



Hydrophobic microenvironment optimization for efficient immobilization of lipases on octadecyl functionalised resins



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ABSTRACT

A novel family of spherical beads made of octadecyl (OD) functionalised methacrylic resins for lipase immobilization was developed. The activity of the immobilized lipase reflected the effect of OD functional groups density (0.3–1.48 mmol OD/g_{dry}) on the hydrophobic microenvironment. The immobilized enzyme activity on Lifetech ECR8806 was outstanding: lipase from *Candida antarctica* B (CALB) showed activity higher than 15,000 PLU/g_{dry}. A significantly high activity was also observed for immobilized lipases from *Rhizomucor miehi* (RM) and *Thermomices lanuginosus* (TL), confirming the positive effect provided by the OD functional groups grafted on the methacrylic polymer backbone.

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1. Introduction

Lipases constitute the most important group of biocatalysts for biotechnological applications. Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are part of the family of hydrolases that act on carboxylic ester bonds. The physiologic role of lipases is to hydrolyse triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol.^{1–6} In addition to their natural function of hydrolysing carboxylic ester bonds, lipases can catalyse esterification, interesterification, and transesterification reactions in non-aqueous media.⁷ A very useful characteristic of lipases is their enantioselectivity intensively exploited in the pharmaceutical industry where they are used for the preparation of single-isomer chiral drugs, either by kinetic resolution of racemic alcohols, acids, esters or amines, or by the desymmetrization of prochiral compounds.⁸ This versatility makes lipases the enzymes of choice for many applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries.⁹

Limitations of the industrial use of these enzymes have mainly been owing to their high production costs as well as the lack of long-term stability under the process conditions and in many cases to difficulties in recovery and recycling. Production costs can be

overcome by molecular technologies, enabling the production of these enzymes at high levels and in a virtually purified form.¹⁰

Immobilising enzyme molecules makes it possible to reuse them in subsequent reaction cycles, resulting in reduced production costs and simplified operations for isolating the products of the enzyme reaction. For this purpose was necessary to research and develop particularly efficient insoluble supports and simple and efficient immobilisation methods. Very often the key to improved operational performances is provided by immobilizing the enzyme on heterogeneous supports by various techniques like covalent binding,^{11–13} entrapment,^{14–16} or adsorption.^{17,18}

Immobilization of lipases requires carriers specifically developed with an enhanced hydrophobic surface and compatible with a hydrophobic environment where lipases typically work. As previously reported, the large hydrophobic area that surrounds the active site of lipases is the one mainly involved in their adsorption on strongly hydrophobic solid surfaces.¹⁹ Thus, lipases react to these surfaces in a way that is similar to the way they recognise natural substrates and therefore undergo interfacial activation during immobilization.²⁰

Octadecyl methacrylates have attracted attention for their particular compatibility with lipase CALB (from *Candida antarctica* B), from *Rhizomucor miehei* (RM) and *Candida rugosa* lipases via interfacial adsorption.²¹ The activity and stability of lipases immobilized on octadecyl methacrylates are higher than values obtained with other conventional immobilized derivatives (e.g., those

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obtained by multipoint covalent attachment). Furthermore, lipases but also other enzymes like transaminases²² adsorbed on hydrophobic supports like octadecyl methacrylates have been described to be very stable against heat and organic solvents inactivation.^{23–25}

The present paper describes the development and application of a novel type of octadecyl hydrophobic carrier, showing that the physical and chemical properties of the resin greatly affect final performances of immobilized enzymes. A range of resins have been developed using two types of polymer backbones: methacrylic and styrenic and varying the density of OD groups grafted on the polymer. The effect of changes in the hydrophobic microenvironment created at polymer level for enzyme immobilization was quantified via the activities of different immobilized lipases. The activities of lipases immobilized on these novel types of carriers were compared to commercially available products, the immobilized enzymes were: lipase CALB a small enzyme (33 KDa) used for a wide range of biotransformations;²⁶ lipase TL (from *Thermomices lanuginosus*) a small spherical enzyme (31 KDa) with an isoelectric point of 4.4 with a mobile lid that covers the active site;^{27–29} lipase RM with a molecular size 31.6 KDa, an isoelectric point of 3.8 and a small lid that covers the active site.^{7,30}

The lipase activity was quantified using model reactions: the propyl laurate synthesis in presence of immobilized CALB or the tristearin depletion in presence of immobilized lipase RM and TL.

For all three immobilized lipases outstanding results were obtained when ECR8806 resins were used, showing improvements of up to four times compared to other enzyme carriers.

2. Results and discussions

2.1. Influence of octadecyl groups density

In order to understand the effect of the hydrophobic microenvironment on lipase immobilization, we have developed two series of resins: one based on hydrophilic methacrylic polymeric backbone and one on a more hydrophobic styrene backbone. The amount of octadecyl groups has been varied in both types of resins in order to evaluate its effect on immobilized lipase performance.

Octadecyl methacrylates commercially available (EC-OD from Resindion) have an initial epoxy methacrylic matrix which is transformed into a resin with the surface covered by octadecyl groups.¹⁹ In our approach, instead of functionalizing directly the epoxy methacrylic resins, we have tuned the amount of functional groups available at the surface of the polymer thus creating during the polymerization process a highly hydrophobic environment optimal for lipase activity and stability. This optimal amount of octadecyl on the accessible surface of the polymer improves the interfacial adsorption of lipases even when compared to multipointly immobilized preparations, in both thermal and organic co-solvent inactivation.²¹ The resins were prepared by suspension polymerisation to have particle size in the range 300–700 μm , with octadecyl group density varying from 0.3 to 1.48 mmol/g_{dry}.

In Table 1 we report on the physical and chemical properties of these preparations made with different amounts of functional groups.

Varying the functional group density of the resin has a strong impact on physical properties of the methacrylic carriers. The increase of octadecyl groups density gives an increased steric hindrance in the resin structure with a consequent reduction of pore volume (from 1.45 mL/g to 0.22 mL/g), pore diameter (from 1134 Å to 398 Å) and surface area (from 351 m²/g to less than 30 m²/g). The increase in resin hydrophobicity and decrease in porosity due to the amount of octadecyl groups is also reflected by the water content which was 70% in D5400/4 and dropped to 56% in ECR8804.

Table 1

Summary of the physical properties of OD functionalised methacrylic resins

Polymer	Functional groups ^a	Pore volume ^b	Pore diameter ^b	Surface area ^c	Water content ^d
	mmol OD/g _{dry}	ml/g	Å	m ² /g	%
ECR8804	1.48	0.22	398	30	56
ECR8806	1.03	0.65	616	116	64
D5400/2	0.59	1.21	1255	244	68
D5400/4	0.3	1.45	1134	351	70
ECR1030M	none	0.53	286	103	60
EC-OD/S ^e	n.d.	0.2–0.4	100–200	60–80	55–65
Lewatit VP 1600 OC ^f	none	0.51	324	74	60

^a Theoretical value.

^b Measured by mercury intrusion.

^c Measured by BET.

^d Measured by infrared balance.

^e Values from supplier.

^f Lewatit VP 1600 OC is the resin used to manufacture Novozym 435.

We have compared the physical properties of the newly prepared octadecyl methacrylates with ECR1030M a non-functionalised divinylbenzene (DVB)/methacrylic resin which proved to be excellent for CALB immobilization, and with EC-OD competitors' resin, as well as with the Lewatit VP 1600 OC used for the manufacture of Novozym 435.^{19,20} It has to be mentioned that there are several techniques for measuring the porosity of the resins giving results that are not always comparable to each other. Therefore the physical characteristics of EC-OD/S might not be directly comparable to those of the Purolite resins.

To verify if the increase in hydrophobicity is a key parameter governing adsorption properties and enzyme performances, in a second set of experiments different resins with a styrene backbone and different concentrations of octadecyl groups were prepared (Table 2). The aim was to evaluate the combined effect of the styrene monomer in the polymer backbone and the various concentrations of OD functional groups on the formation of a hydrophobic microenvironment for lipase immobilization. The new resins were also manufactured by suspension polymerisation using styrene as cross-linker and a hydrophobic monomer. Table 2 reports the physical properties of the obtained resins with particle size in the range 300–710 μm .

Table 2

Summary of the physical properties of OD functionalised styrenic resins

Polymer	Functional groups ^a	Pore volume ^b	Pore diameter ^b	Surface area ^c	Water content ^d
	mmol OD/g _{dry}	ml/g	Å	m ² /g	%
D5399/1	0.808	1.47	1182	379	68
D5399/3	0.940	1.46	1095	302	66
ECR1811	1.039	1.34	1098	257	63
D5399/5	1.135	1.14	1200	232	65

^a Theoretical value.

^b Measured by mercury intrusion.

^c Measured by BET.

^d Measured by infrared balance.

The functional group density was increased from 0.8 mmol/g_{dry} to 1.1 mmol/g_{dry}. The effect of the increase of functional groups caused a decrease in the surface area from 379 m²/g to 232 m²/g. Similarly to methacrylate resins, the increase of OD groups lead also to a decrease both in the pore volume and pore diameter. The water content on the contrary was not significantly affected by the increase in OD groups and was maintained in a range of 63–68%. The smaller variation in the concentration of OD functional group combined with the higher hydrophobicity of the styrene monomer

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