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Enzyme immobilization on epoxy supports in reverse micellar media: Prevention of enzyme denaturation

Lahari Thudi[a], Lakshmi S. Jasti[a], Yalangi Swarnalatha[a], Nitin W. Fadnavis[a,*], Khudbudin Mulani[b], Sarika Deokar[b], Surendra Ponrathnam[b]

[a] Biotransformations Laboratory, Indian Institute of Chemical Technology, Uppal Road, Hyderabad 500007, India
[b] Polymer Science & Engineering, Chemical Engineering Division, National Chemical Laboratory, Pashan Road, Pune 411008, India

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ABSTRACT

Immobilization of enzymes such as α-chymotrypsin (EC 3.4.21.1), yeast alcohol dehydrogenase (YADH) from *Saccharomyces cerevisiae* (EC 1.1.1.1) and glucose dehydrogenase (GDH) from *Gluconobacter cerinus* (EC 1.1.1.119) has been carried out. Copolymers of allyl glycidyl ether (AGE) crosslinked with 25% ethylene glycol dimethacrylate (EGDM) (25 mg, dry wt) were contacted with the enzymes solubilized in reverse micellar media (0.5–5 mg/mL)$_{overall}$ of sodium bis(2-ethylhexyl) sulfosuccinate (AOT) salt in isooctane, and cetyl trimethylammonium bromide (CTAB) in chloroform–isooctane (50:50, v/v). Although the enzymes are readily denatured (>90%) after adsorption on the copolymer in aqueous buffers, no such adsorption-induced denaturation takes place in reverse micelles. α-Chymotrypsin is remarkably stable in AOT reverse micelles when 0.025 M citrate buffer of pH 9.0 containing 2 mM $CaCl_2$ is used in the water pools instead of Tris–HCl buffer of pH 8.5. It was possible to achieve enzyme concentration of 5 mg/mL in 0.3 M AOT at molar ratio of water to surfactant, (W_0), 30 and to obtain α-chymotrypsin loading of 20 mg/g of copolymer. The recovered enzyme solution can be reused with a fresh batch of polymer after supplementing the depleted solution. The immobilized enzyme exhibits excellent stability in aqueous buffers at room temperature and can be recycled several times. YADH is stable in both AOT and CTAB reverse micelles while GDH is stable only in CTAB reverse micelles containing 0.05 M Tris–HCl buffer of pH 8.5. Interestingly, the combination of YADH (2.5 mg/g) and GDH (0.5 mg/g) co-immobilized on the copolymer using CTAB–chloroform–isooctane system can be used for regeneration and recycle of NADPH at least 50 times as exemplified by complete reduction of a prochiral ketoester, ethyl 4-phenyl-2,4-dioxobutyrate (10 mM) to ethyl (*R*)-2-hydroxy-4-phenylbutyrate (HPB ester) using NADPH (0.2 mM).

1. Introduction

Enzyme biocatalysts are finding increasing applications because of the growing demand for biotransformations with a high chemo- and stereospecificity in the fine chemicals industry such as pharmaceuticals, agrochemicals, and health care products [1–4]. Since enzyme recycle is an essential operation of an industrial process, several methods have been developed for immobilization of the enzymes which include simple physical adsorption on carrier matrices such as silica, celite, glass beads, ion-exchange resins; entrapment in cross-linked polymers, gels, microcapsules and semi-permeable membranes and covalent binding to solid support material. In many cases immobilization produces enzyme stabilization through multipoint or multi-subunit attachment on an inert support [5–12]. Among various supports, epoxy supports are most popular [13,14]. Conventionally, the enzyme dissolved in an aqueous buffer is first adsorbed on the epoxy resin. Partitioning of the protein from aqueous phase to the polymer phase is sometimes facilitated by addition of salts [15–17]. Nucleophilic reaction then occurs between the reactive groups such as amino, thiol, phenolic and carboxylate groups of adsorbed enzyme and epoxy groups of the support to provide a covalently bound immobilized enzyme [18–20]. The immobilized enzyme may be further stabilized by crosslinking with glutaraldehyde. The homo-bifunctional reagent may react with primary amino group (e.g., ε-amino groups of lysine residues) of proteins by several means such as aldol condensation or Michael-type addition to form stable inter- and intra-subunit covalent bonds [21]. Several enzymes have been immobilized on epoxy resins, and found to be far more stable than the solubilized counterparts [20,22].

Recently, we have reported interactions of α-chymotrypsin with epoxy-activated allyl glycidyl ether–ethylene glycol dimethacrylate (AGE–EGDM) copolymers [23]. Although the copolymer was

* Corresponding author. Fax: +91 40 27160512.
E-mail addresses: fadnavisnw@yahoo.com, fadnavis@iict.res.in, fadnavis@iictnet.org (N.W. Fadnavis).

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capable of adsorbing large amounts of the enzyme (25%, w/w) without necessity of using salts, almost 80% of the adsorbed enzyme was denatured. This phenomenon of "adsorption induced denaturation", although interesting, was a big deterrent for enzyme immobilization.

In principle, the AGE–EGDM copolymers are excellent candidates for enzyme immobilization. These copolymers are macroporous (pore diameter of 100–150 Å), possess high mechanical strength, good swelling properties, a large number of epoxy groups (300–500 μmoles/g), and can be prepared easily in laboratory. By controlling crosslink density, properties such as specific surface area, pore volume can also be varied according to need. In practice, unfortunately, they cause substantial denaturation and loss of enzyme activity in aqueous solutions. For practical application, it is necessary to recover the expensive enzyme that is not bound to the polymer and reuse it with a fresh batch of polymer. Our studies on interactions of α-chymotrypsin with AGE–EGDM copolymers strongly suggested that the loss of activity was mainly due to two interactions: strong hydrophobic interactions between the protein and polymer surface and, polar interactions of the ester groups present on the polymer with the protein surface. If these interactions are somehow prevented or diluted, it might be possible to use the AGE–EGDM copolymers for enzyme immobilization without serious loss of enzyme activity. The reverse micelles appeared to provide such an alternative.

The reverse micellar systems essentially consist of a solution of a surfactant (50–100 mM) in hydrocarbon solvent containing small amounts (1–3%, v/v) of water. The polar head groups of the surfactant club together due to dipole–dipole and ion–dipole interactions, while the hydrophobic tails extend in the surrounding hydrocarbon solvent. Water added to these solutions is confined near the polar head-groups forming the so-called "water pool" and the proteins can be solubilized in this water pool. An enzyme can be solubilized in these micro droplets without serious loss of its catalytic activity and provide an interesting alternative to aqueous phase enzymatic reactions with hydrophobic substrates [24,25]. It is also possible to prepare organo-gels with highly active enzymes entrapped within the gel [26]. These findings encouraged us to use the reverse micellar media for immobilization of enzymes on AGE–EGDM copolymers. We visualized that the hydrocarbon tails of the surfactant would bind at the hydrophobic domains of the copolymer either as an individual molecule or as a reverse micellar assembly, effectively masking the hydrophobic domains. Similarly, the polar head groups would interact with polar ester groups reducing their direct interactions with enzyme. At the same time, the water-pool would provide a reaction site where the pendant epoxy groups present on the polymer surface would come in contact with the enzyme solubilized in the water pool and a covalent bond formation between polymer and enzyme would take place (Fig. 1).

To the best of our knowledge, very few reports have been made on use of reverse micelles and microemulsions for immobilization of macromolecules on polymer supports [27–29]. Malmsten and Larsson [27] have reported microemulsion system of AOT in isooctane for immobilization of trypsin on glycidyl methacrylate-*co*-1,3-dimethacrylate. In comparison with aqueous phase immobilization, improved protein loading was accomplished in microemulsion. However, observed trypsin activity expected from protein content of the copolymer was substantially lower (<10%) indicating substantial protein denaturation during immobilization.

To gain a better insight to achieve improvement in the efficiency of enzyme binding without loss of enzyme activity, we have carried out a systematic study with three enzymes α-chymotrypsin, yeast alcohol dehydrogenase (YADH) from *Saccharomyces cerevisiae* and glucose dehydrogenase (GDH) from *Gluconobacter cerinus*. Effects of various parameters such as nature and concentration of surfactant,

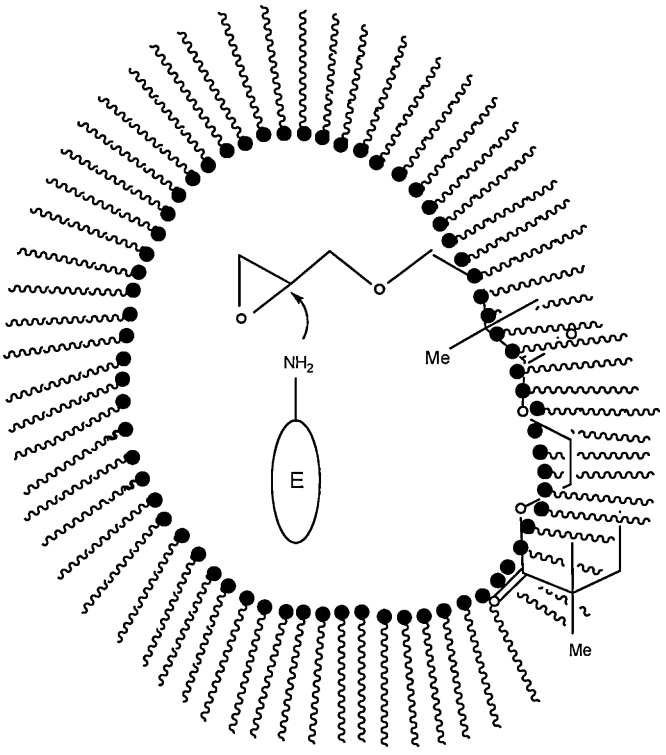


Fig. 1. The rationale of using reverse micellar media for enzyme immobilization.

effect of water content, contact period, protein concentration on immobilization efficiency have been studied.

The amount of water present in the system is usually expressed by the parameter W_0, the molar ratio of water to surfactant ($W_0 = [H_2O]/[\text{surfactant}]$). The protein can be assumed to be dispersed uniformly in the whole of the microemulsion system and express its concentration as "overall" concentration (C_{ov}). On the other hand, the protein is soluble only in water microphase and hence its concentration can also be expressed in terms of "water pool" concentration (C_{wp}). The two types of concentrations are related by Eq. (1).

$$C_{ov} = F_w C_{wp} \quad (1)$$

where F_w is the volume fraction (v/v) of water [30,31]. Since the reverse micellar systems behave as homogeneous solutions for all practical purposes, and we are investigating the relative enzyme activity before and after immobilization, we have chosen to express the concentrations of the substrates and the proteins as "overall" in all cases throughout the manuscript.

Most of the studies have been done with α-chymotrypsin as model system and the results were extended to studies with YADH and GDH. These enzymes were chosen for their utility in biotransformations. For example, α-chymotrypsin is quite useful in peptide synthesis [32,33] while NAD(P)-dependent dehydrogenases are useful catalysts for the synthesis of chiral alcohols and hydroxy acids with high enantioselectivity. However, the coenzyme is required in stoichiometric amount making synthetic applications of redox enzymes prohibitively expensive. For preparative applications, an efficient coenzyme-regenerating step is necessary and several strategies have been developed to achieve this goal [34,35]. One of the popular strategies is to employ a coupled alcohol dehydrogenase (ADH)–glucose dehydrogenase (GDH) system where $NADP^+$ generated during reduction of a ketone by ADH is reduced to NADPH by GDH. Although genetically engineered microbes possessing both ADH and GDH activities work efficiently [36], these are not easily available. As an alternative, it is interesting to immobilize

both the enzymes on a single solid support and use the combined enzyme system for cofactor regeneration.

Compared to α-chymotrypsin (M wt 24,800) [37], the dehydrogenase enzymes are rather large multimeric enzymes. Yeast alcohol dehydrogenase is a tetramer, each subunit consisting of a single polypepide chain of 36 kDa, one coenzyme binding site and one firmly bound zinc atom, which is essential for catalysis [38]. Glucose dehydrogenases also exist mostly in tetrameric forms [39–41]. Prevention of subunit dissociation is very important while immobilizing such multimeric enzymes.

A very interesting strategy described by Fernandez-Lafuente employs a two-step immobilization of multimeric enzymes on heterofunctional supports. These supports possess low density of ionized amino groups and a very high density of stable epoxy groups. The multimeric enzyme is first adsorbed on the support at neutral pH and then covalently immobilized under alkaline conditions. Instead of epoxy, glyoxyl functional groups have also been employed for successful immobilization of multimeric enzymes [42–44]. In the present case we have tried an alternative strategy of using reverse micellar media. We hoped that the subunit dissociation would be minimized in confines of the water pool and provide immobilized multimeric enzymes without denaturation.

2. Materials and methods

2.1. Materials

The enzymes α-chymotrypsin from bovine pancreas (EC 3.4.21.1, cat no. C-4129, type-II, activity >40 units/mg) and alcohol dehydrogenase from *S. cerevisiae* (EC 1.1.1.1, cat no. A 7011, activity >300 units/mg) and reagents N_α-glutaryl-L-phenylalanine p-nitroanilide (GPNA), cetyl trimethylammonium bromide (CTAB), sodium bis(2-ethylhexyl) sulfosuccinate (AOT), Tropaeolin OO indicator were purchased from Sigma–Aldrich, USA. Ethyl 2,4-dioxo-4-phenylbutyrate was prepared in laboratory by known procedure [45]. N-acetyl-L-phenylalanine methyl ester was prepared from L-phenylalanine by a standard procedure [46]. Glucose dehydrogenase (GDH) from *G. cerinus* (EC 1.1.1.119, cat no. 074853, activity 60 units/mg) was purchased from SRL, India. All other reagents and solvents used of analytical grade obtained from Qualigens, India. The porous AGE–EGDM copolymer with mole ratio of the cross-linking co-monomer (ethylene glycol dimethacrylate) to epoxy functional monomer (allyl glycidyl ether) of 0.25:0.75 (AGE-25) was synthesized by suspension polymerization in a jacketed cylindrical polymerization reactor at a constant volume of cyclohexanol as porogen. The copolymer had average particle size of (100–150 μm), average pore diameter of 100–150 Å, pore volume of 0.68 mL/g, a specific surface area of 98 m^2/g and water retention capacity of 5.3 g/g [23]. UV–visible spectrophotometric measurements were performed on Perkin-Elmer Lambda 2 spectrophotometer equipped with temperature control and PECSS software. Laser Raman Spectra were recorded on a Horiba Jobin-Yvon LabRam HR 800 UV Raman spectrometer with a 35 mW internal He–Ne laser source of excitation wavelength 514 nm. All enzyme assays were performed at 25 °C. All experiments were repeated 3 times and were reproducible within (±5%).

2.2. Preparation of reverse micellar solutions

Reverse micellar solutions were prepared by injecting microlitre amounts of aqueous stock solutions of either buffer alone or enzyme in buffer to surfactant solutions. The solutions of cetyl trimethylammonium bromide (CTAB) were prepared in 50–50% (v/v) chloroform–isooctane while that of sodium bis(2-ethylhexyl)sulfosuccinate (AOT) were prepared in isooctane.

2.3. Pre-equilibration of polymer

In a typical experiment, the polymer AGE-25 (50 mg, dry wt) was soaked in appropriate buffer for 4 h and excess water was removed. The polymer was then shaken with reverse micellar solution of required W_0 for 1 h, the supernatant was decanted and the polymer was again equilibrated with a fresh reverse micellar solution. The procedure was repeated till water content in the supernatant (determined by Karl–Fisher titration) remained constant at required W_0.

2.4. Enzyme solubilization in reverse micelles

Reverse micellar solutions of the enzyme were prepared by injecting appropriate amounts of aqueous stock solutions of the enzyme to surfactant solutions. In case of α-chymotrypsin, stock enzyme solutions were prepared in two different buffers (0.05 M Tris–HCl containing 2 mM $CaCl_2$, pH 8.2; and 0.025 M sodium citrate buffer with 2 mM $CaCl_2$, pH 9.0). The solutions of YADH and GDH were prepared in 0.05 M Tris–HCl containing 2 mM $CaCl_2$, pH 8.2. α-Chymotrypsin and YADH were solubilized in AOT-isooctane. In case of GDH, the enzyme was found to be inactive in AOT-isooctane solutions and hence the enzyme solutions were prepared in CTAB dissolved in 50–50% (v/v) chloroform–isooctane where it was found to be as active as in aqueous buffer. For immobilization of a combination of YADH and GDH, reverse micellar solutions in CTAB were prepared individually and mixed prior to immobilization. The final concentration (expressed as overall concentration), of the enzyme ranged from 75 μg/mL to 3 mg/mL.

2.5. Enzyme immobilization in reverse micelles

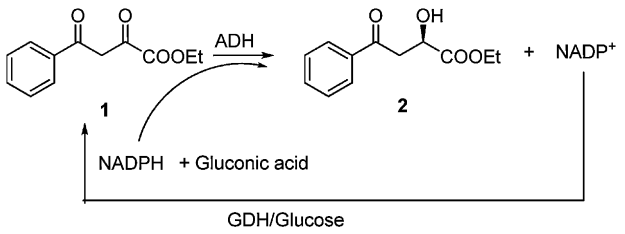
In a typical experiment, surfactant solutions (0.05–0.2 M, 2 mL) with appropriate amounts of buffer were injected with enzyme stock solution to obtain reverse micellar solutions of varying W_0 (5–30). The pre-equilibrated polymer (AGE-25, 25 mg, dry wt) was added and the contents were shaken for 2 h at room temperature on an orbital shaker. The supernatant was then decanted and residual polymer was washed with isooctane till the washings were free of surfactant (determined by titrimetry using Tropaeolin OO indicator) [47]. The polymer was then assayed for enzyme activity.

2.6. Assay of α-chymotrypsin in aqueous medium

α-Chymotrypsin activity was measured in aqueous buffer at 25 °C using N-acetyl-L-phenylalanine methyl ester as substrate [48]. The acid formed due to hydrolysis of the ester was continuously titrated with 0.1 N NaOH. Enzyme activity units are expressed in terms of μmoles of NaOH consumed per minute. The activity for native enzyme was found to be 117 units/mg. It was also confirmed that the presence of surfactant AOT at concentrations up to 2 mM in the assay solution did not cause any decrease in activity of the enzyme for at least 15 min.

2.7. Assay of α-chymotrypsin in reverse micellar medium

α-Chymotrypsin activity in the reverse micellar phase was measured at 25 °C using N_α-glutaryl-L-phenylalanine p-nitroanilide (GPNA) as substrate [49]. The absorbance change at 366 nm was measured for 5 min ($\Delta\varepsilon_{366}$ = 13,000 M^{-1} cm^{-1}). Final overall substrate and enzyme concentrations in cuvette were 0.2 mM 1.3 μM, respectively.



Scheme 1. YADH catalyzed reduction of ethyl 2,4-dioxo-4-phenylbutyrate **1** to ethyl (*R*)-2-hydroxy-4-oxo-4-phenylbutyrate **2** with recycle of NADPH using co-immobilized GDH.

2.8. Assay of YADH in reverse micelles

The activity of YADH solubilized in AOT reverse micelles was measured at 25 °C using buta-2-one as substrate and NADH as cofactor [50]. Enzyme activity was measured from the decrease of absorbance at 340 nm ($\Delta\varepsilon_{340} = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$). Final overall concentrations of the various reactants in the cuvette were: buta-2-one, 0.28 M; NADH, 0.19 mM; and YADH, 0.86 μM, respectively. Similar solutions were made in aqueous buffer for comparative studies.

2.9. Assay of immobilized YADH in aqueous medium

Enzyme activity was measured by following reduction of ethyl 2,4-dioxo-4-phenylbutyrate **1** to ethyl (*R*)-2-hydroxy-4-oxo-4-phenylbutyrate **2** in presence of NADH as cofactor (Scheme 1). A solution of **1** and NADH (10 mM each in 50 mM Tris–HCl buffer, pH 8.5, 2 mL) was stirred with polymer with immobilized enzyme (20 mg) or native enzyme (0.1 mg) on a magnetic stirrer. The conversion was followed by HPLC on C-18 column (Chrompack, 4.6 mm × 250 mm) using mobile phase consisting of 70% acetonitrile–water containing 0.2% formic acid. At flow rate of 0.7 mL/min, the diketo ester was detected at 316 nm while the hydroxy ester **2** was detected at 246 nm. Retention times observed were **1**: 8.4 min; **2**: 5.7 min. The reaction was carried out under non-saturation conditions for the substrate due to its relatively low solubility in water. Under these conditions, the enzymatic reaction follows a first-order kinetics. Since $t_{1/2}$ is a good measure of k_{obsd} for first-order reactions, we have chosen this parameter to estimate enzyme activity. It was observed that $t_{1/2}$ for 100 μg native enzyme under above specified reaction conditions was 53 min and it was linearly proportional to the concentration of enzyme dissolved in the reaction mixture.

2.10. Assay of glucose dehydrogenase in aqueous medium

The activity of GDH was measured by continuous titration of gluconic acid formed due to glucose oxidation catalyzed by GDH at constant pH of 8.5 [50]. The activity was expressed as μmoles of NaOH consumed/min. The activity for native enzyme was found to be 60 units/mg.

2.11. Assay of glucose dehydrogenase in reverse micellar medium

The activity of GDH solubilized in AOT reverse micelles was measured at 25 °C using overall concentrations of glucose (16 mM) and $NADP^+$ (0.2 mM). The enzyme activity was measured from the rate of increase of absorbance at 340 nm ($\Delta\varepsilon_{340} = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$).

2.12. Regeneration and recycle of NADPH by co-immobilized glucose dehydrogenase and yeast alcohol dehydrogenase

A solution of ethyl 2,4-dioxo-4-phenylbutyrate **1** (10 mM), NADPH (0.2 mM) and glucose (25 mM) in Tris–HCl buffer (pH 8.5, 2 mL) was stirred with polymer (20 mg) on which YADH and GDH were co-immobilized. The conversion was followed by HPLC as described earlier in Section 2.9.

2.13. Glutaraldehyde crosslinking of immobilized α-chymotrypsin

The polymer recovered after treatment with enzyme solution as described in Section 2.6 (25 mg, dry wt) was suspended in citrate buffer (1 mL, 0.025 M, containing 2 mM $CaCl_2$ and 200 μL of 0.25% glutaraldehyde, pH 9.0). The contents were shaken for 2 h at room temperature and the supernatant was decanted. The polymer was washed with distilled water and the immobilized enzyme crosslinked with glutaraldehyde was stored in refrigerator.

2.14. Stability of immobilized α-chymotrypsin

Enzyme stability after immobilization on the polymer beads was studied by assaying the enzyme activity of the beads in aqueous buffer using N-acetyl-L-phenylalanine methyl ester as substrate in a packed bed recycle reactor assembly to avoid loss of polymer in a stirred vessel. The recovered and washed beads after reverse micellar immobilization were placed in a Bio Rad Glass Econo column (12 cm × 2 cm) equipped with a filter frit of pore size 28 μm. The substrate (0.05 M) dissolved in Tris–HCl buffer (10 mL, 0.1 M, pH 8.2) was recirculated through the column by means of a peristaltic pump (2 mL/min). The supernatant was analyzed by reverse phase HPLC and time required for 50% conversion ($t_{1/2}$) was recorded. After each cycle, the whole assembly was washed with buffer and a fresh substrate solution was used for next cycle. In this fashion 10 recycles were carried out.

3. Results and discussion

3.1. Enzyme assay procedures

Generally, assay of enzyme activity in reverse micelles can be carried out by small modification of the assay procedure used in aqueous medium. A concentrated enzyme stock solution is prepared in aqueous buffer and required quantities are injected in surfactant solution prepared in hydrocarbon solvent. Although it cannot be assumed that pH of the aqueous buffer used for making enzyme stock solution (pH_{st}) is same in both aqueous and reverse micellar solutions, using the aqueous buffer pH does give defined way to prepare the micelles. A stock solution of substrate is added, the contents are mixed and the reaction is monitored by standard procedure. In most cases, the enzymes follow standard Michaelis–Menten kinetics and provide k_{cat} and K_m values. Here, we would like to point out that the enzymes entrapped in reverse micelles are not exactly "immobilized" like they are on a solid support. The assemblies are dynamic species and the inter-micellar exchange of molecules including proteins is quite fast (10^4–$10^6\ s^{-1}$) [30,31]. Stopped-flow kinetic studies on trypsin and α-chymotrypsin have confirmed that the rate of substrate–enzyme complex formation is not the rate determining step in reverse micelles [51,52]. Also, one needs to be careful while describing activity in reverse micellar solutions, especially when comparing with aqueous solution activities. Substrate partitioning between bulk organic phase and water pool can affect the concentration seen by the enzyme (changes in K_m). Accumulation of a hydrophilic substrates in

the water pool or better availability of a hydrophobic substrate in the reverse micelle than in water can result in observation of higher activity. To avoid ambiguities, spectrophotometric assay procedures were employed for studies in reverse micellar solutions in present investigations. Since the assays had to be performed under conditions of substrate/cofactor concentration at non-saturation levels due to strong absorption in the UV/visible region by the substrate/cofactor at high concentrations, the results of these assays have been used strictly to compare the enzyme behavior under specified conditions of substrate and enzyme concentrations, pH_{st} and W_0 for optimizing conditions for immobilization.

For studies on stability in reverse micelles and activity of the immobilized enzymes, the activities of α-chymotrypsin and GDH were assayed by using titrimetric methods in aqueous buffer where the specific substrates/cofactors can be used at high concentrations (>10 K_m). We have experimentally confirmed that plots of V_{obsd} against [E] are linear for both spectrophotometric and titrimetric assays and the results are complimentary. We have also experimentally confirmed that the presence of surfactant in the assay solutions (up to 3 mM) does not cause any loss of enzyme activity for at least 30 min in both the cases.

In case of YADH, it was not possible to adopt buta-2-one/NADH procedure for immobilized enzyme due to strong adsorption of NADH/NAD^+ on the polymer and unfortunately, a titrimetric method could not be employed since both substrate and product are non-ionic. An alternative procedure was developed based on our experience with reduction of ethyl 2,4-dioxo-4-phenylbutyrate **1** to ethyl (*R*)-2-hydroxy-4-oxo-4-phenylbutyrate **2** by baker's yeast (Scheme 1) [45]. In this procedure the concentration of substrate/product (50 mM) was high enough to overcome any errors due to adsorption on polymer and consistent results (within ±5%) were obtained.

3.2. Effect of water content (W_0) on activity of enzymes

It is well known that activity of an enzyme solubilized in reverse micelles is strongly dependent on the water content of the medium and has been observed for many enzymes in reverse micellar systems with different types of surfactants [30,31]. Increasing the degree of hydration of reverse micelles leads to an increase in the size of inner cavity and the activity of an enzyme in reverse micelle usually reaches a maximum when the size of reverse micelle fits the enzyme [53,54]. Thus, enzyme activities were measured in reverse micellar media at a fixed enzyme concentration and varying water content. The pH of the aqueous buffer used for the preparation of reverse micelles was maintained near known pH-optima of the enzymes (pH 8.2–8.5).

Fig. 2 shows the effect of water content, expressed in terms of W_0, on activity of the three enzymes, α-chymotrypsin, YADH and GDH in reverse micellar systems. Small enzyme like α-chymotrypsin (24 kDa) exhibits optimum activity at low water content (W_0 10) while large enzymes such as YADH (156 kDa) and GDH (104 kDa) show W_0 optimum at W_0 near 30. Our results with α-chymotrypsin and YADH are in excellent agreement with literature reports [49,55–57].

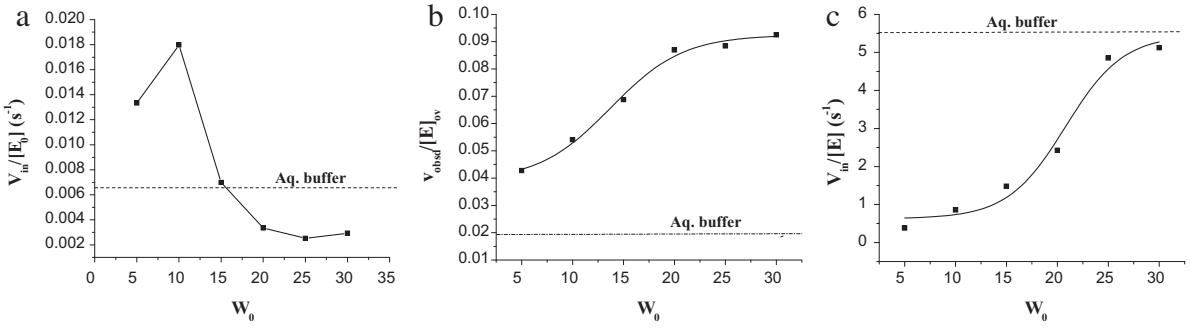


Fig. 2. Activity of enzymes solubilized in reverse micelles as a function of W_0. [Surfactant] = 0.05 M; aqueous stock buffer: 0.05 M Tris–HCl buffer containing 2 mM $CaCl_2$, pH 8.2. (a) α-Chymotrypsin in AOT/isooctane, [E] = 1.3 μM, [GPNA] = 0.2 mM; (b) YADH in AOT/isooctane, [E] = 0.86 μM, [buta-2-one] = 0.28 M, [NADH] = 1 mM; (c) GDH in CTAB/50% (v/v) chloroform–isooctane, [E] = 0.056 μM, [glucose] = 16 mM, [NADP] = 1 mM. Temp. 30 °C.

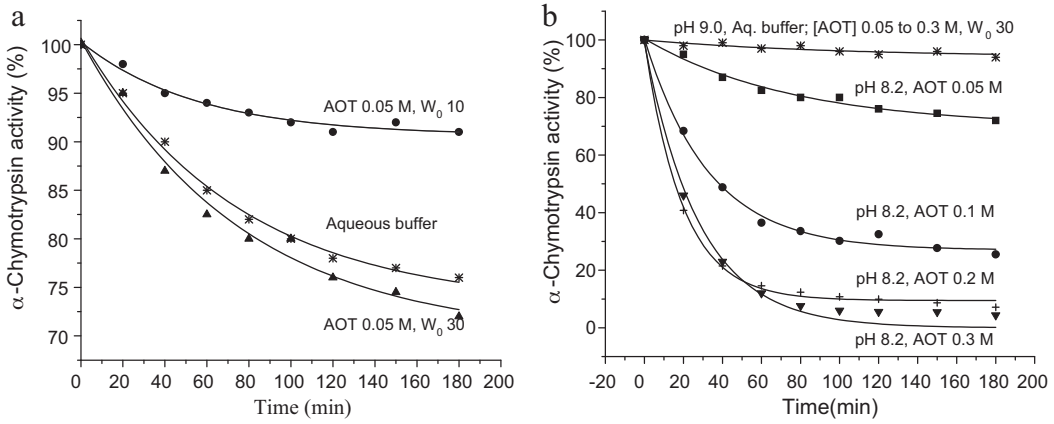


Fig. 3. (a) Stability of α-chymotrypsin solubilized in AOT reverse micelles. (a) (▲) 50 mM AOT, W_0 30; (●) 50 mM AOT, W_0 10; (*) Tris–HCl buffer containing 2 mM $CaCl_2$, pH 8.2. [E] = 0.5 mg/mL, 100% activity = 58 units. (b) Stability of α-chymotrypsin solubilized in AOT reverse micelles as function of pH and AOT concentration at W_0 30. (■) 0.05 M AOT, pH_{st} 8.2; (●) 0.1 M AOT, pH_{st} 8.2; (+) 0.2 M AOT, pH_{st} 8.2; (*) 0.025 M citrate buffer containing 2 mM $CaCl_2$, pH 9.0 and in AOT-isooctane 0.05–0.3 M, pH_{st} 9.0; [E] = 0.75 mg/mL, 100% activity = 88 units.

3.3. Effect of surfactant concentration on stability of enzymes in reverse micelles

Protein solubility in reverse micellar media is limited by the amount of water that is present in the system as well as the amount of enzyme that can be dissolved in water to make aqueous stock solution. By increasing surfactant concentration, it is possible to increase the amount of water (and the enzyme) dissolved in the reverse micellar phase while maintaining a constant W_0. However, under such conditions, the stability of the enzyme becomes an important issue. The effects of surfactant concentration on stability of enzymes in reverse micelles were hence studied.

3.3.1. Effect of surfactant concentration and pH on stability of α-chymotrypsin in AOT reverse micelles

Fig. 3a and b shows the activity of α-chymotrypsin in AOT reverse micelles. In 0.05 M AOT and at pH 8.2, the enzyme is more stable than in water at W_0 10 and retains at least 90% activity after 3 h. Increasing the water content to W_0 30 causes a decrease in enzyme stability and brings it close to that in water. The enzyme loses about 25% of its activity in 3 h in a monophasic manner (Fig. 3a). When incorporated in reverse micelles of higher surfactant concentration, the enzyme activity is lost in a biphasic manner with a fast (k_f) and a slow (k_s) component (Fig. 3b). In the fast component region, the data fits to a first-order rate equation (2).

$$\frac{V}{V_0} = Ae^{-kt} \quad (2)$$

where V_0 is the enzyme activity at time zero and V is the enzyme activity at time 't', k is the rate of deactivation, and A is the pre-exponential factor. It is also observed that the overall rate of deactivation ($0.05 \pm 0.01\ \text{min}^{-1}$) is similar in all AOT solutions, but the quantity of enzyme denatured (pre-exponential factor) increases with surfactant concentration. In the region of slow deactivation, the deactivation rates appear to be similar to that in water ($k = 0.013\ \text{min}^{-1}$; the curves drawn in the figures are for the fitted models). In contrast, at pH 9.0, the enzyme is very stable both in aqueous buffer and reverse micelles at AOT concentrations ranging from 0.05 M to 0.3 M and W_0 10–30. It should be mentioned that at present it is difficult to pinpoint the reason for loss of enzyme activity. It may be caused by actual unfolding of the enzyme, or a slight distortion of the enzyme structure, blocking of the active center or chemical modification of one of the key groups. A similar pH-dependent behavior of α-chymotrypsin in reverse micelles has been reported by Almeida et al. [58]. According to them, the fast deactivation is due to interaction of negatively charged surfactant with a protonable group of the protein with pK_a between 8.6 and 9, most probably N-terminal Ala[149]. This interaction produces a cooperative effect that leads to the breakage of the salt bridge Ile[16]-Asp[194] which in turn results into loss of enzyme activity. At pH 9, this component vanishes and the enzyme deactivation takes place by the same mechanism as that in water such as thermal inactivation and autolysis [59,60]. Although α-chymotrypsin has pH optimum at pH 8, it is more stable at pH 9.0 [61] and $CaCl_2$ provides a conformational stability to the enzyme [60]. Also, one would expect a concentrated α-chymotrypsin stock solution to undergo fast autolysis at pH 9.0, but this does not happen. The enzyme stock solution retains all of its activity for at least 5 h at room temperature. Thus based on above results, we have chosen to carry out immobilization of α-chymotrypsin in reverse micellar media using 25 mM citrate buffer of pH 9 containing 2 mM $CaCl_2$.

3.3.2. Effect of surfactant concentration on stability of YADH and GDH in reverse micelles

Stability of YADH in AOT reverse micelles was studied at W_0 30 as a function of AOT concentration ranging from 0.05 M to 0.3 M

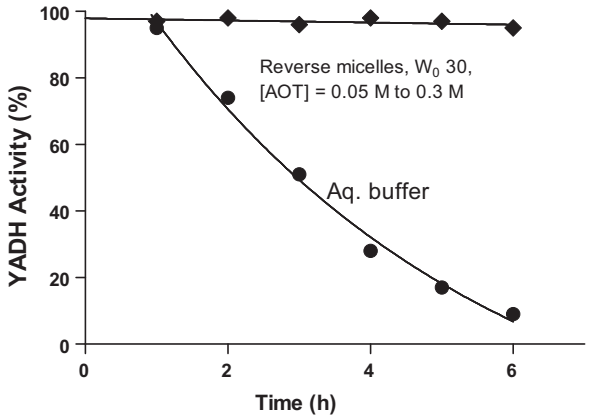


Fig. 4. Stability of YADH solubilized in AOT reverse micelles at W_0 30. [Surfactant] = 0.05–0.3 M; aqueous stock buffer: 0.05 M Tris–HCl buffer containing 2 mM $CaCl_2$, pH 8.2. [E] = 0.86 μM, [buta-2-one] = 0.28 M, [NADH] = 1 mM. 100% activity (V_{obsd}, M s^{-1}) 0.05 M AOT, 1.08×10^{-7}; 0.10 M AOT, 8.95×10^{-8}; 0.2 M AOT, 5.13×10^{-8}; 0.3 M AOT, 4.1×10^{-8}.

(Fig. 4). In aqueous buffer, the enzyme activity is unchanged for 1 h but starts losing its activity in an exponential manner with first-order rate constant of $0.21\ \text{h}^{-1}$. Almost half of its activity is lost in 3 h while the same enzyme is very stable in AOT reverse micelles at W_0 30 (pH 8.5) even at high AOT concentration of 0.3 M.

The enzyme GDH was found to be quite stable at pH 8.5 both in aqueous buffer and in CTAB/chloroform–isooctane reverse micelles for at least 6 h in surfactant concentration range of 0.05–0.3 M probably because it is a glycoprotein and hydrophilic carbohydrate functionalities on its surface protect the enzyme from denaturation.

3.3.3. Effect of water content (W_0) and surfactant concentration on efficiency of α-chymotrypsin immobilization

Effect of W_0 and surfactant concentration on binding of α-chymotrypsin to AGE-25 was studied. The experiments were performed in two ways. In first set, 25 mg (dry wt) pre-equilibrated polymer was used in reverse micellar solution (2 mL). AOT concentration was maintained constant at 0.2 M to ensure solubilization of sufficient protein in the reverse micellar medium (0.5 mg/mL, maximum amount of enzyme that could be solubilized in 0.2 M AOT at W_0 5) and W_0 was varied from 5 to 30. In the second set, the W_0 was kept constant at 30 and surfactant concentration was varied from 0.05 M to 0.2 M. The enzyme concentration was 0.75 mg/mL (maximum amount of enzyme that could be solubilized in 0.05 M AOT at W_0 30). This solution (2 mL) was contacted with 25 mg (dry wt) of polymer pre-equilibrated with appropriate surfactant solutions.

In the first set of experiments, it was observed that water content of the medium had a strong effect on the amount of enzyme bound to the polymer. At low water content of W_0 5, very little enzyme was bound (50 units). The amount of bound enzyme increased at W_0 10 (500 units/g) and then remained unchanged at higher water content (Fig. 5). In the second set of experiments, it was observed that at W_0 30, the amount of enzyme bound to the polymer increased from 500 units/g to 725 units/g with increase in enzyme concentration from 0.5 mg/mL to 0.75 mg/mL. However, the enzyme activity on the beads (725 ± 25 units/g) did not depend on surfactant concentration. It appears that the polymer needs to be sufficiently hydrated for binding with the enzyme. Once it is properly hydrated, the efficiency of enzyme binding depends mostly on the enzyme concentration present in the solution.

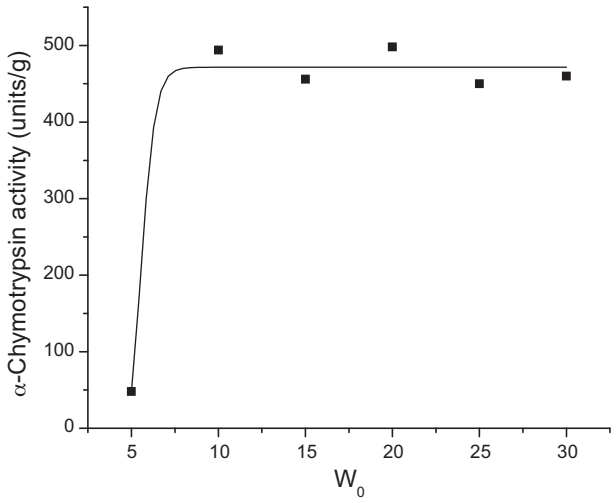


Fig. 5. Effect of W_0 on binding of α-chymotrypsin to AGE–EGDM copolymer in AOT reverse micelles. [AOT] = 0.2 M; aqueous stock buffer: 0.025 M citrate buffer containing 2 mM $CaCl_2$, pH 9.0. [α-Chymotrypsin] = 0.5 mg/mL, [polymer] = 25 mg, reaction volume 2 mL. Temp. 30 °C.

3.4. Effect of enzyme concentration on protein loading

We have studied the binding of α-chymotrypsin at W_0 30 in 0.2 M AOT solution as a function of enzyme concentration at a fixed polymer concentration (25 mg, dry wt). Measurement of enzyme activity in supernatant and that bound to the polymer showed that major amount of protein was bound within first 1 h of contact and reached a plateau after 2 h. The results of bound protein after 2 h contact as a function of enzyme concentration are shown in Fig. 6.

Usually, in aqueous media, a direct reaction between enzyme and epoxy group is very slow, and it is necessary that the polymer first adsorbs the enzyme [18–20]. The binding process is described in terms of equilibrium binding followed by covalent linkage [15]. The overall process can be expressed by Eq. (3) where, the enzyme E is adsorbed on the polymer P in a fast equilibrium process with a dissociation constant Kd, to form a non-covalently bound polymer–enzyme complex E.P. Subsequently, the enzyme reacts with the epoxy group of the polymer to form an irreversible covalent bond.

$$E + P \underset{Kd}{\rightleftharpoons} E.P \xrightarrow{k_1} E - P \quad (3)$$

The results in Fig. 6 show that the amount of bound enzyme increases with enzyme concentration in the reverse micellar solution and appears to follow Eq. (3). However, it is difficult to state with certainty whether the rate of adsorption is indeed faster than the rate of covalent bonding in the reverse micelles and whether the curve in Fig. 6 really describes a standard Langmuir adsorption isotherm. What is interesting here is the fact that assay of enzyme activity in supernatant and that bound on the polymer matrix showed that the binding process was not accompanied by enzyme denaturation. For example, the original reverse micellar solution containing 0.5 mg/mL enzyme shows activity of 117 units for 2 mL. After treatment with polymer (25 mg), the activity loss in the supernatant was 14 units (120 μg) and the assay of the bound enzyme showed that it possesses 13 (±1) units of activity. Under similar conditions in aqueous buffer, the polymer binds 350 μg enzyme/g, but the active enzyme is only 7 units (60 μg). It was possible to achieve enzyme concentration of 5 mg/mL in 0.3 M AOT at W_0 30 and to obtain α-chymotrypsin loading of 2340 units/g (20 mg/g). Considering the number of epoxy groups (300–500 μmoles/g), it appears that the protein is bound only to the epoxy groups that are available on the surface.

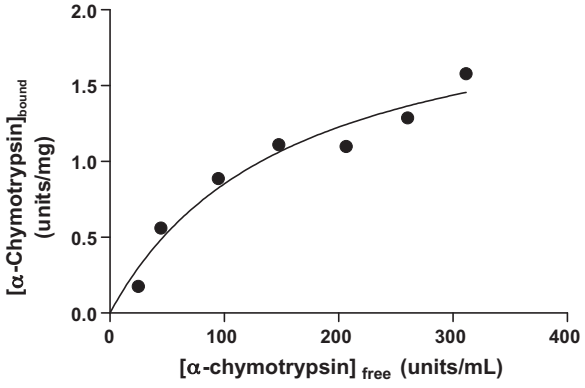


Fig. 6. Binding of α-chymotrypsin to AGE-25 in reverse micelles of AOT in isooctane (0.2 M, W_0 = 30, stock aq. buffer 0.025 M citrate buffer with 10 mM $CaCl_2$, pH 9.0). [Enzyme] = 50–350 units/mL. Wt of polymer = 25 mg (dry wt); total volume 2 mL.

3.5. Raman spectra

The polymer beads possessing immobilized enzyme were crushed to a fine powder and Raman spectra of the bound enzyme were recorded. Fig. 7 shows the Raman amide III band in region 1230–1270 cm^{-1} and amide I band in region 1640–1680 cm^{-1} of native α-chymotrypsin powder (curve a), α-chymotrypsin immobilized on AGE-25 by using AOT reverse micellar medium (curve b) and immobilized on AGE-25 in aqueous buffer (curve c). Absence of significant changes in amide I and amide III bands in curves a and b indicates that the protein binding in reverse micelles does not influence the secondary structure of the protein while in aqueous buffer the enzyme loses its structural integrity on interaction with the polymer.

3.6. Stability of immobilized α-chymotrypsin

Enzyme stability after immobilization on the polymer beads was studied by assaying the enzyme activity of the beads in aqueous buffer using N-acetyl-L-phenylalanine methyl ester as substrate in a packed bed recycle reactor assembly to avoid loss of polymer in a stirred vessel. Fig. 8 shows that α-chymotrypsin immobilized in reverse micellar medium retains 70% activity after 10 recycles (curve b). For comparison, the figure also shows data for activity of the enzyme immobilized in aqueous buffer (curve a). Careful analysis of protein content in the supernatant showed that the enzyme bound via adsorption in aqueous solution is bound superficially and leaches out after each recycle and the enzyme activity decreases to 15% in 10 recycles. In contrast, the enzyme bound in reverse micellar medium is far more stable. The enzyme appears to be bound by a covalent linkage and only traces of bound protein leached out during recycles.

3.7. Improvement of enzyme stability

The stability of the immobilized enzyme was further improved by crosslinking with glutaraldehyde. To optimize the amount of glutaraldehyde sufficient for crosslinking, the beads (25 mg) recovered after enzyme immobilization were suspended in buffer containing glutaraldehyde of varying concentration ranging from 0.01 to 0.1% for 2 h and the residual activity after 10 washes with buffer was measured. Control experiments without polymer showed that the enzyme activity was not decreased in presence of glutaraldehyde. Treatment with 0.05% glutaraldehyde solution was sufficient for enzyme stabilization and its stability was vastly improved after crosslinking (curve c, Fig. 8).

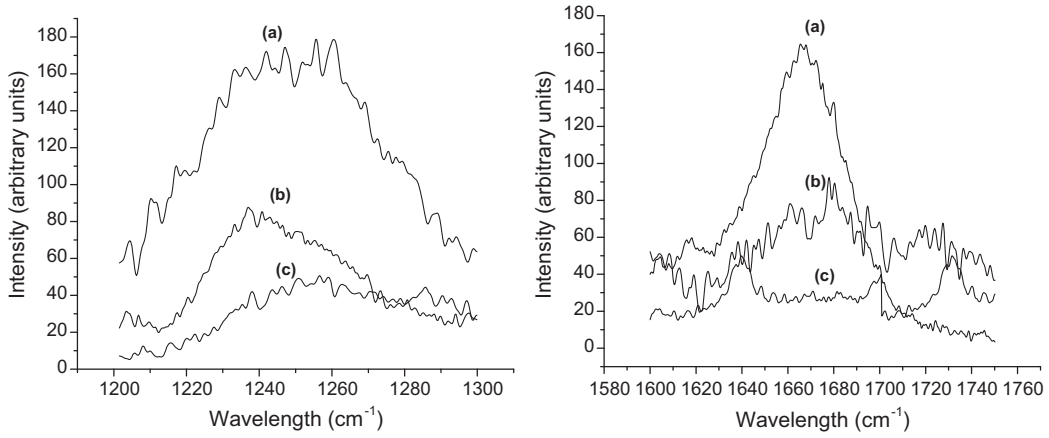


Fig. 7. Laser Raman spectra of (a) native α-chymotrypsin powder, (b) α-chymotrypsin immobilized on AGE-25 in AOT reverse micelles and (c) α-chymotrypsin immobilized on AGE-25 in aqueous buffer.

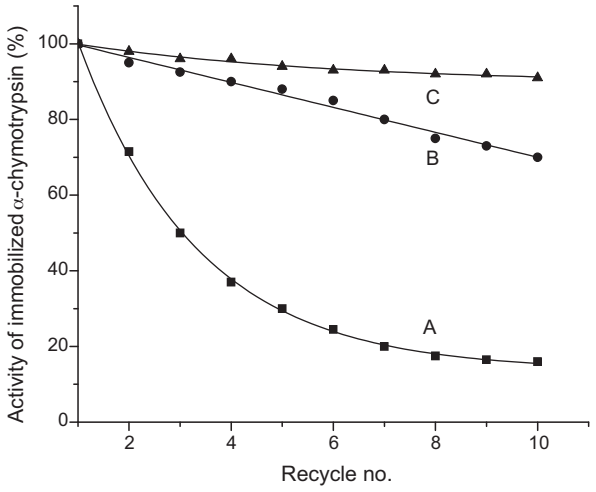


Fig. 8. Recycling of immobilized α-chymotrypsin. (a) (■) Adsorption in aqueous buffer (100% = 28 units); (b) (•) adsorption in AOT reverse micellar solution (100% = 38 units); (c) (▲) adsorption in AOT reverse micellar solution followed by crosslinking with glutaraldehyde (100% = 36 units).

3.8. Immobilization of alcohol dehydrogenase from baker's yeast (YADH) and glucose dehydrogenase (GDH)

Compared to α-chymotrypsin, the dehydrogenase enzymes are rather large multimeric enzymes and subunit dissociation is a matter of concern during their immobilization. Both YADH and GDH have been found to be stable in reverse micelles probably because the subunit dissociation is suppressed in the confines of water pool. Additionally, orientation of the protein towards the polymer surface covered with surfactant may be more advantageous than that in the aqueous phase where the protein–polymer encounter results in severe protein denaturation (>90%, unpublished observations). Thus immobilization of YADH was performed in 0.1 M AOT-isooctane reverse micellar solution (W_0 30, 20 mg polymer, reaction volume 2 mL) while immobilization experiments with GDH were performed in 0.1 M CTAB–chloroform–isooctane system (W_0 30). For co-immobilization, solutions of YADH and GDH (75 μg/mL) were prepared independently in CTAB reverse micellar solution and mixed. Assay of the immobilized enzymes indicated that the individually, enzyme loadings achieved were: YADH 2.7 mg/g ($t_{1/2}$ 98 min, corresponding to 54 μg enzyme on 20 mg polymer) and GDH 3 mg/g (180 units/g). When they were mixed, enzyme loadings obtained were: YADH 2.5 mg/g ($t_{1/2}$ 106 min, corresponding to 50 μg enzyme on 20 mg polymer) and GDH 0.5 mg/g (30 units/g). This aspect of preferential binding is interesting and is being studied further.

3.9. Regeneration and recycle of NADPH with co-immobilized YADH and GDH

The recycle of NADPH by synchronized activity of YADH and GDH immobilized on same polymer support was estimated from conversion of ethyl 2,4-dioxo-4-phenylbutyrate **1** to ethyl (*R*)-2-hydroxy-4-oxo-4-phenylbutyrate **2** using molar ratio of NADPH to substrate of 1:50. The conversion of **1** to **2** was complete in 4 h showing that NADPH turns over at least 50 times in the same batch reactor. The polymer with co-immobilized YADH and GDH was recovered and reused at least 10 times without loss of activity.

3.10. Reuse of supernatant reverse micellar solution containing free enzyme

In all the experiments of enzyme immobilization in reverse micellar media, supernatant solution containing fully active free enzyme was recovered quantitatively. Since binding of the enzyme to the polymer is an equilibrium process, the recovered solution was replenished with addition of more enzyme and reused with a fresh batch of polymer. In this manner, it was possible to use most of the original enzyme for immobilization.

4. Conclusions

In conclusion, enzymes can be successfully immobilized on AGE-25 copolymer by using reverse micellar medium instead of aqueous buffer without loss of enzyme activity. Immobilized enzymes are more stable than their soluble forms and can be further stabilized by crosslinking with glutaraldehyde. The oxido-reductases such as YADH and GDH can also be immobilized with good activity, opening up the possibilities of employing coupled-enzyme method for not only recycling of NADPH but also the enzymes for synthesis of chiral alcohols.

Acknowledgements

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