Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

Purification of Glucose Oxidase and β-Galactosidase by Partitioning in a PEG-Salt Aqueous Two-Phase System in the Presence of PEG-Derivatives

Shrikant B. Dhoot[a]; Juned M. Dalal[a]; Vilas G. Gaikar[a]
[a] Institute of Chemical Technology, University of Mumbai, Matunga, Mumbai

To cite this Article Dhoot, Shrikant B. , Dalal, Juned M. and Gaikar, Vilas G.(2007) 'Purification of Glucose Oxidase and β-Galactosidase by Partitioning in a PEG-Salt Aqueous Two-Phase System in the Presence of PEG-Derivatives', Separation Science and Technology, 42: 8, 1859 — 1881
To link to this Article: DOI: 10.1080/01496390701310355
URL: http://dx.doi.org/10.1080/01496390701310355

Separation Science and Technology, 42: 1859–1881, 2007

ISSN 0149-6395 print/1520-5754 online
DOI: 10.1080/01496390701310355

Purification of Glucose Oxidase and β-Galactosidase by Partitioning in a PEG-Salt Aqueous Two-Phase System in the Presence of PEG-Derivatives

Shrikant B. Dhoot, Juned M. Dalal, and Vilas G. Gaikar
Institute of Chemical Technology, University of Mumbai, Matunga, Mumbai

Abstract: Purification of glucose oxidase from *Aspergillus niger* and that of β-galactosidase from *Kluyveromyces lactis* have been attempted using poly(ethylene glycol) (PEG)-sodium sulfate aqueous two phase system (ATPS) in the presence of PEG-derivatives, i.e. PEG-Coomassie brilliant blue G-250 and PEG-benzoate, PEG-palmitate and PEG-TMA, respectively. The enzymes showed poor partitioning towards the PEG phase in comparison with other proteins in ATPS containing no ligands. Selective partitioning of other proteins was observed towards the PEG phase in the presence of PEG-benzoate and PEG-palmitate enriching β-galactosidase in the salt phase whereas in the case of glucose oxidase, PEG-Coomassie brilliant blue G-250 derivative worked as a better affinity ligand for other proteins. A 19-fold purification was obtained with the PEG dye derivative after 5 stage cross extractions with 80% recovery of glucose oxidase and an enrichment factor upto ~7 for β-galactosidase with the PEG-TMA derivative. The interaction of PEG-benzoate and PEG-TMA ligands with the active site of β-galactosidase has been evaluated by molecular modeling. The effect of the molecular weight of glucose oxidase on its partitioning was confirmed as the molecular simulation shows strong affinity interaction of PEG-glucoside with the enzyme.

Keywords: Aqueous two-phase extraction, *A. niger*, glucose oxidase, *K. lactis*, β-galactosidase, PEG-derivatives, molecular modelling

Revised 9 August 2006, Accepted 5 February 2007

Address correspondence to Vilas G. Gaikar, Institute of Chemical Technology, University of Mumbai, Matunga, Mumbai 400019. Tel.: 91-22-24145616, 19; Ext.: 286; Fax: +91-22-24145614; E-mail: v.g.gaikar@udct.org or v.g.gaikar@gmail.com

INTRODUCTION

The impetus for research and development in bioseparations has been given by complexity in the downstream processing of pharmaceutical and biological products. These products are often present at low concentrations in complex mixtures containing other similar materials. Almost 50 to 90% of the production cost of a typical biological product resides in its purification strategy. Most of these valuable bio-products are vulnerable to enzymatic degradation and physical damage due to extreme pH, temperature, shear conditions, and solvent used in purification process.

Aqueous two-phase systems (ATPS) formed by mixtures of either two hydrophilic polymers or a polymer and a salt provide advantages of biocompatibility, easy processing, and high sensitivity in recognition of any ligand-protein interaction (1–4). The partitioning of proteins in a biphasic system depends on the concentration and molecular weight of the polymer(s), the concentration and type of the salt and pH (5, 6). The partitioning coefficient of a target protein, many a times, is not always different enough from that of other proteins in the crude fermentation broth to allow an efficient separation in a single stage. By introducing an affinity ligand, the partitioning of the ligand-binding protein, however, can be changed (6, 7). This affinity partitioning principle has mostly been carried out with poly(ethylene glycol) bound affinity ligands (7–10).

A number of enzymes have been studied for purification using ATPS loaded with affinity ligands. In some instances, e.g. glucose-6-phosphate dehydrogenase, phosphofructokinase and penicillin G acylase, the change in the partition coefficient of the enzymes has been as large as 3 to 4 orders of magnitude (11, 12). Other enzymes which have been purified using affinity ATPS are alcohol dehydrogenase, amylase, glucoamylase, formate dehydrogenase, catalase, etc. (13). ATPS with ligands are promising for the extraction and the partitioning of the products in opposite phases. The affinity partitioning principle makes use of biospecific ligands (PEG-NADH, PEG-NAD), pseudo-biospecific ligand (PEG-benzoate, PEG-dye), hydrophobic ligand (PEG-palmitate), and ionically interacting ligands (PEG-trimethyl amine, PEG-sulphate) etc. (14–16).

In this paper, we report the purification of glucose oxidase and β-galactosidase from fermentation broths of *Aspergillus niger* and *Kluyveromyces lactis*, respectively, using the PEG-4000-sodium sulfate ATP system. The partitioning of the enzymes is studied in the presence of different PEG derivatives to exploit specific interactions in the system.

MATERIALS AND METHODS

Materials

Organic solvents, PEG-4000, and sodium sulfate were purchased from S. d. fine Chemicals, Mumbai. Coomassie brilliant blue G-250 was obtained

form SRL, Mumbai. Peroxidase, sucrose, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, citric acid, sodium nitrate, and calcium carbonate, 2-mercaptoethanol, and o-nitrophenyl β-D-galactopyranoside (ONPG) were purchased from Sigma (St. Louis, MO, U.S.A.) and Himedia Lab Ltd., Mumbai. Corn steep liquor was obtained from Anil starch, Ahmedabad. All other chemicals were of analytical grade.

Experimental Details

Synthesis of PEG Derivatives

The PEG derivatives. i.e. PEG-TMA, PEG-benzoate, and PEG-palmitate, were prepared by adopting procedures available in literature (12, 17). The PEG-Coomassie brilliant blue G-250 derivative was also prepared following a reported procedure (18). PEG-4000 (5 g) and the dye (0.18 g) were dissolved in 10 cm^3 of water. Subsequently, 0.02 g of KOH was added to the solution and the mixture was heated at 80–90°C for 4 hr in a constant temperature water bath. On addition of chloroform (100 cm^3) to the mixture at room temperature (30°C) the unreacted dye separated out. The thick residue was further treated on a rotavac to remove water and then dissolved in methanol by slightly warming the suspension. The undissolved impurities were removed by filtration. The filtrate was kept at 0°C for 48 hr. The product precipitated as a dark blue colored residue which was separated and washed with excess methanol and kept in a dessicator for drying.

For preparation of the PEG-glucoside derivative, PEG and glucose in the proportion of 2:1 molar ratios were reacted in an aqueous medium in the presence of p-toluene sulfonic acid (PTSA) (1% w/v) as a catalyst at 70°C with continuous stirring for 2 hr. The reaction mixture was then neutralized and the excess of water was removed. The thick syrup like liquid was then kept overnight in the dessicator for drying. The dry product was extracted with dichloromethane (DCM) and recovered by removing DCM by simple distillation. The Fehlings test was performed to confirm the formation of glycoside derivative (19).

Production of Glucose Oxidase

A fungal strain of *Aspergillus niger* (NCIM 545), obtained from National Chemical Laboratory, Pune (India), was grown in an optimized medium of sucrose (7% w/v), KH_2PO_4, $MgSO_4 \cdot 7H_2O$, citric acid (0.025% w/v), $FeCl_3 \cdot 6H_2O$ (0.001% w/v), sodium nitrate (0.4% w/v), calcium carbonate (2% w/v), and CSL (2% w/v) at pH 7 and temperature 25–30°C. The fermentation was carried out for 48 hr on a rotary shaker at 250 rpm. After fermentation the cells were harvested by centrifugation for 30 min at 10,000 rpm. The supernatant was used for the extraction of glucose oxidase.

Production of β-Galactosidase

For the production of β-galactosidase, *Kluyveromyces lactis* was grown on a liquid medium containing lactose (2.5%), malt extract (0.25%), yeast extract (0.25%), urea (0.25%), KH_2PO_4 (0.25%), $MgSO_4 \cdot 7H_2O$ (0.06%), $CaCl_2 \cdot 2H_2O$ (0.01%) at pH 7.0 and room temperature on a rotary shaker (175 rpm) for 18 hr.

In both the cases the fermentation medium was sterilized by autoclaving at 120°C (15 psi steam) for 20 min. A 2 cm^3 microbial suspension in a physiological saline solution (0.90% w/v) was prepared from a fully grown culture on the slant and used as an inoculum.

β-Galactosidase, being an intracellular enzyme, had to be recovered by subjecting the cells to ultrasonication. The cells were first harvested by centrifuging the cell suspension at 10,000 rpm for 20 min. The cells were collected, washed with phosphate buffer (pH 7.0), and again re-suspended in a fresh phosphate buffer solution at pH 7.0. This suspension was then sonicated at 50 MHz for 20 min. in "Dakshin" ultrasonic horn in an ice bath so that the temperature of the broth during the sonication did not rise above 10°C. The resultant broth with the cell lysate was centrifuged at 10,000 rpm for 20 min. The clear cell free supernatant was used as a crude extract for further 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 crude extract. The equilibrium phase diagram of PEG-4000/sodium sulphate/water system has been reported (20). In other partitioning experiments, a part of the PEG was replaced by a PEG derivative. The resultant solutions were equilibrated by mixing and then the phases were separated by centrifugation. After the phases were separated, the volume of each phase was noted and both phases were analyzed for the enzyme activity and protein content.

Determination of Glucose Oxidase, β-Galactosidase Activity and Protein Content

Glucose oxidase activity was measured by *o*-dianisidine-peroxidase method (21). The activity of the β-galactosidase was measured by ONPG assay method (22).

The total protein concentration was measured by the Bradford method (23). In the presence of the PEG-dye, the proteins partitioned in the top phase were estimated from the mass balance.

Multiple Extractions

Multiple extractions were carried out in a cross-current mode to study the effective separation of the enzymes. The optimized concentrations of PEG-TMA and PEG-dye derivatives in the total PEG was used for these studies for β-galactosidase and glucose oxidase, respectively.

Molecular Modeling Study

Hyperchem version 7.0, (Hypercube Inc.) was used to study the interactions of the enzymes with PEG derivatives in an aqueous environment. The docking of the ligand into the substrate binding pocket of β-galactosidase (or glucose oxidase) with the MM method was carried out to form the non-covalently bonded Michaelis complex using the AMBER-96 force field. The minimum energy of the ligand, PEG, and active site was calculated under the solvated conditions. In the interaction studies, two structures, i.e. the enzyme and the ligand were brought together and the optimization of the complex was performed starting with several different initial conditions. The lowest energy configuration was selected as the final conformation.

Partitioning Study in ATPS

The partition coefficient of proteins between the two phases, K_P, is defined as a ratio of the concentration of the proteins (mg/cm^3) in the top PEG phase to that of the proteins in the bottom salt phase. Similarly for the enzyme, the partition coefficient, K_E, defined as the ratio of activities (units/cm^3) in the top PEG and bottom salt phases.

The % enzyme recovery (% R_e) in each phase of the ATP system is defined as

$$\% \text{ Re} = \frac{A}{A_i} \times 100 \tag{1}$$

where A is the activity in either phase after partitioning while A_i is the initial activity in the solution (or broth) before the extraction.

RESULTS AND DISCUSSION

Partitioning in PEG-Na_2SO_4 Two-Phase System without PEG-Derivatives

In the absence of any PEG-derivative in the standard ATP system of PEG-4000 (10% w/w) and sodium sulphate (7.5% w/w), both, total proteins and the enzyme(s) (glucose oxidase as well as β-galactosidase), showed a

strong preference for the salt-rich phase. This showed no separation of the enzyme from other proteins as nearly all the proteins and the enzymes tend to stay in the bottom phase. Both the enzymes have high molecular weight (glucose oxidase: 160 kD and β-galactosidase: 540–600 kD). The excluded volume effect of PEG in the top phase restricts the transfer of enzyme(s) to the PEG phase. The increase in molecular weight of PEG is known to reduce the partition coefficient of high molecular weight enzymes such as alcohol dehydrogenase (ADH) (150 kD) from 1.6 to 0.05 (24).

The partitioning studies for β-galactosidase were carried out at different concentrations of PEG-4000 and Na_2SO_4. As the salt concentration was increased, both total proteins and the enzyme partitioned relatively more to the PEG rich phase. Both the enzyme and the total proteins therefore show salting-out at higher salt concentrations and subsequently a slightly increased partitioning towards the PEG phase. At 16% (w/w) Na_2SO_4 and 14% (w/w) PEG-4000, the maximum difference in the K values of the enzyme and total proteins was observed (Table 1).

Partitioning of Glucose Oxidase and Total Proteins in the Presence of PEG-Derivatives

PEG-Glucoside as Affinity Extractant

Since glucose is a natural substrate for glucose oxidase, the PEG-glucoside derivative was expected to interact very selectively with the enzyme. The increasing percentage of PEG-glucoside in the total PEG content of the system showed a slightly decreased enzyme activity in the salt phase indicating its transfer to the top PEG phase. However, the enzyme activity in the top PEG phase was much lower than that estimated from the balance on the enzyme activity. The decreased activity of the enzyme in the PEG phase having PEG glucoside could be due to specific and strong interaction of PEG glucoside with the active site of the enzyme. We show later by molecular simulation that the PEG-ligand indeed shows specific interaction

Table 1. Effect of different phase composition on the partitioning of proteins and β-galactosidase

Na_2SO_4 (%w/w)	K_{enzyme}	$K_{protein}$
10	0.16	0.58
12	0.20	1.2
14	0.28	1.19
15	0.32	1.19
16	0.41	1.6

K = Partition coefficient.

with the enzyme. The limited transfer of the enzyme, from the activity balance in the salt-rich phase, however, still showed its poor partitioning towards the PEG phase.

On further increasing the PEG-glucoside content, beyond 6% of the total PEG of the system the activity of the enzyme remained constant in the PEG phase although its activity balance in the salt phase indicated it transferred more to the PEG phase. The activity balance in the two phases did not match the initial total activity in all the cases. At the same time the partitioning of proteins towards the top phase also increased slightly (Fig. 1).

PEG-Dye as Affinity Extractant for Other Proteins

Since, the enzyme remains substantially in the bottom phase even in the presence of a specific ligand (PEG-glucoside), it was decided to use the PEG derivatives which can preferentially bind to other proteins and transfer them to the PEG phase. In such a case, the enzyme can be recovered in purified form in the salt phase. The dye, Coomassie brilliant blue G-250, which is routinely used for protein estimation, was considered for the purpose (23).

The effect of PEG-Coomassie blue G-250 (PEG-dye) content in the total PEG as phase forming polymer on the partitioning of proteins and the enzyme is shown in Fig. 2. When the PEG-dye content in the total PEG of the system

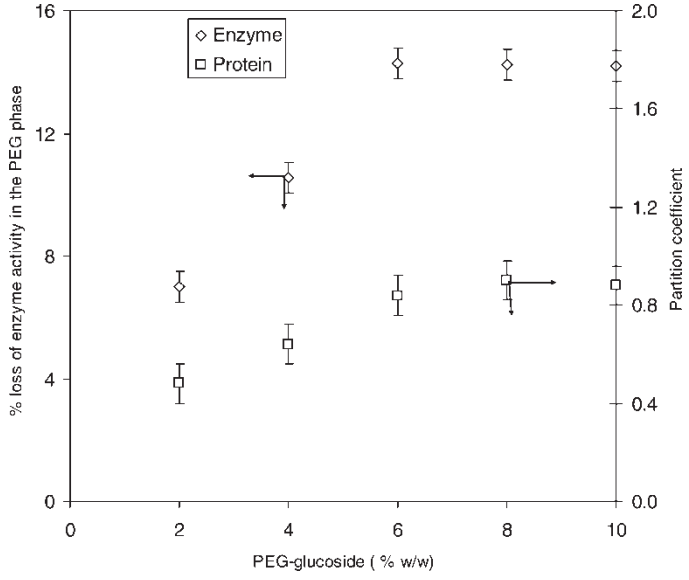

Figure 1. Loss of enzyme activity in PEG phase and partitioning in PEG-glucoside ATPS (pH 5.5). ◇: Enzyme (glucose oxidase), □: Protein.

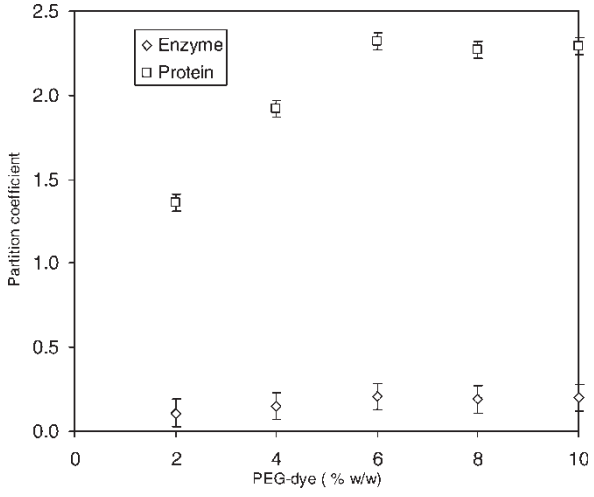

Figure 2. Partitioning of glucose oxidase and proteins in PEG-dye ATPs (pH 5.5). ◇: Enzyme (glucose oxidase), □: Protein.

was increased, from 2% to 10% (w/w), the partition coefficient of the proteins increased from 0.2 to 2.3 transferring majority of the proteins to the top PEG phase. Above 6% of the PEG-dye content of total PEG, the protein transfer remained constant. The K values for the proteins, therefore, showed a typical saturation behavior. But most importantly, the presence of the PEG-dye in the PEG phase resulted in specific extraction of other proteins into the upper phase without affecting the enzyme's partition behaviour. The effectiveness of the PEG-dye expressed as $\Delta \log K_{proteins}$ i.e. ($\log K_{with\ ligand} - \log K_{without\ ligand}$), increases with increasing the PEG-dye content in the system (Table 2).

Coomassie brilliant blue G-250 dye binds to proteins at argenine, tryptophan, tyrosine, and phenylalanine residues in the anionic form (23). The increase in the partitioning of proteins to the top phase occurs because of the combined effect (electrostatic as well as hydrophobic) of the dye. The effect of hydrophobicity of the proteins and of ATP forming polymers on the partitioning of proteins was shown to be important by Johansson and Shanbhag (8). In the presence of high salt concentrations the hydrophobic interactions are more effective in such a system (24).

Glucose oxidase showed a marginal increase in its K values with the increasing PEG-dye content of the system (Fig. 2). But the maximum amount of the enzyme partitioned to the top phase was still just 11%. In order to reduce even this loss of enzyme to the top phase, the partitioning studies were conducted at different pH conditions using the 6% PEG-dye system. Figure 3 shows that at pH 3.8, the maximum transfer of proteins (nearly 58%) was in a single stage with approximately 4% enzyme getting

Table 2. Comparison of log K values of proteins in a standard two-phase system and affinity two-phase system (glucose oxidase)

$\log K_{proteins}$ in standard system		$\log K_{proteins}$ and $\triangle \log K$ value for protein in affinity system with varying concentration of dye ligand at pH 5.5		
pH	$\log K_{proteins}$	Ligand concentration in % of total PEG	$\log K_{proteins}$	$\Delta \log K$
3.8	−1.78	2	0.30	1.6
4.5	−1.77	4	0.65	1.95
5.0	−1.77	6	0.84	2.14
5.5	−1.30	8	0.81	2.11
6.0	−1.42	10	0.82	2.12

$\Delta \log K$ ($=\log K_{proteins} - \log K_{enzyme}$) shows the efficiency of affinity ATPs.

transferred to the top phase. On further increasing the pH of the system, the enzyme partitioning to the top phase also increased but a reverse trend was observed for the proteins. The isoelectric point (pI) of the glucose oxidase obtained from *A. niger*, which is composed of at least six components, ranges from pH 3.9 to 4.3. The dye in the solution remains in the cationic form (23). Hence with increasing pH, i.e. above its pI, the net charge on

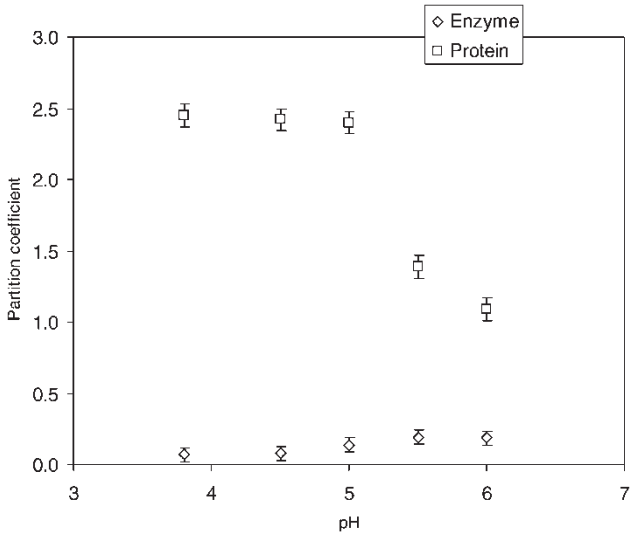

Figure 3. Effect of pH on partitioning of enzyme and protein in PEG-dye ATPs (PEG-dye, 6% w/w). ◇: Enzyme (glucose oxidase), □: Protein.

the enzyme becomes negative and the dye remains in a cationic form which becomes responsible for the slight increase in the partition coefficient of the enzyme towards the PEG phase containing the PEG-dye derivative. The opposite behavior of the proteins also can be explained on the basis of the dye's electrostatic interaction with the proteins. The PEG bound dyes are known to strongly interact with proteins at low pH conditions (25, 26). These results show that proteins can be selectively extracted into the PEG-phase in the presence of the PEG-dye derivative at low pH conditions, leaving the enzyme in the salt phase. The dye derivative remains in the top phase of the system and the results indicate the change in the partition behavior has to be because of the presence of the PEG-dye in the PEG phase.

Partitioning of β-Galactosidase and Proteins in the Presence of PEG-Derivatives

Partitioning of Enzyme and Proteins using PEG-Benzoate

Partitioning studies conducted by replacing a part of total PEG content with PEG-benzoate revealed that as the PEG-benzoate content was increased the partitioning of the bulk proteins increased towards the top PEG rich phase (Figs. 4 and 5). At high salt concentrations, as in the polymer-salt ATP system, the proteins with large and exposed hydrophobic surface residues,

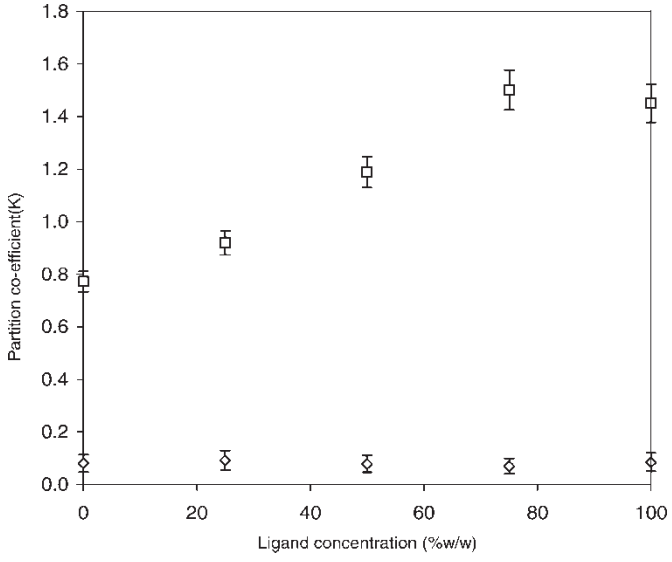

Figure 4. Partitioning of enzyme and proteins in PEG-benzoate (PEG-Bz) substituted ATPS. ◇: Enzyme (β-galactosidase), □: Protein.

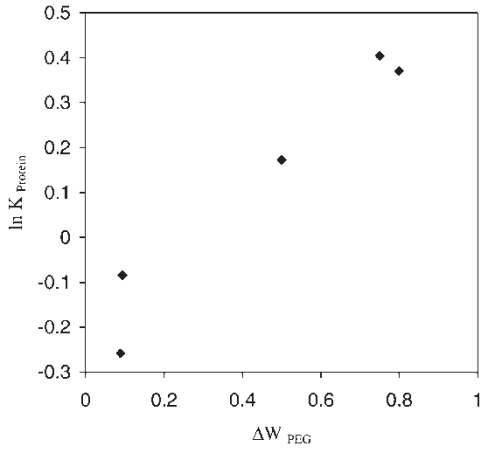

Figure 5. Plot of ln $K_{Protein}$ vs the phase composition difference in the PEG in the top and bottom phases for different concentrations of PEG-benzoate.

experience the "salting out" effect and hence show an affinity for the PEG-benzoate rich PEG phase. Salts such as phosphates and sulfates, by their desolvation effect, facilitate the approach of the ligand to the hydrophobic regions on the protein surface. With the increased concentration of the ligand, more molecules become available to bind to the proteins. Only other proteins are selectively partitioned to the PEG phase indicating relatively higher hydrophobic residues on their surfaces. Figure 4 shows that the enzyme, however, remains in the bottom salt phase. This has been attributed to its high molecular weight and thus exclusion from the PEG rich phase as well as due to the poor hydrophobic surface nature. Figure 5 correlates the partition behavior to the phase composition difference in the system indicating substantial effect of the presence of the PEG-derivative on the partitioning behavior of the proteins. The increased percentage of the PEG derivative also increases the phase difference of the system. The percentage recovery and the enrichment factor for the enzyme in the bottom phase are shown in Fig. 6. A 3.5 purification fold was observed in a single stage operation when 75% of the PEG was replaced with PEG-benzoate. No specific or non-specific interaction is, therefore, expected between the PEG-benzoate and the enzyme.

Partitioning of the Enzyme and Proteins using PEG-Palmitate

The fatty acid derivatives of PEG also show increased hydrophobic affinity towards the proteins (27). PEG-palmitate was, therefore, used for the partitioning studies at different concentrations. The partition coefficient of the proteins was much higher in the presence of the PEG-palmitate (Figs. 7 and 8), clearly indicating the hydrophobic effect of the PEG derivative.

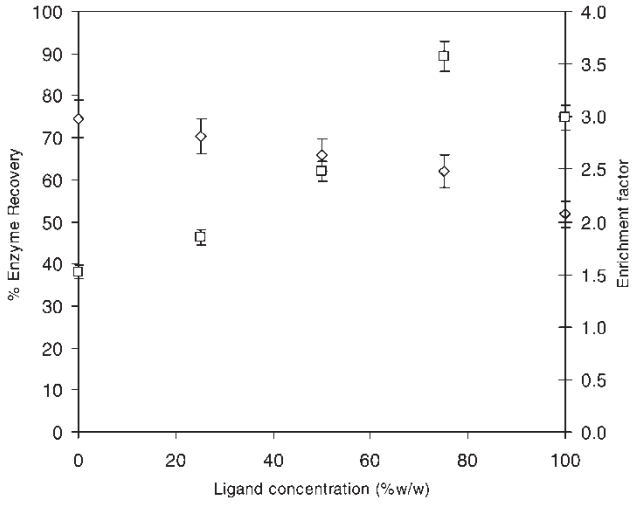

Figure 6. Percent enzyme recovery and enrichment factor in bottom phase with PEG-Bz substituted ATPS. ◇: % Enzyme recovery (β-galactosidase), □: Enrichment factor.

As shown earlier in Fig. 5, the presence of PEG-palmitate increases the phase composition difference in the system which can be related to the partitioning of proteins (Fig. 8). The maximum partitioning of the proteins occurred at 30% of the PEG-palmitate in the total PEG. The partitioning of the enzyme

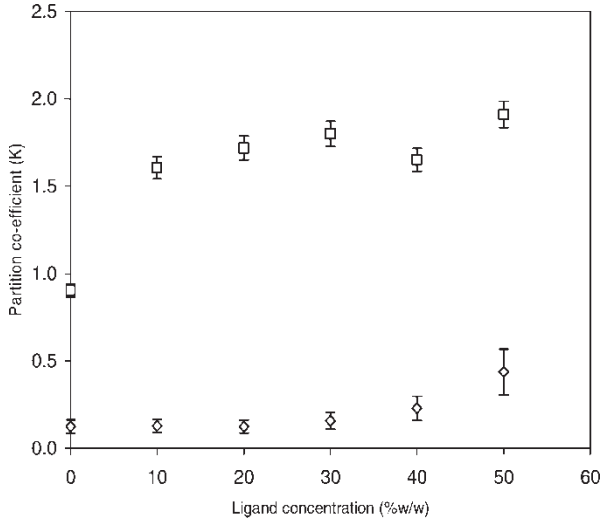

Figure 7. Partitioning of enzyme and proteins in PEG-palmitate substituted ATPs. ◇: Enzyme (β-galactosidase), □: Protein.

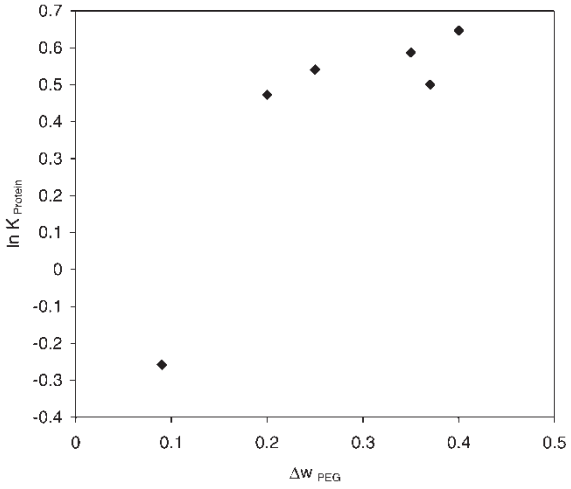

Figure 8. Plot of ln $K_{Protein}$ vs the phase composition difference in the PEG in the top and bottom phases for different concentrations of PEG-palmitate.

showed, on the other hand, no significant effect of increasing content of PEG-palmitate in the total system. The partitioning of a protein depends on the hydrophobicities of the individually exposed amino acid residues on the protein surface. Proteins with exposed hydrophobic surfaces had been selectively partitioned earlier towards the PEG phase using the palmityl derivative of PEG (27). The amino acid composition of the peptide chains of β-galactosidase shows relatively high levels of glutamic acid (124) and aspartic acid (105) and lower levels of hydrophobic residues like valine (64), isoleucine (38), and phenylalanine (38) (28). Hence, their interaction with the hydrophobic side chains of PEG-palmitate is expected to be less prominent. Moreover, salts facilitate firm binding of the hydrophobic patches of a protein with the hydrophobic ligands. The enrichment factor of 3.1 in a single stage was obtained with optimum ligand concentration i.e. 30% in the total PEG content (Fig. 9).

Partitioning of the Enzyme and Proteins using PEG-TMA

The partitioning with increasing concentration of PEG-TMA was conducted to evaluate the importance of electrostatic interactions. Figure 10 shows that as the PEG-ligand content was increased from 10% to 80% of the total PEG, the partitioning of the proteins to the top phase also increased. PEG-TMA is a positively charged ligand and shows electrostatic affinity for the proteins which are not negatively charged at the experimental pH. The presence of some negatively charged residues like aspartic acid and glutamic acid on the surface of bulk proteins is supposed to be responsible

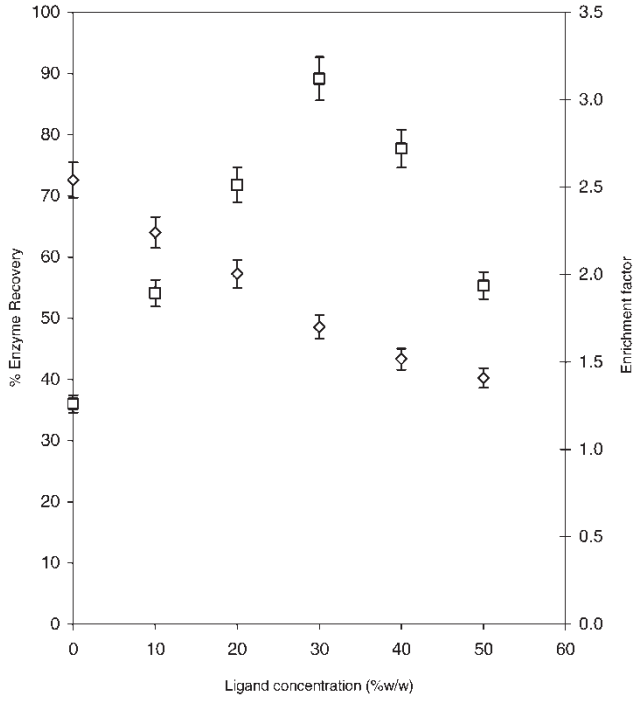

Figure 9. Percent enzyme recovery and enrichment factor in PEG-palmitate substituted ATPS. ◇: % Enzyme recovery (β-galactosidase), □: Enrichment factor.

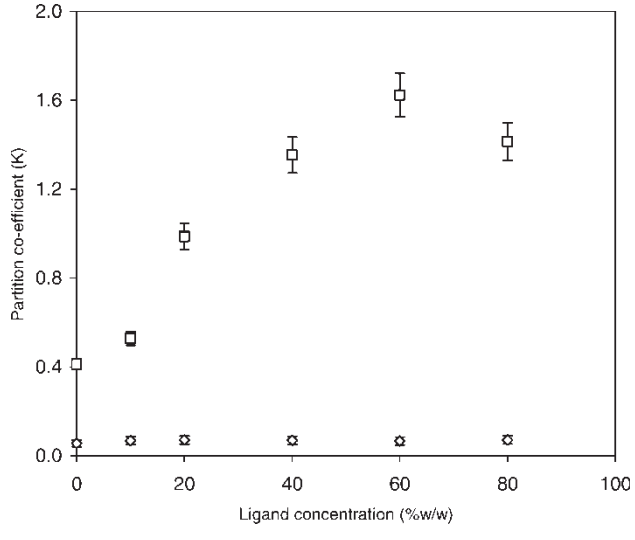

Figure 10. Partitioning of enzyme and proteins in PEG-trimethyl amine (PEG-TMA) substituted ATPS. ◇: Enzyme (β-galactosidase), □: Protein.

for the increase in the partition coefficient of the proteins. The pI of β-galactosidase is between 4.1–4.5 and hence at the pH of fermentation broth (pH 7.0), it is negatively charged. But still there is no significant partitioning of the enzyme to the top phase where the positively charged PEG-TMA is present. It is obvious that because of a very high molecular weight of the enzyme it is excluded by the PEG phase. The results of pH studies on partitioning of the enzyme and proteins are shown in Fig. 11.

Multistage Cross-Current Extraction

The total proteins showed higher partition coefficient in the presence of different PEG derivatives while the enzymes still remained in the bottom phase. This fact was exploited to purify the enzymes by conducting multiple extractions in a cross-current mode, where the maximum proteins were extracted into the top PEG phase, leaving the enzymes in the purer form in the bottom salt phase. For these multiple extractions, the % extraction has been defined with respect to the enzyme activity (or protein concentration) in the salt raffinate phase obtained from the previous extraction stage.

Glucose Oxidase

The other proteins showed a much higher partition coefficient in the affinity PEG (PEG-dye)-salt system than that of the enzyme. Figure 12 shows the

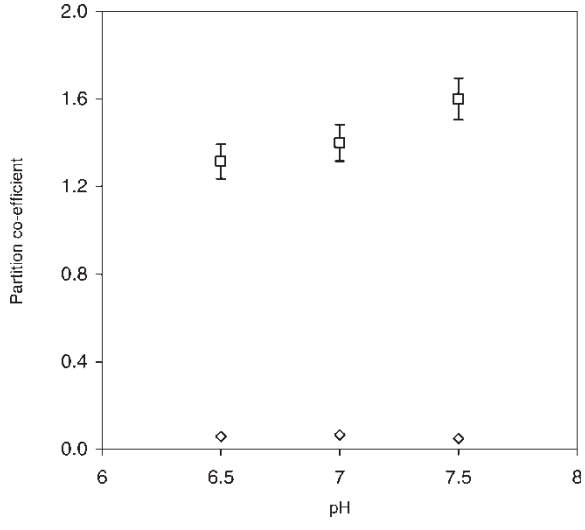

Figure 11. Effect of pH on partitioning in presence of PEG-TMA. ◇: Enzyme (β-galactosidase), □: Protein.

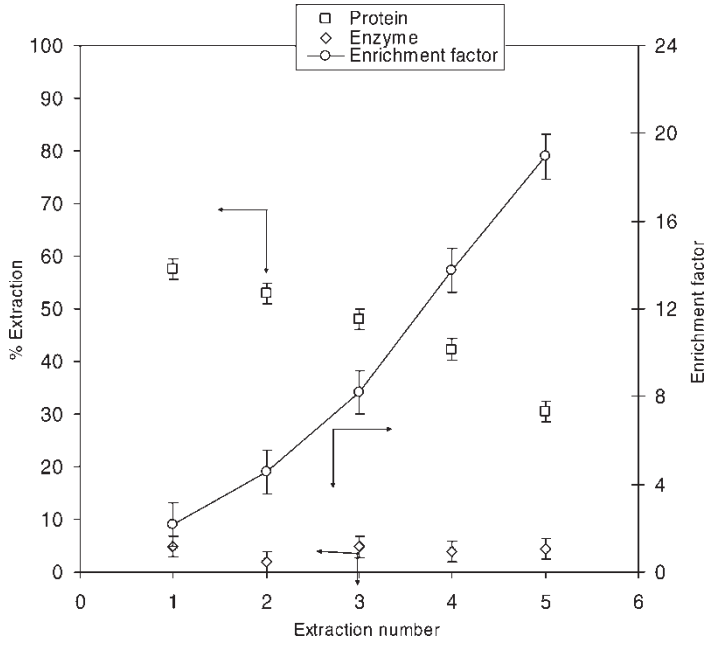

Figure 12. Percent extraction of protein and glucose oxidase and enrichment factor of glucose oxidase at each stage in multistage cross-current extraction from PEG-dye ATPS (PEG-dye 6% w/w and pH 3.8). ◇: % Enzyme extracted, □: % Protein extracted, ○: Enrichment factor.

extraction of proteins in a system containing PEG-dye (6% w/w) after each stage of extraction along with the respective enrichment factor. With increasing extraction cycles, the proteins partitioning towards the top phase decreased moderately after each stage. After five repeated extractions, $\sim 80\%$ of the initial glucose oxidase activity of the broth remained in the bottom salt phase with $\sim$19 fold purification.

The electrostatic interactions prevail at low ionic strength and hydrophobic interactions at high salt concentrations (29). Therefore, the high salt concentrations used in the system were also responsible for forcing the hydrophobic interactions. Salts, such as phosphates and sulfates, promote the hydrophobic affinity (30). However, the dye based ligands are known to combine ionic and hydrophobic interactions in a way that is not easily predictable. Water in deep grooves of biomolecules may have substantially higher fugacity than in the bulk solution. Displacement of such water, at high ionic strength, can add to the solvent entropic effect and thus contribute to the enhanced ligand binding (31). The antichaotropic salts, by their desolvation effect, facilitate the immobilized ligand approach into the regions that are otherwise not easily accessible to the solutes. The displacement of water is a prerequisite for short range forces like those between dipole-dipole,

dipole-induced dipole, and charge transfer. The salt used in this experimental system, according to Hofmeister series, is antichaotropic, i.e. water structuring. These salts provide an advantage that they frequently stabilize the proteins in the separation medium at high concentrations.

β-Galactosidase

Figure 13 shows the partitioning of the proteins in a system containing PEG with PEG-TMA, at pH between 7–7.5, after each stage of extraction in a multiple extraction system. After five repeated extractions, the amount of enzyme that remained in the bottom salt phase was very high, i.e. the final enzyme recovery was 75–80%, with ~7 fold purification. At high salt concentrations the electrostatic interactions are diminished. So after each extraction stage, the increasing concentration of the salt in the bottom phase reduces the electrostatic interaction between PEG-TMA and bulk proteins. It is also possible that the proteins, remaining in the bottom phase after the extraction compared to the initial crude broth, did not show affinity towards the ligand and hence contribute to the decreased partitioning of the proteins with increasing number of the extraction cycles.

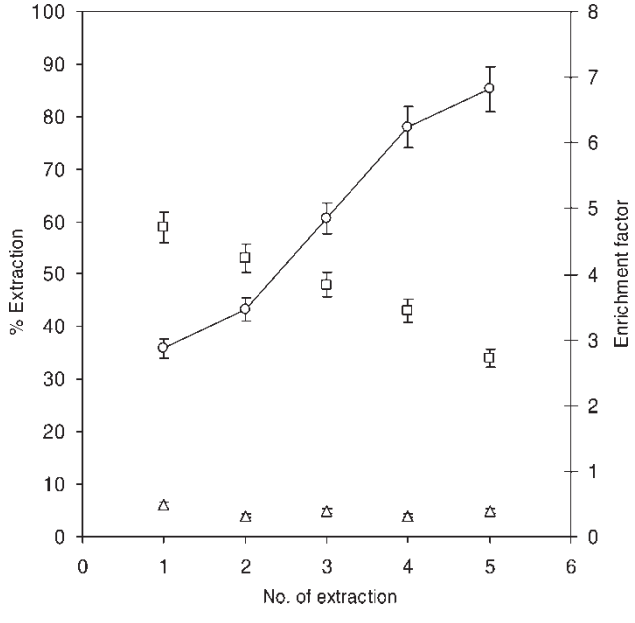

Figure 13. Effect of multiple extractions on the partitioning of enzyme (β-galactosidase) and proteins in PEG-TMA (60% w/w) substituted ATPS. ◇: % Enzyme extracted, □: % Protein extracted, ○: Enrichment factor.

Molecular Modeling

Glucose oxidase showed no preferential partitioning to the top PEG phase even when a specific PEG-glucoside derivative was used. In order to verify that the molecular weight of the enzyme is important in its partitioning behavior, affinity interactions of PEG and PEG-glucoside with the active site of glucose oxidase were evaluated by molecular simulation. A small chain of PEG of molecular weight 300 was used to mimic the large polymer. As only a part of the affinity ligand was responsible for the interactions with the protein, this part was considered to be sufficient for the energy minimization and further interaction studies. *A. niger* glucose oxidase structure was obtained from the protein data bank (32). The enzymatic reaction of glucose oxidase is divided into two steps. In the reductive half reaction, β-D-glucose is converted to *d*-gluconolactone whereas in the oxidative half reaction the enzyme is oxidized by molecular oxygen yielding hydrogen peroxide. Hence, the active site region was restricted to residues being potentially involved in the redox reaction i.e. His 516, His 559, and Glu 412 (Fig. 14a). The immediate neighbors of the catalytically active part which do not participate in a direct way in a chemical reaction (Tyr 68, Phe 414, Trp 426) were included in the active site to take into account the influence of the protein environment on the active site. The interaction studies of PEG-glucoside and enzyme active site were conducted in solvated conditions. Periodic boundaries were applied by filling the space around the ligand and glucose oxidase by discrete water molecules.

The energy minimization process involved systematically altering the coordinates of the ligand until the minimum energy was reached. The glucose oxidase active site and ligand were brought together in solvated conditions and the optimization of the complex was conducted for different orientations of the ligand. The lowest energy configuration was selected as the most stabilized configuration of the enzyme-ligand complex.

The energy of interaction of the glucose oxidase with different ligands was estimated from the difference in energy of the associated complex from the total energy of the individual species in the complex. From Fig. 14b it is clear that no specific interaction of PEG with the active site takes place though it contains the terminal hydroxyl group. The interaction energy of the solvated complex of the glucose oxidase and PEG was estimated -936.9 kJ/mol.

The optimized structure of the PEG-glucoside and glucose oxidase complex is shown in Fig. 14c. The interaction energy was found to be -4647.7 kJ/mol. Despite such high interaction energy which indicates very specific interaction between two species, the enzyme was not partitioned to that extent to the PEG phase suggesting the molecular weight of the enzyme has a crucial role in deciding its partitioning. The optimized structure of the hydrated complex of the enzyme-ligand shows the formation of three hydrogen bonds where glucose is directed roughly between His 516 and His 559.

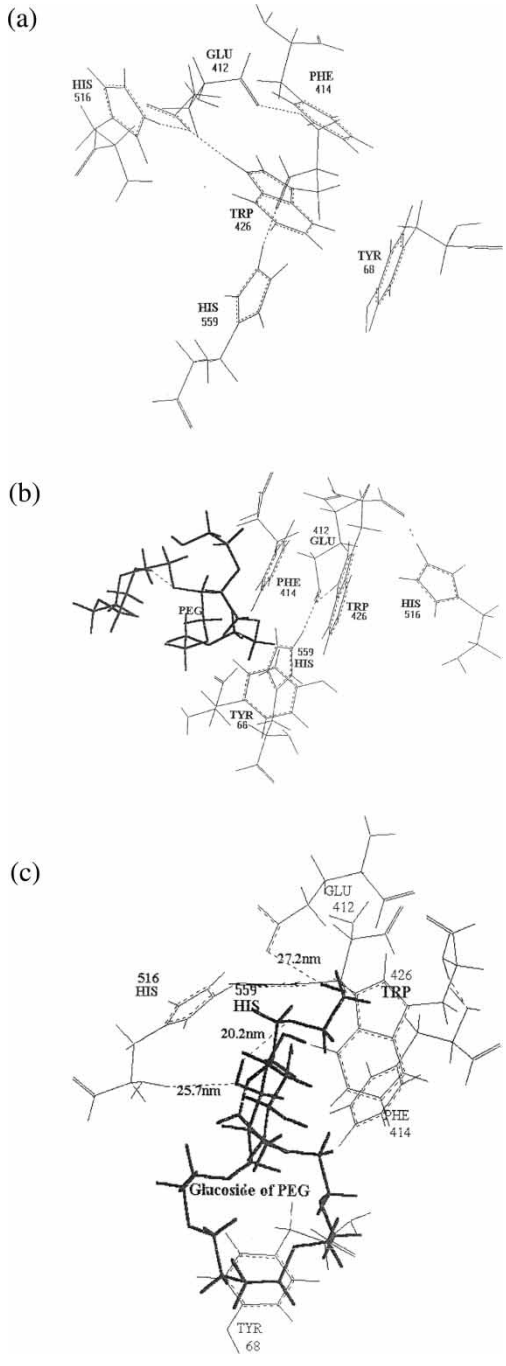

Figure 14. Optimized structure of glucose oxidase active site (a); PEG and glucose oxidase active site (b); PEG-glucoside and glucose oxidase active site (c).

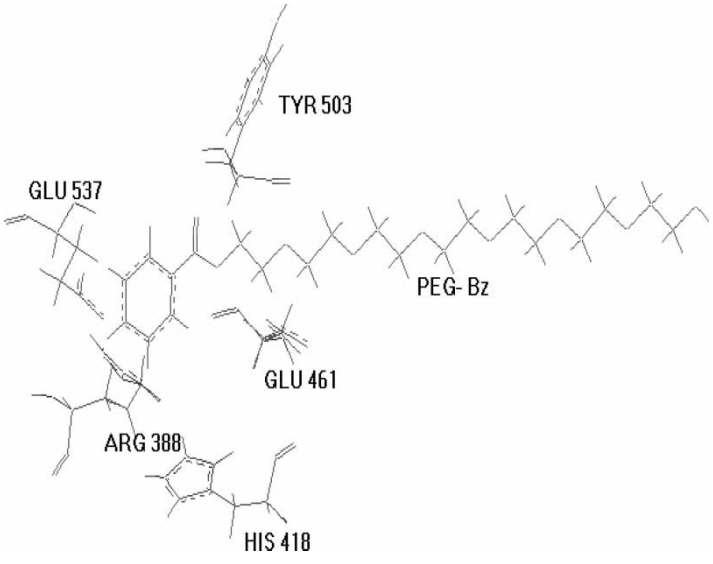

Figure 15. Optimized structure of PEG-benzoate and β-galactosidase active site.

β-Galactosidase

The interaction energy of the solvated complex of the β-galactosidase active site and PEG was estimated to be -3267 kJ/mol. PEG-benzoate and PEG-TMA also have been evaluated to verify possible interactions of the ligand with active site of the β-galactosidase. Figure 15 shows the optimized structure of the enzyme active site and PEG-benzoate molecule.

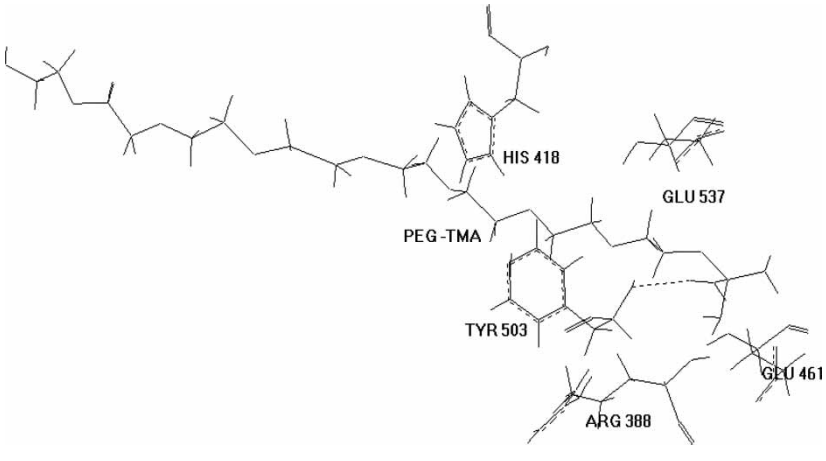

Figure 16. Optimized structure of PEG-TMA and β-galactosidase active site.

There is no specific interaction of the ligand with the active site of the enzyme. The interaction energy was -40.32 kJ/mol. Figure 16 shows the optimized structure of the enzyme active site with PEG-TMA. The amino group of the tyrosine residue formed a hydrogen bond with terminal -NH_3 of the ligand. The interaction energy of the solvated complex of the enzyme and PEG-TMA was estimated -84.4 kJ/mol. These studies showed that despite the possible interactions of the β-galactosidase with the polymer, the volume exclusion effect ensured that the enzyme was excluded from the PEG phase.

CONCLUSION

The enzymes, glucose oxidase and β-galactosidase, are recovered from the fermentation broth of *A. niger* and *K. lactis,* respectively, in a few cross-current extraction stages, using PEG-dye and PEG-TMA as affinity ligands, respectively. A $\sim$19 fold purification with $\sim$80% recovery of glucose oxidase whereas a $\sim$7 fold purification with similar percentage recovery was obtained for β-galactosidase. The molecular simulation confirmed the specific interaction of PEG-glucoside with the enzyme active site revealing that the molecular weight of the enzyme has a crucial role in its partitioning. The molecular modelling study with PEG-benzoate and PEG-TMA for β-galactosidase also indicate the prominent effect of the size of the partitioning enzyme(s).

REFERENCES

1. Mattiasson, B. (1983) Applications of aqueous two-phase systems in biotechnology. *Trends Biotechnol.*, 1: 16–20.
2. Hustedt, H. et al. (1985) Protein recovery using two-phase systems. *Trends Biotechnol.*, 3: 139–144.
3. Johansson, G. and Joelsson, M. (1985) Preparation of cibacron blue F3G-A (polyethylene glycol) in large scale for use in affinity partitioning. *Biotechnol. Bioeng.*, 27: 621–625.
4. Sharp, K.A., Brooks, D.E., and Fisher, D. (1985) Theoretical aspect of partitioning. In *Partitioning in Aqueous Two Phase System: Theory, Methods, Uses, and Applications to Biotechnology*; Walter, H., Brooks, D.E., and Fisher, D. (eds.); Academic Press: London, 11–84.
5. Albertsson, P.A. (1971) *Partition of Cell Particles and Macromolecules*, 2nd Ed.; Almqvist and Wiksell: Stockholm.
6. Johansson, G. (1984) Affinity partitioning. *Methods in Enzymol.*, 104: 356–364.
7. Flanagan, S.D. and Barondes, S.H. (1975) Affinity partitioning: purification of proteins using two-phase systems. *J. Bio. Chem.*, 250: 1484–1489.
8. Johansson, G. and Shanbhag, V.P. (1984) Affinity partitioning of proteins in aqueous two-phase systems containing polymer bound fatty acids. *J. Chromatogr.*, 284: 63–72.

9. Chaabouni, A. and Dellacherie, E. (1979) Affinity partition of proteins in aqueous two-phase systems containing PEG-bound ligand and charged dextran. *J. Chromatogr.*, 171: 135–143.
10. Kula, M.R., Kroner, K.H., and Hustedt, H. (1982) Purification of enzymes by liquid-liquid extraction. In *Advances in Biochemical Engineering*; Fiechter, A. (ed.); Springer-Verlag: Berlin/New York, Vol. 24, 73–117.
11. Johansson, G. and Andersson, M. (1984) Parameters determining affinity partitioning of yeast enzyme using polymer bound triazine dye ligand. *J. Chromatog.*, 303: 39–51.
12. Gavasane, M.R. and Gaikar, V.G. (2003) Aqueous two-phase affinity partitioning of penicillin acylase from *E.coli* in presence of PEG-derivatives. *Enz. Microbiol. Technol.*, 32: 665–675.
13. Clark, W.M. and Sandler, S.I. (1988) Affinity partitioning and its potential in biotechnology. *Sep. Sci. Technol.*, 23: 761–783.
14. Xu, Y. et al. (2003) Liquid-liquid extraction of enzymes by affinity aqueous two-phase systems. *Brazilian Archives Biology Technol.*, 46: 741–750.
15. Mosbach, K. (1974) AMP and NAD as general ligands. In *Methods Enzymology—Affinity Techniques in Enzyme Purification: Part B*; Jakoby, W.B. and Wilchek, M. (eds.); Academic Press: NY, Vol. 34, 229.
16. Mondal, K. and Gupta, M.N. (2006) The affinity concept in bioseparation: Evolving paradigms and expanding range of applications. *Biomol. Eng.*, 23: 59–76.
17. Harris, J.M. and Yalpani, M. (1985) Polymer ligands used in affinity partitioning and their synthesis. In *Partitioning in Aqueous Two Phase System: Theory, Methods, Uses, and Applications to Biotechnology*; Walter, H., Brooks, D.E., and Fisher, D. (eds.); Academic Press: London, 590.
18. Harris, J.M. et al. (1984) Synthesis and characterisation of poly(ethylene glycol) derivative. *J. Poly. Sci: Polym Chem.*, 22: 341–352.
19. Bachkov, A.F. and Zaikov, G.M. (1979) *Chemistry of the O-Glycosidic Bond*; Pergaman press: Oxford, 177–201.
20. Pathak, S.P. et al. (1991) New salt-polyethylene glycol systems for two-phase aqueous extraction. *Chem Eng J.*, 46 (1): B31–B34.
21. Assay method of glucose oxidase (Sigma-Aldrich, USA product catalogue for glucose oxidase).
22. Dickson, R.C., Dickson, L.R., and Markin, J.R. (1979) Purification and properties of inducible beta-galactosidase isolated from yeast *Klyuveromyces lactis*. *J. Bacteriol.*, 137: 51–61.
23. Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principles of protein-dye binding. *Anal. Biochem.*, 72: 248–254.
24. Kuboi, R., Wang, W.H., and Komasawa, I. (1992) Aqueous two-phase extraction of dehydrogenases using triazine dyes in PEG/phosphate systems. *Chem. Eng. Sci.*, 47: 113–121.
25. Dean, P.D.G. and Watson, D.H. (1979) Protein purification using immobilized triazine. *Dyes. J. Chromatog.*, 165: 301–319.
26. Kopperschlager, G., Bohme, H.J., and Hofmann, E. (1982) Cibacron blue F3G-A and related dyes as ligands in affinity chromatography. In *Advances in Biochemical Engineering*; Ghose, K., Fiechter, A., and Blakebrough, N. (eds.); Springer-Verlag: Berlin/New York, Vol. 25, 101–138.

27. Shanbagh, V.P. and Axelsson, C.G. (1975) Hydrophobic interaction determined by partitioning in aqueous two-phase systems. Partition of proteins in systems containing fatty acid esters of poly(ethylene glycol). *Eur. J. Biochem.*, 60: 17–22.
28. Flickinger, M.C. and Drew, S.W. (1999) *Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation*; Wiley-Interscience, Vol. 3, 1291–1305.
29. Hjerten, S. (1973) Some general aspects of hydrophobic interaction chromatography. *J. Chromatog.*, 87: 325–331.
30. Porath, J., Sundberg, L., Fornstedt, N., and Olsson, I. (1973) Salting-out in amphiphilic gels as a new approach to hydrophobic adsorption. *Nature*, 245: 465–466.
31. Porath, J. (1987) Metal ion–hydrophobic, thiophilic, and electron governed interactions and their applications to salt-promoted protein adsorption chromatography. *Biotech. Progress*, 3: 14–21.
32. http://www.Rcsb.org/pdb/cgi/result Browser. Cgi (24th Feb. 2003/11.00 hours).