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

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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

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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,

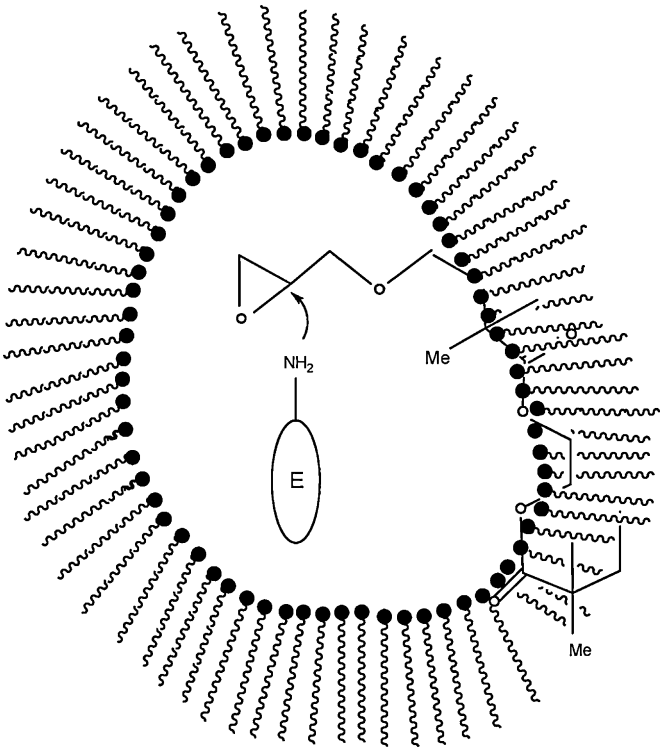

Fig. 1. The rationale of using reverse micellar media for enzyme immobilization.

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