Available online at www.sciencedirect.com
SCIENCE DIRECT
Enzyme and Microbial Technology 39 (2006) 259–264
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

# Effect of lipase–lipase interactions in the activity, stability and specificity of a lipase from *Alcaligenes* sp.

Lorena Wilson [b], José M. Palomo [a], Gloria Fernández-Lorente [a], Andrés Illanes [b], José M. Guisán [a,*], Roberto Fernández-Lafuente [a,*]

[a] *Departamento de Biocatálisis, Instituto de Catálisis (CSIC), Campus UAM Cantoblanco, 28049 Madrid, Spain*
[b] *School of Biochemical Engineering, Universidad Católica de Valparaíso, Chile*

Received 18 August 2005; accepted 24 October 2005

**Abstract**

It has been found that the lipase QL from *Alcaligenes* sp. presents a tendency to form very strong bimolecular aggregates (as shown by gel filtration experiments). The addition of detergents (e.g., Triton X-100) is an easy way to break this aggregate. Soluble enzyme in absence of Triton (that is, forming a dimer) was more stable than the enzyme in the presence of Triton. The lack of Triton effect on the stability of immobilized preparations of monomeric enzyme suggests that its main effect is the breakage of the aggregate. The enzyme was immobilized on supports activated with glutaraldehyde in the presence and absence of Triton X-100, to immobilized monomer, or dimers, respectively, and we have found that the properties of the immobilized preparations were very different (after exhaustive washing to eliminate the remaining Triton). When the enzyme was immobilized under conditions where the enzyme tended to form aggregates, stability was much higher, activity was also higher, and the specificity and enantioselectivity of the enzyme were quite different than when the enzyme was immobilized in the presence of Triton. The addition of detergent to the enzyme preparation produced in absence of Triton promoted a release of around 50% of the protein to the supernatant. This suggested that we can immobilize the dimer or the monomer depending on the immobilization conditions. Thus, the control of the lipase–lipase interaction during immobilization dramatically alters the final biocatalyst properties.


*Keywords:* Lipase QL from *Alcaligenes* sp.; Interfacial activation; Lipase–lipase interaction; Protein immobilization

## 1. Introduction

Lipases are very relevant enzymes from both a physiological and a biotechnological point of view. In addition to their natural function (hydrolysis of fats and oils), lipases are also able to catalyze the regio- and enantioselective hydrolysis or synthesis of many esters, which are very different from their natural substrates [1–3]. Thus, lipases are able to recognize very different substrates but, at the same time, are able to catalyze very highly selective reactions.

Lipases display a peculiar mechanism of action, "interfacial activation" [4–9]. Lipases may exist in two different forms. One of them, where the active centre of the lipase is secluded from the reaction medium by a polypeptide chain called "lid", is considered to be inactive (closed form). The other one, presenting the lid displaced and the active centre exposed to the reaction medium, is considered to be active (open form). In aqueous, homogeneous media, lipase molecules exist in equilibrium between these two forms, with this equilibrium shifted towards the closed form. This interchange between open and closed forms is accompanied by complex conformational changes of the lipase [4–9].

In the presence of any hydrophobic interface, the open form of the lipase becomes adsorbed on it and the equilibrium is shifted towards the open form of the lipase [10–18].

Recently, it has been shown that most lipases have a natural trend to form biomolecular aggregates, by adsorption of open lipases on open lipases via the large hydrophobic pocket formed around the active centre. These aggregates present completely different catalytic properties when compared to the individual lipase molecule [19,20]. In fact, this interaction may be used to purify lipases by specific adsorption [21] and even to immobilize them [22]. Thus, this possibility should be considered during any lipase study.

* Corresponding authors. Tel.: +34 91 585 4809; fax: +34 91 585 4760.
*E-mail addresses:* jmguisan@icp.csic.es (J.M. Guisán), rfl@icp.csic.es (R. Fernández-Lafuente).

0141-0229/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2005.10.015

Lipase QL is an extracellular enzyme produced by the strain *Alcaligenes* sp. with a molecular weight of 31,000 Da. It is inhibited by cationic detergents and activated by non-ionic detergents (specifications of the supplier Meito Sangyo Co. Ltd.). Lipase QL is an interesting but not very well characterized lipase. It has been used to catalyze the acylation of primary and secondary alcohols [23–25] and the production of different key intermediates for the preparation of several pharmaceutical products [26,27]. The main feature of this enzyme is its very high stability [28]; therefore, it may be interesting to know the reasons for this extremely high thermostability.

## 2. Materials and methods

### 2.1. Materials

Lipase from *Alcaligenes* sp. (QL) was from Meito Sangyo Co. Ltd. (Tokyo, Japan). Glyoxyl-agarose 6BCL and 10BCL were kindly donated by the company Hispanagar SA (Burgos, Spain). Octyl-agarose 4BCL was purchased from Pharmacia Biotech (Uppsala, Sweden). *p*-Nitrophenyl propionate, *R*- and *S*-glycidyl butyrate, Triton X-100, α-hydroxyphenylacetic acid methyl ester and polyethyleneimine (MW 25,000) were purchased from Sigma Chemical Co. (St. Louis, USA). 2-*O*-Butyryl-2-phenylacetic acid was prepared as described previously [29]. Other reagents and solvent used were of analytical grade.

### 2.2. Gel filtration of Alcaligenes sp. lipase

Gel filtration analyses were performed using a glass column packed with beaded Agarose 10BCL (column size: 10 mm × 509 mm; column bed volume: 40 mL). The eluting buffer used was 100 mM sodium phosphate, pH 7; all separations were carried out at 25 °C with a flow rate of 1.23 mL/min, and lipase QL concentration was 0.285 mg protein/mL.

Where indicated, 0.1% (v/v) Triton X-100 was added to the elution buffer. The column was equilibrated by passing 400 mL of the appropriate buffer. The eluted solution was collected in 1 mL aliquots and the enzymatic activity determined as described below.

The molecular weight of lipase QL preparations was estimated from a calibration curve plotted using standard proteins: penicillin G acilase (PGA) 90 kDa, bovine serum albumin (BSA) 67 kDa and lipase of *Candida antarctica* B (CAL-B) 33 kDa.

### 2.3. Enzymatic activity assay

This assay was performed by measuring the increase in the absorbance at 348 nm produced by the released *p*-nitrophenol in the hydrolysis of 0.4 mM pNPP in 25 mM sodium phosphate buffer at pH 7 and 25 °C. To initialize the reaction, 0.05 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. One international unit of pNPP activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol pNPP/min (IU) under the conditions described above.

### 2.4. Thermal inactivation of soluble enzyme

To check their thermal stability, different lipase preparations were incubated at pH 7.0 or 8.5 at 70 °C. Where indicated, 0.05% (v/v) Triton X-100 was added to the solution buffer. Samples were withdrawn periodically and the activity was measured using pNPP assay. The experiments were carried out by triplicate and error was never over 5%.

### 2.5. Immobilization of lipase QL on octyl-agarose support

One gram of octyl-agarose support was added to 16 mL of 25 mM sodium phosphate buffer lipase solution (0.05 mg protein/mL) at pH 7. The mixture was then stirred at 25 °C and 250 rpm for 4 h. After that, the solution was removed by filtration and the supported lipase washed several times with distilled water.

### 2.6. Immobilization of lipase QL on glutaraldehyde-agarose (Glut-M and Glut-BA QL preparations)

Five grams of activated agarose gel modified with glutaraldehyde was added to 50 mL of 25 mM sodium phosphate buffer lipase solution (0.285 mg protein/mL) at pH 7 in the absence or in the presence of 0.1% Triton X-100. The mixture was then stirred at 25 °C and 250 rpm for 1 h. After that, the liquid phase was removed by filtration. Fifty millilitres of sodium bicarbonate buffer 100 mM, pH 10, containing 1 mg/mL of sodium borohydride was added to the immobilized preparation. The mixture was then stirred at 25 °C for 30 min. After that, the liquid phase was removed by filtration and the supported lipase washed properly with distilled water to remove the reduction agent excess and keep it at 4 °C.

### 2.7. Enzymatic hydrolysis of R- and S-glycidyl butyrate

Two hundred and fifty milligrams of immobilized preparation was added to 10 mL of substrate 10 mM in 25 mM sodium phosphate buffer at pH 7, acetonitrile 5% (v/v). The mixture was then stirred at 25 °C and 250 rpm. A pH-stat Mettler Toledo DL50 graphic was used to maintain the pH value constant during the reactions. The conversion was analyzed by RP-HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450) using a Kromasil $C_{18}$ (25 cm × 0.4 cm) column. Products were eluted at flow rate of 1.5 mL/min using acetonitrile–10 mM ammonium phosphate buffer at pH 2.95 (35:65, v/v) and UV detection performed at 225 nm.

The enantiomeric ratio (*E*) was calculated directly from the ratio between the reaction rates of both isomers (using hydrolysis degrees between 10 and 20% where the enzyme kinetics is in the first-order region).

### 2.8. Enzymatic hydrolysis of α-hydroxyphenylacetic acid methyl ester or 2-O-butyryl-2-phenylacetic acid

Five hundred milligrams of immobilized preparation was added to 3 mL of substrate 10 mM α-hydroxyphenylacetic acid methyl ester or 0.5 mM 2-*O*-butyryl-2-phenylacetic acid, at 25 °C in 25 mM sodium phosphate buffer, pH 7, under continuous stirring. A pH-stat Mettler Toledo DL50 graphic was used to maintain the pH value constant during the reactions. The conversion was analyzed by RP-HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450) using a Kromasil $C_{18}$ (25 cm × 0.4 cm) column. Products were eluted at flow rate of 1.5 mL/min using acetonitrile–10 mM ammonium phosphate buffer at pH 2.95 (35:65, v/v) and UV detection performed at 225 nm in the case of 2-*O*-butyryl-2-phenylacetic acid, and (25:75, v/v) and UV detection performed at 254 nm to α-hydroxyphenylacetic acid methyl ester.

At different conversion degrees, the enantiomeric excess of the released acid was analyzed by Chiral Reverse Phase HPLC. The column was a Chiracel OD-R, the mobile phase was an isocratic mixture of 5% acetonitrile and 95% $NaClO_4/HClO_4$ 0.5 M at pH 2.3 and the analyses were performed at a flow of 0.5 mL/min by recording the absorbance at 225 nm.

The enantiomeric ratio (*E*) was calculated directly from the ratio between the reaction rates of both isomers (using hydrolysis degrees between 10 and 20% where the enzyme kinetics is in the first-order region).

### 2.9. Effect of the presence of Triton X-100 on the activity of the different soluble preparations

A standard enzyme solution was prepared (1 g/L of lipase QL) in 5 mM sodium phosphate buffer at pH 7 and Triton X-100 (concentration range between 0 and 2%, v/v). After 15 min at 25 °C and stirring, the activity was measured.

### 2.10. SDS-PAGE experiments

Soluble enzyme and the octyl-agarose immobilized preparation were boiled in 1 vol of 2% sodium dodecylsulfate (SDS) [13]. Then, SDS-PAGE analysis