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Journal of Molecular Catalysis B: Enzymatic 11 (2001) 649–656

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Biocatalyst engineering exerts a dramatic effect on selectivity of hydrolysis catalyzed by immobilized lipases in aqueous medium

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Abstract

It has been found that enantioselectivity of lipases is strongly modified when their immobilization is performed by involving different areas of the enzyme surface, by promoting a different degree of multipoint covalent immobilization or by creating different environments surrounding different enzyme areas. Moreover, selectivity of some immobilized enzyme molecules was much more modulated by the experimental conditions than other derivatives. Thus, some immobilized derivatives of *Candida rugosa* (CRL) and *C. antarctica*-B (CABL) lipases are hardly enantioselective in the hydrolysis of chiral esters of (*R*,*S*)-mandelic acid under standard conditions (pH 7.0 and 25°C) ($E < 2$). However, other derivatives of the same enzymes exhibited a very good enantioselectivity under nonstandard conditions. For example, CRL adsorbed on PEI-coated supports showed a very high enantio-preference towards *S*-isomer ($E = 200$) at pH 5. On the other hand, CABL adsorbed on octyl-agarose showed an interesting enantio-preference towards the *R*-isomer ($E = 25$) at pH 5 and 4°C. These biotransformations are catalyzed by isolated lipase molecules acting on fully soluble substrates and in the absence of interfacial activation against external hydrophobic interfaces. Under these conditions, lipase catalysis may be associated to important conformational changes that can be strongly modulated via biocatalyst and biotransformation engineering. In this way, selective biotransformations catalyzed by immobilized lipases in macro-aqueous systems can be easily modulated by designing different immobilized derivatives and reaction conditions.

Keywords: Selective hydrolysis; Immobilized lipases; Modulation of selectivity of lipases; Mandelic acid

1. Introduction

1.1. Immobilized versus soluble lipases catalyzing biotransformations in macro-aqueous systems

Most of lipase biotransformations reported in literature are carried out by using high concentrations

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1381-1177/01/$ - see front matter
PII: S1381-1177(00)00080-1

of soluble preparations of lipase, very frequently even nonpurified ones. Because of the trend of lipases to be activated (adsorbed) on hydrophobic structures [1], many of these soluble biotransformations in macro-aqueous systems may be strongly influenced by lipase–lipase or lipase–protein interactions even acting on fully soluble substrates. In addition to that, in some cases over-saturated substrate solutions are used and, thus, soluble lipases may undergo interfacial activation on drops of immiscible substrates [2,3]. Nevertheless, under these complex conditions very good selectivities have been reported for many biotransformations catalyzed by lipases [4,5].

However, when these biotransformations are carried out by immobilized lipase preparations, the enzyme is working under very different conditions (Fig. 1). On one hand, lipase molecules are now fully dispersed on the supports and, hence, they cannot undergo any lipase–lipase or lipase–protein interactions. On the other hand, when using over-saturated concentrations of substrate, drops of immiscible substrate cannot penetrate inside the porous structure of the supports and, hence, immobilized lipase molecules are only acting on soluble substrate molecules, without any interfacial activation promoted by those drops [1].

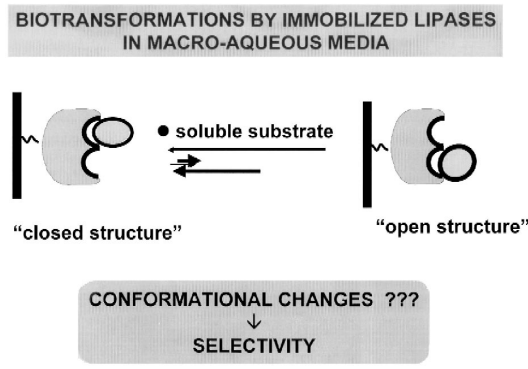


Fig. 2. Selectivity of immobilized lipases in macro-aqueous systems may be strongly influenced by the transition between their closed-inactive and their open-active structures.

In this way, the catalytic behavior of immobilized lipases in macro-aqueous environments can be quite different from the behavior of high concentrations of free enzyme acting on over-saturated substrate solutions (Fig. 1).

Molecules of immobilized lipases in macro-aqueous systems should mainly display their "closed structure" in a partial equilibrium with the "open one(s)" [2,3] (Fig. 2). In this way, these biotransformations, in addition to their industrial interest, may

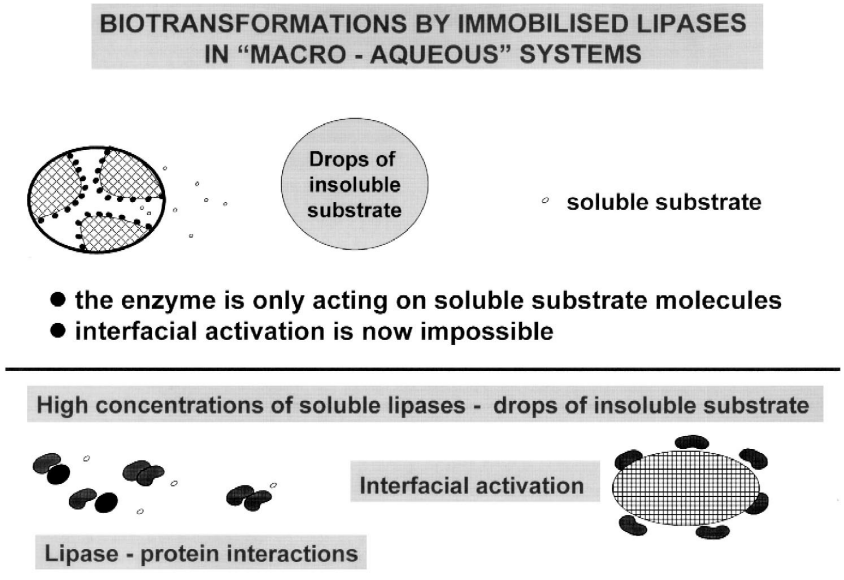


Fig. 1. Immobilized versus soluble lipases in catalyzing biotransformations in macro-aqueous systems.

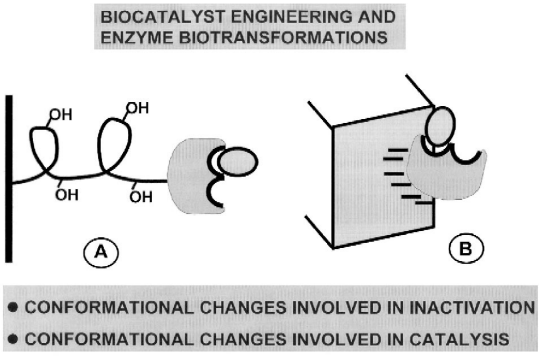


Fig. 3. Possible modulation of conformational changes involved in activity — stability of lipases via controlled and directed immobilization: (A) "pure immobilization" via mild covalent attachment through long and inert spacer arms may preserve intact the properties corresponding to soluble lipases; (B) "multipoint immobilization" via areas very related to the active center can strongly alter enzyme properties.

represent a very clear example of selective biotransformations catalyzed by enzyme molecules undergoing dramatic conformational changes. From here, it can be expected that these conformational changes may be important in the control of enzyme selectivity.

1.2. Modulation of conformational changes of enzymes via biocatalysts engineering

For technological and economical reasons, the industrial performance of many biotransformations has to be done by using immobilized enzyme derivatives. Thus, the possibility to use these strictly necessary immobilization techniques as additional tools to also alter the selectivity of enzymes appears as a quite exciting approach.

The development of different protocols for enzyme immobilization may exert a very significant influence in the conformational changes related to enzyme inactivation and enzyme catalyst (Fig. 3). For example, an enzyme that is immobilized through a very mild chemical modification of only one residue and secluded from the support through a long and hydrophilic spacer arm is very likely to suffer identical conformational changes as the soluble enzyme [6]. Then, both soluble and immobilized enzyme should exhibit identical activity-stability properties. However, in some other cases, it is possible to immobilize an enzyme through several surface residues attached to the support via very short spacer arms [7]. If the groups involved in the immobilization are close to the active center, then conformational changes involved in both inactivation and in catalytic action could be greatly restricted with regard to the corresponding soluble enzyme. Moreover, the activity-selectivity, sensitivity to the reaction conditions and stability properties of these enzyme preparations could be strongly altered [7–9].

1.3. Enantioselective hydrolysis of esters of (R,S)-mandelic acid

Optically pure isomers of *R* and *S* mandelic acid and their esters are very useful in organic synthesis. Esters of the *R*-isomer may be used for enzymatic synthesis of the antibiotic cefamandole [10] and optically pure acids may be used in the resolution of racemates [11]. An optically pure nonhydrolyzed ester can be easily obtained by enantioselective hydrolysis of the other ester even with immobilized lipases showing moderate enantioselectivity (e.g., E ca. 20–30). Moreover, when lipases exhibit a very high enantioselectivity ($E > 100$) both optically pure ester and pure acid can be obtained (Fig. 4). By using this interesting enantioselective hydrolysis, we have evaluated the activity and selectivity of very different derivatives of very different microbial and mammalian lipases under different experimental conditions. The most interesting results obtained with

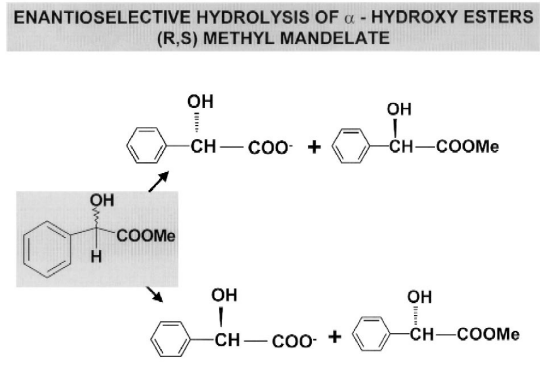


Fig. 4. Enantioselective hydrolysis of (*R,S*)-methyl mandelate catalyzed by different derivatives of lipase from *Candida antarctica* (fraction B). Experiments were carried out as described in Methods.

Candida rugosa lipase (CRL) and *C. antarctica* (fraction B) lipase (CABL) are reported here.

2. Materials and methods

2.1. Materials

Standards corresponding to the pure *R*- and *S*-isomer and the racemic mixture of (*R,S*)-ethyl and methyl mandelates were obtained from Sigma-Aldrich. Deca-octyl-Sepabeads and Amino-epoxy-Sepabeads were gently gifts of Resindion (Mitsubishi Chem., Milan, Italy) and amino-agarose 6BCL [12] was kindly donated by Hispanagar (Burgos, Spain). Octyl agarose 4BCL was purchased from Pharmacia Biotech (Upsala, Suede). The lipases from *C. rugosa* and from *C. Antarctica* (fraction B) were commercially available (Roche Diagnostics). Glutaraldehyde agarose was prepared as previously described [13]. Other reagents and solvents used were of analytical grade.

2.2. Immobilization of lipases

The derivatives of CRL and CABL were prepared following the procedures previously described for: (i) immobilization through long spacer-arm of dextran covalently attached to the internal surface of agarose [6]; (ii) multipoint covalent immobilization on agarose gel activated with glutaraldehyde [13]; (iii) and (iv) interfacial adsorption on hydrophobic supports bearing octyl chains [14]. In addition to that, lipases were also ionically adsorbed on solid supports (e.g., polymeric resins) fully coated by a flexible bed of polyethilenimine (PEI) [15]. Finally, lipases were immobilized on amino-epoxy-Sepabeads by incubation of the enzyme and the support at pH 7 for 24 h [16]. A schematic representation of the main immobilized lipase molecules is given in Fig. 5.

A moderate amount of lipases (2 mg of protein/ml of activated supports) was offered to the activated supports. More than 99% of the offered lipase was immobilized on all supports. Since lipases are poorly active towards esters of mandelic acid, diffusional limitations do not affect both the activity and selectivity of these derivatives.

2.3. Enantioselectivity studies

Evaluations of hydrolytic activities and enantioselectivity were performed by using 10 ml of 10 mM (*R,S*)-mandelic acid esters in 25 mM sodium phos-

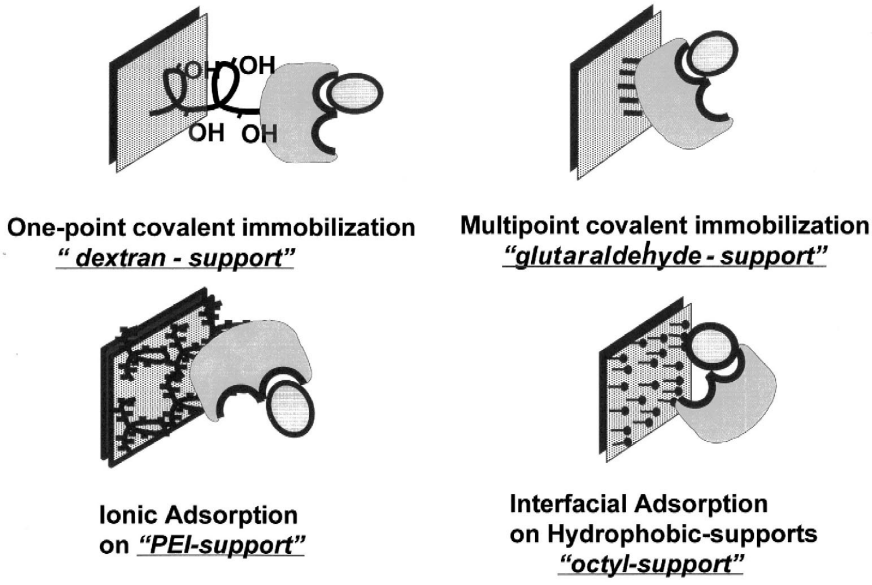


Fig. 5. Different immobilized lipase derivatives.

phate buffer at pH 7.0 and 25°C under mechanic stirring (standard conditions). Other experiments were carried out at different pH and temperatures (see Results). During the reaction courses, the pH value was maintained constant by automatic tritation and the enzymatic activity (μmol of substrate hydrolyzed per minute per mg of immobilized protein) was evaluated from the NaOH consumption. The degree of hydrolysis was followed by reverse-phase HPLC (Spectra Physic SP 100) coupled with an UV detector (Spectra Physic SP 8450) at 225 nm. The column was a C18 ODS, 25 × 0.4 cm, the mobile phase was a mixture of acetonitrile (60%) and water (40%) and the analyses were performed at a flow rate of 1.0 ml/min. In this way, both the decrease of the peak corresponding to the ester and the increase of the peak corresponding to the acids could be analyzed. At different conversion degrees, the enantiomeric excess of the remaining esters was analyzed by Chiral Reverse Phase HPLC. The column was a Chiracel OD-R, the mobile phase was a mixture of 40% acetonitrile and 60% water and the analyses were performed at a flow of 0.5 ml/min. Enantioselectivity was expressed as E value calculated from the enantiomeric excess of the remaining ester and the conversion degree according to the formula previously reported by Chen et al. [17].

3. Results

3.1. Enantioselective hydrolysis of methyl mandelate by immobilized derivatives of C. rugosa lipase

The enantioselectivity of different immobilized derivatives of *C. rugosa* lipase (CRL) towards the hydrolysis of (*R*,*S*)-methyl mandelate is given in Fig. 6. CRL immobilized on amino-epoxy-Sepabeads hardly showed enantioselectivity for this hydrolysis under standard conditions (pH 7.0, 25°C). However other derivatives showed interesting enantiopreference for the hydrolysis of the *S*-isomer. In fact, the enzyme adsorbed on PEI-support exhibited an enantioselectivity of 21.

Moreover, all derivatives were sensitive to experimental conditions (pH, temperature, ionic strength, presence of cosolvents, detergents and so on). However, the degree of sensitivity was again very different for different derivatives. Thus, CRL immobilized

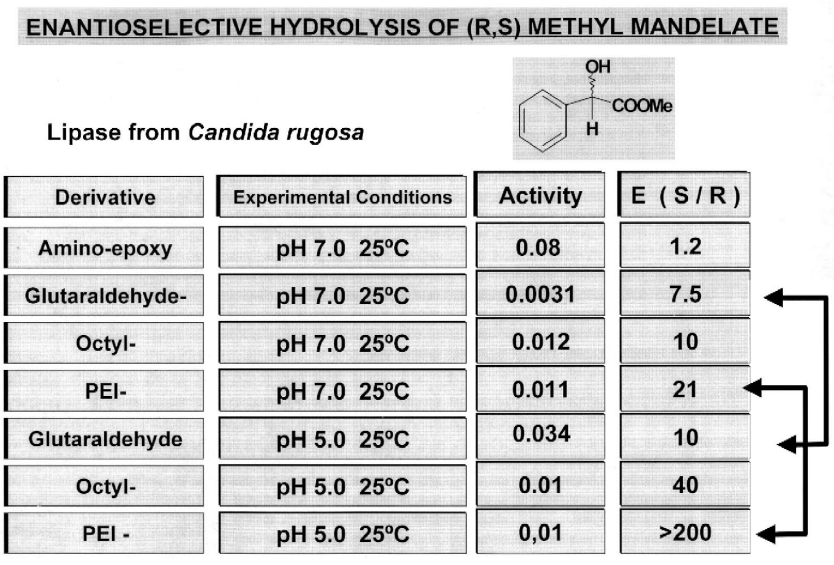


Fig. 6. Enantioselective hydrolysis of (*R*,*S*)-methyl mandelate catalyzed by different derivatives of lipase from *C. rugosa*. Experiments were carried out as described in Methods.

on glutaraldehyde supports slightly increased its enantioselectivity when lowering the pH from 7 to 5.0 (from 7.5 to 10) while enantioselectivity of the enzyme adsorbed on PEI (the best derivative under standard conditions) was greatly increased (from 21 to more than 200) when performing the same change in experimental conditions. In this way, the same lipase (CRL) can display an extremely different enantioselectivity (from 1.2 to more than 200) by simply changing the immobilization protocol and the reaction conditions. In fact, under optimal conditions and using optimal derivative, *R*-methyl mandelate with an ee higher than 99% could be obtained after 52% of hydrolysis of the racemic (*R,S*)-methyl mandelate.

3.2. Enantioselective hydrolysis of methyl and ethyl esters of (R,S)-mandelic acid by different immobilized derivatives of C. antarctica lipase (fraction B)

C. antarctica lipase (fraction B) immobilized on glutaraldehyde hardly showed enantioselectivty in the hydrolysis of (*R,S*)-ethyl mandelate (under standard conditions) (Fig. 7) . However, other derivatives (for example, the enzyme adsorbed on hydrophobic supports) showed a certain enantioselectivity for the *R*-isomer (opposite to *C. rugosa* lipase) ($E = 4.4$) . The enantioselectivity of these derivatives was further increased when using methyl esters instead of the ethyl one, when lowering the pH value (from 7.0 to 5.0) and when lowering the temperature (from 25°C to 4°C), to reach an interesting enantioselectivity of 25.

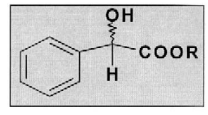
4. Discussion

Enatioselectivity of immobilized lipases during biotransformations in macro-aqueous systems could be strongly modulated by preparing different immobilized derivatives. That is, immobilized preparations in which the enzyme became immobilized on the support through different areas of its surface, with a different rigidity (multipoint attachment) or surrounded by a different micro-environment.

Under standard experimental conditions (pH 7.0, 25°C), the best derivatives of *C. rugosa* lipase (CRL) were one order of magnitude more enantioselective than the worst ones. Moreover, some derivatives (usually the more enantioselective ones) were much more sensitive to slight alterations in the reaction

ENANTIOSELECTIVE HYDROLYSIS OF (R,S) MANDELATES

Lipase from *Candida antarctica* (fraction B)



Derivative	R	Experimental Conditions	Activity	E (R / S)
Glutaraldehyde	- CH_2 - CH_3	pH 7.0 25°C	7	1.5
Octyl -	"	"	14	4.0
"	- CH_3	"	3.7	6.6
"	"	pH 5.0 25°C	0.22	10
"	"	pH 5.0 4°C	0.1	25

Fig. 7. Enantioselective hydrolysis of (*R,S*)-methyl mandelate catalyzed by different derivatives of lipase from *C. antarctica* (fraction B). Experiments were carried out as described in Methods.

conditions than the other ones. In this way, by lowering pH to 5.0 and temperature to 4°C an additional increase in enantioselectivity of one order of magnitude could be promoted. Some immobilized derivatives of *C. rugosa* and *C. antarctica* (fraction B) lipase were hardly enantioselective under standard conditions ($E < 2$). However, other derivatives of the same enzymes under slightly modified experimental conditions exhibit very interesting enantiselectivities ($E = 200$ for CRL towards the *S*-isomer of methyl mandelate and $E = 25$ for CAB towards the *R*-isomer of the same chiral ester).

Commercial lipase preparations (e.g., the one from *C. rugosa*) were usually composed by different isoenzymes and isoforms and they also contained some contaminant hydrolases [18–20]. From this point of view, we could assume that dramatic changes observed in enantioselectivity of different derivatives could be associated to the preferential immobilization of different hydrolase fractions on the different derivatives. In fact, Lalond et al. [21] have shown that the crystallization of *C. rugosa* lipase removed contaminating hydrolases and dramatically increased enzyme enantioselectivity. However, more than 99% of the activity of soluble enzyme (towards methyl mandelate) has now become immobilized on all different supports and several derivatives have a very similar activity even exhibiting a very different enantioselectivity. In this way, the very different enantioselectivity observed for these different lipase derivatives may be mainly associated to differences in the mechanism of opening of the active center of the same lipase that was immobilized in very different fashion.

A similar modulation of selectivity of enzymes by preparing different immobilized derivatives has been observed for penicillin G acylase [8] and for Pseudomonas fluorescens lipase [9]. Moreover, other relevant parameters (regioselectivity, symmetry) could be greatly modified by using different derivatives of very different lipases in macro-aqueous systems (Guisan et al, in preparation). Under these conditions, immobilized lipases are only acting on fully soluble substrates and in the absence of any kind of interfacial activation. That is, immobilized lipases are fully surrounded by water and undergoing a complex conformational change between their closed-inactive and their open-active structures.

The possibility of modulating the selectivity of lipases has been largely discussed in literature. Genetic engineering (random or site-directed mutagenesis) and reaction engineering have been found to be very useful tools. However, the modulation of lipase selectivity via controlled immobilization has not yet been clearly established. Now, immobilization becomes much more than a need, it also becomes a powerful tool to improve enzyme selectivity. In general, we can assume that any biotransformation where the enzyme is undergoing important conformational changes during catalysis should be strongly modulated via the design of controlled protocols of enzyme immobilization (via different areas of the protein, with different rigidity, etc.). The immobilization of the enzymes through areas more or less connected or related to the active center should promote attentions on those conformational changes and, hence, in the activity and selectivity of the enzyme.

Acknowledgements

This project has been founded by VITA INVEST, by the Spanish CICYT (QUI97-0506-C03-02), and by the European Community (BIO4-CT96-0005). We are very grateful to Resindion (Mitsubishi Chem.) for the kind supply of Sepabeads and to Hispanagar for the kind donation of agarose gels. We are very grateful to Mr. Daminati and Mr. Miyata (Resindion — Mitsubishi Chem.), and Mr. Armisen (Hispanagar) for their very helpful suggestions.

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