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Single-step purification of different lipases from *Staphylococcus warneri*

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ABSTRACT

Three different lipases from the extract crude of *Staphylococcus warneri* have been purified by specific lipase–lipase interactions using different lipases (TLL, RML, PFL, BTL2) covalently attached to a solid support as adsorption matrix. BTL2 immobilized on glyoxyl-DTT adsorbed selectivity only a 30 kDa lipase from the crude, which was desorbed by adding 0.1% triton X-100. Using glyoxyl-PFL as matrix, two new lipases (28 and 40 kDa) were adsorbed, and completely pure 40 kDa lipase was obtained after desorption using 0.01% triton, whereas 28 kDa lipase was desorbed after the incubation of the lipase matrix with 3% detergent. When using other matrixes as glyoxyl-TLL or glyoxyl-RML, different lipases were adsorbed. This methodology could be a very efficient and useful method to purify several lipases from crude extracts from different sources.

1. Introduction

Lipases are very relevant enzymes from both a physiological and a biotechnological point of view. Although their natural function is the hydrolysis of triglycerides, these enzymes are one of most used enzymes because they can recognize many substrates different from their natural ones [1–8].

This is possible, thanks to their peculiar catalytic mechanism, the so called interfacial activation. In homogeneous aqueous media, these enzymes exist in a certain equilibrium between two different conformations, mainly as closed conformation, where the active site is completely isolated from the reaction medium by an oligopeptide chain called flap or lid, and the minority open conformation where this flap is displaced and 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]. Thus, in the presence on hydrophobic interfaces, drops of oil (its natural substrate) [12,13], hydrophobic support surfaces [14,15], gas bubbles [16], hydrophobic proteins (fungal hydrophobins) [17], lipopolysaccharides [18], etc., the equilibrium is shifted towards the open conformation and is kept adsorbed on the interface. Furthermore, the mechanism also occurs directly between lipase molecules which have a natural tendency to form bimolecular aggregates, by means of interactions between the hydrophobic surfaces surrounding the active centers [19,20].

Considering that normally the crude extracts of lipase preparations contain other proteins, such as esterases and in many cases other lipases, the development of efficient purification methods will permit to obtain high final purification yields, avoiding possible undesired reaction in applications and increasing the final activity towards non-natural substrates.

In this manuscript, we have tried to use this interfacial activation capacity as a tool to purify lipases. The methodology 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* the similar mechanism involving the bimolecular aggregates' formation.

This idea has been initially demonstrated in our group using lipase from *Pseudomonas fluorescens* as a matrix [21] for purification of extract crude and commercial solutions of lipases. Here in this manuscript we have demonstrated the generality of the methodology using other different lipases from different sources as an adsorbent matrix in order to purify different lipases existing in a crude extract of *Staphiloccocus warneri*.

The structural analysis of the surface [22] of the different lipases has determined the existence of lysine residues (Lys) normally in the opposite side of the active center, whereas the area near the active center has almost no Lys residues (Fig. 2). Thus, the immobilization of these enzymes on glyoxyl-agarose (i.e., via the richest lysine area) [23] should yield an orientation of the enzyme with the active center exposed to the reaction medium.

However, analyzing the crystal structure of BTL2 it is possible to observe that lysines are near to the active site in this case, there-

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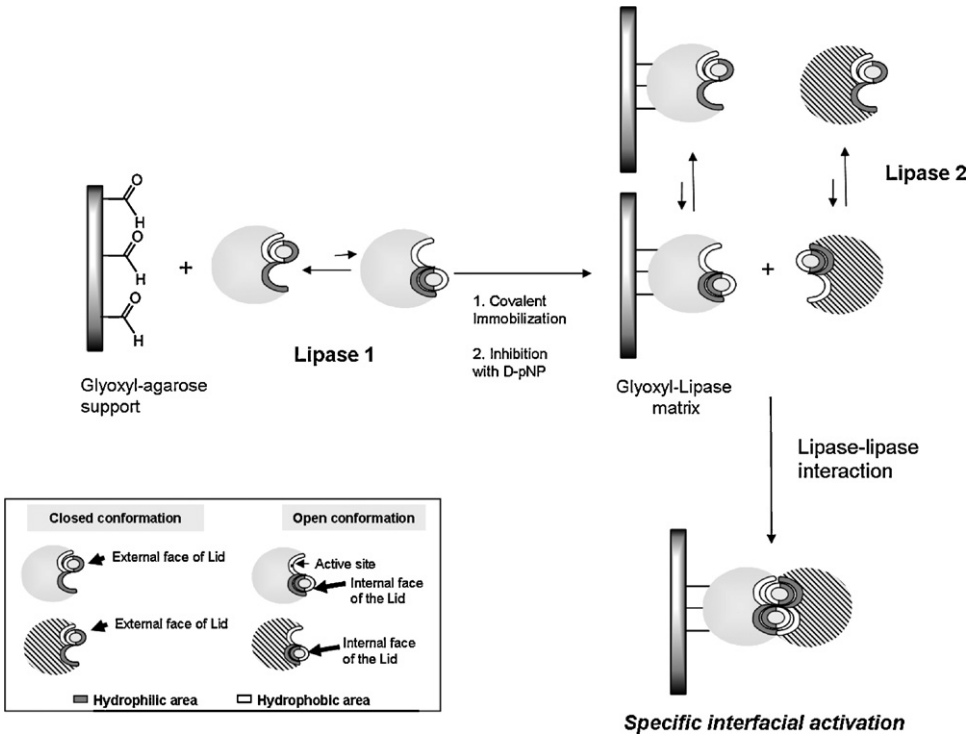

Fig. 1. Representation of the specific adsorption of lipases on an immobilized lipase matrix.

fore the best orientation should be from the terminal amino group (Fig. 3). Therefore a methodology of immobilization on glyoxyl at pH 8 incubated with DTT will be the best one to obtain an immobilization from this point with a final inert support surface [24]. The mechanism proposal of both immobilization strategies is shown in Fig. 4.

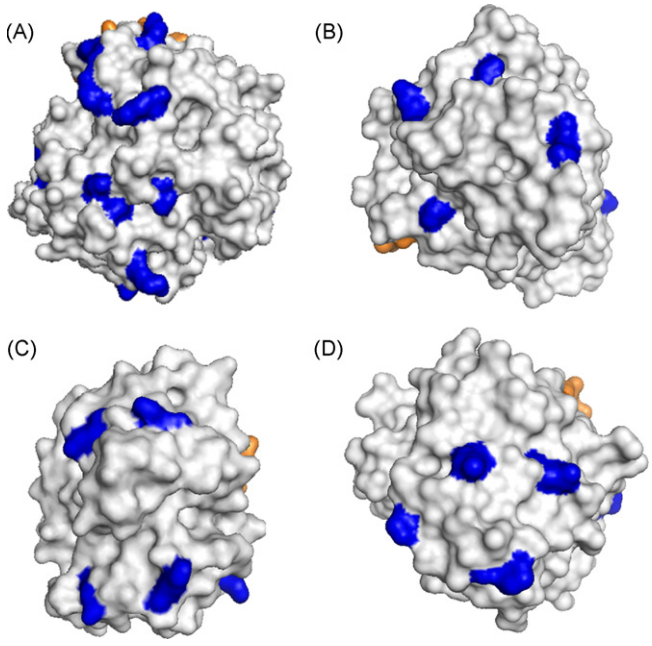

Fig. 2. Representations of the crystal structure surface of several lipases. (A) CAL-A, (B) PFL, (C) TLL, (D) RML. Lysine residues present in the enzyme surface (blue) and the amino acid residues constituting the lid (orange). Structure was obtained from PDB data bank with following codes: 2VEO (CAL-A), 2LIP (PFL), 1TIB (TLL) and 3TGL (RML). Figure was drawn using Pymol 0.99 program. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2. Materials and methods

2.1. Materials

The lipase from *Pseudomonas fluorescens* (PFL) PS "Amano" (lot. LPSA21250457) was purchased from Amano Enzyme Inc. (NAGOYA, Japan). The lipases from *Thermomyces lanuginosa* (TLL), *Candida antarctica* (fraction A) (CAL-A) and *Rhizomucor miehei* lipase (Palatase 20000L) (RML) were from Novo Nordisk (Denmark). The lipase from *Geobacillus thermocatenulatus* (BTL2) was obtained in our lab as previously described [25]. Crosslinked agaroses (6%) were kindly donated by Hispanagar S.A. (Burgos, Spain). Triton X-100, dithiothreitol (DTT), p-nitrophenylpropionate (pNPP) and diethyl-p-nitrophenylphosphate (D-pNP) were from Sigma (St. Louis, MO).

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 (U) under the conditions described above.

2.3. Preparation of the crude of S. warneri

A microorganism isolated from an abattoir fat-rich wastewater of Brazil, and very recently identified as *S. warneri* EX17 [26], was kept at 4 °C on tributyrin agar plate containing (g/L): peptone, 5; yeast extract, 3; tributyrin, 10. Batch cultivations were carried in a 5 L working volume stirred bioreactor (Biostat B model, B.

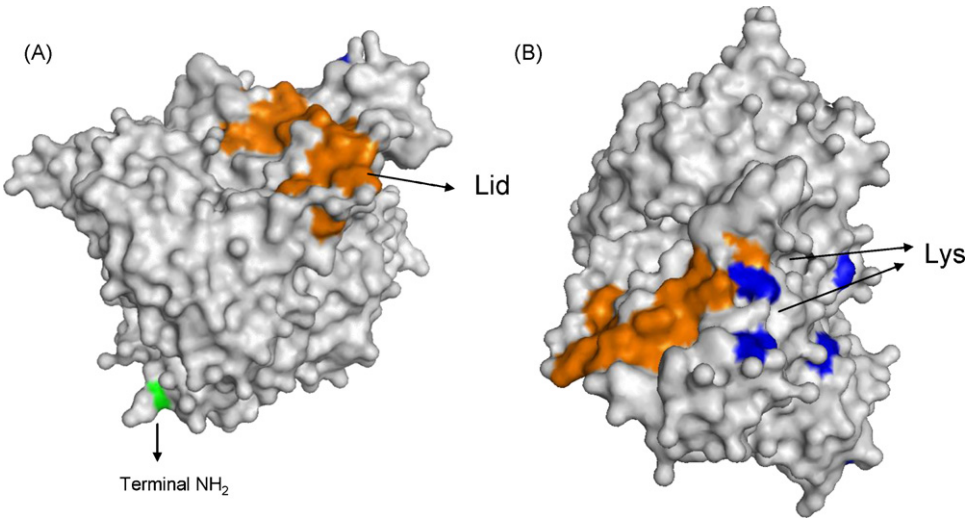

Fig. 3. Representations of the crystal structure surface of the open conformation of *G. thermocatenulatus* lipase. (A) The side selected constituted the terminal amine (green) and the amino acid residues constituting the lid (orange); (B) lateral side of the protein. Lysine residues present in the enzyme surface (blue). Figure was drawn using Pymol 0.99 program. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Braun Biotech International, Germany) filled with 4 L of medium. The culture medium and bioreactor conditions were optimized in previous studies to produce the extracellular lipase [26,27]. The culture medium contained, in g/L: peptone, 10; raw yeast extract, 5; glycerol, 30; olive oil, 6 and soybean oil, 5. The cultures were carried out with an oxygen volumetric mass transfer coefficient (k_La) of 38 h^{-1} by 12 h at pH 7 and 37 °C. The culture broth was centrifuged (3000 × *g* for 20 min at 4 °C) to recover the supernatant, and this was clarified by washing with *n*-hexane (1:1, v/v) to remove oils contained in the culture medium. The aqueous phase, called crude protein extract, was used for further studies.

2.4. Preparation of the different matrix glyoxyl-lipase

2.4.1. Purification of lipases

All lipases were purified previously using the interfacial adsorption method on octyl-agarose support as previously described [28]. In all cases 10 mg lipase/g support were immobilized.

After, lipases were eluted from the support by using a 25 mM sodium phosphate buffer solution containing 1% Triton X-100 obtaining a final solution of 1 mg_{lipase}/mL.

2.4.2. Immobilization of lipases on glyoxyl-agarose

In each case, 10 mL of desorbed lipase solution (1 mg_{lipase}/mL) was dissolved in 10 mL of 100 mM sodium bicarbonate pH 10.2 and the pH was adjusted at pH 10.15. After that, 1 g glyoxyl-agarose was added and the reaction was maintained during 24 h at 25 °C. Finally the enzyme-support multi-interaction was ended by adding 1 mg of sodium borohydride per milliliters of suspension during 45 min [23].

In the case of BTL2, 10 mL of desorbed lipase solution (1 mg_{lipase}/mL) was dissolved in 10 mL of 25 mM sodium phosphate pH 8.3 with 50 mM DTT and the pH was adjusted at pH 8.0. After that, 1 g glyoxyl-agarose was added and the reaction was maintained during 2 h at 25 °C. Finally the enzyme-support multi-interaction was ended by adding 1 mg of sodium borohydride per milliliters of suspension during 30 min [23]. Immobilization yield of each lipase is shown in Table 1.

2.4.3. Irreversible inactivation of immobilized lipases in the presence of diethyl-p-nitrophenylphosphate (D-pNP)

0.4 g of glyoxyl-lipase immobilized preparations were dissolved in 5 mL of 25 mM sodium phosphate buffer solution at pH 7 and 25 °C to 1.45 mM of inhibitor (D-pNP). The reaction was

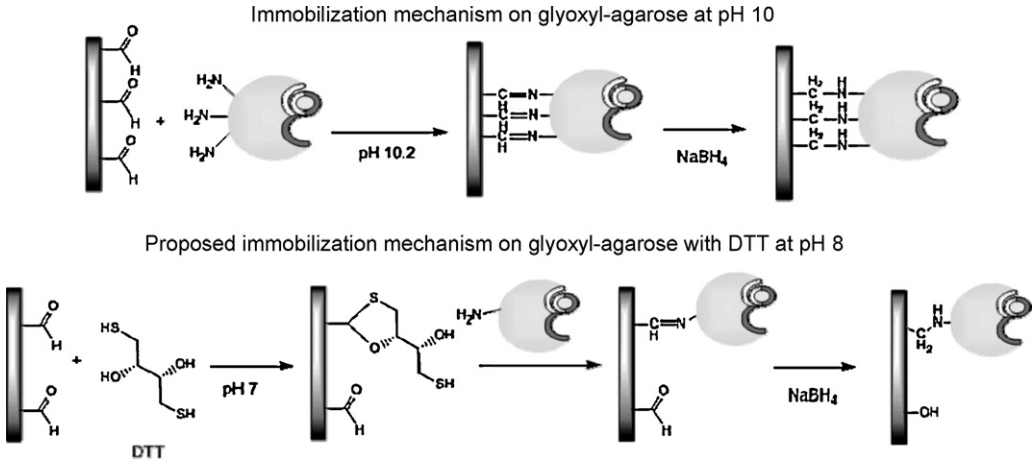

Fig. 4. Immobilization mechanism of lipases on glyoxyl-agarose at pH 10 or with DTT at pH 7.

Table 1
Preparation of different lipase matrices.

Lipase	Support	Immobilization (%)
P. fluorescens lipase (PFL)	Glyoxyl-agarose	83
T. Lanuginosa (TLL)	Glyoxyl-agarose	100
R. miehei lipase	Glyoxyl-agarose	87
C. antarctica (fraction A) (CAL-A)	Glyoxyl-agarose	100
G. thermocatenulatus (BTL2)	Glyoxyl-agarose	100
G. thermocatenulatus (BTL2)	Glyoxyl-DTT-agarose	100

maintained until the activity of the immobilized enzyme was zero.

2.5. Immobilization of lipases on the different glyoxyl-lipases

In a standard experiment, one gram of the different glyoxyl-lipase preparations (or octyl-agarose) was added to a 15 mL solution of *S. warneri* crude (0.426 mg/mL) at pH 7 and at 25 °C. 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 pNPB assay. After immobilization, the different adsorbed lipase preparations were thoroughly washed with distilled water.

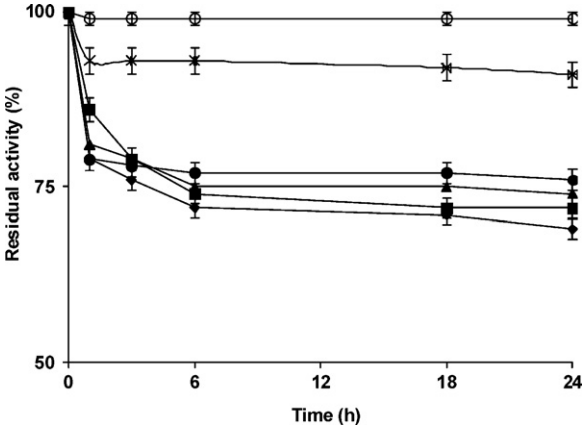

Fig. 5. Immobilization course of different lipases from *Staphilococcus warneri* on different glyoxyl-lipase supports. Supernatants from adsorption of extract crude of *S. warneri* in glyoxyl-PFL (triangles); glyoxyl-RML (squares); glyoxyl-TLL (triangles); glyoxyl-DTT-BTL2 (circles); glyoxyl-BTL2 (empty circles), glyoxyl-CAAL (asterisk).

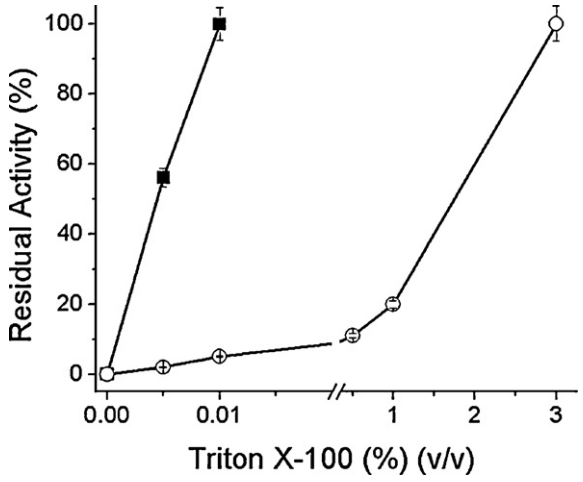

Fig. 7. Desorption of lipases adsorbed on glyoxyl-PFL by progressive addition of triton X-100. 45 kDa lipase (squares), 28 kDa lipase (circles)). Experiments were performed at 25 °C and pH 7.

2.6. Lipase desorption

The different lipases were desorbed from the different glyoxyl-lipase matrixes by using different percentage (v/v) of Triton X-100 in 25 mM sodium phosphate buffer for 30 min incubation each time.

2.7. SDS-PAGE analysis

SDS-PAGE electrophoresis was performed according to Laemli's method [29] in a SE 250-Mighty Small II electrophoretic unit (Hoefer Co.) using gels of 14% polyacrylamide in a separation zone of 9 cm × 6 cm and a concentration zone of 5% polyacrylamide. The gels were stained following the Coomassie method and the silver staining method [30]. Molecular weight markers were the LMW kit (14,400–94,000 Da) from Pharmacia.

Table 2
Desorption of the *S. warneri* lipase adsorbed on glyoxyl-DTT-BTL2 by progressive addition of Triton X-100. Experiments were performed at 25 °C and pH 7.

Triton (%)	Lipase desorption (%)
0.01	78
0.05	83
0.1	100

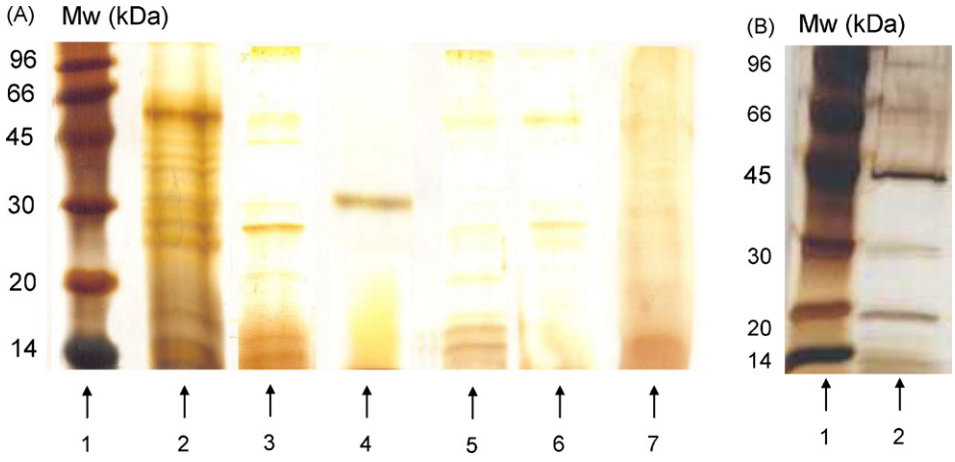

Fig. 6. SDS-PAGE analysis of SW on different lipase preparations. (A) Lane 1 – low molecular weight marks. Lane 2 – crude extract of *S. warneri*. Lane 3 – *S. warneri* adsorbed on glyoxyl-PFL. Lane 4 – *S. warneri* adsorbed on glyoxyl-DTT-BTL2. Lane 5 – *S. warneri* adsorbed on glyoxyl-RML. Lane 6 – *S. warneri* adsorbed on glyoxyl-TLL. Lane 7 – *S. warneri* adsorbed on glyoxyl-CAL-A. (B) Lane 1 – low molecular weight marks. Lane 2 – *S. warneri* adsorbed on octyl-agarose.

Table 3
Summary of the purification factor, yield or specific activity for the three lipases purified from *S. warneri*.

Lipase	Total activity (U/mL)	Specific activity (U/mg)	Purification factor	Purification yield (%)
Crude extract	0.88	0.18	–	–
45 kDa	0.41	2.3	13.0	46
28 kDa	0.67	4.7	26.4	75
30 kDa	0.70	6.1	34.0	79

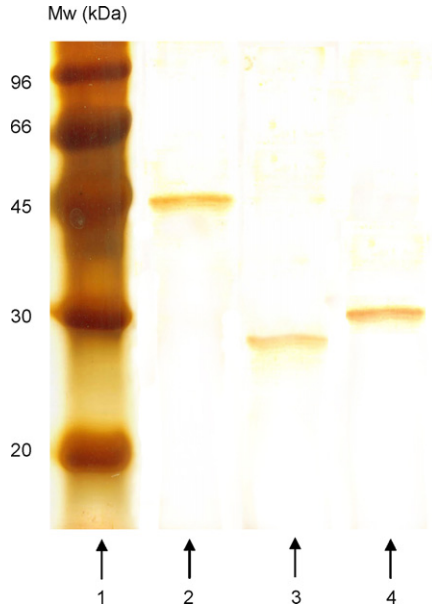

Fig. 8. SDS-PAGE analysis of desorbed lipases from *S. warneri* adsorbed to different glyoxyl-lipase matrixes. Lane 1 – low molecular weight marks. Lane 2 – 45 kDa lipase (SWL) desorbed from glyoxyl-PFL-SW with 0.01% triton X-100. Lane 3 – 28 kDa lipase desorbed from glyoxyl-PFL-SW with 3% triton X-100. Lane 4 – 30 kDa lipase desorbed from glyoxyl-DTT-BTL2-SW with 0.1% triton X-100.

3. Results and discussion

3.1. Immobilization of different lipases from S. warneri crude on different glyoxyl-lipase preparations by lipase–lipase interaction

15 mL solution of *S. warneri* crude (0.426 mg/mL) was offered to the different lipase matrixes (glyoxyl-PFL, glyoxyl-DTT-BTL2, glyoxyl-BTL2, glyoxyl-CAL-A, glyoxyl-RML, glyoxyl-TLL) at pH 7 and at 25 °C.

Using glyoxyl-PFL, glyoxyl-RML or glyoxyl-TLL around 25% of esterase activity (using pNPB as substrate) of *S. warneri* was absorbed on the lipase matrix at low ionic strength after 5 h, whereas the protein adsorption percentage was only of 5% with glyoxyl-CAL-A preparation after incubation for 24 h (Fig. 5). When glyoxyl-BTL2 was used as matrix, 100% of the activity was maintained on the supernatant probably because BTL2 did not present the adequate orientation after immobilization in glyoxyl-agarose at pH 10, because the immobilization involves the Lys area near to the lid as shown in Fig. 3, avoiding the complete free movement of the lid, and therefore not being a good adsorbent matrix. Therefore, in this case a new kind of matrix was prepared. The BTL2 was immobilized on glyoxyl in the presence of DTT at pH 8 involving an immobilization from the terminal amine and permitting an immobilized enzyme with the active site oriented to the medium to be used as a matrix. Thus glyoxyl-DTT-BTL2 exhibited 25% immobilization also, same as the previous matrices (Fig. 5).

Furthermore, it was interesting to note that the 30 kDa lipase, the only lipase previously described from *S. warneri* [26], was the unique protein adsorbed on the glyoxyl-DTT-BTL2 matrix from the quite crude extract by SDS-PAGE analysis as shown in Fig. 6a. Two different proteins were adsorbed using glyoxyl-PFL matrix, mainly one with 45 kDa and another with approximately 28 kDa, as shown in the SDS-PAGE (Fig. 6a) which presented esterase activity against α-naphtyl acetate in a native SDS-PAGE (data not shown). The glyoxyl-RML or glyoxyl-TLL preparations adsorbed several proteins after incubation with the *S. warneri* crude, being much less specific (Fig. 6a) in the same way that using hydrophobic supports such as octyl-agarose (Fig. 6b).

Therefore, the use of different lipases with different hydrophobic areas surrounding the active site show very different selectivities in the adsorption of different proteins (lipases) from a crude extract.

3.2. Desorption profile of the different adsorbed lipases

After the adsorption step, two lipases matrixes (glyoxyl-PFL and glyoxyl-DTT-BTL2) were selected to perform the purification of the three lipases containing on crude of *S. warneri*. The final purification step consisted of the elution of the enzymes from the solid support by treatment with a neutral detergent, Triton X-100.

Fig. 7 shows the desorption profile of the different lipases adsorbed on glyoxyl-PFL. A complete elution of 45 kDa lipase was achieved after the addition of very low amount of detergent (0.01%). In the case of 28 kDa protein, it was necessary to added up to 3% detergent to complete elution of the lipase from the support.

Using glyoxyl-DTT-BTL2, an elution profile was studied to determine the amount necessary to desorb the 30 kDa lipase. In this case, 0.1% Triton X-100 was enough to obtain 100% of the protein on solution (Table 2).

Thus, after a gradient of detergent was easily to obtain in pure form two new lipases of 45 and 28 kDa, and 30 kDa lipase as shown in SDS-PAGE (Fig. 8). The purification factor, yield and specific activity of the new purified lipases are described in Table 3.

4. Conclusion

These studies have demonstrated that, besides PFL, other lipases may be used for the selective adsorption of lipases, doing general this methodology very likely by the interaction between the hydrophobic areas surrounding the active center of most lipases.

This methodology has permitted to easily purify and separate three new lipases from the extract crude of the *S. warneri*.

The 30 kDa lipase was selectively adsorbed as the unique protein on glyoxyl-BTL2 by specific interfacial adsorption whereas other two lipases (28 and 45 kDa) were adsorbed on glyoxyl-PFL. After, a simple detergent elution, all three lipases were completely purified on solution with purification factors from 13 up to 34.

Therefore, this simple method could be very useful to separate and purify new lipases from different crude extracts from different sources, e.g. extract from very extreme environments where we can find lipases with very interesting biotechnological applications.

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