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Concanavalin a Immobilized Monosize and Magnetic Poly(glycidyl Methacrylate) Beads for Use in Yeast Invertase Adsorption

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Concanavalin A (Con A) immobilized monosize and magnetic poly(glycidyl methacrylate)[m-poly(GMA)] beads were investigated for specific adsorption of yeast invertase from aqueous solutions. m-Poly(GMA) beads (1.6 μm in diameter) were prepared by dispersion polymerization in the presence of Fe_3O_4 nanopowder. The epoxy groups of m-poly(GMA) beads were opened by base catalyses. Then, Con A was immobilized by covalent binding onto the beads. Con A immobilization amount was 12.5 mg/g. The invertase adsorption capacity of the m-poly(GMA)/Con A beads was 111 mg/g. The maximum invertase adsorption on the m-poly(GMA)/Con A beads was observed at pH 5.5. The optimum activity for both free and adsorbed invertase was observed at 50°C. V_{max} values were determined as 330 U/mg and 292 U/mg enzyme, for free and adsorbed invertase, respectively. K_M values were found to be the same for free and adsorbed invertase (20 mM). Adsorption of invertase via Con A improved the pH stability of invertase. Thermal stability also increased with adsorption. The adsorbed enzyme activity was found to be quite stable in repeated experiments.

Keywords: Enzyme immobilization, magnetic beads, poly(glycidyl methacrylate), concanavalin A, invertase

1 Introduction

Affinity purification of glycoenzymes and glycopeptides on immobilized lectins is widely employed for preparative purposes (1–4). The solution to be fractionated is applied to a column of the immobilized lectin, and the unbound materials are washed off with an appropriate solution (5). The specifically bound glycosylated enzymes are then eluted with a solution of the sugar for which the lectin is specific. Concanavalin A (Con A), one of the most widely used lectins, is well known to interact very strongly with the α-D-mannose and α-D-glucose containing glycoconjugates on cell surface (6). It exhibits a series of remarkable properties. It has been used as haemaglutinin for the studies of cell surface and cell division, to examine the nature of the carbohydrate residues responsible for blood group specificity and to distinguish between malignant or abnormal and normal cells (7). Therefore, immobilized Con A can be used for histochemical detection of sugar chains on the cell surface (8, 9). Another biospecific route tried in the separation of glycoenzymes has been the preparation of insoluble complexes of these enzymes with Con A.

Invertase (β-D-fructofuranosidase fructohydrolase, EC 3.2.1.26) is a highly efficient enzyme that has been described as specific for converting sucrose to glucose and fructose (10). The hydrolized sugar mixture obtained by invertase has the advantage of being colorless in contrast to the colored products obtained by acid hydrolysis (11). Immobilization of invertase on corn grits, gelatine and various agarose polymers has already been achieved, while its immobilization onto hydrogel polymer has been limited. The latter have good chemical properties and mechanical stability and are not susceptible to microbial attack (12–14).

Micron-sized magnetic beads are currently enjoying a fairly ample range of applications in many fields including biotechnology, biochemistry, colloid sciences and medicine (15–20). The magnetic character implies their response to a magnet, making sampling and collection easier and faster, but their magnetization disappears once the magnetic field is removed. Magnetic beads promise to solve many of the problems associated with chromatographic separations in packed bed and in conventional fluidized bed systems (21). Magnetic carriers combine some of the best

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characteristics of fluidized beds (low pressure drop and high feed-stream solid tolerances) and of fixed beds (absence of particle mixing, high mass transfer rates, and good fluid-solid contact) (22). Magnetic separation is relatively rapid and easy, requiring simple apparatus, as compared to centrifugal separation. Recently, there has been increasing interest in the use of magnetic carriers in biomolecule coupling, protein purification and extracorporeal therapy for several diseases (23). Magnetic carriers can be produced using inorganic materials or a number of synthetic and natural polymers. High mechanical resistance, insolubility and excellent shelf life make inorganic materials ideal as carriers. The main disadvantage of inorganic supports, however, is their limited functional groups for ligand immobilization. Magnetic carriers can be porous or non-porous (24). Magnetic carriers are more commonly manufactured from polymers since they have a variety of surface functional groups which can be tailored to use in specific applications. Poly(hydroxyethyl methacrylate), poly(vinyl alcohol), poly(acrylamide), poly(vinyl butyral), poly(methyl methacrylate), superparamagnetic poly(vinyl acetate-divinyl benzene), agarose, histidine containing poly(ethylene glycol dimethacrylate), poly(styrene-divinyl benzene) and alginate beads in the size range of 50–300 μm in diameter are typical polymeric carriers which are used in different applications (25–38).

In this study, Con A immobilized monosize and magnetic poly(glycidyl methacrylate) [m-poly(GMA)] affinity beads are used for adsorption of yeast invertase. m-Poly(GMA) beads were produced by dispersion polymerization of GMA. The effects of adsorption process on the enzyme activity, the operational thermal storage and the specificity of invertase were investigated. Kinetic parameters and inhibition studies were also performed for both free and adsorbed invertase.

2 Experimental

2.1 Materials

Invertase (β-D-fructofuranosidase fructohydrolase, E.C.3.2.1.26, Grade VII from baker's yeast), Concanavalin A (Con A, M:104, Jack Bean from Canavalia Ensiformis), sucrose, glucose, fructose, 3,5- dinitrosalycilic acid and magnetic nanopowder (Fe_3O_4, diameter: 20–50 nm) were all obtained from Sigma (St. Louis, MO, USA) and used as received. Glycidyl methacrylate (GMA, Fluka A.G., Buchs, Switzerland) was purified by vacuum distillation and stored in a refrigerator until use. Azobisisobutyronitrile (AIBN) and poly(vinyl pyrrolidone) (MW. 30,000, BDH Chemicals Ltd., Poole, England) were selected as the initiator and the steric stabilizer, respectively. AIBN was recrystallized from methanol. Ethanol (Merck, Germany) was used as the diluent agent without further purification. All other chemicals were guaranteed or analytical grade reagents commercially available and used without further purification. Before use the glassware was rinsed with deionised water and dried in a dust-free environment. All water used in the experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed bed system.

2.2 Synthesis of m-poly(GMA) Beads

m-Poly(GMA) beads were synthesized as previously described elsewhere (39). A typical procedure applied for the dispersion polymerization of m-poly(GMA) is given below. The monomer phase was comprised of 40 ml GMA, 250 mg AIBN and 1 g nanopowder (Fe_3O_4). The resulting medium was sonicated for 5 min at 200 W in an ultrasonic water bath (Bransonic 2200, England) for the complete dissolution of AIBN in the polymerization medium. Poly(vinyl pyrrolidone) (4.0 g) was dissolved in 50% (V/V) aqueous ethanol solution and placed in a polymerization reactor. The reactor content was stirred at 500 rpm during the monomer addition completed within 5 min and the heating was started. The reactor was purged with bubbling nitrogen for 5 min. Then, the sealed reactor was placed in a shaking water bath at room temperature. The initial polymerization time was defined when the reactor temperature was raised to 65°C. The polymerization was allowed to proceed under nitrogen atmosphere at 65°C for 4 h. After completion of the polymerization period, the reactor content was cooled down to room temperature and centrifuged at 5000 rpm for 10 min for the removal of dispersion medium. m-Poly(GMA) beads were redispersed within 10 mL of ethanol and centrifuged again under the same conditions. The ethanol washing was repeated three times for complete removal of unconverted monomer and other components. Finally, m-poly(GMA) beads were redispersed within 10 mL of water (0.1%, by weight) and stored at room temperature.

The morphology and size of the m-poly(GMA) beads were observed in a scanning electron microscope (JEOL, JEM 1200EX, Tokyo, Japan). m-Poly(GMA) beads were dried at room temperature and coated with a thin layer of gold (about 100 Å) in vacuum and photographed at $\times 1000$ magnification. The bead size was determined by measuring at least 100 beads on photograph taken on a SEM.

2.3 Concanavalin A Immobilization

To immobilize Con A, firstly, epoxide groups on the m-poly(GMA) beads were activated by alkaline hydrolysis. A typical procedure can be given as: one gram of m-poly(GMA) beads was added into 4 M NaOH solution for 6 h while shaking continuously. Then, Con A immobilization was carried out by using 25 mL of Con

A solution containing 100 μM $CaCl_2$, 100 μM $MnCl_2$, 2% (w/v) glucose. The pH of the solution was adjusted by phosphate buffer (0.1 M and 7.0). The m-poly(GMA) beads was shaken in a water bath (T: 4°C) for 24 h. Con A immobilized beads were filtered and washed with distilled water, acetate buffer and phosphate buffer several times untill all the physically adsorbed Con A molecules were removed. The amount of Con A immobilization on the m-poly(GMA) beads was determined by measuring the decrease of Con A concentration and also by considering the Con A molecules adsorbed non specifically (amount of Con A adsorbed on the unmodified beads) by measuring the absorbance at 280 nm. The leakage of Con A from the beads was followed by treating the beads with phosphate buffer solution for 24 h at room temperature. The released amount of Con A after this treatment was measured spectrophotometrically at 280 nm with a molar absorptivity of 14.0 for a 1% solution of Con A.

2.4 Invertase Adsorption Studies

Invertase adsorption of the plain m-poly(GMA) and m-poly(GMA)/Con A beads were studied at various pHs in acetate (0.1 M pH 4.0–5.5) and in phosphate buffer solutions (0.1 M, pH 6.0–8.0). In order to determine the adsorption capacities of m-poly(GMA)/Con A beads, the concentration of invertase in the medium was varied in the range of 0.25–5.0 mg/mL. The adsorption experiments were conducted for 2 h at 25°C, while shaking continuously. At the end of this period, the enzyme adsorbed beads was removed from the enzyme solution and was washed with the same buffer three times. Invertase concentrations were determined using absorbance at 280 nm before and after adsorption processes. The amount of adsorbed invertase was calculated from mass balance.

2.5 Activity Assays of Free and Adsorbed Invertase

The activities of free and adsorbed invertase preparations were determined by measuring the amount of glucose liberated from the invertase-catalyzed hydrolysis of sucrose per unit time. In the determination of the activity of the free enzyme, the reaction medium consisted of acetate buffer (2.5 ml, 50×10^{-3} M, pH 5.0) and sucrose (0.1 ml, 300×10^{-3} M). Following a preincubation period (5 min at 35°C), the assay was started by the addition of the enzyme solution (0.1 ml, 10 mg/ml) and incubation was continued for 5 min. In order to terminate the enzymatic reaction, the reaction medium was then placed in a boiling water bath for 5 min.

The same assay medium was used to determine the activity of the adsorbed enzyme. The enzymatic reaction was started by the incubation of 0.5 g m-poly(GMA)/Con A/invertase beads into the assay medium (10 ml) and was carried out at 35°C with shaking in a water bath. After 15 min, the reaction was terminated by removal of the m-poly(GMA)/Con A/invertase beads from the reaction mixture.

Sucrose hydrolysis performances of the free and adsorbed enzyme preparation was determined by measuring glucose content of the medium. Assay mixture contained glucose oxidase (GOD, EC 1.1.3.4) (25 mg), peroxidase (POD, EC 1.11.1.7) (6 mg) and *o*-dianisidine (13.2 mg) in phosphate buffer solution (100 ml, 0.1 M, pH 7.0). An aliquot (2.5 ml) of enzymatically hydrolyzed sample was mixed and then incubated in a water bath at 35°C for 30 min. After addition of sulfuric acid solution (1.5 ml, 30%) absorbance was measured by a UV/Vis spectrophotometer (Shimadzu, Model 1601, Tokyo, Japan) at 530 nm. The activity of the adsorbed invertase preparation was presented as a percentage of the activity of free enzyme of same quantity.

These activity assays were carried out in the temperature range of 20–80°C to determine the temperature profiles of free and adsorbed enzyme. Optimum pH was determined by individually changing conditions of the invertase activity assay. pH of the solution was changed between 4.0–6.0 (acetate buffer) and 6.0–8.0 (phosphate buffer). The results of dependence of temperature, storage stability and repeated run are presented in a normalized form with the highest value of each set being assigned the value of 100% activity.

2.6 Determination of the Kinetic Constants

The kinetic parameters K_m and V_{max} were determined by measuring initial reaction rates at different conditions given above for adsorbed and free invertase with sucrose. For this purpose, free invertase (0.6 mg) or invertase adsorbed m-poly(GMA)/Con A beads (0.6 mg) were added to 50 ml sucrose solution of different concentrations between 25×10^{-3}–250×10^{-3} M and initial activities were determined as described above. K_m and V_{max} values were calculated from Lineweaver-Burk plots.

2.7 Incubation Period and Substrate Specificity

The incubation period of free and adsorbed enzyme were determined by measuring the residual activity of the enzyme exposed to three different time (in a range 1–5 min) at the same enzyme assay conditions. The specificity of enzyme activity was investigated by exposing the enzyme to other disaccarides, maltose and lactose. In place of sucrose solution in assay procedure, same amount of disaccharide was used as substrate.

2.8 Storage Stability

The activity of free and adsorbed invertase in acetate buffer (0.3 M, pH 5.5) was measured in a batch-operation mode at 4°C under the experimental conditions given above for 25 days for free enzyme and 50 days for adsorbed enzyme. Thermal stability of free and adsorbed invertase were

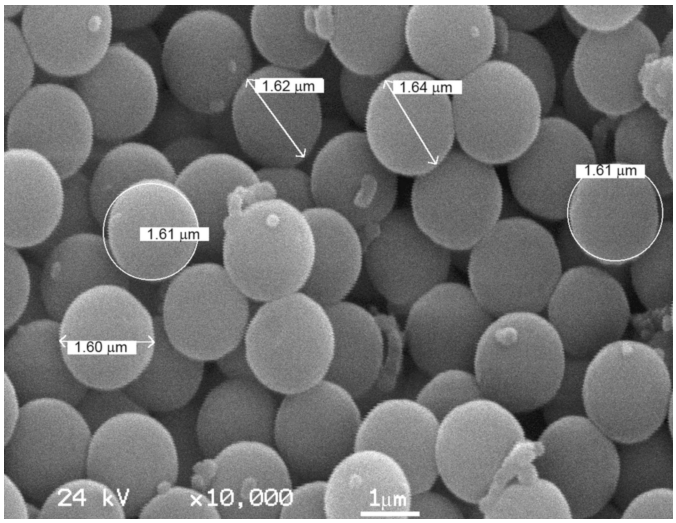


Fig. 1. SEM micrograph of m-poly(GMA) beads.

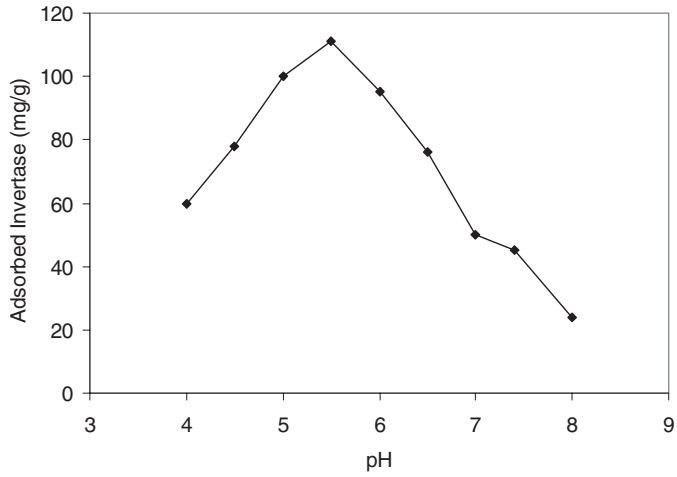


Fig. 2. Effect of pH on invertase adsorption onto m-poly(GMA)/Con A beads; Con A loading.12.5 mg/g; invertase concentration: 3 mg/ml; T: 20°C.

carried out by measuring the residual activity of the enzyme exposed to three different temperatures (50–70°C) in acetate buffer (0.3 M, pH 5.5). After every 10 min time interval, a sample was removed and assayed for enzymatic activity as described above.

3 Results and Discussion

3.1 Properties of m-poly(GMA) Beads

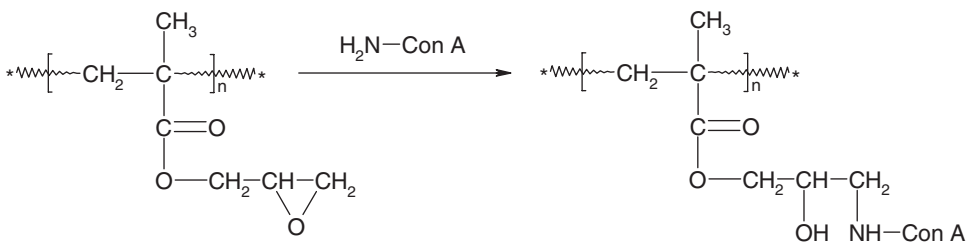
In our previous study, the preparation of m-poly(GMA) beads (RSD <1%, 1.62 μM in diameter) were described in details (39). The morphology and structure of the beads were observed by SEM picture as shown in Figure 1. As seen here, the m-poly(GMA) beads were highly uniform in size. m-Poly(GMA) beads are hydrophilic networks capable of imbibing large amounts of water, yet remain insoluble and preserve their three-dimensional shape. Micron sized beads have gaining more attention because reducing bead size to 0.1–2.0 μm, increase the protein loading capacity (40).

3.2 Concanavalin A Immobilization

Con A was used for adsorption of invertase due to the specific interactions via carbohydrate residues of proteins. Scheme 1 shows the Con A immobilization reaction on the beads. Con A was covalently immobilized onto the beads through a coupling reaction between the free amine groups of Con A and the epoxy groups of the beads. Ligand leakage is a serious problem in biotechnological applications due to the undesired reactions and the lost of efficiency for the adsorption (41). Con A leakage was investigated in PBS solution. Con A leakage was not observed from m-poly(GMA)/Con A beads. Of course, the reactive character of the selected polymeric matrix allowed the direct attachment of Con A without the requirement of an additional activation protocol. The Con A immobilization capacity of the beads was determined to be 12.5 mg Con A/g beads at the immobilization conditions (pH 7.0; 24 h, 4°C).

3.3 Invertase Adsorption Studies

The effect of pH on the adsorption of invertase onto the m-poly(GMA)-Con A beads was studied in the pH range 4.0–8.0 and the effect of pH on adsorption are presented



Sch. 1. Con A immobilization reaction on the m-poly(GMA) beads.

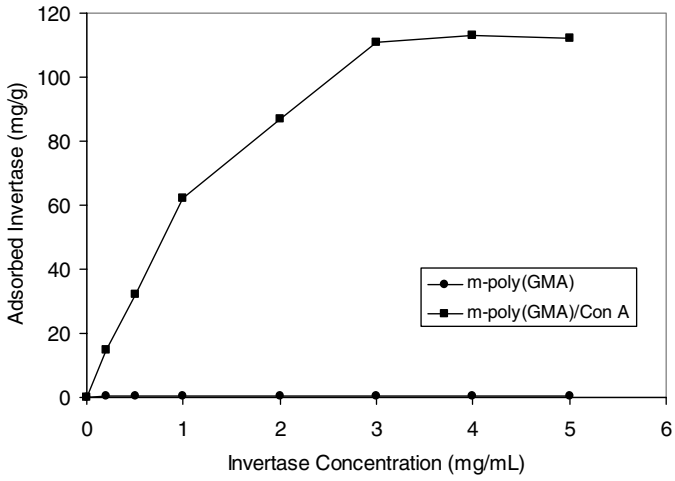


Fig. 3. Effect of invertase concentration on invertase adsorption onto m-poly(GMA)/Con A beads; Con A loading: 12.5 mg/g, pH: 5.5; T: 20°C.

in Figure 2. The maximum adsorption was observed at pH 5.5. The decrease in the invertase adsorption capacity in more acidic and more alkaline regions can be attributed to electrostatic repulsion effects between the opposite charged groups. Proteins have no net charge at their isoelectric points, and therefore the maximum adsorption from aqueous solutions is usually observed at their isoelectric points. The isoelectric pH of invertase is 4.7.

The adsorption isotherm of invertase is presented for m-poly(GMA)/Con A beads in Figure 3. An increase in invertase concentration in the adsorption medium led to an increase in adsorption efficiency but this leveled off at invertase concentration of 3.0 mg/ml. Maximum invertase adsorption obtained for m-poly(GMA)/Con A beads was 111 mg/g. This could be due to the specific interactions between invertase and immobilized Con A molecules. Non-specific invertase adsorption of m-poly(GMA) beads was 0.45 mg/g which was obtained by using plain m-poly(GMA) beads without Con A immobilization.

3.4 Kinetic Constants

Kinetic parameters, the Michaelis constant (K_m) and the maximal initial rate of the reaction (V_{max}) for free and adsorbed invertase preparations were determined using sucrose as a substrate (Table 1). For both free and adsorbed enzymes, K_m was found to be 20 mM, whereas V_{max} was calculated as 330 U/mg enzyme for free enzyme and 292 U/mg enzyme for adsorbed enzyme, respectively. The same K_m values of both enzyme preparations may be explained by no diffusional limitations imposed on the substrate. The substrate molecules easily access to the active binding sides of the invertase immobilized magnetic beads. V_{max}/K_m values were so closed to each other, 16.5 $U.mg^{-1}.mM^{-1}$ for free invertase and 14.6 $U.mg^{-1}.mM^{-1}$ for adsorbed invertase. This also shows a small decrease in enzyme affinity and higher accessibility of substrate molecules to the active site of the adsorbed enzyme.

Table 1. Properties of free and adsorbed invertase onto m-poly(GMA)/Con A beads

Parameters	K_M (mM)	V_{max} (U mg^{-1} enzyme)	V_{max}/K_M
Free Enzyme	**20**	**330**	**16.5**
$CuSO_4$ (inhibitor)	20	93	4.7
Adsorbed Enzyme	**20**	**292**	**14.6**
$CuSO_4$ (inhibitor)	38	287	7.6

Inhibitions of free invertase and adsorbed invertase by metal ions were investigated by using Cu(II) ions as inhibiting agent (i.e., Cupric sulfate). Cu(II) ions have shown a non-competitive inhibiting activity for free enzyme. Although there is no structural similarities between sucrose and cupric sulfate, non-competitive inhibition was determined. The situation depends on the binding of Cu(II) ions onto electron donor side chain, especially histidine of invertase. For adsorbed invertase, mixed type inhibition was determined. In addition, we determined that 2 mM cupric sulfate can inhibit 50% of free enzyme whereas no inhibition occurs for adsorbed enzyme in same inhibitor concentration.

3.5 Effect of pH and Temperature on the Catalytic Activity

The effect of pH on the sucrose hydrolysis ability of both free and immobilized invertase is shown in Figure 4. There is a maximum at pH 5.5 for free and adsorbed enzyme. But it is very clearly observed that adsorbed enzyme is more stable below and above pH 5.5 than free enzyme because of adsorption onto the beads.

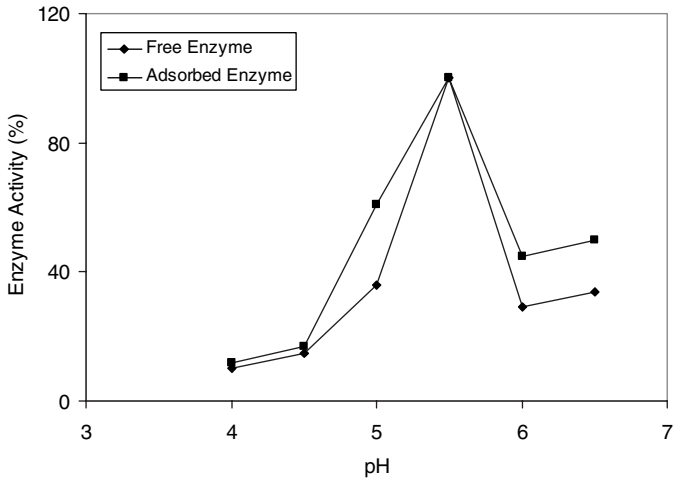


Fig. 4. pH profiles of the free and immobilized invertase; Con A loading: 12.5 mg/g; sucrose concentration: 0.01 mg/ml; T: 20°C.

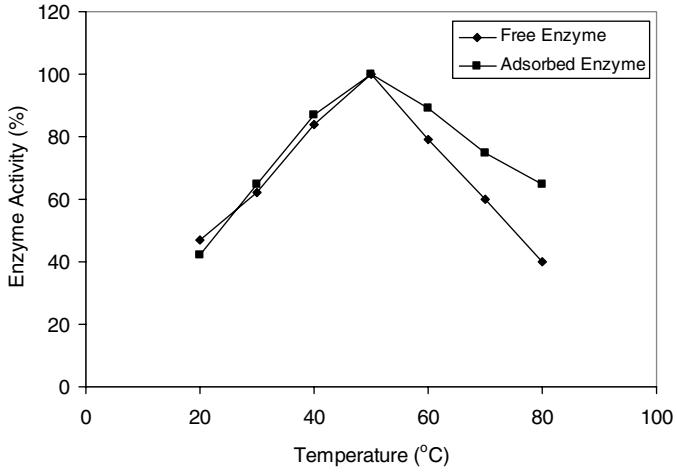


Fig. 5. Temperature profiles of the free and immobilized invertase; Con A loading: 12.5 mg/g; sucrose concentration: 0.01 mg/ml, pH: 5.5.

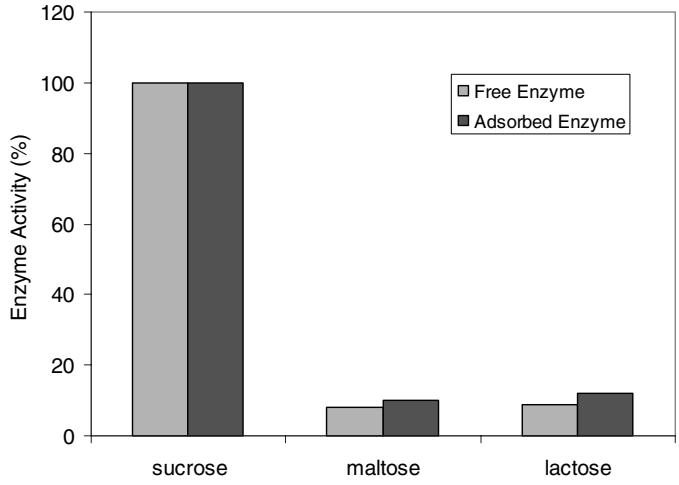


Fig. 7. Substrate specificity of free and adsorbed invertase.

Figure 5 shows the effect of temperature on the sucrose hydrolysis. For both preparation, free and adsorbed invertase, the maximum activity was observed at 50°C, the activities obtained in a temperature range of 20–80°C and expressed as percentage of the maximum activity. An increase in the relative activity was observed with the increasing temperature. The activity of free enzyme decreased at temperatures higher than 50°C, probably due to thermal deactivation. However, the activity of adsorbed enzyme gave slow decrease. Thus, it is concluded that adsorbed enzyme is more stable than free enzyme at higher temperature and the adsorption caused a significant improvement in the thermal stability of invertase.

3.6 Incubation Period and Substrate Specificity

The effect of the incubation period on the activities of both free and adsorbed invertase was given in Figure 6. As seen in the figure, free enzyme lost its activity in 5 min about 50%, although, adsorbed invertase has an ability to keep its activity. It can be explained by an increase in stability of the enzyme because of immobilization onto the beads.

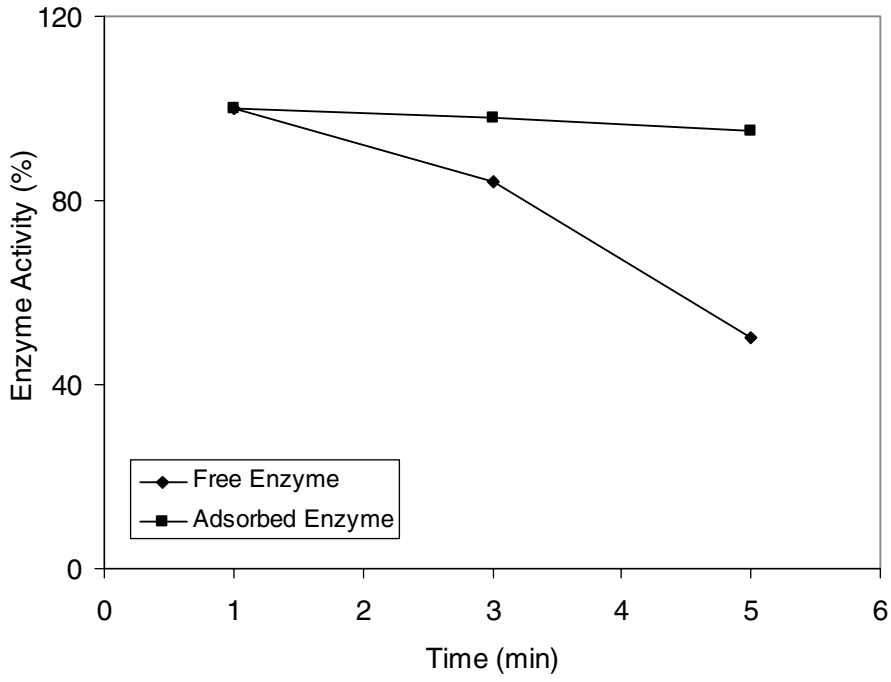


Fig. 6. Effect of incubation period on enzyme activity.

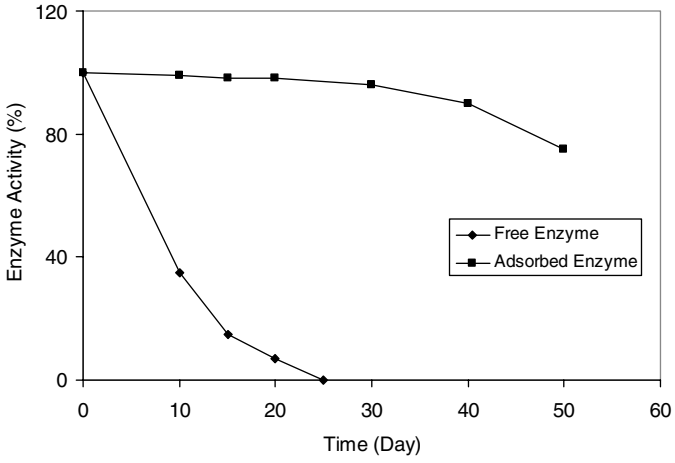


Fig. 8. Storage stability of free and adsorbed invertase.

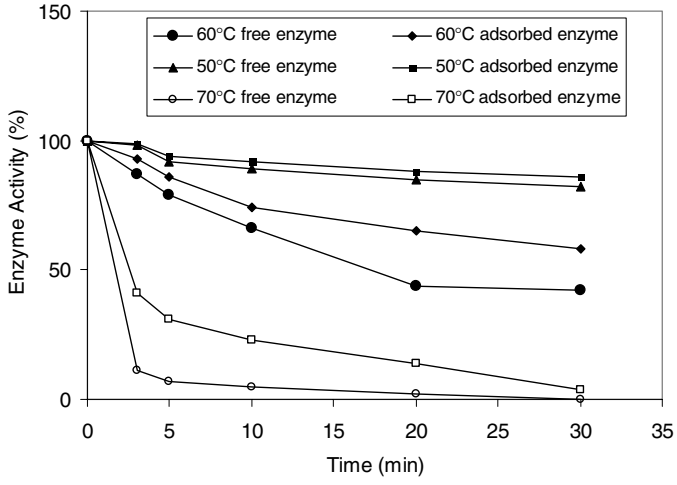


Fig. 9. Effect of temperature on the stability of free and adsorbed invertase.

The substrate specificity of free and adsorbed invertase was determined using maltose and lactose as comparative substrates (Figure 7). The activity on sucrose was accepted as 100% and compared to the activity of other substrates. As seen in figure, both enzyme preparations, free and adsorbed invertase, more specifically hydrolyzed sucrose molecules than maltose and lactose. Hydrolyzing activity of adsorbed invertase on maltose and lactose was slightly higher than free invertase. Enzymes have a long amino acid chain consist of several recognition sides for the substrate molecules. During the adsorption process, some conformational changes on invertase can be occurred. So, the substrate specificity of the adsorbed enzyme was slightly changed. In the present work, substrate specificity of the adsorbed invertase decreased just 2% for maltose and 3% for lactose.

3.7 Storage Stability

An aliquot (2.5 ml) of enzymatically hydrolyzed sample was mixed and then incubated in a water bath at 35°C for 30 min.

Free and adsorbed invertase preparations were stored in acetate buffer (0.3 M, pH 5.5) at 4°C and the activity measurements were carried out for a period of 25 days for free enzyme and 50 days for adsorbed enzyme (Figure 8). No enzyme release was observed. The free enzyme lost its all activity within 25 days. Adsorbed preparation of m-poly(GMA)/Con A lost only 4% of its activity during the same period. This decrease in enzyme activity was explained as a time-dependent natural loss in enzyme activity and this was prevented to a significant degree upon adsorption. After 50 days, adsorbed enzyme preserved its activity up to 75%. It can be concluded that adsorption process of invertase made it more stable than free form.

3.8 Thermal Stability

Thermal stability experiments were carried out with free and adsorbed enzymes, incubated in the absence of substrate at the various temperatures for 30 min. Figure 9 shows the heat inactivation curves between 50–70°C for the free and adsorbed enzymes, respectively. At 50°C, the adsorbed and free enzyme retained their activity about to a level 86% and 82%, respectively. At 60°C, the adsorbed and free invertase retained their activity about to a level 58% and 42%, respectively. At 70°C, the adsorbed invertase retained its activity 11%, whereas free enzyme lost its all activity. The adsorbed form inactivated at a much slower rate than the native form. These results showed that, the activity of the adsorbed preparation is more resistant than that of the soluble form against heat and denaturing agents.

4 Conclusions

In this study, adsorption of invertase onto Con A immobilized m-poly(GMA) beads has been investigated. The magnetic monosize beads were prepared by dispersion polymerization method with presence of magnetic nanopowder. The most important advantage of using these particles is no need for activation step for immobilization of affinity ligand, Con A. As known, use of magnetic beads in bioprocesses has many advantages. They can be easily separated from reaction medium by applying a magnetic field. And also, the use of magnetic beads reduces capital and operation costs. In the study, we have aimed to find a cost effective and magnetic hydrophilic affinity beads having high adsorption capacity for immobilization of enzymes. In light of the results, we concluded that m-poly(GMA)/Con A beads can be used for adsorption of invertase and have an economical potential to use as enzymatic reactor.

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