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Use of immobilized lipases for lipase purification via specific lipase–lipase interactions

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Abstract

Lipase from *Pseudomonas fluorescens* (PFL), an enzyme with a great tendency to yield bimolecular aggregates, was immobilized via multipoint covalent attachment on glyoxyl-agarose in the presence of Triton X-100. This strategy permitted to obtain the enzyme with the active center oriented towards the reaction medium. This immobilized enzyme presents the capacity of specifically adsorbing PFL molecules, that can be easily desorbed by the use of detergents. More interesting, the enzyme was also able to adsorb other lipases. That is, the lipase from *Bacillus thermocatenulatus* (BTL2) cloned in *Escherichia coli* was selectively adsorbed on this immobilized enzyme, enabling a very simple purification strategy. Similar results were achieved with some other lipases (those from *Rhizomucor miehei* (RML), *Rhizopus oryzae* (ROL), and *Humicola Lanuginosa* (HLL)). In all cases, the enzyme could be easily desorbed by incubation with Triton X-100. The matrix could be used several cycles without any detrimental effect on the adsorption capacity.
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Keywords: Immobilized enzymes; Lipases; Enzymes

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 triglycerides), lipases are able to recognize many substrates different from their natural ones, catalyzing the regio- and enantioselective hydrolysis or synthesis of many esters and the resolution of racemic mixtures [1–8].

On the other hand, lipases present a peculiar action mechanism, the so-called interfacial activation. In aqueous media they exist in a closed and inactive conformation, where the active site is completely isolated from the reaction medium by an oligopeptide chain called flap or lid. This conformation exists in a partial equilibrium with an open and active conformation, where the lid is displaced, stabilized by ionic interactions or hydrogen bonds with a part of the surface of the lipase allowing the access of the substrate to the active site [9–11]. Moreover, these enzymes are adsorbed on very different hydrophobic interfaces: drops of oil (its natural substrate) [12,13], hydrophobic support surfaces [14–18], gas bubbles [19], hydrophobic proteins (fungal hydrophobins) [20], lipopolysaccharides [21], etc., shifting the equilibrium towards the open conformation. In fact, it has been recently reported that most lipases have a natural tendency to form bimolecular aggregates, by means of interactions between the hydrophobic surfaces surrounding the active centers [22,23].

In this manuscript, we have tried to use this capacity as a new tool to purify lipases. The hypothesis is that the immobilization of a lipase on a support with the active center exposed to the medium would permit the adsorption of other lipase molecules (Fig. 1) via a similar mechanism to the one that yields bimolecular aggregates.

The structural analysis of the surface [24] of the lipase from *Pseudomonas fluorescens* determined the existence of lysine residues (Lys) in the opposite side of the active center (Fig. 2A), whereas the area near the active center has almost no Lys residues (Fig. 2B). Immobilization of this en-

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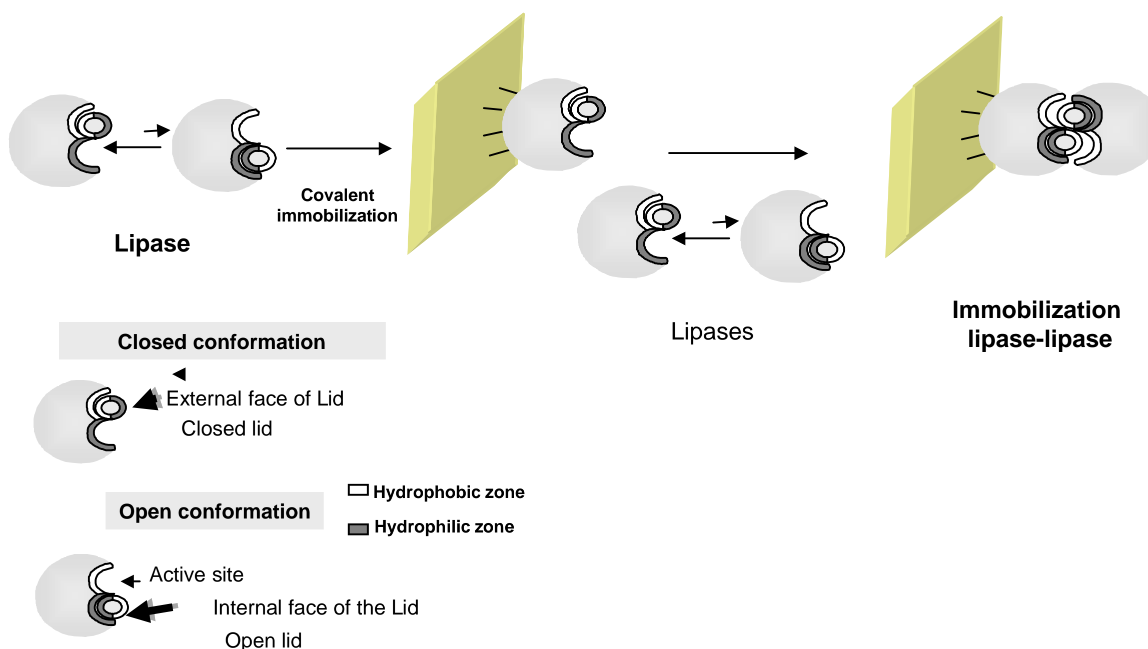

Fig. 1. Representation of the ideal preparation of new matrix to immobilize lipases.

zyme on glyoxyl-agarose was chosen by several properties of the system, that permit to have a stable enzyme with the active center oriented toward the medium. Immobilization occurs via weak Shitf's bases, that require the reaction with no ionized lysines (that is, immobilization only occurs at alkaline pH value). This weak energy makes necessary the establishment of several simultaneous support-protein bonds to fix the protein on the support, and make that the protein orientation regarding the support occurs by the richest lysine areas [25]. Moreover, this support has been used to yield a very intense enzyme-support multipoint attachment between the enzyme and the support, and a fully inert surface after immobilization. Thus, many enzymes have been stabilized using this technique, e.g., Penicillin G acylase from *Escherichia coli* [26] and *K. citrophila* [27], trypsin [28], chymotrypsin [29], alcalase [30], carboxypeptidase A [31], FNR NADP-reductase [32], esterase [33], thermolysin [34], DAAO [35], catalases [36,37], and lipases from different sources [18,38], urokinase [39], L-aminoacylase [40], chitosinase [41]. The final reduction of the immobilized

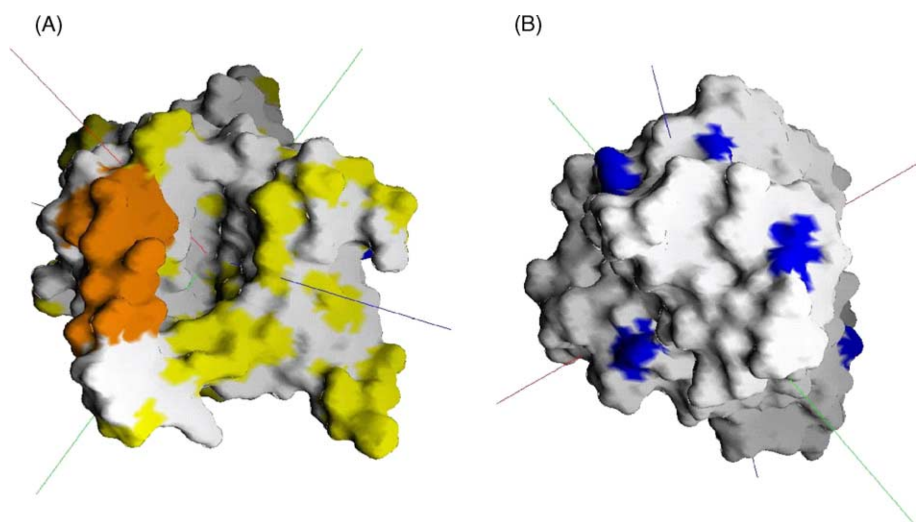

Fig. 2. Representations of the crystal structure surface of the open conformation of *P. fluorescens* lipase. (A) The side selected constituted the active site pocket and the lid. The amino acid residues constituting the lid (Thr137 to Thr150) (in orange), hydrophobic residues (in yellow). (B) The opposite side of the oxoanion hole. Lysine residues present in the enzyme surface (blue). Figure was drawn with the program GRASP using the PDB structure from the protein data bank encode 2LIP [21].

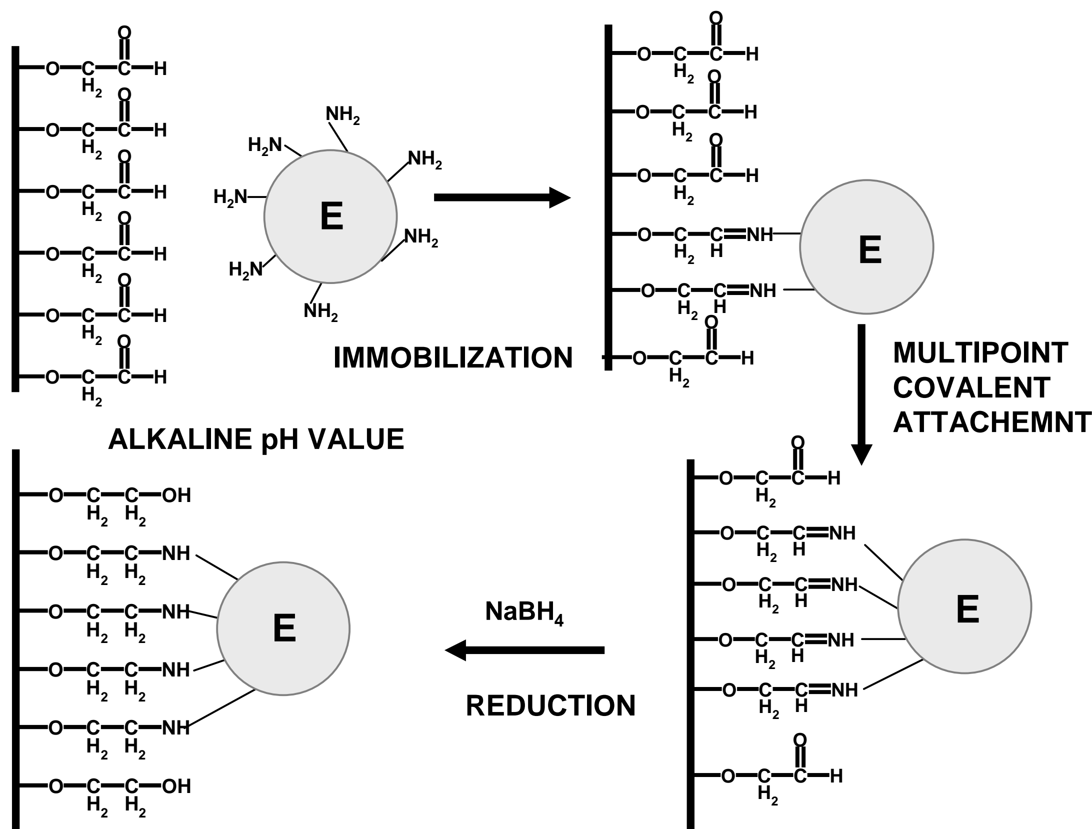

Fig. 3. Immobilization of enzymes on glyoxyl-agarose supports.

preparations with sodium borohydride permits to have very stable secondary amino bonds and very inert hydroxy groups in the support surface (Fig. 3).

Additionally, it was possible to observe that this lipase has not hydrophobic areas on the enzyme surface, except for the one generated by the internal face of the lid and the active center (Fig. 2).

In this way, we have chosen to study the adsorption of lipases from different sources on PFL immobilized on glyoxyl-agarose.

2. Experimental

2.1. Materials

The lipase from *P. fluorescens* lipase (PFL) PS "Amano"(lot LPSA21250457) was purchased from Amano Enzyme (Nagoya, Japan). The lipases from *Humicola lanuginosa* (Novozym 871, HLL), *Candida antarctica (fraction B)* and *Rhizomucor miehei* lipase (Novozym 388, RML) were from Novo Nordisk (Denmark). The lipase from *Bacillus thermocatenulatus* (BTL2) was kindly donated by Marisa Rua (University of Vigo, Spain). This lipase was obtained from an extract of *E. coli* [42] and previously centrifuged. The lipase *Rhizopus oryzae* was from Fluka. Glyoxyl-cross-linked agarose (4%), having 40 μmol/mL of support were kindly donated by Hispanagar (Burgos, Spain) and prepared as previously described [43]. Triton X-100, *p*-nitrophenylpropionate (pNPP) and were from Sigma (St. Louis, MO, USA).

2.2. Activity determination assay

This assay was performed by measuring the increase in absorbance at 348 nm produced by the release of *p*-nitrophenol (pNP) in the hydrolysis of 0.4 mM pNPP in 25 mM sodium phosphate buffer at pH 7 and at 25 °C. To initialize the reaction, 0.05 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. The experiments were carried out in triplicate, and experimental error was never over 5%. One international unit of pNPP activity was defined as the amount of enzyme that is necessary to hydrolyze 1 μmol of pNPP per minute (IU) under the conditions described above.

2.3. Preparation of the matrix glyoxyl-PFL

The commercial extract of PFL was dissolved to give 25 mg extract per milliliter in 5 mM sodium phosphate, submitted to gentle stirring during 20 min at 25 °C and at pH 7, and centrifuged at 12 000 rpm for 30 min. The protein concentration in the commercial preparation was determined by the Bradford method [44]. Hundred milliliter of this preparation (25 mg/mL) of commercial PFL contained 0.15 mg

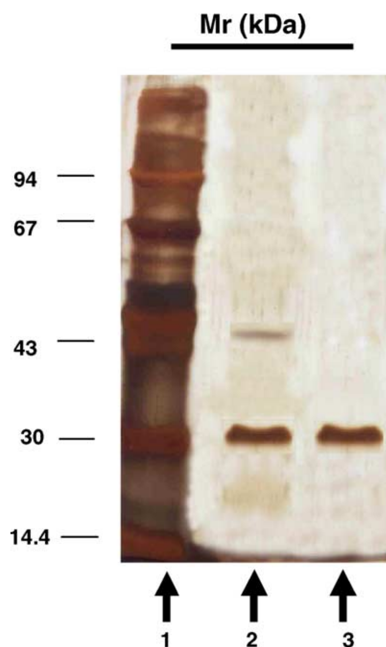

Fig. 4. SDS-PAGE gels of different PFL preparations. Lanes: 1: low-molecular mass marks; 2: commercial preparation of PFL; 3: PFL adsorbed on glyoxyl-PFL kDa = Kilodatton.

of protein per milliliter. Six gram of glyoxyl-agarose [43] was added to the enzyme solution to obtain an immobilized preparation with a final loading of 2.5 mg prot/g support in the presence of 1% of Triton to prevent dimerization. The pH was increased to 10.5. After 24 h, 1.5 mg of PFL per gram of support was immobilized (60%) and the enzyme-support multi-interaction was ended by adding 1 mg of sodium borohydride per mL of suspension during 30 min [45].

2.4. Immobilization of lipases on glyoxyl-PFL

In a standard experiment, 1 g of glyoxyl-PFL was added to a solution of 5 mL of the lipase preparation in 30 mL of 25 mM sodium phosphate buffer at pH 7 and at 25 °C to obtain immobilized preparations with a final enzyme loading of 1 mg/mL. A blank suspension was prepared by adding 1 g of reduced glyoxyl-agarose at pH 7. Periodically, the activity of both the suspensions and supernatants was analyzed using the previously described pNPP assay. After immobilization, the adsorbed lipase preparation was washed 10-fold with 10 volumes of distilled water.

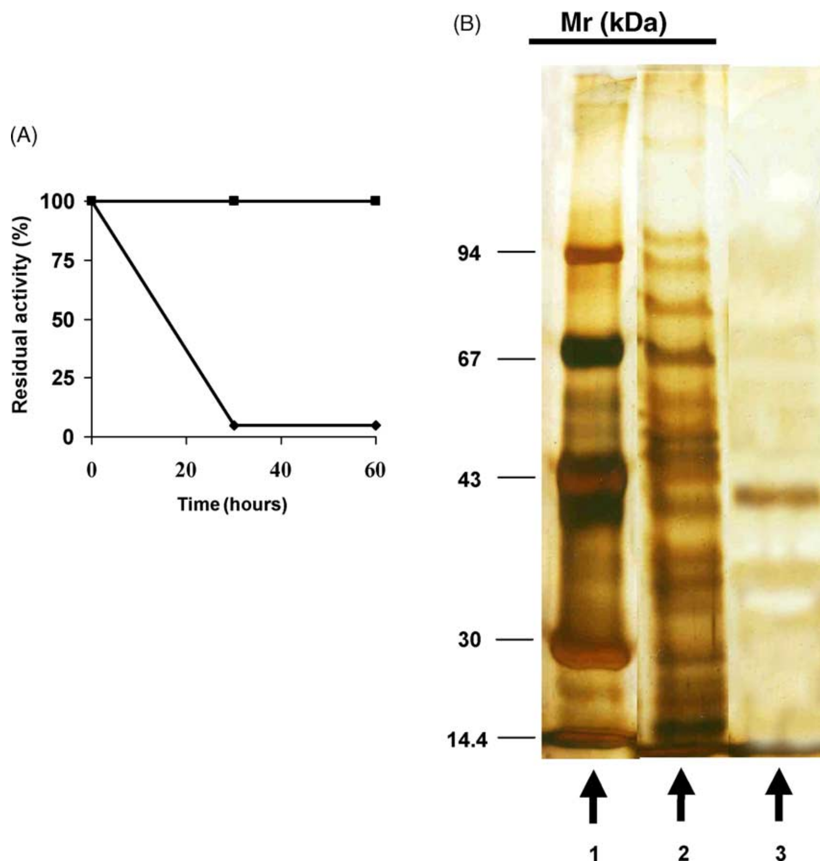

Fig. 5. (A) Immobilization course of *B. thermocatenulatus* lipase (BTL2) on glyoxyl-PFL support. Supernatant of the immobilization suspension (rhombus), supernatant of the blank suspension (squares). The experiments were performed as described in Section 2. (B) SDS-PAGE electrophoresis of BTL2 preparations. Lanes: 1: Low-molecular mass marks; 2: crude extract of BTL2; 3: BTL2 adsorbed on glyoxyl-PFL.

2.5. Sodium dodecayl sulfate-polyacrylamide electrophoresis analysis(SDS-PAGE)

SDS-PAGE electrophoresis was performed according to Laemli's method [46] in a SE 250-Mighty Small II electrophoretic unit (Hoefer Co.) using gels of 12% polyacrylamide in a separation zone of 9 cm × 6 cm and a concentration zone of 5% polyacrylamide. The gels were stained following the silver staining method [47]. Molecular mass markers were the LMW kit (M_r) (14 400–94 000) from Pharmacia.

3. Results

3.1. Immobilization of lipases on glyoxyl-PFL by lipase–lipase interaction

First of all, 1 mg PFL per g of support was offered on the immobilized PFL, bearing in mind the tendency of this enzyme to form bimolecular structures [22,23]. The immobilization course revealed the adsorption of around 40% of the offered enzyme in 1 h. Moreover, SDS-PAGE analysis of this composite (Fig. 4) showed a single band, indicating that only the lipase, and not the contaminant enzymes, was adsorbed on the immobilized lipase. After that, the lipase was desorbed by incubation in 0.5% Triton X-100. Several adsorption–desorption cycles could be performed without detecting any change in the adsorption–desorption courses.

Next, lipases from different sources were offered to this glyoxyl-PFL matrix at pH 7, 25 °C and 50 mM sodium phosphate buffer.

A sample of 1 mg protein per gram support of the lipase from *B. thermocatenulatus was* offered to the immobilized PFL, and 95% of the activity was absorbed (Fig. 5A). In addition, it was interesting that the lipase was the unique protein adsorbed on this matrix from the quite crude extract, as shown in Fig. 5B.

The lipase from *R. miehei* (RML) was adsorbed very quickly on this immobilized enzyme, immobilizing 89% of the enzyme in 2 h (Fig. 6a). In the case of the lipase from *H. lanuginosa* (HLL), 71% of the enzyme was immobilized on this matrix (Fig. 6b). The lipase from *R. oryzae* (ROL) was the quickest adsorbed on this glyoxyl-PFL matrix since 75% of the offered enzyme has been immobilized in 30 min (Fig. 6c).

However, the lipase of *C. antarctica (fraction B)* was not adsorbed on the glyoxyl-PFL, probably due to the fact that this lipase do not form bimolecular aggregates [22,23] since present a very small hydrophobic zone surrounding the active site [48].

This adsorption of different lipases on the support was selective in all cases for the lipases, since in the analysis by SDS-PAGE (Fig. 7) only the lipases were adsorbed on the immobilized PFL.

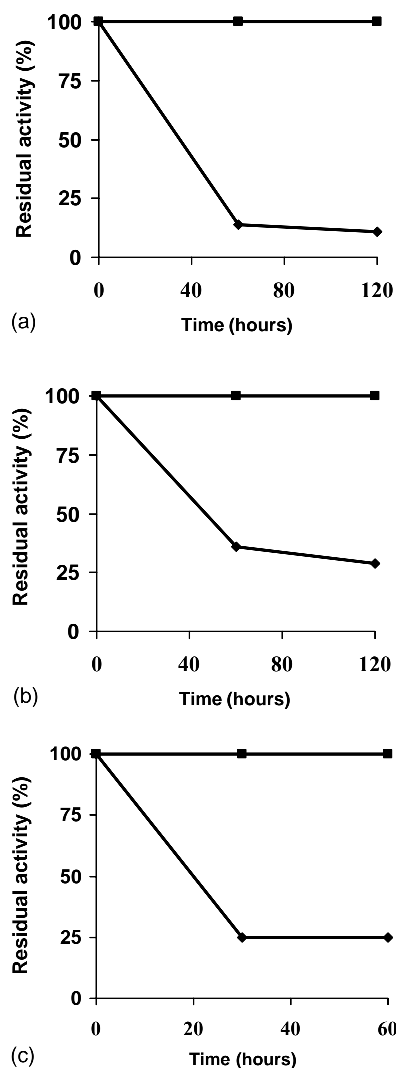

Fig. 6. Immobilization course of different lipases on glyoxyl-PFL support. (a) *R. miehei* lipase, (b) *H. lanuginose* lipase, (c) *R. oryzae* lipase. Supernatant of the immobilization suspension (rhombus), supernatant of the blank suspension (squares). The experiments were performed as described in Methods.

3.2. Desorption of adsorbed lipases

The enzymes could be easily and fully desorbed from the support by using this detergent. Fig. 8 shows the desorption of the different lipases adsorbed on glyoxyl-PFL. In the case of RML, it was necessary to use 0.2% of detergent, while 0.6% Triton was necessary to release BTL2. It was necessary 0.9% to full desorbe ROL, or even 1% to desorb HLL from the support. Thus, this interaction is not only selective but also quite strong. Obviously, bearing in mind that the adsorption was specific; the only protein desorbed from this matrix was the adsorbed lipase (results not shown).

Therefore, this strategy can be used as a very simple method to adsorb/desorbe lipases getting the purify lipase. The matrices were used 10-fold without detecting any difference in the adsorption–desorption of the lipases. Storage

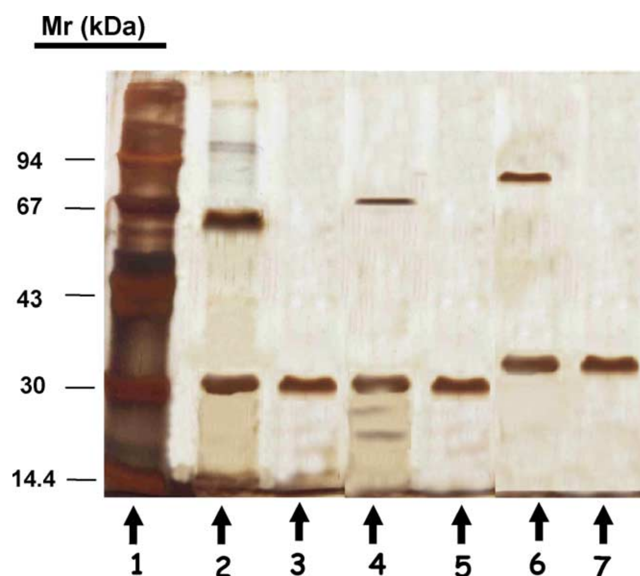

Fig. 7. SDS-PAGE analysis of different lipase preparations. Lanes 1: Low-molecular-mass marks; 2: commercial preparation of RML; 3: RML adsorbed on glyoxyl-PFL; 4: commercial preparation of HLL; 5: HLL adsorbed on glyoxyl-PFL; 6: commercial preparation of ROL; 7: ROL adsorbed on glyoxyl-PFL.

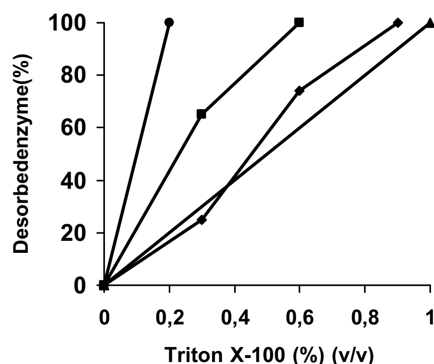

Fig. 8. Desorption of lipases adsorbed on glyoxyl-PFL by progressive addition of Triton X-100. ROL (circles), BTL2 (squares), RML (rhombus), HLL (triangles). Experiments were performed at 25 °C and pH 7.

in 0.5% sodium azide at 4 °C permitted to keep unaltered the matrix properties during 4 months.

4. Conclusion

These studies have demonstrated that immobilized lipases may be used for the selective adsorption of other lipases, very likely by the interaction between the hydrophobic areas surrounding the active center of most lipases. In this way, lipase X–PFL dimers have been formed and broken several times, by using Triton X-100 as detergent, showing that this can be a very simple method to immobilize lipases and to purify them.

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References

[1] M.T. Reetz, C.J. Rüggeberg, M.J. Dröge, W. J Quax, Tetrahedron 58 (2002) 8465.
[2] R.D. Schmid, R. Verger, Angew. Chem. Int. Ed. Engl. 37 (1998) 1609.
[3] M. Murakami, H. Kamaya, C. Kaneko, M. Sato, Tetrahedron: Asymmetry 14 (2003) 201.
[4] C.-H. Wong, G. M. Whitesides, Enzymes in Synthetic Organic Chemistry, Pergamon Press, Oxford, 1994.
[5] R.J. Kazlauskas, U.T. Bornscheuer, Biotransformations with Lipases in Biotechnology, 1998.
[6] G. de Gonzalo, R. Brieva, V.M. Sánchez, M. Bayod, V. Gotor, Tetrahedron: Asymmetry 14 (2003) 1725.
[7] K. Farber, Biotransformations in Organic Chemistry, Springer, Berlin, 1997, p. 92.
[8] F.L. Solares, M. Diaz, R. Brieva, V. Sanchez, M. Bayod, V. Gotor, Tetrahedron: Asymmetry 13 (2002) 2577.
[9] L. Sarda, P. Desnuelle, Biochim. Biophys. Acta 30 (1958) 513.
[10] L. Brady, A.M. Brzozowski, Z.S. Derewenda, E. Dodson, G. Dodson, S. Tolley, J.P. Turkenburg, L. Christiansen, B. Huge-Jensen, L. Norskov, L. Thim, U. Menge, Nature 343 (1990) 767.
[11] A.M. Brzozowski, Z.S. Derewenda, G.G. Dodson, D.M. Lawson, J.P. Turkemburg, F. Bjorkling, B. Huge-Jensen, S.S. Patkar, L. Thim, Nature 351 (1991) 491.
[12] U. Derewenda, A.M. Brzozowski, D.M. Lawson, Z.S. Derewenda, Biochemistry 31 (1992) 1532.
[13] M. Basri, K. Ampon, W.M.Z. Yunus, C.N.A. Razak, A.B. Salleh, J. Am. Oil Chem. Soc. 72 (1995) 407.
[14] R. Fernández-Lafuente, P. Armisén, P. Sabuquillo, G. Fernández-Lorente, J.M. Guisán, Chem. Phys. Lipids 93 (1998) 185.
[15] B. Al-Duri, E. Robinson, S. McNerlan, P. Bailie, J. Am. Oil Chem. Soc. 72 (1995) 1351.
[16] P. Sabuquillo, J. Reina, G. Fernández-Lorente, J.M. Guisán, R. Fernández-Lafuente, Biochim. Biophys. Acta 1388 (1998) 337.
[17] M. Sugiura, M. Isobe, Chem. Pharm. Bull. 24 (1976) 72.
[18] J.M. Palomo, G. Muñoz, G. Fernández-Lorente, C. Mateo, R. Fernández-Lafuente, J.M. Guisán, J. Mol. Catal. B: Enzyme 19–20 (2002) 279.
[19] M. Sugiura, M. Isobe, Biochim. Biophys. 397 (1975) 412.
[20] M.A. Taipa, K. Liebeton, J.V. Costa, J.M. Cabral, K.E. Jaeger, Biochim. Biophys. Acta 1256 (1995) 396.
[21] J.M. Palomo, M. Peña, G. Fernández-Lorente, C. Mateo, A.G. Pisabarro, R. Fernández-Lafuente, L. Ramirez, J.M. Guisán, Biomacromolecules 4 (2003) 204.
[22] J.M. Palomo, M. Fuentes, G. Fernández-Lorente, C. Mateo, J.M. Guisán, R. Fernández-Lafuente, Biomacromolecules 4 (2003) 1.
[23] G. Fernández-Lorente, J.M. Palomo, M. Fuentes, C. Mateo, J.M. Guisán, R. Fernández-Lafuente, Biotechnol. Bioeng. 82 (2003) 232–237.

[24] K.K. Kim, H.K. Song, D.H. Shin, K.Y. Hwang, S.W. Suh, Structure 5 (1997) 173.
[25] O. Abian, C. Mateo, M. Bernedo, E. Cuenca, M. Fuentes, J.M. Palomo, et al., J. Biotechnol., Submmited for publication.
[26] G. Alvaro, R.M. Blanco, R. Fernández-Lafuente, J.M. Guisán, Appl. Biochem. Biotechnol. 26 (1990) 210.
[27] J.M. Guisán, G. Alvaro, R. Fernández-Lafuente, Biotechnol. Bioeng. 42 (1993) 455.
[28] R.M. Blanco, J.J. Calvete, J.M. Guisán, Enzyme Microb. Technol. 11 (1989) 353.
[29] J.M. Guisán, A. Bastida, A.C. Cuesta, R. Fernández-Lafuente, C.M. Rosell, Biotechnol. Bioeng. 39 (1991) 75.
[30] P.W. Tardioli, J. Pedroche, R.L. Giordano, R. Fernández-Lafuente, J.M. Guisán, Biotechnol. Progr. 19 (2003) 352.
[31] P.W. Tardioli, R. Fernández-Lafuente, J.M. Guisán, R.L. Giordano, Biotechnol. Prog. 19 (2003) 565.
[32] T. Bes, C. Gomez-Moreno, J.M. Guisán, R. Fernández-Lafuente, J. Mol. Catal. 98 (1995) 161.
[33] R. Fernández-Lafuente, D.A. Cowan, A.N.P. Wood, Enzyme Microb. Technol. 17 (1995) 366.
[34] J. M Guisán, E. Polo, J. Agudo, M.D. Romero, G. Alvaro, M.J. Guerra, Biocatal. Biotrans. 15 (1997) 159.
[35] L. Betancor, A. Hidalgo, G. Fernández-Lorente, C. Mateo, V. Rodríguez, M. Fuentes, R. Fernández-Lafuente, J.M. Guisán, Biotechnol. Prog. 19 (2003) 784.
[36] L. Betancor, A. Hidalgo, G. Fernández-Lorente, C. Mateo, V. Rodríguez, M. Fuentes, R. Fernández-Lafuente, J.M. Guisán, Biotechnol. Prog. 19 (2003) 763.
[37] A. Hidalgo, L. Betancor, F. Lopez-Gallego, R. Moreno, J. Berenguer, R. Fernández-Lafuente, J. M Guisán, Enzyme Microb. Technol. 33 (2003) 278.
[38] J.M. Palomo, R.L. Segura, G. Fernandez-Lorente, M. Pernas, M.L. Rua, M. Guisán, R. Fernández-Lafuente, Biotechnol. Prog. 20 (2004) 630.
[39] C.-W. Suh, G.-S. Choi, E.-K. Lee, Biotechnol. Appl. Biochem. 37 (2003) 149.
[40] H.S. Toogood, I.N. Taylor, R.C. Brown, S.J.C. Taylor, R. McCague, J.A. Littlechild, Biocatal. Biotrans. 20 (2002) 241.
[41] T. Kuroiwa, S. Ichikawa, S. Sato, S. Mukataka, Biotechnol. Bioeng. 84 (2003) 121.
[42] M.L. Rúa, C. Schmidt-Dannert, S. Wahl, A. Sprauer, R. D Schmid, J. Biotechnol. 56 (1997) 89.
[43] J.M. Guisán, Enzyme Microb. Technol. 10 (1988) 375.
[44] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[45] R.M. Blanco, J. M Guisán, Enzyme Microb. Technol. 11 (1989) 360.
[46] U.K. Laemmli, Nature 227 (1970) 680–685.
[47] R.C. Switzer, C.R. Merril, S. Shifrin, Anal. Biochem. 98 (1979) 231.
[48] J. Uppenberg, M.T. Hansen, S. Patkar, T.A. Jones, Structure 2 (1994) 293.