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Dry entrapment of enzymes by epoxy or polyester resins hardened on different solid supports

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ABSTRACT

Embedding of enzymes was performed with epoxy or polyester resin by mixing in a dried enzyme preparation before polymerization was started. This fast and low-cost immobilization method produced enzymatically active layers on different solid supports.

As model enzymes the well-characterized *Thermomyces lanuginosus* lipase and a new threonine aldolase from *Ashbya gossypii* were used. It was shown that *T. lanuginosus* lipase recombinantly expressed in *Aspergillus oryzae* is a monomeric enzyme with a molecular mass of 34 kDa, while *A. gossypii* threonine aldolase expressed in *Escherichia coli* is a pyridoxal-5′-phosphate binding homotetramer with a mass of 180 kDa.

The enzymes were used freeze dried, in four different preparations: freely diffusing, adsorbed on octyl sepharose, as well as cross-linked enzyme aggregates or as suspensions in organic solvent. They were mixed with standard two-component resins and prepared as layers on solid supports made of different materials e.g. metal, glass, polyester. Polymerization led to encapsulated enzyme preparations showing activities comparable to literature values.

1. Introduction

Immobilization is an opportunity to increase the half-life of enzymes, to re-use the biocatalyst and furthermore to enable a long-term processing. Additionally, fixed biocatalysts show improved long-term performance under optimal chemical synthesis conditions, e.g. in organic solvents, at higher temperature or extreme ionic strength. Therefore, immobilization can lower the costs for enzymes in industrial application [1–5].

In the last decades new immobilization methods were established as well as new matrices were applied to modify enzyme characteristics and optimize immobilization yield. Combining for example the geometry, as well as physical and chemical properties of a support of a resin can change the properties of an enzyme in the desired way. Meso- and macroporous resins based on polystyrene [6], methacryl [7], or epoxide [8], can be functionalized by different groups to introduce ionic, hydrophobic or absorptive interactions. Furthermore, covalent immobilization can be realized using glyoxyl- [9], epoxy- [7] or glutaraldehyde [10] groups. In general, these resins are used in a porous bead-based form where pore size can be altered appropriate to the molecular size of the enzyme and the substrate/product as well as providing a hydrophilic and protective environment inside pores or fissures [3,11]. Concerning the method of entrapment, such inorganic or organic polymeric substances have also been used for enzyme [12] and cell [8] immobilization too.

Approximately 80% of industrial processes using immobilized enzymes make use of hydrolases [13]. In particular, lipases are used in the detergent, food, leather, textile, oil and fat, cosmetic, paper, and pharmaceutical industries [14]. Lipases are hydrolytic enzymes cleaving or synthesizing ester-bonds preferentially at an interface of long-chain (≥10 carbon atoms) triacylglycerols. The high interest level of industry depends on the wide range of properties of lipases such as positional specificity, fatty acid specificity, thermal stability, and activity in organic solvents [15]. One prominent representative

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with a broad application spectrum is the *Thermomyces lanuginosus* Lipase (TLL), recombinantly produced in *Aspergillus oryzae*. Due to its robustness, well-studied structure and kinetics it is already industrially applied as a soluble as well as immobilized enzyme [16].

Beside hydrolases, oxidoreductases and lyases are found in industry [17]. In the production of β-hydroxy-α-amino acids (L-threonine, L-serine and 3-hydroxyproline), which are precursors for active substances in the pharmaceutical (e.g. anti-cancer drugs, antibiotics and immunosuppressors) and agrochemical industries, L-threonine aldolase (TA) using pyridoxal-5′-phosphate (PLP) as a cofactor is of interest. The enzyme catalyzes L-threonine cleavage into glycine and acetaldehyde as well as the reverse that means CC coupling reactions [18,19]. Overexpression of TA in the filamentous fungus *Ashbya gossypii* leads to an increase in glycine production, which in turn increases riboflavin production [20]. Immobilization of TA has already been achieved on several carriers such as Eupergit CM [21].

The potential of enzyme immobilization is under-utilized due to several drawbacks such as the time-intensive laborious development of an appropriate immobilization method, cost-intensive materials and limited enzyme load. In the present study an enzyme entrapment method was developed for immobilizing water-free enzyme preparations, either lyophilized or suspended in an organic solvent. The water dry enzyme molecules were mixed with standard polymeric glue based on either polyester or epoxy. Supports differing in chemical composition and geometry were coated with the enzyme/polymer mix. After polymerization these enzyme reaction units could be used for activity assays. Two very different enzymes were applied, namely the recombinant TLL and the TA from *A. gossypii* expressed in *E. coli*. Enzyme characterization showed their differences in structure and size as well as cofactor dependence. TLL was used as a model to compare different immobilization methods including the new dry entrapment. The immobilisates were analyzed with respect to enzyme activity recovery and their activity towards different substrates. With respect to show broader prospective application, TA was applied to the dry entrapment method analyzing both enzyme activity and recovery.

The received results confirm the development of a new immobilization method that is easy to apply to very different enzymes on varying supports. The dry entrapment needs no specific design of support material or enzyme and is therefore time- and cost-saving.

2. Methods

2.1. Enzymes

As models for the newly developed immobilization method, two very different enzymes were used and characterized: the NOVAREKO (NovaBiotec® Dr. Fechter GmbH, Berlin) preparation, a *T. lanuginosus* lipase (TLL) recombinantly produced by *A. oryzae*, and *A. gossypii* threonine aldolase (TA) recombinantly produced in *Escherichia coli*.

2.1.1. Threonine aldolase overexpression and purification

The *GLY1* (gene accession number: AJ005442) gene encoding the TA was amplified using the matrix Yep352GB07-1, a *Saccharomyces cerevisae*/*E. coli* shuttle vector including parts of the genomic library of *A. gossypii*. Primers were constructed according to Monschau et al. [20] with some changes to introduce additional restriction sites for *BsaI* to ensure easy cloning into pASK-IBA43 (IBA, Germany), a commercial expression vector for *E. coli*. The amplicon was purified and inserted into this vector following manufacturer instructions. The integration into *E. coli* BL21 was carried out by heat shock generating the *E. coli* BL21 pAF *Gly1* strain. The expression of TA fused C-terminal to a Strep-tag was induced by the addition of anhydrotetracycline as described in the customer instructions of IBA (Germany). After French Pressing the cell pellet the cleared supernatant was applied to a Strep-Tactin column for purification (IBA, Germany).

2.1.2. Determination of molecular mass

The molecular mass of the enzyme preparations was determined by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE).

2.1.3. Chromatographic methods

Purification of TLL was carried out by Fast Protein Liquid Chromatography (FPLC) using Q-Sepharose. The main elution fractions bearing lipase activity were pooled and applied to size exclusion chromatography. For this purpose Sepharose G-100 beads (Pharmacia, Sweden) were used in a 0.05 M Tris–acetate buffer, pH 7.5, containing 0.1 M NaCl with a flow rate of 18 ml/h. Fractions of 2.3 ml were collected and analyzed by UV spectroscopy and lipase activity determination. Bovine serum albumin (69 kDa), carbonic anhydrase (28.8 kDa), and cytochrome c (12.4 kDa) were applied as standard molecules in a separate run on the column.

For size determination and analysis of the quaternary structure of TA from *A. gossypii*, a sepharose G-100 bead column in 0.05 K/Na-Phosphate buffer pH 7.0 and 0.3 M NaCl at a flow rate of 10 ml/h was used. Fractions of 2 ml were collected and analyzed by UV spectroscopy at 280 nm; additionally, enzyme activity was determined. Standard molecules were mixed with the TA preparation and run in the same performance (alkoholdehydrogenase (150 kDa); albumin from chicken egg (45 kDa); cytochrome c (12.4 kDa)).

2.1.4. Pyridoxal-5′-phosphate dependence

An important co-factor in amino acid synthesis and degradation is pyridoxal-5′-phosphate (PLP). To analyze if the TA of *A. gossypii* is dependent on PLP an absorption spectrum of the enzyme solution between 300 nm and 650 nm was carried out. Enzyme activity was measured in the presence and absence of PLP.

2.1.5. Isoelectric focusing (IEF) and activity staining

To determine the isolectric point of recombinant TLL, IEF was carried out as described by Stahmann et al. [22]. The 5% (v/v) polyacrylamide gel was completed with 6% (v/v) ampholytes ranging from pH 3 to 5 (Serva, Germany). Subsequently, the proteins were either stained with Coomassie brillant blue R250 or esterase activity was visualized as described by Barig et al. [23].

2.1.6. Enzyme activity and concentration

For each experiment a minimum of two separate trials was carried out, determining the mean value for presentation. If more than two individual experiments were done the standard deviation is indicated.

Lipase activity was determined by colorimetric assay using *para*-nitrophenyl palmitate (pNPPalm) as substrate as previously described by Barig et al. [23]. To show hydrolytic activity on short chain molecules, *p*-nitrophenyl propionate (pNPProp) was applied. To this end, 10 μl of solution A (7.8 mg pNPProp in 1 ml 100% ethanol) and 990 μl of solution B (0.1 M Na-Phosphate buffer pH 7.4) were mixed with 100 μl of enzyme solution and the released *p*-nitrophenol was analyzed spectrophotometrically at 405 nm for 4 min at 30 °C. One unit (U) was defined as the amount of enzyme needed to liberate 1 μmol *p*-nitrophenol ($\varepsilon = 12{,}500\ M^{-1}\ cm^{-1}$) per minute under the previously described conditions.

Lipase activity on natural substrates was determined by titrimetry using the autotitrator TIM 854 Titration Manager (Radiometer Analytical S.A., France). For this purpose, the substrate solution (0.5 g gum arabicum, 20 ml 0.9% NaCl solution, 3 ml H_2O, 400 μl substrate, e.g. rape seed oil, triolein, tributyrin) was dispersed with Ultraturrax® for 30 s at 12,500 rpm and the pH was set at 7. The solution was stirred at 400 rpm and reaction was initiated by the addition of 1 ml of enzyme solution. Free fatty acids were continuously titrated by 10 mM NaOH. One unit (U) was determined as the amount of enzyme needed to liberate 1 μmol of free fatty acid per minute.

TA activity was determined either spectrophotometrically using the substrate analogue phenylserine or by high-performance liquid chromatography (HPLC) determining the amount of released glycine from threonine as described by Monschau et al. [24]. For spectrophotometric detection the substrate solution was composed of 0.9% DL-threo-β-phenylserine (Sigma–Aldrich Chemie GmbH, Germany) and 0.05 mM pyridoxal-5′-phosphate (PLP) (Sigma, Germany) in 250 mM HEPES/NaOH buffer pH7. Reaction was performed by mixing 980 μl of pre-heated (28 °C) substrate solution intensively with 20 μl enzyme solution. The release of benzaldehyde ($\varepsilon = 1400\ M^{-1}\ cm^{-1}$) was detected by spectrophotometric measurement at 279 nm for a period of 15 min at 28 °C. One unit (U) was defined as the amount of enzyme needed to liberate 1 μmol of benzaldehyde per minute [25].

Protein concentration was determined by the method of Bradford [26].

2.2. Immobilization

Different immobilization methods were carried out to compare or combine them with the newly developed dry entrapment method.

2.2.1. Cross-linked enzyme aggregates (CLEAs)

The TLL was immobilized by forming enzyme aggregates and subsequent cross-linking with glutaraldehyde as described by Lopez-Serrano et al. with some variations [27]. For a single preparation of cross-linked TLL, 0.5 ml lipase solution was mixed with 0.875 ml 0.1 M Na-Phosphate buffer pH 7, 0.125 ml of a 50 mg/ml SDS solution and 1 ml of 55% ammonium sulfate in the same buffer system. These components were stirred for 2 h at room temperature to ensure precipitation. To cross-link the aggregated lipase, 4 μl of 50% glutaraldehyde were added and stirred overnight at 4 °C. Three washing steps using ddH_2O ensured removal of the reacting agents. The CLEAs were suspended in 5 ml ddH_2O and stored at 4 °C.