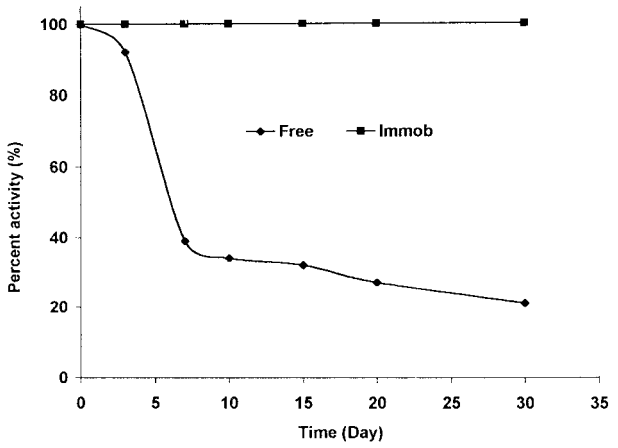

Fig. 5. Effect of storage on the activity of free and immobilized invertase.

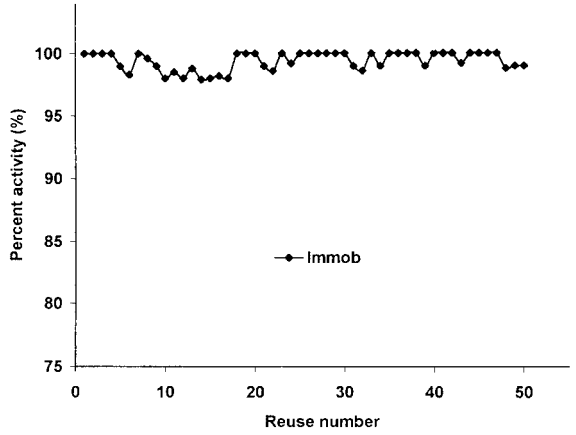

Fig. 6. Effect of repeated use on the activity of immobilized invertase.

covalently-bound, respectively (Fig. 5). Preparations of β-fructofuranosidase, chemically attached to both polystyrene beads and tubes, have been used over a period of several weeks without any significant loss in activity, the preparations being stored in water at 4°C when not in use. A preparation of polystyrene-bead-supported enzyme did, however, lose all its activity after 3 months when stored in the dry state (Filippusson & Hornby, 1970).

3.6. Repeated use capability

The immobilized sample was used repeatedly 50 times within 5 days and the measured activities are presented in Fig. 6. It has been observed that, after the 50th use, immobilized enzyme retained about 98% of its original activity.

4. Conclusion

Invertase was covalently immobilized on a new matrix material that has good mechanical properties. Upon immobilization on a granular dimer acid-co-alkyl polyamine support, by using CDI, the immobilized enzyme achieved very high storage stability and reusability. Previous reports with resigns have shown that the higher activity and larger amount of immobilized enzyme obtained tended only to be achieved for an ion-exchange resin with a charge opposite to that of the enzyme (Imai et al., 1986). These properties prove the usefulness of the examined materials in continuous reactors in biotechnological applications.

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