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Invertase reversibly immobilized onto polyethylenimine-grafted poly(GMA–MMA) beads for sucrose hydrolysis

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Abstract

The epoxy group containing poly(glycidyl methacrylate-co-methylmethacrylate) poly(GMA–MMA) beads were prepared by suspension polymerisation and the beads surface were grafted with polyethylenimine (PEI). The PEI-grafted beads were then used for invertase immobilization via adsorption. The immobilization of enzyme onto the poly(GMA–MMA)–PEI beads from aqueous solutions containing different amounts of invertase at different pH was investigated in a batch system. The maximum invertase immobilization capacity of the poly(GMA–MMA)–PEI beads was about 52 mg/g. It was shown that the relative activity of immobilized invertase was higher then that of the free enzyme over broader pH and temperature ranges. The Michaelis constant (K_m) and the maximum rate of reaction (V_{max}) were calculated from the Lineweaver–Burk plot. The K_m and V_{max} values of the immobilized invertase were larger than those of the free enzyme. The immobilized enzyme had a long-storage stability (only 6% activity decrease in 2 months) when the immobilized enzyme preparation was dried and stored at 4 °C while under wet condition 43% activity decrease was observed in the same period. After inactivation of enzyme, the poly(GMA–MMA)–PEI beads can be easily regenerated and reloaded with the enzyme for repeated use.
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Keywords: Invertase; Immobilized enzyme; Affinity beads; Adsorption; Enzyme reactor

1. Introduction

Many methods of immobilization have been tried, ranging from covalent attachment to adsorption or physical entrapment [1–7]. Among the various immobilization techniques available, adsorption may have a higher commercial potential than other methods because the adsorption process is simpler, less expensive, retains a high catalytic activity, and most importantly the support could be repeatedly reused after inactivation of the immobilized enzyme [5–10]. A number of methods for reversible immobilization of enzymes have been reported in the literatures [2,11,12]. However, the adsorption is generally not very strong and some of the adsorbed enzyme is desorbed during washing and operation. For this reason, reversible enzyme immobilization via adsorption requires a strong hydrophobic or ionic interaction between the enzyme and support [13–17]. Desorption of enzymes from the polyethylenimine coated support was found to require the use of denaturing conditions (under low pH and high ionic strength), but this desorption would be necessary after inactivation of the enzyme upon use [16–21].

In this study, poly(GMA–MMA) beads were synthesized from the monomers glycidyl methacrylate and methylmethacrylate and cross-linked with ethyleneglycol dimethacrylate. The epoxy groups of poly(GMA–MMA) beads were used for attachment of PEI. The polycationic amino groups of the PEI are used for the reversible immobilization of invertase. The invertase is a carboxylic group rich acidic protein. Therefore, the enzyme "invertase" was selected as a negatively charged protein to evaluate its immobilization on support coated with an opposite charged polymer. The immobilization of invertase via ionic adsorption onto the poly(GMA–MMA)–PEI beads from aqueous solutions containing different amounts of enzyme at different pH was investigated in a batch system. The optimum pH and temperature for the free and immobilized enzymes, as well as operational stability in a continuous system, were investigated.

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2. Experimental

2.1. Materials

Invertase (β-fructofuranosidase, EC 3.2.1.26, Grade VII from baker's yeast), glucose oxidase (GOD, EC 1.1.3.4. Type II from *Aspergillus niger*), peroxidase (POD, EC 1.11.1.7, Type II from horseradish), bovine serum albumin (BSA), *o*-dianisidine dihydrochloride, sucrose, glucose, polyethylenimine (PEI), polyvinyl alcohol (PVA), α,α′-azoisobisbutyronitrile (AIBN), bovine serum albumin (BSA) were obtained from Sigma Chem. Co. Glycidyl methacrylate (GMA), methylmethacrylate (MMA) and ethyleneglycoldimethacrylate (EGDMA) were obtained from Fluka AG (Switzerland). The monomers GMA and MMA were distilled under reduced pressure in the presence of hydroquinone and stored at 4 °C until use. All other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany).

2.2. Preparation of poly(GMA–MMA) beads

Poly(GMA–MMA) beads were prepared via suspension polymerisation. The aqueous continuous phase was comprised of 0.2 M NaCl. The organic phase contained GMA (7.5 ml), MMA (10 ml), EGDMA (7.5 ml; cross-linker) and isopropyl alcohol (15 ml, containing 5.0% polyvinyl alcohol as stabilizer) were mixed together with 0.5 g of AIBN in 30 ml of toluene. The polymerisation reactor was placed in a water-bath and heated to 65 °C. The reactor was then equipped with a mechanical stirrer, nitrogen inlet and reflux condenser. The polymerisation mixture was placed into a dropping funnel and was introduced drop wise into the reactor in about 10 min during stirring at 200 rpm under a nitrogen atmosphere. The polymerisation reaction was maintained at 75 °C for 2.0 h and then at 85 °C for 2.0 h. After the reaction, the beads were filtered under suction and washed with distilled water and ethanol. The product was dried in a under vacuum oven. The beads were sieved and 50–150 μm size of fraction was used in further reactions.

2.3. Grafting of poly(GMA–MMA) beads with polyethylenimine

Functional epoxy group carrying poly(GMA–MMA) beads (10 g) were equilibrated in phosphate buffer (50 mM, pH 8.0) for 2 h, and transferred to the same fresh medium containing PEI (1.0% (v/v)). Grafting of PEI on the poly(GMA–MMA) beads surface was carried out at 65 °C for 5 h, while continuously stirring the reaction medium. After this period, the poly(GMA–MMA)–PEI beads were removed from the reaction medium and washed with 1.0 M NaCl and then phosphate buffer (0.1 M, pH 7.0).

2.4. Immobilization of invertase onto PEI-grafted poly(GMA–MMA) beads

Immobilization of invertase on the poly(GMA–MMA)–PEI via adsorption was studied at various pH values, in either acetate (25 ml, 50 mM, pH 4.0–5.5) or phosphate buffer (25 ml, 50 mM, pH 6.0–7.0). The initial concentration of invertase was 1.0 mg/ml in each buffer solution and poly(GMA–MMA)–PEI beads (0.3 g) was used. In the second set experiments, the initial concentration of invertase was changed between 0.25 and 2.00 mg/ml in the immobilisation medium. In this case, invertase was dissolved in acetate buffer (25 ml, 50 mM, and pH 5.5) and PEI-grafted poly(GMA–MMA) beads (0.3 g) were added. The immobilization experiment was conducted for 2 h at 20 °C while continuous stirring. At the end of this period, the enzyme-immobilized beads were removed from the enzyme solution. The amount of protein in the enzyme preparations and the wash solution were determined by spectrofluorimetry (excitation at 280 nm and emission at 340 nm) using a (Jasco, Model FP 750, Tokyo, Japan) spectrofluorimeter. A calibration curve was prepared from crystalline invertase as a standard (0.02–0.2 mg/ml) and was used in the calculation of enzyme concentration.

2.5. Activity assays of free and immobilized invertase

The activities of both free and immobilised enzyme were determined as described previously [7,9]. The activity–pH profiles of the free and immobilized invertase were studied in acetate buffer (50 mM) in the pH range 4.0–5.5 and in phosphate buffer (50 mM) in the range pH 6.0–8.0. The effect of temperature on the free and immobilized invertase was studied in acetate (50 mM, pH 5.5) and phosphate buffer (50 mM, pH 6.0), respectively. The immobilised invertase activity was terminated by decantation of the reaction mixture into another glass. The results of dependence of pH, temperature and storage are presented in a normalized form with the highest value of each set being assigned the value of 100% activity. The activities of the free and the immobilized invertase were expressed in μmol glucose/min/mg of enzyme and μmol glucose/min/mg enzyme-immobilized invertase on the poly(GMA–MMA)–PEI beads, respectively.

Sucrose hydrolysis performance of the free and immobilized enzyme preparations was determined by measuring the glucose content of the medium according to a method described previously [22] using an UV–vis spectrophotometer (Shimadzu, Model 1601, Tokyo, Japan), at 525 nm.

2.6. Determination of the kinetic constants

K_m and V_{max} values of the free enzyme were determined by measuring initial rates of the reaction with sucrose (30–300 mM) in acetate buffer (50 mM, pH 5.5) at 35 °C. K_m and V_{max} were calculated from the data obtained after 5 min.

2.7. Storage stability measurements of free and immobilized enzymes

The storage stability of invertase immobilized poly(GMA–MMA)–PEI beads was tested in both dry and wet states. For the wet state storage, the enzyme-immobilized poly(GMA–MMA)–PEI beads were stored in phosphate buffer solution at 4 °C. For dry storage, the enzyme-beads were left to

dry in vacuum desiccators for 24 h at 4 °C under reduced pressure, and then stored in a sealed bottle at the same temperature. The activities of the immobilized invertase were determined for both storage conditions as described above for a storage period of up to 2 months. The residual activity was defined as the fraction of total activity recovered after immobilization of invertase on the poly(GMA–MMA)–PEI beads compared with the same quantity of free enzyme.

2.8. Reusability of poly(GMA–MMA) beads

In order to determine the reusability of the poly(GMA–MMA)–PEI beads for repeated enzyme immobilization, adsorption and desorption cycle of invertase was repeated six times by using the same poly(GMA–MMA)–PEI beads. Enzyme desorption were performed in a KSCN solution (1.0 M, pH 8.0, 1.0 ml). The enzyme adsorbed beads were placed in the desorption medium while stirring at 100 rpm at 25 °C for 3 h. The beads were removed from desorption medium washed several times with acetate buffer (50 mM, pH 5.5) and were then reused in the enzyme immobilization.

2.9. Characterization of poly(GMA–MMA)

2.9.1. Determination of the epoxy groups content

The available epoxy groups content of the poly(GMA–MMA) beads was determined by pyridine–HCl method as described previously [23]. Briefly, the pyridine–HCl solution was prepared by mixing 16 ml HCl with 984 ml pyridine. A 1.0 g beads was transferred in 50 ml pyridine–HCl solution and refluxed for 20 min. After cooling down the solution, the amount of available epoxy groups was determined by titration of pyridine–HCl solution with 0.1 M NaOH.

2.9.2. Determination of the free amino groups content

The content of amino groups in the final poly(GMA–MMA)–PEI support, i.e. those that remained free in PEI after its covalent bonding to poly(GMA–MMA), was determined by potentiometric titration. For that 1 g poly(GMA–MMA)–PEI beads was transferred in HCl solution (0.1 M, 20 ml) and it was then incubated in a shaking water-bath at 35 °C for 6 h. After this reaction period, the final HCl concentration in the solution was determined by a potentiometric titration with 0.05 M NaOH solution.

2.9.3. Scanning electron microscopy

The dried poly(GMA–MMA) beads were coated with gold under reduced pressure and their scanning electron micrographs were obtained using a JEOL (Model JSM 5600, Japan) scanning electron microscope.

2.9.4. Surface area measurement

The surface area of the poly(GMA–MMA)–PEI beads sample was measured with a surface area apparatus (BET method).

2.9.5. Determination of the water content

The poly(GMA–MMA) and PEI-grafted poly(GMA–MMA) beads were allowed to soak in distilled water for 24 h, swollen beads (∼1 g) were weighed after removing the excess

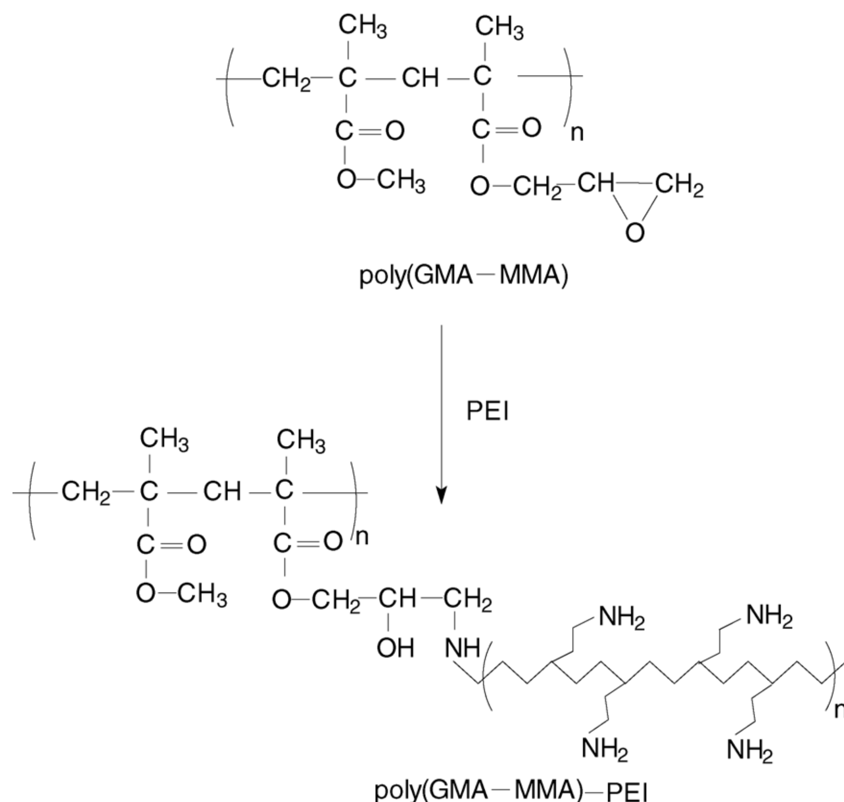

Fig. 1. The representative chemical structure of the support.

water, dried in vacuum oven at 60 °C for 24 h until constant weight.

3. Results and discussion

3.1. Properties of poly(GMA–MMA) beads

The representative chemical structure of polycationic polymer (i.e. PEI)-grafted poly(GMA–MMA) is presented in Fig. 1. The amount of epoxy groups available in the initial poly(GMA–MMA) was determined to be 1.44 mmol/g beads. Epoxy group carrying supports are able to form covalent linkages with different side chain groups (amino, thiol, phenolic ones) on the macromolecule structure under suitable experimental conditions (e.g. pH 7.0) [8]. The polyethylenimine polymer was linked to the acrylic backbone by an amide bond formed by the reaction of the $-NH_2$ with the epoxy group of GMA. The free amino groups content of the poly(GMA–MMA)–PEI beads were determined by potentiometric titration and the amounts of free amino groups on the polyethylenimine-grafted beads were found to be 1.69×10^{-3} mmol/g beads.

The water content of the poly(GMA–MMA) and PEI-grafted poly(GMA–MMA) beads were determined as 34% and 41%, respectively. These are moderate swelling degrees and suitable to use as column packing material for application of immobilized enzyme in a continuous flow system. Scanning electron microscopy (SEM) micrographs presented in Fig. 2A and B. The SEM micrographs show that the beads have a porous surface structure. The porous surface properties of the poly(GMA–MMA) beads would favour higher immobilization capacity for the enzyme due to increase in the surface area. The surface area of the poly(GMA–MMA) beads was measured by BET method and was found to be 11.4 m^2/g beads. The surface properties of the poly(GMA–MMA) beads would favour higher immobilization capacity for PEI and the enzyme due to increase in the surface area.

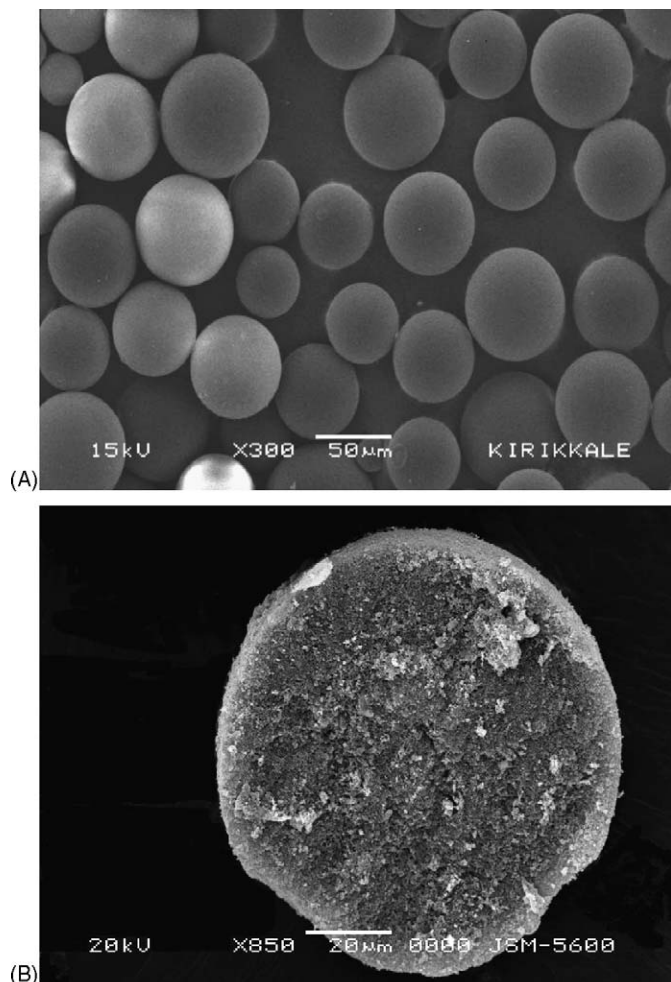

Fig. 2. SEM micrographs of poly(GMA–MMA) beads: (A) magnification 300×; (B) cross section magnification 850×.

3.2. Immobilization of invertase onto PEI-grafted poly(GMA–MMA) beads

In order to maximize the immobilized invertase onto PEI-grafted poly(GMA–MMA) beads; medium pH and initial enzyme concentration were changed for each individual set of batch immobilization experiments. Although insignificant, the amount of enzyme desorbed into the washing solution was also accounted for the calculation of immobilization efficiency of invertase on the poly(GMA–MMA)–PEI beads. The amount of immobilized invertase on the poly(GMA–MMA)–PEI beads is expressed as the weight of immobilized protein per gram beads (mg/g).

The maximum invertase immobilization was obtained at pH 5.5 about 52 mg/g beads (Fig. 3). Significantly lower invertase immobilization was observed for the poly(GMA–MMA)–PEI beads in the other studied pH regions. Invertase is an acidic protein with a pI value of 4.5 and it contains large number of hydroxyl, carboxyl and amino groups. In the present study, the maximum adsorption was observed at pH 5.5, and was shifted about 1.0 unit to less acidic pH values of the pI value of invertase. At this pH, invertase is negatively charged. On the other hand, the pK_a value of the amino groups of PEI is about 7.1 and it has positive charge at pH 5.5. The maximum adsorption at pH

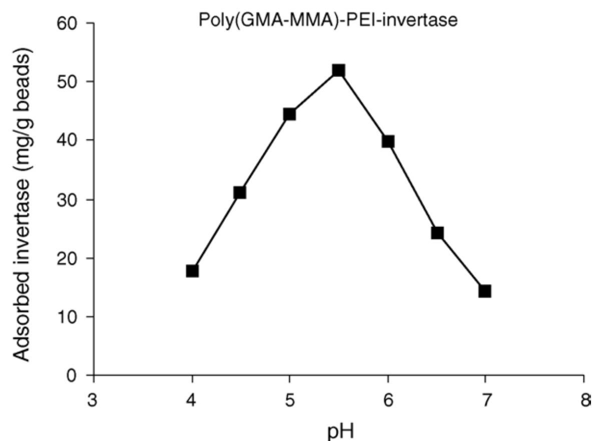

Fig. 3. Effect of pH on invertase immobilization via adsorption on the poly(GMA–MMA)–PEI beads. The immobilization of invertase on the beads at various pH values was studied under the following conditions: initial concentration of enzyme 1.0 mg/ml; temperature 20 °C.

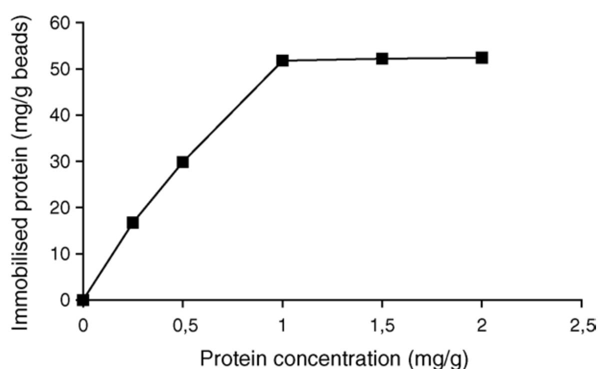

Fig. 4. Effect of initial invertase concentration on the enzyme loading on the poly(GMA–MMA)–PEI beads. The experimental conditions are: pH 5.5; initial concentration of enzyme varied between 0.25 and 2.0 mg/ml; temperature 20 °C.

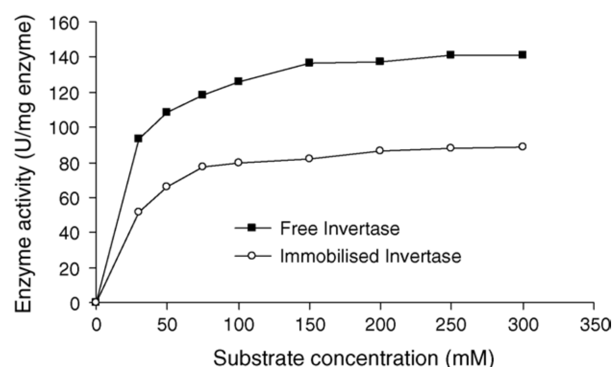

Fig. 5. The substrate saturation curves of free and immobilized invertase at different sucrose concentrations.

5.5 could result from preferential interaction between invertase and grafted cationic PEI molecules. Additional interactions may result from hydrogen bonding and ion-exchange effects, the latter was caused between the amino groups of the PEI and the carboxylic acid side-chains of the enzyme molecules.

As seen in Fig. 4, an increase in protein concentration in the medium led to an increase in immobilization efficiency but this levelled off at an invertase concentration of 1.0 mg/ml. As presented in Fig. 4 with increasing enzyme concentration in solution, the amount of invertase adsorbed per unit area by poly(GMA–MMA)–PEI beads increases almost linearly up to 1.0 mg/ml. It becomes constant when the enzyme concentration is greater than 1.0 mg/ml. This could be explained by saturation of interacting groups of the grafted PEI molecules with the adsorbed invertase molecules, which achieve maximum immobilization capacity.

3.3. Biochemical properties of free and immobilized invertase

When a biocatalyst is immobilized, kinetic parameters K_m and V_{max} undergo variations with respect to the corresponding parameters of the free form, revealing an affinity change for the substrate. These variations are attributed to several factors such as protein conformational changes induced by the support, steric hindrances and diffusional effects. These factors may operate simultaneously or separately, alternating the microenvironment around the bound enzyme. In the construction of an enzyme reactors and biosensors, it is very important to know the variations in the apparent kinetic parameters that appear as a result of immobilization. The substrate saturation curves of both free and immobilised invertase are presented in Fig. 5. The maximum reaction rate (V_{max}) and Michaelis-Menten constants (K_m) for the free and immobilised invertase were calculated from reciprocal plot of the data (Lineweaver–Burk plot). These were, therefore, determined in this study as well. For the free enzyme, K_m was found to be 17 mM, whereas V_{max} value was calculated as 148 U/mg proteins. Kinetic constants of the immobilized invertase were also determined in the batch system (Table 1). The apparent K_m value was found to be 29 mM for the adsorbed invertase on the poly(GMA–MMA)–PEI beads. The V_{max} value of immobilized enzyme was estimated from the data as 97 U/mg adsorbed protein onto poly(GMA–MMA)–PEI beads. As expected, the K_m and V_{max} values were significantly affected after immobilization on to the PEI-grafted beads. The reason for the difference in K_m values between the free and the immobilized invertase could be due to extensive interaction of ionic groups of invertase with the PEI molecules or large areas of contact between individual enzymes and the support surface causing deformation on the enzyme conformation. A comparison with the results obtained by Bayramoğlu et al. [7] for the covalently immobilised invertase on the surface of the poly(2-hydroxyethyl methacrylate–glycidyl methacrylate) film, the K_m value of the immobilized enzyme was increased from 14 to 38 mM compared to the free form. The decrease in V_{max} value as a result of immobilization should be related with the increase in K_m value, since an increase in the K_m value leads a decrease in the affinity of the enzyme for its substrate [7,24–27]. Furthermore, the immobilization of the enzyme also occurred inside the porous space of the beads, so increasing mass transfer resistance. In any case, immobilized invertase showed lower specificity constants compared to its free counterpart.

3.4. Effect of pH and temperature on the catalytic activity

The change in optimum pH depends on the charge of the enzyme and/or of the water insoluble matrix. This change is useful in understanding the structure–function relationship of enzyme and to compare the activity of free and immobilized

Table 1
Properties of the free and immobilized invertase onto poly(GMA–MMA)–PEI beads

Form of enzyme	K_m (mM)	V_{max} (U/mg enzyme)	Enzyme loading (mg enzyme/g beads)	Recovered activity (%)
Free invertase	17	148	–	100
Immobilized invertase	29	97	52	66

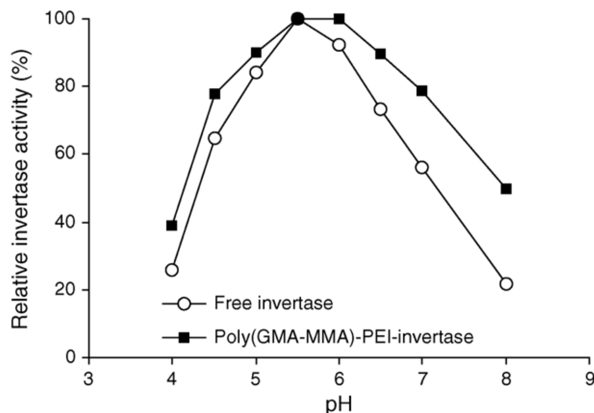

Fig. 6. pH profiles of the free and immobilized invertase preparations.

enzyme as a function of pH. The effect of pH on the activity of the free and immobilized invertase preparations for sucrose hydrolysis is presented in Fig. 6. The pH value for optimum activity for the free invertase was found to be at 5.5. On the other hand, the optimal pH for the immobilized invertase was extended between pH 5.5 and 6.0. This extension is possibly due to the secondary interactions (i.e. ionic and polar interactions, hydrogen binding) between the enzyme and the PEI incorporated matrix. Furthermore, the pH profiles of the immobilized invertase display significantly improved stability on both sides of the optimum pH value, in comparison to that of the free form, which means that the immobilization method preserved the enzyme activity in a wider pH range. These results could probably be attributed to the stabilization of invertase molecules resulting from multipoint ionic complex formation with the grafted PEI molecules [17].

As seen in Fig. 7, the activity of the free invertase is strongly dependent on temperature, with the optimum temperature being observed at about 35 °C. The optimum reaction temperature for immobilized invertase was between 35 and 40 °C, and the temperature profiles of the immobilized invertase were broader than that of the free counterpart. The increase in optimum temperature for the immobilised enzyme is probably a consequence of enhanced thermal stability. One of the main reasons for enzyme immobilization is the anticipated increase in its stability to various deactivating force due to restricted conformational mobility of the molecules following immobilization [17,26–28].

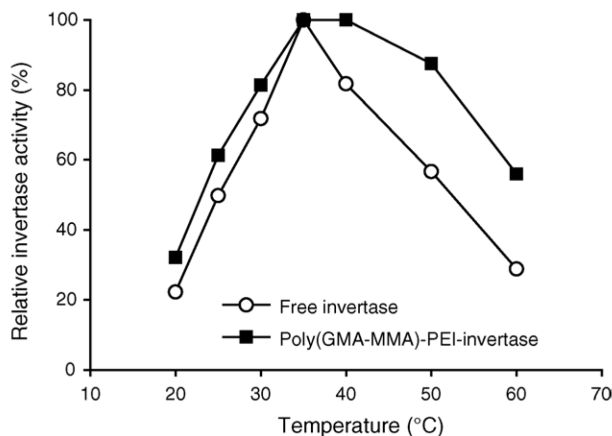

Fig. 7. Temperature profiles of the free and immobilized invertase preparations.

The results for the temperature range from 20 °C to optimum temperatures are also used for the calculation of activation energy (from Arrhenius plots). The plots for both the enzymes were linear and the calculated values of activation energy were about 17.75 and 13.47 kcal/mol for the free and immobilised invertase, respectively. The lower value of activation energy obtained for the immobilised invertase could be resulted from the internal diffusion limitation of the substrate "sucrose" into the porous space of the support-enzyme system. Similar results were reported previously [29–32]. For example, Sharp et al. reported that β-galactosidase immobilised onto porous cellulose sheets had a smaller activation energy than that of the free enzyme, and their system was slightly limited by the internal diffusion [29]. Kitano et al. [30], Ramachandran and Perlmutter [31] and Bille et al. [32] reported that the activation energy for the immobilised enzyme was lower than that of the free enzyme because the internal diffusion of the substrate into the enzyme-carrier system was rate-limiting step. In contrast, cases where enzyme activation energy increased after immobilisation have also been reported [32,33].

It should be noted that the pH and temperature profiles (Figs. 6 and 7) of the free invertase are different from those reported in our previous studies [7,9]. The enzyme "invertase" was commercially obtained from chemical company at different time interval. The commercial enzyme preparations can be prepared from different strain of the same microorganisms, purified under different experimental conditions or prepared from different batches. For these reasons and others, the experimental results could be slightly different for the numerical data and the pH and temperature profiles for the enzyme 'invertase'.

3.5. Repeated loading of poly(GMA–MMA) beads with the enzyme

The adsorption capacity of the support was not changed during successive repeated loading of invertase on poly(GMA–MMA)–PEI beads. Enzyme activity of the preparations did not significantly change during the adsorption–desorption cycles (lost above its original capacity about 7% ends of the six uses). This result showed that the PEI-grafted poly(GMA–MMA) beads can be repeatedly used in enzyme immobilization without significant detectable losses in its initial adsorption capacity. The result also indicate that KSCN is a suitable desorption agent for the PEI-grafted poly(GMA–MMA)–PEI beads and allows repeated use of the poly(GMA–MMA)–PEI beads in this study.

3.6. Storage stability of the invertase preparations

In general, if an enzyme is in solution, it is not stable during storage, and the activity is gradually reduced. The free and the immobilized invertase preparations were stored in both dry and wet states. In dry storage condition, only a 6% decrease in activity was detected during a 2-month storage period (Fig. 8). Under the wet storage conditions, the activity loss of the immobilized invertase was about 43% in the same storage period. The free

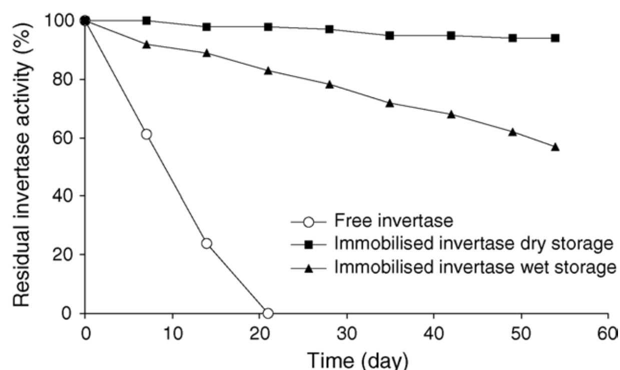

Fig. 8. Storage stabilities of the free and immobilized invertase under wet and dry conditions.

enzyme lost all its activity within third week. Thus, the immobilized invertase exhibits higher storage stability than that of the free form (Fig. 8). The higher stability of the immobilized invertase could be attributed to the prevention of thermal denaturation as a result of multipoint attachment of invertase molecules on the PEI-grafted poly(GMA–MMA) beads. As previously reported hydrogel carrier such as carboxymethylcellulose, and poly(2-hydroxyethyl methacrylate) provides a protective microenvironment for enzymes and yield higher stabilities [17,34]. On the basis of these observations, PEI-grafted poly(GMA–MMA) beads support should provide a stabilization effect, minimizing possible distortion effects imposed from aqueous medium on the conformational structure of the immobilized enzyme. The generated multipoint ionic interactions between enzyme and PEI-grafted poly(GMA–MMA) beads should also convey a higher conformational stability to the immobilized enzyme. Thus, the PEI-grafted poly(GMA–MMA) beads and the immobilization method provides higher shelf life compared to that of its free enzyme.

4. Conclusion

The poly(GMA–MMA) beads were prepared form GMA and MMA monomers in the presence of an initiator AIBN via suspension polymerisation. The polyethylenimine polymer was grafted on the beads as a cationic polymer ligand. The desired amount of enzyme can be loaded on the beads by changing the initial concentration of enzyme in the immobilization medium. The immobilized invertase retained much of their activity in wider ranges of temperature and pH than that of the free form. The storage stability of the immobilized invertase was also increased at 4 °C with respect to the free enzyme. After inactivation of enzyme upon use, the adsorbed enzyme can be desorbed from the poly(GMA–MMA)–PEI beads with KSCN. The regenerated poly(GMA–MMA)–PEI beads can be reused for the reversible immobilization of same or different enzyme. On the basis of the above explanations, the PEI-grafted poly(GMA–MMA)–PEI beads have numerous advantages such as: (i) the reactive beads can be prepared without any activation steps and no need any toxic substance for surface modifications; (ii) it can be easily used for enzyme immobilization under mild experimental conditions; (iii) the poly(GMA–MMA) beads has high compatibility with grafted PEI molecules for biomolecules and also has high enzyme binding capacity due to the large number of polycationic binding sites on the beads surface; the desired amount of epoxy and amino groups can be created on the beads surface by changing the monomer ratio in the initial polymerisation mixture; (iv) in addition, the reusability of the poly(GMA–MMA)–PEI beads support may provide economic advantages for large-scale biotechnological application. A high storage stability obtained with the immobilized invertase indicates that the stability of invertase was increased upon immobilization on the poly(GMA–MMA)–PEI beads.

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