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Characterization and application of a sterol esterase immobilized on polyacrylate epoxy-activated carriers (Dilbeads™)

P. Torres [a], A. Datla [b], V.W. Rajasekar [b], S. Zambre [b], T. Ashar [b], M. Yates [a], M.L. Rojas-Cervantes [c], O. Calero-Rueda [d], V. Barba [d], M.J. Martínez [d], A. Ballesteros [a], F.J. Plou [a,*]

[a] *Departamento de Biocatálisis, Instituto de Catálisis y Petroleoquímica, CSIC, Cantoblanco, 28049 Madrid, Spain*
[b] *Research Support International Ltd., DIL Complex, Thane West, India*
[c] *Departamento de Química Inorgánica y Química Técnica, Facultad de Ciencias, UNED, 28040 Madrid, Spain*
[d] *Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain*

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Abstract

The sterol esterase from the ascomycete *Ophiostoma piceae* was immobilized on novel polyacrylate-based epoxy-activated carriers (Dilbeads™). Six supports with particle sizes between 120 and 165 μm were prepared varying the composition of monomers, cross-linkers and porogens. Their surface areas and porosities were determined by N_2 adsorption and mercury intrusion porosimetry. The pore volumes ranged from 0.63 to 1.32 cm^3/g, but only Dilbeads™ RS and NK had narrow pore size distributions (with maxima at 33.5 and 67.0 nm, respectively). The distribution of the enzyme in the support was studied by fluorescence confocal microscopy. The immobilized esterase on Dilbeads™ TA showed a significant pH and thermal stability and was assayed in the continuous hydrolysis of cholesteryl esters –present in the pulp industry process waters.

Keywords: Covalent immobilization; Epoxy carriers; Cholesterol esterase; Confocal microscopy; Pitch control

1. Introduction

For the industrial development of biocatalytic processes an effective immobilization method is commonly required to allow the reuse of enzymes or continuous processing [1,2]. Different strategies have been proposed to immobilize enzymes, based on adsorption [3,4], covalent binding [5], granulation [6], entrapment in polymers [7] and cross-linking of enzyme crystals or protein aggregates [8].

Covalent immobilization has the advantage of forming strong and stable linkages between the enzyme and the carrier that result in robust biocatalysts [5]. Covalent attachment also eliminates the loss of activity caused by enzyme leakage from the support. Different materials, e.g. cross-linked dextrans (Sepharose), polysaccharides (agarose) or porous silica can be chemically activated by different approaches to covalently attach enzymes [9]. However, the number of commercialized activated carriers for covalent immobilization is relatively small compared with available enzyme adsorbent materials.

Epoxy(oxirane)-activated materials, such as Eupergit C [10,11] or Sepabeads [12,13] are very attractive because of their high reactive groups density and the simple chemistry for covalent attachment of the enzyme to the support. In this work we describe for the first time a new class of epoxy-activated supports (Dilbeads™), based on the combination of three or four types of acrylic monomers with varying porogen concentration, prepared with a range of pore sizes to cover the spatial requirements of different enzymes. These materials are rigid and do not swell in

* Corresponding author. Tel.: +34 91 5854869; fax: +34 91 5854760.
E-mail address: fplou@icp.csic.es (F.J. Plou).
URL: http://www.icp.csic.es/abg (F.J. Plou).

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water as occurs with the polyacrylamide-based Eupergit C. Dilbeads™ are epoxy(oxirane)-activated, so they bind enzymes as a function of pH through different nucleophiles: at neutral or slightly alkaline pH with the thiol groups, at pH > 8 with the amino groups and at pH > 11 with phenolic groups of tyrosines [12]. The spatial distribution of the enzyme in the carrier bead was studied by confocal laser scanning microscopy, which non-invasively gathers optical slices from a macroscopic bead.

To test the applicability of these carriers for enzyme immobilization, we have covalently attached to Dilbeads™ the sterol esterase from the ascomycete *Ophiostoma piceae,* which can be used for hydrolysis of cholesteryl esters during pulp manufacture [14]. These colloidal particles accumulate in pulp or machinery forming "pitch deposits" or remain suspended in the process waters. The application of enzymes for the biotreatment of wastewaters (enzymatic bioremediation) is rapidly expanding as it offers some advantages compared with other technologies [15].

2. Experimental

2.1. Materials

Cholesterol, cholesteryl oleate, *p*-nitrophenyl butyrate, polyoxyethylene 10 tridecyl ether (Genapol X-100), fluorescein isothiocyanate and hydranal-composite 5 were purchased from Sigma. Sterol esterase was produced as described elsewhere [16]. All other reagents and solvents were of the highest available purity and used as purchased.

2.2. Synthesis and characterization of Dilbeads™ carriers

All the carriers were synthesized in 500 ml flat-bottom cylindrical reactors, previously purged with nitrogen, fitted with a reflux condenser, a mechanical stirrer and a thermometer. Suspension polymerization was carried out to produce different types of polymer beads, by using a judicious mix of acrylate monomers, cross-linkers and porogens. After the polymerization, the beads were filtered and washed thoroughly. Dilbeads™ is a proprietary product of Fermenta Biotech Ltd., and the process of manufacture is detailed in an Indian patent application [17]. The polymer beads formed by the above proprietary process were further used in the immobilization experiments.

Mercury intrusion porosimetry analyses of the supports were performed using a Fisons Instruments Pascal 140/240 porosimeter. To ensure that the samples were moisture free, they were dried at 100 °C overnight prior to measurement. The recommended values for the mercury contact angle (141°) and surface tension (484 mN/m) were used to evaluate the pressure/volume data by the Washburn equation, assuming a cylindrical pore model [18]. The particle size distributions of the supports were determined by analysis of the intrusion curve, which in the case of a finely divided powder gives information on the interparticle porosity. From the total porosity of the material – assuming spherical particles – the packing factor and subsequently the particle size distribution were calculated according to the Mayer–Stowe theory [19]. The specific surface area (S_{BET}) of the supports was determined from analysis of nitrogen adsorption isotherms at −196 °C, using a Micromeritics ASAP 2010 device. The samples were previously degassed at 100 °C for 12 h to a residual vacuum of 5×10^{-3} torr, to remove any loosely-held adsorbed species. Water content of the supports was assayed using a DL31 Karl–Fisher titrator (Mettler). Scanning electron microscopy (SEM) was performed using an XL3 microscope (Philips) on samples previously metallized with gold.

2.3. Preparation and characterization of immobilized sterol esterase

Dilbeads™ (1 g) were mixed with 5 ml of crude sterol esterase in 0.3 M potassium phosphate buffer (pH 8.0). The mixture was incubated for 72 h at 4 °C with roller shaking. The biocatalyst was then filtered using a glass filter (Whatman), washed (3 × 10 ml) with 1 M potassium phosphate buffer (pH 8.0), dried under vacuum and stored at 4 °C.

To characterize the distribution of sterol esterase in Dilbeads™, we used fluorescence confocal microscopy with proteins previously labeled with fluorescein isothiocyanate (FITC). The protein to be labeled was first dissolved in 0.05 M carbonate buffer, pH 9.0, to a final concentration of approx. 4.5 mg/ml. FITC dissolved in dimethylformamide was then added to a FITC/protein ratio of 5 μg/mg. The reaction was then performed at room temperature for 1 h. The labeled protein was purified from unbound FITC by gel chromatography using a pre-packed PD-10 column (Amersham Biosciences). Fluorescence confocal microscopy was performed with a Leica TCS SP2 confocal laser scanning microscope (CLSM) equipped with nine excitation lines and software for image processing. A 40.0 × 1.25 oil immersion objective was used for all measurements and the pinhole aperture was set to 1.50 Airy (122 μm). The laser provided excitation of FITC at 488 nm and emitted fluorescent light was detected at 520 nm.

The pH stability was measured at 25 °C in 20 mM citrate–phosphate–borate buffer at different pH values. In the case of immobilized enzyme, the amount of biocatalyst added was adjusted to approx. 0.5 mg per ml. The remaining activity was measured using the standard activity assay with *p*-nitrophenyl butyrate (pNPB) after different times in reaction. The thermostablity studies were performed in 20 mM citrate-phosphate-borate buffer (pH 6.0) incubating the enzyme (0.5 mg immobilized biocatalyst per ml) at 45 or 60 °C. The remaining activity was measured with pNPB at different times. The activity was assayed spectrophotometrically following *p*-nitrophenol release ($\varepsilon_{410} = 15200$ $M^{-1}\,cm^{-1}$) from 1.5 mM pNPB in 20 mM citrate-phosphate-borate buffer (pH 7.2).