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Aqueous Two-Phase Partitioning of Glucose Isomerase from *Actinoplanes missouriensis* in the Presence of PEG-Derivatives and its Immobilization on Chitosan Beads

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Abstract: Purification of glucose isomerase by its partitioning in a PEG-salt aqueous two-phase system (ATPS) in the presence of PEG derivatives has been studied. Selective partitioning of the proteins was observed towards the PEG phase containing PEG-benzoate and PEG-palmitate, enriching glucose isomerase in the salt phase. Cross-current extraction in 4 stages in the presence of PEG-palmitate gave an enrichment factor of ~5 for the enzyme. After initial purification with ATPS, glucose isomerase was immobilized on cross-linked chitosan beads. The immobilized enzyme was stable over a wider pH range (5.2–9.0) and showed an optimum pH of 6.5

Keywords: Aqueous two-phase systems, *A. missouriensis*, glucose isomerase, chitosan, PEG, PEG-derivatives

INTRODUCTION

Aqueous two- phase systems (ATPS) provide a benign method for separation of bioproducts with certain advantages, in particular, the biocompatibility and

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reliable scale-up to the industrial level (1). The ATPS have been useful for bulk separations of biological materials, recovery of a variety of intracellular enzymes from disrupted cell broth (2), purification of interferon from mammalian cell cultures (3), extractive fermentation (4) and affinity purification of enzymes and proteins (5).

The polyethylene glycol (PEG)/salt ATPS are particularly useful because of their low cost and ease of handling (6). In ATPS, the separation is achieved by unequal distribution of solutes between the phases which is influenced by molecular weight of the polymer, type and concentration of salts, pH, temperature, and the presence of the affinity ligands (7). More extreme partitioning of bioproducts can be obtained by introducing a polymer carrying covalently bound charged groups or affinity ligands (6–8).

This investigation focuses on the partitioning behavior of glucose isomerase, obtained by fermentation using *Actinoplanes missouriensis*, in a PEG-4000/sodium sulphate two-phase system, in the presence and absence of certain ionic and hydrophobic PEG derivatives. Glucose isomerase (GI) [E.C. 5.3.1.18], is an intracellular enzyme produced by several microorganisms belonging to *Actinoplanes, Arthobacter, Bacillus, Lactobacilli* and *Streptomyces* genera (9). Enzymes purified from these organisms exhibit pH and temperature optima between, 7.0 and 8.0 and 55°C and 60°C, respectively. The enzyme catalyzes the reversible isomerization of glucose to fructose and this allows their commercial use in producing High Fructose Syrup (HFS) (10). *Actinoplanes missouriensis* NRRL B- 3342 was reported to be the best in terms of the enzyme yield (11). The enzyme from *Actinoplanes missouriensis* appeared to be most stable of all known GI because it reportedly retains its activity at 90°C for a reasonable time period (12). The enzyme from *Actinoplanes missouriensis* has also been reported to give higher fructose production per unit volume with glucose as the substrate from a mixture of D-glucose and D-xylose indicating higher affinity for D-glucose (12). The enzyme is conventionally purified by precipitation followed by DEAE cellulose ion exchange chromatography (13).

Glucose isomerase is usually available in the immobilized form for the reactions. These preparations, by entrapment in gel or binding to solid supports, are relatively stable for continuous operations (14). Enzyme immobilization techniques provide, in addition to the desired reuse of the enzyme, advantages such as product separation and continuous operation. For successful development and application of an immobilized biocatalyst, the enzyme support is generally considered as the most important component. Chitosan is an ideal support material for enzyme immobilization because of its hydrophilicity, biocompatibility, biodegradability, and anti-bacterial property (15). The macromolecule is derived chemically by deacetylation of natural polymer chitin. Furthermore, chitosan exhibits a considerable protein binding capacity and the immobilized enzyme remains considerably active (16).

MATERIALS AND METHODS

Materials

A bacterial strain of *Actinoplanes missouriensis* (NCIM 2838) was procured from the National Chemical Laboratory (NCL), Pune, and used for producing the enzyme.

Glucose, sucrose, yeast extract, malt extract, peptone, dipotassium hydrogen phosphate, magnesium sulphate, and sodium hydroxide were purchased from HiMedia Lab. Ltd., Mumbai. PEG-4000, sodium sulphate, benzoyl chloride, palmitic acid, trimethylamine, glutaraldehyde, maleic anhydride, $CoCl_2 \cdot 6H_2O$, and all other necessary salts were purchased from S.D. Fine Chemicals, Mumbai. Coomassie Brilliant blue G-250 was purchased from the SISCO Research Laboratory, Mumbai. Dichloromethane (DCM), methanol and chloroform were obtained from Merck (I) Ltd., Mumbai. Thionyl chloride and perchloric acid were obtained from Thomas Baker Ltd., Mumbai. All other chemicals were of analytical grade. Distilled water was used for all the experiments.

EXPERIMENT DETAILS

Synthesis of PEG Derivatives

For preparing PEG-glucosides, PEG-4000 and glucose, in 2:1 molar ratio, were reacted in an aqueous solution in the presence of 1% (w/w) *p*-toluene sulfonic acid as a catalyst at 70°C with continuous stirring for two hours following a reported procedure (17). The reaction mixture was then neutralized and the excess water was removed on a rotavac. The thick syrup like liquid was kept overnight in the desiccators for drying. The product was then extracted with dichloromethane (DCM) to separate unreacted glucose. DCM was recovered by simple distillation. The residue was dried and the yield obtained was 68%. Fehlings test was performed to confirm the formation of glucoside (18).

The other derivatives of PEG, i.e. PEG-benzoate and PEG-palmitate were prepared by following a procedure as reported in literature (19).

Production of Glucose Isomerase

The bacterial strain *Actinoplanes missouriensis* was grown on a liquid medium containing glucose (1%), sucrose (2%), yeast extract, and malt extract (each 0.3%), peptone (0.5%). K_2HPO_4, $MgSO_4$ (each 0.1%) and $CoCl_2 \cdot 6H_2O$ (0.015%) at pH 7.0 and a room temperature of 30°C. The pH was adjusted with 0.1 N NaOH. A 50 cm^3 of medium in a 250 cm^3 conical flask was

sterilized by autoclaving at 121°C (15 psi steam) for 20 minutes and 1.25 cm^3 of bacterial suspension in a physiological saline solution (0.85% w/v), prepared from a fully-grown culture, was used to inoculate the medium. The fermentation was carried out for 96 hours on a rotary shaker at 180 rpm.

At the end of the fermentation period, the cell suspension (broth) was processed for the recovery of the enzyme. Since, glucose isomerase is an intracellular enzyme, the cells were harvested by centrifuging the cell suspension at 10,000x g for 30 minutes. The cells were then washed with 0.2 $mol.dm^{-3}$ maleate buffer (pH 6.8) and resuspended in a fresh 0.2 $mol.dm^{-3}$ maleate buffer (pH 6.8) solution. For the pH dependence studies, the cells were resuspended in 0.2 $mol \cdot dm^{-3}$ maleate and tris maleate buffer solutions in the pH range 5.2–6.8 and 6.8–8.0, respectively. This suspension was then sonicated at 50 MHz for 10 minutes in a "Dakshin" ultrasonic horn in an ice bath with frequent interruptions so that the temperature of the broth during the sonication did not rise beyond 12°C. The resultant broth with the cell lysate was centrifuged at 10,000x g for 15 minutes. The clarified extract was used as a crude extract for the partitioning studies.

Preparation of Aqueous Two-Phase System

The two-phase systems were prepared by dissolving PEG-4000 (10% w/w) and then sodium sulphate (7.5% w/w) directly in the extract obtained from the above step. The equilibrium phase diagram of PEG-4000/sodium sulphate/water has been reported by Phatak et al. (20) while phase diagrams in the presence of PEG-benzoate and PEG-palmitate are reported in the literature. (8) In the partitioning experiments involving PEG glucoside the concentration of PEG-glucoside used from 4–10% of total PEG(10%w/w), PEG-Benzoate (PEG-Bz), the concentration of PEG-Bz used from 10–40% that of the total PEG(10%w/w) and for PEG-palmitate the concentration of PEG-palmitate used from 10–25% that of the total PEG(10%w/w). This mixture was mixed well on a vortex mixer so that the PEG dissolved completely giving a clear and a colorless solution. To this homogenous solution 7.5% w/w of sodium sulphate was added and the solution was mixed to dissolve the salt. The resultant solutions were equilibrated by mixing and then the phases were separated by centrifugation. After the phase separation, the volume of each phase was noted and samples from the upper and lower phases were withdrawn for determination of glucose isomerase activity and the protein content.

DETERMINATION OF GLUCOSE ISOMERASE ACTIVITY AND PROTEIN CONTENT

The enzyme activity was assayed as described by Hu (21) with a slight modification. The assay mixture contained 4.5 cm^3 of 0.2 $mol \cdot dm^{-3}$ maleate buffer

(pH 6.8); 5.0 cm^3 substrate containing 2 $mol \cdot dm^{-3}$ glucose, 0.1 $mol \cdot dm^{-3}$ $MgSO_4 \cdot 7H_2O$, 0.01 $mol \cdot dm^{-3}$ $CoCl_2 \cdot 6H_2O$; and 0.5 cm^3 of the enzyme extract. The reaction mixture was incubated at 65°C for 60 minutes. The reaction was stopped by the addition of 1.0 cm^3 of 0.2 $mol \cdot dm^{-3}$ perchloric acid. The reaction mixture was vortexed and cooled down to room temperature of 30°C. An aliquot of the mixture was diluted with distilled water a 100 times. The fructose formed due to the isomerization of glucose was estimated by a cysteine HCl -carbazole- sulphuric acid method (22).

One unit of the enzyme activity is defined as the amount of the enzyme required to produce one μmole of fructose per minute per cm^3. The assay of the immobilized enzyme was carried out in a similar way. The total protein content was measured by the Bradford method (23).

Immobilization of the Enzyme on Chitosan Beads

The partially purified enzyme was chemically immobilized on chitosan beads using glutaraldehyde. Aqueous solutions of glutaraldehyde at different concentrations (1–6%) were prepared and added to the suspension of chitosan beads in 50 $mmol \cdot dm^{-3}$ maleate buffer at pH 6.8. After 2 hours of agitation at 100 rpm in a rotary shaker, the beads were separated by filtration and washed with the same buffer solution till no glutaraldehyde was detected in the washings. The activated beads were added to a partially purified solution of glucose isomerase and stirred at 50 rpm for 1hour, 2 hours, 3 hours, and 4 hours. The beads were again washed with the maleate buffer and 10% sodium borohydride solution was added drop-wise under agitation followed by filtration and washing with the buffer. The protein content and the enzyme activity of the supernatant, and the activity of the immobilized enzyme were determined separately.

To determine the optimum pH of the free and immobilized enzymes, the enzyme activity was assayed between pH 5.2 to 9.0, using the method described earlier, in 0.05 $mol \cdot dm^{-3}$ maleate, phosphate, and alkaline borate buffers in the pH ranges 5.2–6.8, 6.8–8.0, and 8.0–9.0, respectively. The stability of the enzyme as a function of pH was determined by measuring the residual activity of free and immobilized enzymes at each pH after 1 hour storage at room temperature of 30°C.

RESULTS AND DISCUSSION

Partitioning of Enzyme and Proteins in PEG-4000/Sodium Sulphate/Water ATPS

Studies with the standard aqueous two-phase system consisting of PEG-4000 (10% w/w) and sodium sulphate (7.5% w/w) showed preferential partitioning

of both the proteins and the enzyme towards the salt-rich phase, in the absence of any PEG derivative. (Fig. 1) Since proteins show a different partitioning behavior depending on the charge on the proteins, their partitioning was also conducted at different pH conditions. Increasing the pH, the partition coefficients (K_D) for both proteins and the enzyme increased slightly. The maximum K values for the enzyme and proteins were, however, only 0.05 and 0.13, respectively. The vertical bars in Fig. 1 indicate a variation in the partition coefficient from the repeated runs under identical conditions. No separation of the enzyme from other proteins is, therefore, possible with the standard PEG-4000/Na_2SO_4 ATPS as nearly all the proteins and the enzyme tend to stay in the salt rich phase. The molecular weight of the enzyme is 172 kD. Because of the excluded volume effect of PEG the enzyme does not partition to the PEG phase. It is known that, in the same manner as two polymers with high molecular weights do not co-exist in the solution, the enzyme gets excluded from the PEG phase (7).

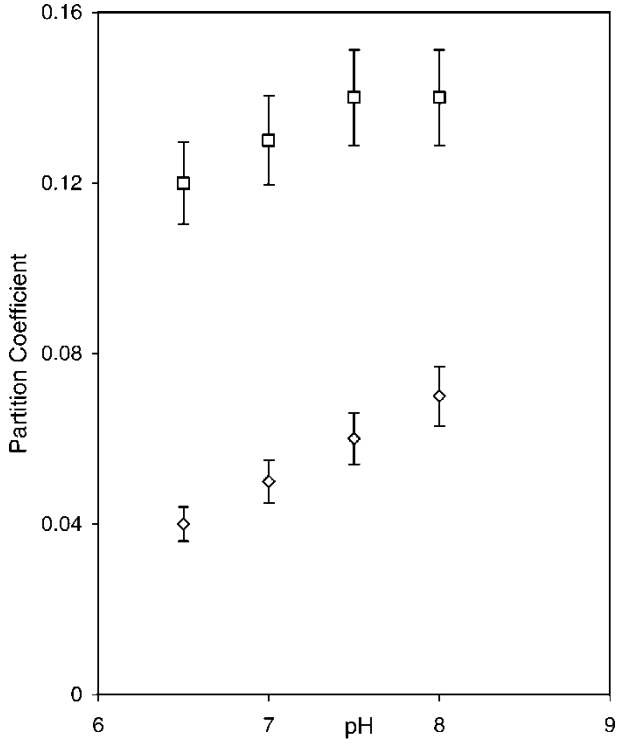

Figure 1. Partition coefficients of enzyme and protein in standard two-phase system [PEG-4000 10% (w/w) and sodium sulphate 7.5% (w/w)]. ◇ (Enzyme); □ (Protein).

Partitioning of Enzyme and Proteins in the Presence of PEG-Derivatives

Partitioning in the Presence of PEG-Glucoside

Since glucose is a natural substrate for glucose isomerase, it was expected that the PEG-glucoside derivative could interact specifically with the enzyme. The partitioning experiments, in the presence of increasing concentration of PEG-glucoside, showed a decreased enzyme activity in the salt-rich phase indicating the possible transfer of the enzyme to the PEG phase. However, the enzyme activity in the PEG phase was lower than that estimated from the activity balance. The maximum transfer of the enzyme to the PEG phase was, also, just 10%. (Fig. 2a) One of the possible reasons for the decreased activity of the enzyme in the PEG phase was thought to be the specific interaction of PEG-glucoside with the enzyme. However, as the concentration of PEG-glucoside increases, the partition coefficient of protein also increases (Fig. 2a).

Partitioning in the Presence of Hydrophobic PEG Derivatives

PEG-Bz is one of the hydrophobic derivatives previously used for the partitioning study of *Penicillin acylase* (8). It was reported that the benzoate group of PEG-Bz interacts in a specific manner with the active site of the enzyme. While in the present study with PEG-Bz it was found that as the concentration of PEG-Bz was increased, the partition coefficient of the proteins increased significantly. The partition coefficient of the protein increased from 0.15 to 0.55 (Fig. 2b). On the other hand, partitioning of the enzyme was not affected at all by PEG-Bz. The PEG derivative, therefore, caused the selective partitioning of other proteins towards the PEG phase. The maximum enrichment factor was 2.2 with 72% enzyme recovery in the salt-rich phase in single extraction step (Fig. 3a).

The fatty acid derivatives of PEG show an affinity towards the proteins mainly because of the hydrophobic interactions due to the long hydrophobic chain. The hydrophobic partitioning depends on the hydrophobicity of the individually exposed amino acids on the protein surface. Proteins with large exposed hydrophobic surfaces are known to selectively partition towards the PEG phase in the presence of PEG-palmitate (24). Shanbagh and Axelsson had used a PEG pamitate derivative in the partitioning study of Bovine serum albumin, B-lactoglobulin, and Cohemoglobin. They all show an increase in the partition coefficient with the increase in the PEG-palmitate concentration. The PEG-palmitate was, therefore used for the partitioning studies at different concentrations. The partition coefficient of the proteins was increased by an order of magnitude, from 0.13 to 1.81, clearly indicating selective transfer of the proteins due to the hydrophobic effect (Fig. 2c). The maximum transfer of the proteins occurred at the 20% ligand concentration in total PEG. While in study of Gavasane and Gaikar, who

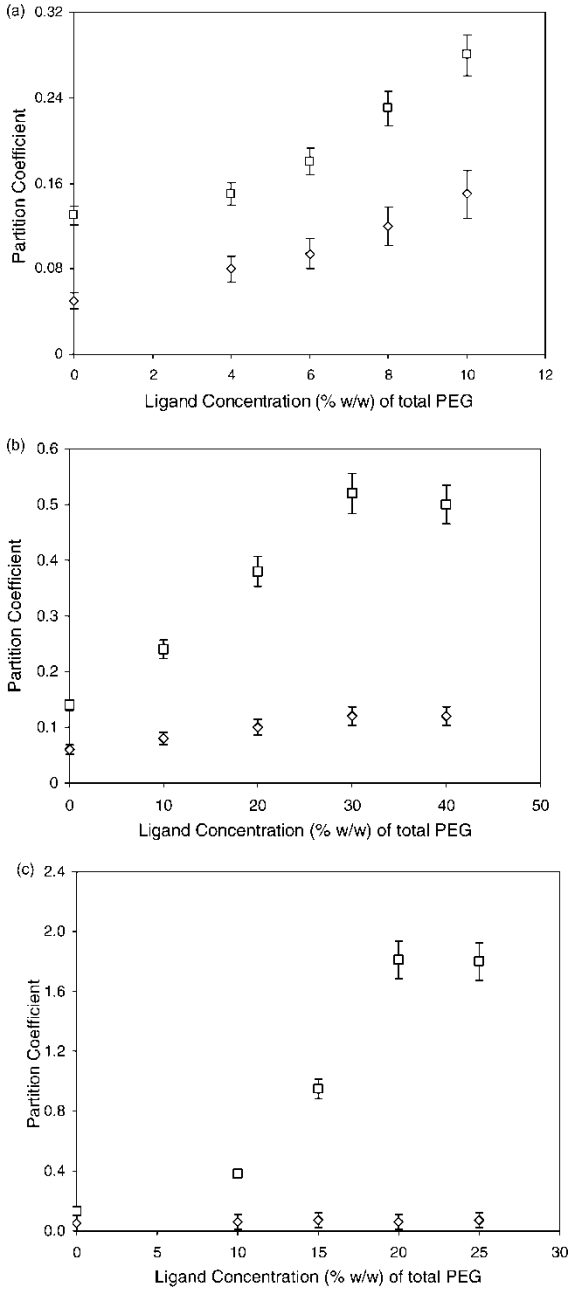

Figure 2. (a) Partitioning of enzyme and proteins in PEG-Glucoside substituted ATPS. ◇ (Enzyme); □ (Protein). (b) Partitioning of enzyme and protein in PEG-Benzoate (PEG-Bz) substituted ATPS. ◇ (Enzyme); □ (Protein). (c) Partitioning of enzyme and proteins in PEG-palmitate substituted ATPS. ◇ (Enzyme); □ (Protein).

used PEG-palmitate for the partitioning study of penicillin acylase, it was observed that up to certain concentration of PEG-palmitate enzyme selectively partitioning towards the PEG phase while after this concentration the protein also starts the partitioning towards the PEG-phase. At the same time, in the present study the partitioning of the enzyme showed no effect at all with respect to the increasing PEG-palmitate content. The surface hydrophobicity of glucose isomerase is very low, as the amino acids, composition of

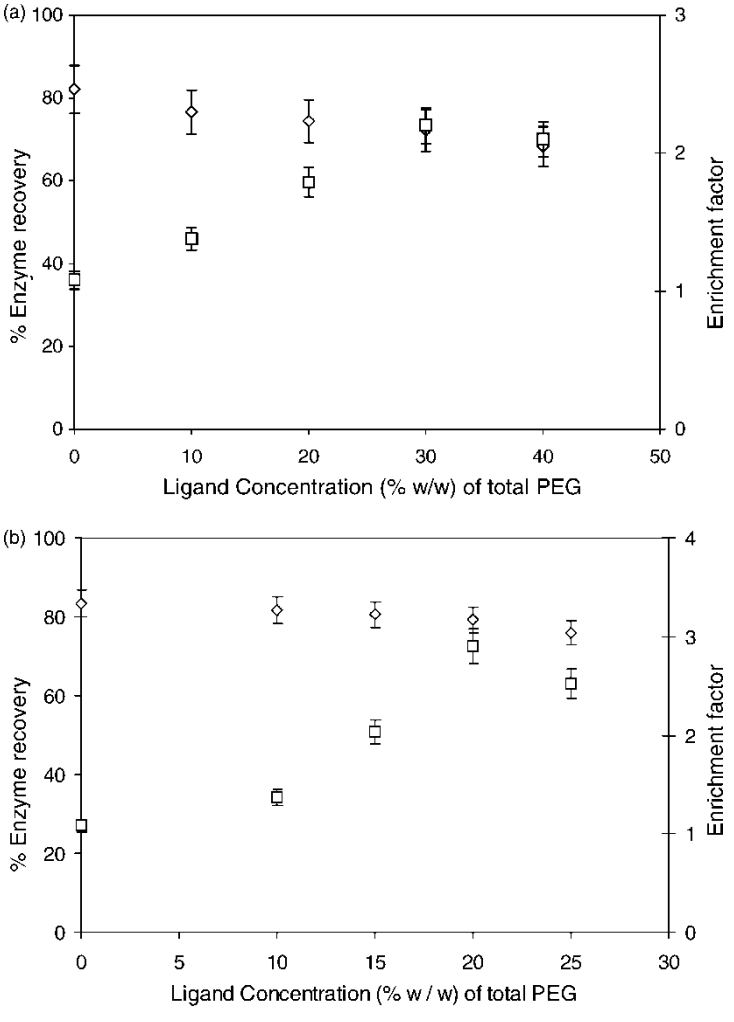

Figure 3. (a) Enzyme recovery and enrichment factor in bottom phase with PEG-Bz substituted ATPS. ◇ % Enzyme recovery; □ Enrichment factor. (b) Enzyme recovery and enrichment factor in PEG-palmitate substituted ATPS. ◇ % Enzyme recovery; □ Enrichment factor.

the peptide chain of the enzyme shows a lower percentage of apolar amino acids. The active site of the enzyme contains aspartic acid, histidine, glutamic acid, and lysine (25). Thus, from the single amino acid hydrophobicity scale (Gly < Ala < Leu < Phe < Tyr < Trp) it can be said that the enzyme contains less hydrophobic amino acids and, therefore, its interaction with the palmitate derivative of PEG was minimum (26). In the presence of salts, the hydrophobic interactions become more effective and facilitate firm binding of hydrophobic patches of other proteins with the hydrophobic ligand. The maximum enrichment factor with 20% PEG-palmitate was 2.9 with 79% enzyme recovery in the salt-rich phase in a single stage operation (Fig. 3b).

The partitioning of the enzyme and the proteins was further studied at different pH conditions, using the optimum 20% concentration of PEG-palmitate in the total PEG. With increasing the pH, the partition coefficient of the proteins increased upto a certain pH (7.5) and thereafter remained constant (Fig. 4a). But the partition coefficient of the enzyme remained almost constant. The maximum enrichment factor of the enzyme at pH 7.5 was $\sim$4 with 77% recovery in the salt rich phase in a single stage extraction (Fig. 4b). The enzyme loss in all these experiments which remained more or less constant, however, could not be explained. A possibility exists that a part of the enzyme does get transferred to the PEG phase in the presence of these derivatives and its activity is possibly lost on interaction with the hydrophobic surfaces of the PEG derivatives.

As the PEG-palmitate gave a good enrichment factor and an appreciable enzyme recovery, it was used for all further studies. Multiple extractions, in a cross-current manner, were conducted to extract the maximum amount of the bulk proteins into the PEG phase, leaving the enzyme in a purer form in the salt-rich phase.

Figure 5a shows the partitioning of the proteins in the system 20% PEG-palmitate in the total PEG, after each stage of the multiple extractions. With each extraction stage, the protein's partitioning towards the top phase decreased moderately. After four repetitive extractions, the amount of the enzyme remaining in the salt-rich phase was very high, i.e. its recovery was 73% with $\sim$5.5 fold purification (Fig. 5b). These results suggest that ATPS in the presence of PEG-palmitate can be used to obtain glucose isomerase in the purified form.

Immobilization of the Enzyme

The effect of glutaraldehyde concentration was first investigated in the concentration range of (1–6%) to select the optimum concentration of the glutaraldehyde that gives the highest activity of the immobilized enzyme. Concentration of an activating agent plays a very important role in the immobilized enzyme activity and immobilization yield. Figure 6 shows the

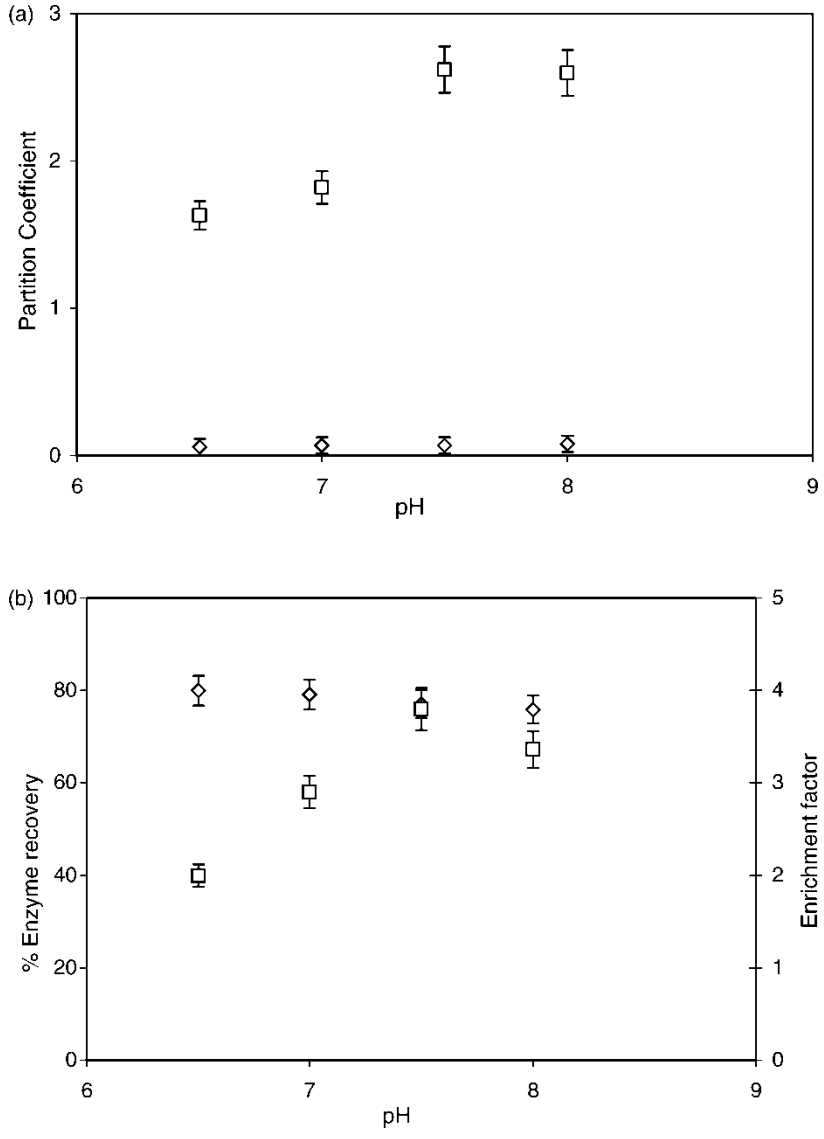

Figure 4. Effect of pH on partitioning of enzyme and proteins in PEG- Palmitate (20%) substituted ATPS. ◇ (Enzyme); □ (Protein). (b) Enzyme recovery and enrichment factor in bottom phase with PEG-palmitate (20%) substituted ATPS. ◇ % Enzyme recovery; □ Enrichment factor.

maximum activity of the immobilized enzyme at 4% (w/w) glutaraldehyde. At low concentrations of glutaraldehyde, the aldehyde groups may involve more in cross-linking amino groups of the chitosan and leaving very few free aldehyde groups for the enzyme immobilization. At higher

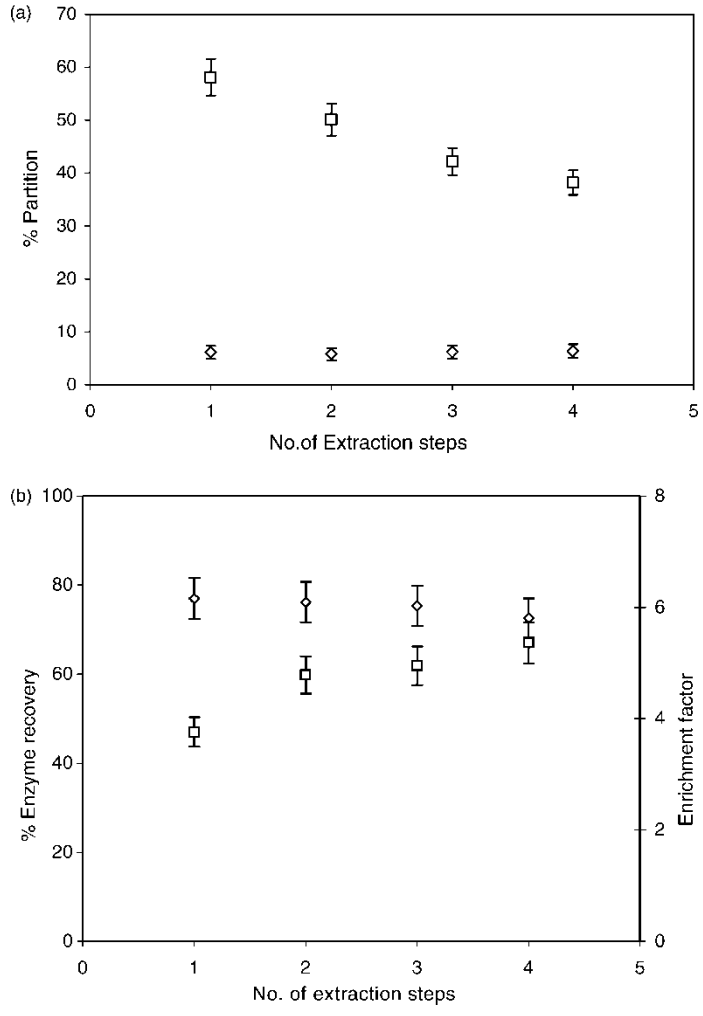

Figure 5. Effect of multiple extractions on the partitioning of protein and enzyme in PEG-palmitate (20%) substituted ATPS. ◇ Enzyme; □ Protein. (b) Effect of multiple extractions on percentage enzyme recovery and enrichment factor in PEG-palmitate substituted ATPS. ◇ % Enzyme recovery; □ Enrichment factor.

glutaraldehyde concentrations, a large number of free aldehyde groups is available for the reaction with the amino group of the enzyme. Glutaraldehyde has also been reported to react nonspecifically with amino groups of some peptides, the sulfidryl group of the cysteine, the α-amino group of lysine, and the phenolic and imidazole rings of the histidine (27). It is well–documented that the active site of the glucose isomerase contains amino acid residues such as aspartic acid, glutamic acid, histidine, and

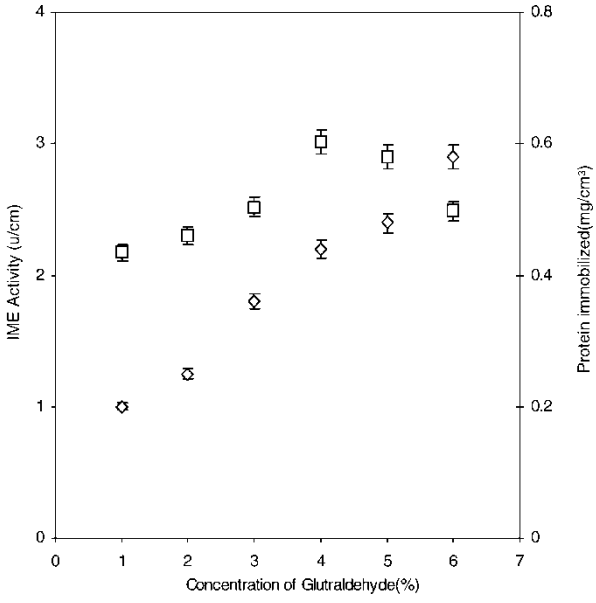

Figure 6. Effect of glutaraldehyde concentration on immobilized enzyme activity ◇ Protein (mg/cm^3); □ IME (u/cm^3).

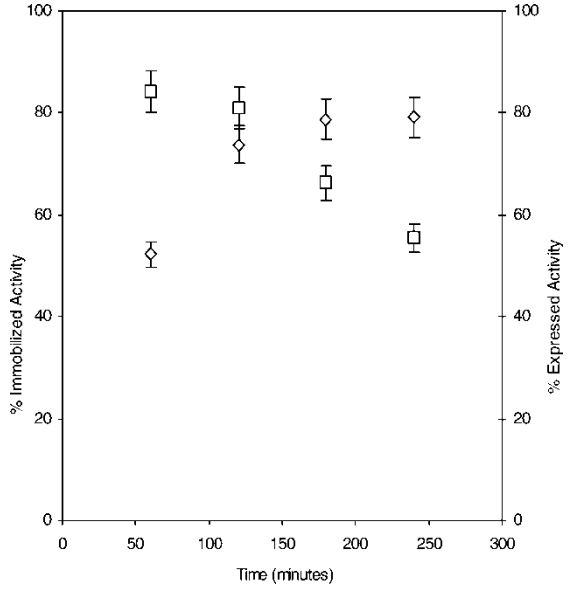

Figure 7. Effect of immobilization time on enzyme activity. ◇ % Immobilized activity; □ % Expressed activity.

lysine (25). The reaction of the glutaraldehyde with these amino acids at the active site of glucose isomerase may cause conformational changes in the enzyme. At higher concentrations of glutaraldehyde, due to more free reactive groups, multiple point attachments increase and may cause more conformational change which might lead to a loss of the enzyme activity on immobilization.

For optimization of the immobilization time, the activated chitosan beads were incubated with the enzyme solutions in different tubes. The enzyme activity of supernatant and that of immobilized enzyme beads were checked from separate tubes at different time intervals. The immobilized activity was calculated from the mass balance of the supernatant activity after immobilization. The expressed activity is the actual activity of the immobilized beads. When the enzyme solution was incubated for immobilization for a longer time, no further increase in the activity of the immobilized enzyme was found. Figure 7 shows that the activity expressed by the immobilized enzyme decreased due to a greater density of bonds per enzyme on increasing time and consequently due to the possible molecular structure deformation.

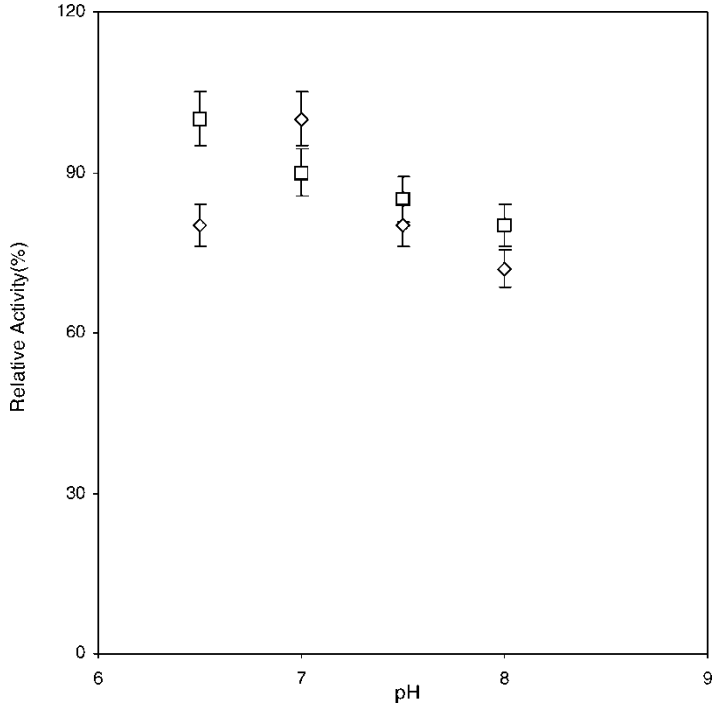

Figure 8. Effect of pH on immobilized glucose isomerase activity. ◇ Free enzyme; □ Immobilized enzyme.

The activity profiles of the native and the immobilized glucose isomerase are presented in Fig. 8. The maximum activity was observed at pH 6.5 and 7.0 for the immobilized and natives enzymes, respectively. The enzyme immobilized on chitosan beads, exhibited a shift in optimal pH of about 0.5 units towards the acidic pH, indicative of the matrix behaving as a polycation (28). It may be assumed that the hydrogen and hydroxyl ions are distributed differently between the area close to the surface and the remainder of the solution, with negative charges clustering close to the immobilized enzyme. A microenvironment is thus formed close to the immobilized enzyme with a higher pH than that of the external solution so that the optimal pH becomes lower than that of the free enzyme. The effect of pH on glucose isomerase stability expressed as the residual activity after one hour of storage at a given pH is shown in Fig. 9. The immobilized enzyme in the acidic range is an attractive property for the enzyme applications because of the use of neutral or lower pH in the isomerization of glucose, prevents formation of D-pscicose (29).

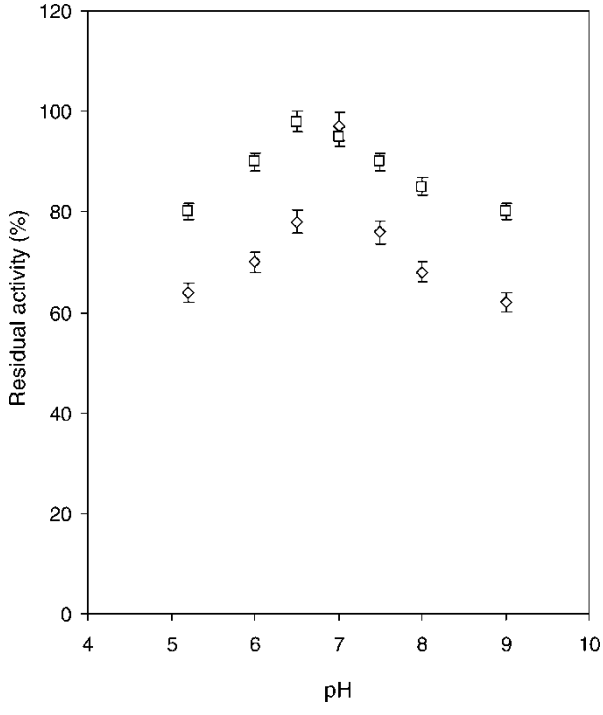

Figure 9. Effect of pH on the stability of immobilized glucose isomerase activity. ◇ Free enzyme; □ Immobilized enzyme.

CONCLUSION

The purification of glucose isomerase has been carried out in a standard PEG-4000/salt system in presence/absence of PEG derivatives. As total proteins and enzyme showed preference to the bottom salt rich phase the use of affinity partitioning in the presence of different PEG derivatives has been found to be useful. The crucial role of the molecular weight of the enzyme and that of the polymer in partitioning was confirmed using a biospecific PEG derivative like PEG-glucoside. Different PEG derivatives were used to purify the enzyme by partitioning other bulk proteins to the top phase. The bulk proteins showed the maximum partition coefficient in the PEG-palmitate substituted two-phase system. An enrichment factor $\sim$5.5 and 73% enzyme recovery in the bottom salt rich phase was achieved after fourth extractions in a cross current mode with the PEG-palmitate derivative. Following the bulk purification with ATPS, the enzyme glucose isomerase was immobilized on chitosan beads. The enzyme, after immobilization studies, was found to be stable in the pH range 5.2–9.0.

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