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Biocatalysis

Immobilization of unspecific peroxygenases (EC 1.11.2.1) in PVA/PEG gel and hollow fiber modules

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ABSTRACT

The immobilization of enzymes has many advantages, such as higher stability, easier handling, and reuse of the catalyst. Here we report, for the first time, two effective methods for the immobilization of unspecific peroxygenase (UPO; EC 1.11.2.1). This biocatalyst type comprises heavily glycosylated heme-thiolate proteins that catalyze various biotechnologically relevant oxyfunctionalizations. Both the encapsulation in cryogel and the retention of the enzyme in hollow fiber modules were found to be efficient methods for their immobilization. After encapsulation, the enzyme still exhibited 60% of its initial activity. Interestingly, we did not find differences in the kinetic parameters of free and immobilized UPOs. In long-term experiments, the conversion of the pharmaceutical diclofenac with immobilized UPOs in different reactor types yielded between 62 mg and 154 mg of the major human drug metabolite 4′-hydroxydiclofenac. The maximal total turnover number was about 60-fold higher compared to the free enzyme. A test over 5 months showed that storage of encapsulated UPOs in non-polar solvents (e.g., cyclohexane) helps to preserve the enzyme stability and increases their relative activity (by about ~150%, in the case of diclofenac hydroxylation). In addition to the hydrophilic substrate diclofenac, encapsulated UPOs also oxidized the hydrophobic model compound cyclohexane.

1. Introduction

The drive toward green modern developments in biotechnology, has paved the way for the broader application of biocatalysis in organic synthesis and industrial processes. The use of biocatalysts has the advantage of mild reaction conditions (room temperature, aqueous solvents, atmospheric pressure, etc.) [1]. In 2011, a new group of peroxide-utilizing oxidoreductases, the unspecific peroxygenases (UPOs), were approved under EC 1.11.2.1 in the enzyme nomenclature system. The first UPO was described in 2004 (for the time being as a fungal haloperoxidase) for the basidiomycete *Agrocybe aegerita* [2]. Similar enzymes were found in other fungi in the following years (e.g., in *Coprinellus radians*, *Coprinopsis verticillata* [3], and *Marasmius rotula*) and turned out to represent a multifunctional superfamily of biocatalysts, which act as functional hybrids of peroxidases and cytochrome P450 monooxygenases [4–8]. UPOs are extracellular, highly glycosylated proteins and thereby rather stable. However, since they are produced in N-rich complex media, their separation and purification is laborious and time-consuming, which makes their application barely rentable on an industrial scale at the moment. There are different ways to overcome this shortage. On the one hand, protein engineering and over-expression in suitable hosts (first promising results in this direction have been achieved with *Aspergillus* and *Saccharomyces*) [9,10] and immobilization of the enzymes on the other hand could contribute to solve this problem. Enzymes can be immobilized by a number of techniques, such as by the carrier-binding method, cross-linking, entrapment in lattice or capsules or enzyme retention by ultrafiltration membranes [11].

Immobilization by means of biomass entrapment within various hydrogels is generally one of the most successful approaches for preparing immobilized biocatalysts [12–14]. A number of gel matrices have been tested as possible carriers and gel-forming agents (e.g., alginate, carrageenan, agar, gelatin, collagen, polyacrylates, polyurethanes, etc.) [14], but many of them exhibit stability problems (temperature, solvents, etc.) which result in a loss of enzyme activity during the encapsulation process [15]. Cryogels are among the most suitable carriers; their unique porous structure, which

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results from the freezing–thawing of concentrated aqueous polymer solutions, in combination with their suitable physicochemical properties (such as elasticity and high mechanical stability), have motivated their wide use as matrices for the immobilization of whole cells and also of enzyme proteins [14,16,17].

In this paper, we focus on two procedures of UPO immobilization: encapsulation in polyvinyl alcohol/polyethylene glycol gel in a freezing-thawing process and retention of the protein in hollow fiber modules. Both methods may be particularly suitable because they are not affected by the high level of UPO glycosylation [18,19], which is not the case for all other immobilization methods tested so far [15]. Most immobilization methods base on linkage processes between the matrices and the protein structure. Often the only free positions of highly glycosylated enzymes are near the channel to the active site, which leads to an increased enzyme deactivation rate.

2. Material and methods

2.1. Reactants

The analytical standard for 4′-hydroxydiclofenac [2-(2,6-dichloro-4-hydroxy-phenylamino) phenyl acetic acid] was purchased from BIOZOL Diagnostica Vertrieb GmbH (Echnig, Germany); 3,4-(methylenedioxy) nitrobenzene (nitrobenzodioxole, NBD) was purchased from TCI Deutschland GmbH (Eschborn, Germany); and polyvinylalcohol (PVA, Mowiol 10-98), polyethylenglycol (PEG 1000), silicon oil (−60 to 200 °C), diclofenac [2-(2,6-dichlorophenylamino) phenyl acetic acid)], and all other chemicals were purchased from Sigma–Aldrich (Schnelldorf, Germany).

2.2. Enzymes

The unspecific peroxygenases of *Agrocybe aegerita* (*Aae*UPO) and *M. rotula* (*Mro*UPO) were produced and purified as described previously [2,20]. The specific activities were 93 U mg^{-1} and 25 U mg^{-1}, respectively; 1 U catalyzes the oxidation of 1 μmol of 3,4-dimethoxybenzyl alcohol to 3,4-dimethoxybenzaldehyde in 1 min at 23 °C at pH 7 [2].

2.3. Encapsulation of peroxygenase in PVA/PEG-gel beads

The method was optimized according to Hischer et al. [21] and Steinsiek [22]. A mixture of 10 g PVA Mowiol 10-98 (polymerization degree: 1400) and 10 g PEG 1000 was dissolved in water, stirred for 1 h at 90 °C and cooled down to room temperature. Afterwards, 300 mg of NaOH pellets were dissolved in 1 mL of water and added to the mixture dropwise under continuous stirring (0.5 h, saponification of the mixture). One part of the mixture was adjusted with concentrated acetic acid to pH 7.0 and the other part to pH 5.5. Then, 6 mL of the enzymes [160 units of *Aae*UPO in potassium phosphate buffer (PPB, 100 mM, and pH 7) or *Mro*UPO in PPB (100 mM and pH 5.5)] were added to 40 mL of the solution.

In experiments with cyclohexane as substrate, glucose oxidase (GO_x) was co-immobilized for gentle dosage of hydrogen peroxide (H_2O_2). In this case, 1600 units of GO_x were added together with the buffered UPO solution.

Preparation of the gel beads occurred via dropwise addition of the solution (with a multipette) into silicon oil (cooled continuously to −35 °C in ethanol/dry ice). The volume of one drop (about 40 μL) was chosen according to findings of a previous study [22]. The frozen drops in silicon oil were slowly thawed in the fridge overnight. Next day, the gel beads were washed with and stored in cyclohexane [23], except in the long-term stability study (see below).

2.4. Properties of immobilized enzyme

Density of the gel beads (~4 mm i.d.) was calculated to be 1.05 g cm^{-3} (5 beads originating from 40 μL had a mass of 210 mg). The enzyme activity was calculated as follows: 46 mL of the solution were amended with 160 U of UPO, which yielded a theoretical load of 0.14 U per bead. Activity changes during repeated reactions or in stability tests were determined by the nitrobenzodioxole (NBD) assay [24]. The reaction mixture contained 500 μL of PPB pH 7, 100 μL of NBD solution (5 mM), 390 mL of deionized water, and one gel bead. The reaction was started with 1 mM H_2O_2 and the absorption measured after 10 min of reaction by a spectrophotometer at 425 nm (ϵ_{425} = 9.700 M^{-1} cm^{-1}).

2.5. Peroxide dosage optimization

For this experiment, 24 beaker glasses (5 mL) were filled with 500 μL of PPB (pH 7 and 100 mM). Five washed beads with immobilized *Aae*UPO were put into each beaker and supplemented with 100 μL diclofenac solution (5 mM in 50% v/v ACN) and 100 μL of ascorbic acid (40 mM). The beakers were divided into eight group of three. To the beakers of the first four groups, H_2O_2 was added over 10 min in five portions every 2 min (final peroxide concentrations: 1 mM, 2 mM, 4 mM, and 8 mM). To the beakers of the next two groups, H_2O_2 was continuously supplied with a syringe pump over 30 min (final peroxide concentrations: 10 mM and 16 mM). The last two groups of beakers were continuously supplied with H_2O_2 via a syringe pump over 60 min (final peroxide concentrations: 16 mM and 32 mM).

Similar experiments were carried out with nitrobenzodioxole (NBD) and H_2O_2 or *m*CPBA (*meta*-chloroperoxybenzoic acid) as co-substrates. The reaction mixtures were prepared as follows: 10 beakers (5 mL) were filled with 1 mL of PPB (pH 7 and 100 mM), 600 μL of deionized water and 200 μL of NBD (5 mM in ACN). To the first two beakers, five beads of immobilized *Aae*UPO were added and the reaction started by addition of 200 μL H_2O_2 (200 mM) or *m*CPBA (200 mM). After 10 min the beads were transferred from the first to the second set of beakers and this procedure was repeated additional three times with the same five beads. After 10 min, the reaction mixtures were transferred into quartz cuvettes and the concentration of nitrocatechol measured at 425 nm (quantification was done as described previously) [24], respectively.

2.6. Characterization of immobilized peroxygenase

To determine the long-term storage parameters of encapsulated UPO, 100 washed beads with immobilized *Aae*UPO were divided into four fractions and stored at 11 °C in different solvents: PPB (pH 7 and 100 mM), *n*-hexane, cyclohexane, and dichloromethane. Enzyme activity was measured on day 1, 2, 6, 13, 20, 27, 42, 65, and 160 using the NBD assay described above [24]. Reactions were carried out in 56-well plates with 1-mL cavities that contained 500 μL PPB (pH 7 and 5.5 for *Aae*UPO and *Mro*UPO, respectively), 390 μL H_2O, 100 μL NBD (5 mM), one bead (0.14 U UPO) and started by addition of 10 μL H_2O_2 (100 mM). Reaction mixtures were stirred with a magnet stirrer for 10 min and subsequently measured using a multimode reader (Tecan Infinity M200, Tecan Group Ltd., Männedorf, Germany). All experiments were carried out in triplicate. To verify the positive effect of organic solvents on the enzyme activity, the experiment was repeated with PPB (pH 7) and cyclohexane. The beads were treated as described before, but the first activity test was already started 30 min after incubation of the beads with either buffer or cyclohexane. To verify the calculated