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Modulation of a lipase from *Staphylococcus warneri* EX17 using immobilization techniques

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ABSTRACT

This research describes the immobilization on glyoxyl, cyanogen bromide or octyl agarose beads of a purified lipase from *Staphylococcus warneri* strain EX17 (SWL), and the effect on its properties. The immobilization on glyoxyl-agarose at pH 10 and 25 °C, conditions in which the enzyme is readily inactivated, required the stabilization of the soluble enzyme. This was attained by the addition of 25% glycerol. Using this additive, immobilization on glyoxyl-agarose beads proceeded very quickly with good activity retention around 80%. This was the most stable preparation under thermal inactivation at pH 5, 7 and 9, in the presence of either cosolvents or detergents. This preparation was hyperactivated by concentrations of Triton X-100, which would produce negative effects over enzyme activity when using the other SWL preparations. Immobilized SWL preparations hydrolyzed different chiral esters, such as (±)-methyl mandelate, (±)-2-*O*-butyryl-2-phenylacetic acid, and (±)-2-hydroxy-4-phenyl-butyric acid ethyl ester, being its specificity depended on the immobilization protocol. The enantiospecificity was also strongly modulated by the immobilization. Thus, using HPBEt as substrate, octyl-SWL exhibited an opposite enantiospecificity to the other two biocatalysts. This preparation was the most enantioselective in the hydrolysis of (±)-2-*O*-butyryl-2-phenylacetic acid ($E = 56.3$).

1. Introduction

The immobilization of enzymes is a convenient way to use them as industrial biocatalysts, especially when the enzymes are stable enough and can be reused for several reaction cycles [1,2]. Moreover, if the immobilization protocol is properly designed to give an intense multipoint covalent attachment, the immobilization may be a powerful way to improve the enzyme rigidity, which increases enzyme stability against any distorting agents [3,4]. However, to obtain an intense multipoint covalent attachment is not easily achieved, requiring the selection of a suitable support and the use of proper immobilization conditions [5]. Immobilization of many enzymes in glyoxyl-agarose beads has produced high stabilization factors, 2–5 orders of magnitude [6].

Lipases are the most broadly used enzymes in biocatalysis in order to produce very different compounds, from structured oils to fine chemicals [7–9]. One particular feature of lipases is their conformational changes in structure during catalysis, changing between a closed form, where the active centre is secluded from the medium by a oligopeptide chain called flap or lid, and an open form where this lid is shifted and the active centre is exposed [10,11]. In contact with hydrophobic surfaces, such as oil drops [11], the open form of lipase is adsorbed, involving the large hydrophobic surface formed by the inner side of the lid and the active centre: the so-called interfacial activation of lipases.

That makes that in the specific case of lipases, the interfacial activation of the lipase by adsorption of the open form of the enzyme on hydrophobic supports at low ionic strength may be used as an immobilization method [12]. This simple strategy allows the preparation of hyperactivated lipases and, in many cases, some stabilization of the enzyme may be achieved. The structural changes that the lipases suffer might alter its selectivity. Therefore, immobilization of lipases by adsorption or covalent bonding involving different protein surface areas, which may cause different enzyme rigidity or microenvironment, has proved to be in many cases a powerful tool to improve the enzyme selectivity [3,13].

Four different lipases from *Staphylococcus warneri* M strain have been deposited in the protein databases (accession numbers

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BAD90560, BAD90561, BAD90562 and BAD90565) [http://blast.ncbi.nlm.nih.gov]. For *S. warneri* 863 strain, two lipases, SWL1 and SWL2, have been already purified and partially characterized [14,15], being SWL2 96% identical to the protein deposited under accession number BAD90565. In this research, we have used an extracellular lipase from *S. warneri* strain EX17 that shows 100% of identity with a protein fragment of the lipase deposited under accession number BAD90562 (results not shown). Our aims were to immobilize this lipase using different protocols: adsorption on hydrophobic support [12], limited covalent attachment, and multipoint covalent attachment.

Moreover, we have checked if the specificity of this lipase may be also easily modulated [3]. As model substrates, three different ester have been chosen: (±)-2-*O*-butyryl-2-phenylacetic acid, (±)-methyl mandelate, and (±)-(R,S) 2-hydroxy 4-phenyl butanoic acid ethyl ester (HPBEt).

The two first substrates are precursors of mandelic acid, having the chiral centre in the nucleophile or in the acyl donor site. (*R*)-Mandelic acid or esters used for the synthesis of very interesting cephalosporin antibiotics such as cephamandole and cephonicid [16]. (*R*)-Mandelic acid is a key intermediate in the synthesis of the styryl lactone, as goniodiol, which exhibits a potent and selective cytotoxicity against human lung carcinoma with no significant toxicity against brine shrimp [17]. Enantiomerically pure acids may also be used in the resolution of racemates by selective precipitation [18]. (*R*)-HPBEt is a chiral building block useful for the synthesis of different ACE inhibitors [19].

2. Materials and methods

2.1. Materials

Lipase from *S. warneri* EX17 (SWL) was produced as previously described [20,21]. (±)-2-*O*-butyryl-2-phenylacetic acid, (±)-methyl mandelate, ethanolamine hydrochloride, hexadecyltrimethylammonium bromide (CTAB), Triton X-100, and *p*-nitrophenyl butyrate (p-NPB) were from Sigma. (R,S) 2-hydroxy 4-phenyl butanoic acid ethyl ester (HPBEt) was kindly supplied by VITA Invest. 1,4-Dioxane, diethyleneglycol dimethyl ether (diglyme), and dimethyl formamide (DMF) were from Fluka. Butyl-toyopearl 650M was from Tosoh Corporation. Octyl-sepharose CL-4B and cyanogen bromide activated Sepharose 4B (CNBr) were purchased from GE Healthcare. Cross-linked agarose (10 BCL) was kindly donated by Hispanagar S.A. (Burgos, Spain) and its modification to glyoxyl-agarose (activated with 200 μmol/g of support) was performed as described elsewhere [22]. Other reagents and solvents used were of analytical or HPLC grade.

2.2. Methods

The experiments were carried out at least by triplicate and the standard error was always under 5%.

2.2.1. Lipase activity assay

This assay was performed by measuring the increase in the absorbance at 348 nm produced by the release of *p*-nitrophenol in the hydrolysis of 0.4 mM pNPB in 25 mM sodium phosphate at pH 7 and 25 °C, using a themostatized spectrum with continuous magnetic stirring. To initiate the reaction, 0.05 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. In some instances, detergents were added to the reaction mixtures. One unit of pNPB activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol of pNPB/min (U) under the conditions described above.

2.2.2. Purification of SWL

The purification was carried out following a previously described procedure [23] by the interfacial activation against hydrophobic supports. The crude SW protein extract was diluted with 10 mM sodium phosphate pH 7.0. Butyl-toyopearl was added and maintained under continuous stirring. Periodically, the activities of suspensions and supernatants were measured by using the pNPB assay. After completion of the adsorption process, the support with the adsorbed proteins was vacuum filtered using a sintered glass funnel and abundantly washed with distilled water. SWL was desorbed from the hydrophobic support by suspending the immobilized enzyme in a relation 1:10 (w/v) in 25 mM sodium phosphate pH 7.0 at 4 °C containing 0.5% of Triton X-100. Only one protein band was detected by SDS-PAGE (with a volumetric activity of 0.824 U/mL) [23].

2.2.3. Immobilization of SWL

In all cases, the experiments show the activities of a suspension where the support is inert agarose but under identical conditions to the immobilization experiment (control suspension), the activity of the suspension with the support where we intend to immobilize the enzyme (immobilization suspension) and the supernatant of this suspension (taken by using a tip-filter) (immobilization supernatant of the immobilization suspension).

The presence of detergent during the covalent immobilization ensures the immobilization of individual lipase molecules and avoids the artifacts that lipase–lipase interactions may generate [24–28].

2.2.3.1. On octyl-agarose. Purified SWL was diluted with 10 mM sodium phosphate pH 7 to reduce the concentration of Triton X-100, and it allows the absorption of the lipase via interfacial activation [12] on octyl-agarose support at 4 °C and pH 7, for 1 h. The adsorbed enzyme derivative was prepared using 1 g of octyl-agarose support and 100 mL of diluted purified SWL. After immobilization, the SW adsorbed on octyl agarose beads preparation was recovered by filtration and washed with abundant water. This immobilized enzyme was called octyl-SWL.

2.2.3.2. On CNBr-activated support. CNBr-activated supports are among the most used to covalently immobilize protein via their amino groups [29]. Purified SWL was covalently immobilized on CNBr-activated support at 4 °C and pH 7, for 15 min to reduce the possibilities of getting a multipoint covalent attachment between enzyme and support. The enzyme-support reaction was ended by incubating the support with 1 M ethanolamine at pH 8 for 2 h. During the immobilization and further blocking of the support, the suspension was submitted to continuous gentle stirring. Finally, the immobilized SWL preparation was vacuum filtered using a sintered glass funnel and washed with abundant water to eliminate the detergent. This immobilized enzyme was called CNBr-SWL. This preparation, that should be immobilized in the support by just some few covalent attachments (ideally just one), will be a very good reference to the soluble enzyme, but in the absence of any intermolecular interaction that may alter the results [3].

2.2.3.3. On glyoxyl-agarose beads. The pH of the purified SWL was adjusted with 1 M sodium bicarbonate at pH 10 to obtain a final concentration of 100 mM. The immobilized enzyme derivative was prepared using 1 g of glyoxyl-support and 10 mL of purified SWL in the presence or absence of 25% of glycerol. The mixture was maintained at 25 °C during the specified times. As reaction end-point, solid sodium borohydride was added to a concentration of 1 mg/mL and the mixture was maintained at 25 °C under very gentle stirring. After 30 min, the immobilized and reduced derivatives were

washed thoroughly with distilled water. This immobilized enzyme was called Gx-SWL.

2.2.4. Effect of pH and temperature on the stability of different SWL immobilized preparations

The different SWL preparations were incubated in 25 mM sodium acetate pH 5 and 55 °C, 25 mM sodium phosphate pH 7.0 and 60 °C, or 25 mM sodium bicarbonate pH 9 and 50 °C. Samples were periodically withdrawn using a pipette with a cut-tip and under vigorous stirring to have a homogeneous biocatalyst suspension. The activity was measured using the pNPB assay described above. The experiments were carried out in triplicates and the plotted results are the mean of these repetitions. Stabilization is given as the ration of the half-live of the problem derivative divided by that of the preparations that is being compared to it.

2.2.5. Inactivation of different SWL immobilized preparations in the presence of organic cosolvent

Enzyme derivatives were washed with a mixture of 50% of solvent: 50% of 50 mM sodium phosphate aqueous solution at final pH 7 and 4 °C. Subsequently, the enzyme derivatives were resuspended in the same solution and incubated at 4 °C. Samples were periodically withdrawn, and the activity was checked following the above described assay. Experiments were carried out in triplicates and the plotted results are the mean of these repetitions.

2.2.6. Effect of detergents on the activity and stability of different SWL immobilized preparations

To evaluate the effect of detergents on lipase activity, the lipase activity was assayed with different detergents at different concentrations. To determine the stability of SWL immobilized preparations, they were incubated in the presence of various detergents at 25 °C for 1 h, at specified concentrations. The residual lipase activity was determined under standard assay conditions.

2.2.7. Enzymatic hydrolysis of esters

The activities of different immobilized preparations of SWL were analyzed in the hydrolysis reaction of three different chiral esters: (±)-methyl mandelate (**1**), (±)-2-*O*-butyryl-2-phenylacetic acid (**2**), and (±)-2-hydroxy-4-phenyl-butyric acid ethyl ester (**3**). Substrates (**1**) and (**3**) were dissolved to a concentration of 1 mM, in 2 mL of 25 mM sodium dihydroxy phosphate pH 7 and at 25 °C. Then, 0.5 g of immobilized SWL preparation was added to these esters solutions. Substrate (**2**) was dissolved to a concentration of 1 mM in 2 mL of 25 mM sodium acetate pH 5 or 7, in both cases at 25 °C. Then, 0.5 g of immobilized SWL preparation was added to these solutions.

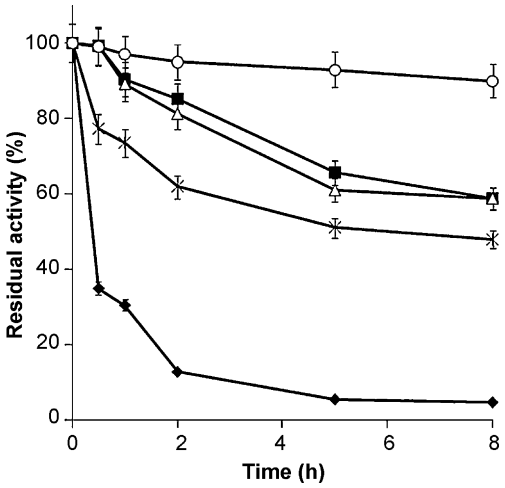

Fig. 1. Stability of soluble SWL at pH 10 under different experimental conditions. (♦) 25 °C; (■) 4 °C; (○) 4 °C in the presence of 25% of glycerol; (△) 25 °C in the presence of 25% of glycerol; (*) 25 °C in the presence of 25% of polyethylene glycol.

The degree of hydrolysis was analyzed by reverse-phase HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450). For these assays a Kromasil C18 (250 mm × 4.6 mm, 5 μm Ø) column was used, the mobile phase was 20% acetonitrile/80% 10 mM ammonium dihydroxy phosphate (v/v) pH 2.9, for (**1**); (35:65, v/v) for (**2**); and (40:60, v/v) for (**3**) at 1.5 mL/min, and UV detection was performed at 225 nm for all the substrates.

2.2.8. Determination of enantiomeric excess and enantioselectivity

The enantiomeric excess of the released acid (ee_p) (at conversions between 10 and 15%) was analyzed by chiral reverse-phase HPLC. The column was a Chiracel OD-R, the mobile phase was an isocratic mixture of acetonitrile:$NaClO_4$ 0.5 M, (20:80, v/v) for compound (**3**), (5:95, v/v) for compound (**2**), and an isocratic mixture of acetonitrile:10 mM ammonium dihydroxy phosphate (30:70, v/v) for compound (**1**). The final pH of the three mobile phases was adjusted to 2.3 with HCl and the flow was 0.5 mL/min, performing the UV detection at 210 nm.

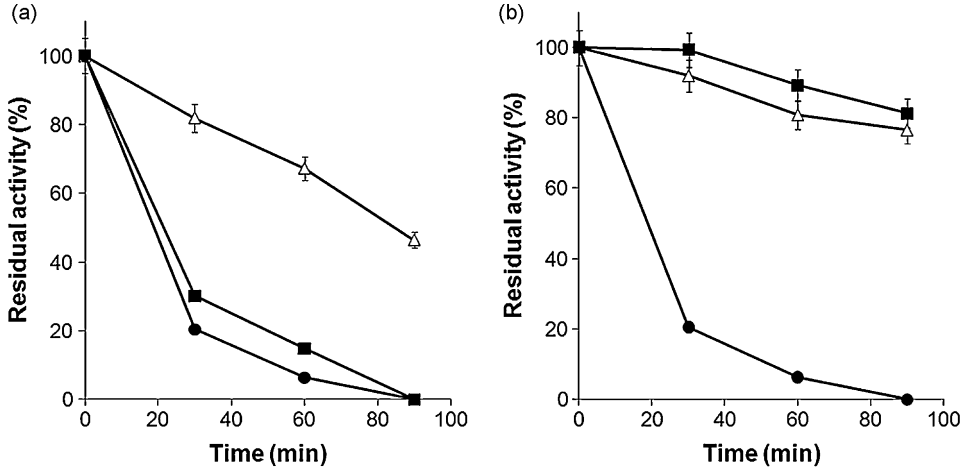

Fig. 2. Immobilization course of SWL on glyoxyl-agarose at pH 10 and 25 °C: (a) in the absence of glycerol and (b) with 25% of glycerol. (■) Control suspension; (△) immobilization suspension; (●) supernatant of the immobilization suspension. Results are the mean of triplicates.

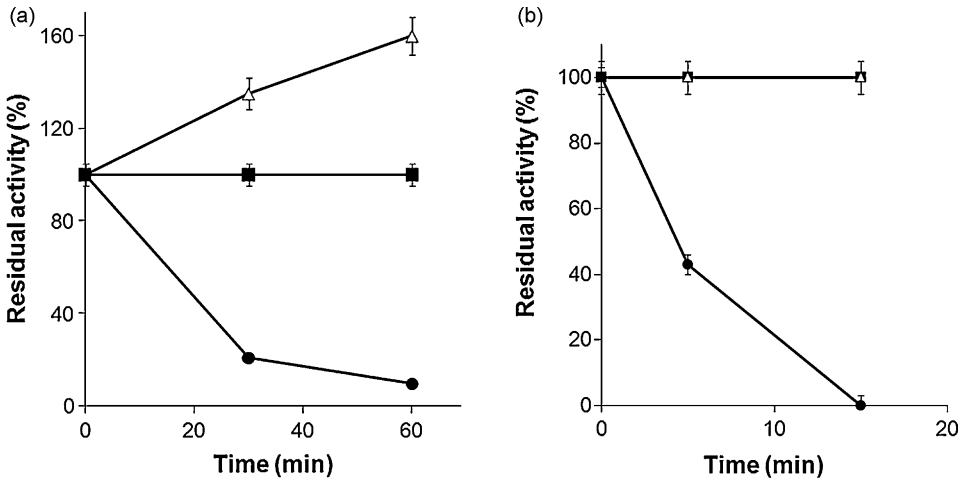

Fig. 3. Immobilization course of SWL on: (a) octyl-agarose and (b) CNBr-agarose. (■) Control suspension; (△) immobilization suspension; (●) supernatant of the immobilization suspension. Results are the mean of triplicates.

The enantiomeric ratio (E) was calculated in all cases using Eq. (1) reported by Chen et al. [30]:

$$E = \frac{\ln[1 - c(1 + ee_p)]}{\ln[1 - c(1 - ee_p)]} \quad (1)$$

where c is the conversion ratio of reaction; and ee_p is the enantiomeric excess of the formed enantiomer. The values included in the tables were calculated at values of c of 10, 20 and 30%, and they are given as the mean values.

3. Results

3.1. Stability of SWL at pH 10

The immobilization of SWL on glyoxyl-agarose needs to be performed at pH 10 [22], and 25 °C is the recommended temperature in order to have good enzyme-support multi-point reaction [5]. However, under these conditions, SWL activity was decreased by more than 70% after 30 min (Fig. 1). Temperature reduction to 4 °C produced a slower inactivation, with only 30% of activity loss after 6 h. It would, therefore, be interesting to find some additives capable to stabilize the enzyme under these conditions, which could favor multipoint immobilization. Poly-hydroxyl compounds have been described as stabilizers of many enzymes [31–33]. Thus, the effect of 25% (v/v) glycerol or 25% (v/v) polyethylene glycol on the enzyme stability at pH 10 and 25 °C was analyzed. It was found that these compounds stabilized the enzyme, being glycerol the best stabilizing agent. For instance, using this stabilizing reagent, the reduction on SWL activity at 25 °C and pH 10 was similar to that at 4 °C in absence of this additive, while in the presence of 25% glycerol, loss of SWL activity was negligible at 4 °C. This stabilizing agent was utilized also in the immobilization of penicillin G acylase on glyoxyl-agarose [3].

3.2. SWL immobilization on glyoxyl-agarose

Fig. 2 shows the course of immobilization of SWL on glyoxyl-agarose at 25 °C and pH 10 in the presence or absence of 25% glycerol. Rates of immobilization were similar in both cases and full immobilization was achieved after 90 min of enzyme-support incubation. After this time, in the absence of glycerol, the immobilized enzyme retained around 45% of its initial activity, while the soluble enzyme was fully inactivated under these conditions. This suggested that the immobilized enzyme was already stabilized, immediately after immobilization, against the inactivation at pH 10. This is relevant considering that at the same time the enzyme was reacting with the support, which might produce some enzyme distortion. Apparently, the enzyme-support reaction was far less negative for the enzyme activity than the effect of the pH 10. When the immobilization was performed in the presence of glycerol, the retained activity was around 80%. This preparation was selected for all other assays.

3.3. SWL immobilization on octyl and CNBr agarose

Fig. 3a shows the immobilization on octyl agarose. The immobilization proceeds rapidly and increased enzyme activity by a 1.6-fold factor. This result agrees with previous results of immobilization of other lipases in octyl agarose. The immobilization of SWL on CNBr was slower (Fig. 3b), and the immobilized enzyme activity remained unaltered. This suggested a very mild immobilization.

3.4. Stability of the different enzyme preparations

Fig. 4 shows the effect of the incubation at high temperature on the activity of the three preparations at different pH values. The Gx-SWL preparation was the most stable one: the stabilization factor was 40-fold at pH 7 and 9, and reached 160-fold at pH 5 when compared with octyl-SWL. At pH 5 and 7, the stability of CNBr-SWL and octyl-SWL biocatalysts was similar, while at pH 9, octyl-SWL was 10-fold less stable. These results contrast with previous findings for many other lipases, for which the interfacially activated enzymes were more stable than the standard covalent and even the glyoxyl preparations. However, it should be considered that the stability of the open form stabilized by the hydrophobic surface or the stability of the enzyme in the standard opening–closing equilibrium, apparently in this case the open form is less stable.

Fig. 5 shows the stability of the covalently immobilized SWL preparations in the presence of different organic solvents. In these experiments, octyl-SWL was not studied because this immobilization system can release some lipase molecules to the medium in the presence of cosolvent, making complex the understanding of the phenomena occurring. Both preparations of covalently immobilized SWL was quite stable at 4 °C in the presence of dioxane and dimethyl formamide (Fig. 5a and c respectively). Gx-SWL retained over 80% in both solvents while CNBr-SWL retained around 60% after 4 h. The stability in diglyme was slightly lower, and the glyoxyl preparation was found to be the most stable.

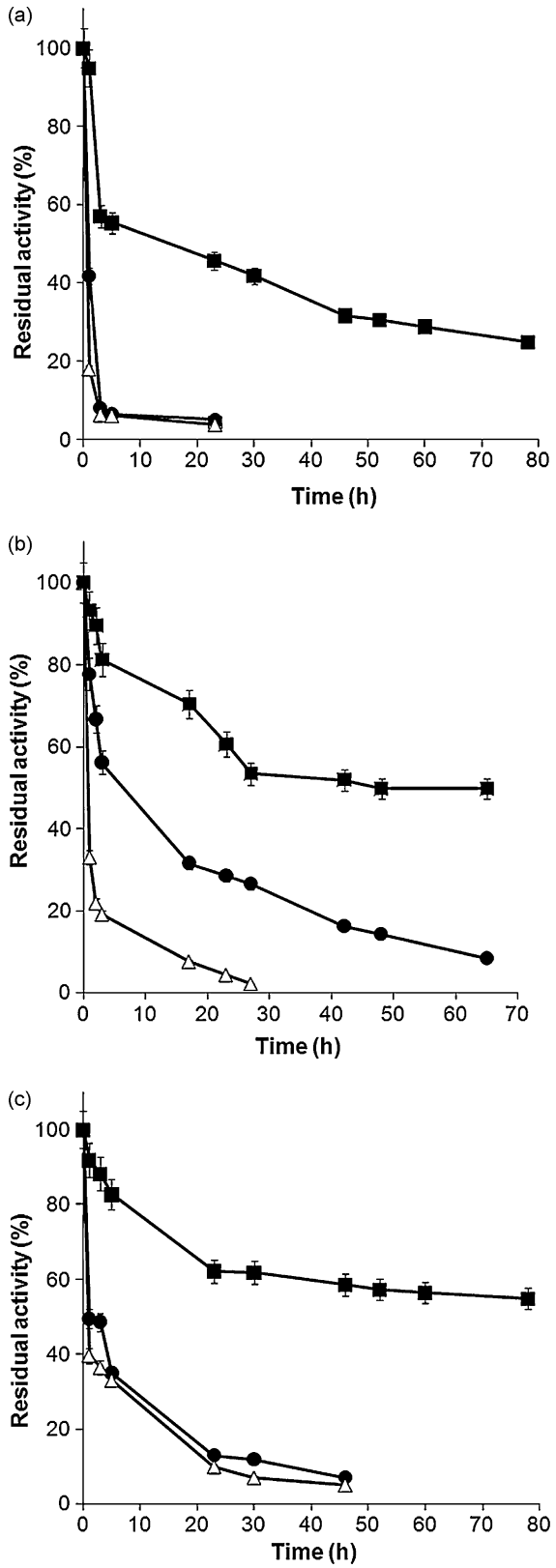

Fig. 4. Inactivation courses of different SWL immobilized preparations at: (a) 60 °C and pH 7.0; (b) 50 °C and pH 9.0; (c) 55 °C and pH 5.0. (●) CNBr-SWL; (△) Octyl-SWL; (■) Gx-SWL. Results are the mean of triplicates.

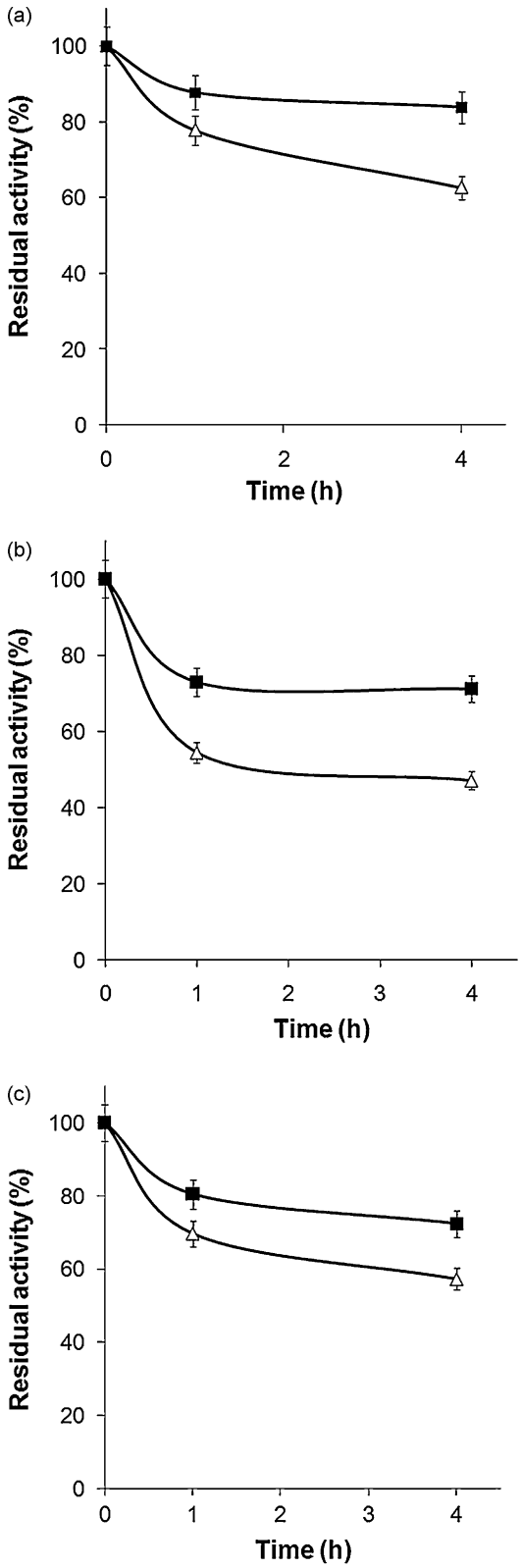

Fig. 5. Inactivation courses of SWL preparations in the presence of 50% of cosolvent at pH 7 and 4 °C. (a) Dioxane; (b) diglyme; (c) DMF. (△) CNBr-SWL; (■) Gx-SWL. Results are the mean of triplicates.

Table 1
Effect of detergents on the stability of SWL preparations.

Detergent (0.5%)	Residual activity[a] (%)	
	CNBr-SWL	Gx-SWL
Control	100	100
Triton X-100	74.8 ± 4	174.6 ± 5
CTAB	30.8 ± 2	55.2 ± 3
SDS	7.2 ± 2	6.6 ± 0.8

[a] The preparations were incubated by 1 h in each detergent and the activity was measured after this time.

3.5. Effect of the detergent on the enzyme activity and stability

Detergents have been described to have a complex effect on lipases. They can stabilize the open form of lipases, increasing their activity; but they also may have a negative effect on the lipase stability and even act as competitive inhibitors [34]. Moreover, detergents have been proposed as a simple way to modulate the enzyme selectivity [35]. This means that enzyme molecules with different rigidity, may present a different behaviour when incubated in the presence of detergents.

Fig. 6 shows the effect of different detergent in the activity of the covalent preparations of SWL. Octyl-SWL cannot be used in this case because of the risk of enzyme desorption. Triton X-100 enhanced enzyme activity for CNBr-SWL and Gx-SWL preparations, but their behaviours were quite different. CNBr-SWL increased the activity 2.5 times at 0.1% Triton X-100 concentration, while with 0.5% the activity increased 2 times, and with 1% the activity was similar to that of the enzyme in absence of detergent. Using Gx-SWL, the maximal activity was obtained at 0.5% concentration, over 2.2 times higher than the activity in the absence of detergent, and at 1% the activity was 1.7 times higher.

CTAB caused a decrease in the CNBr-SWL activity even at the lowest concentration studied (0.01%), while at 1% the residual activity was around 20% of that in absence of detergent. Using Gx-SWL, 0.1% CTAB produced a slight increment of enzyme activity, although at higher concentrations it produced a decrease in enzyme activity to values similar of those observed for CNBr-SWL. Finally, SDS was found to be deleterious for the activity of both immobilized enzyme preparations, with no significant differences observed between the two preparations.

To understand whether the main effect produced by the detergents was inhibition or inactivation, the immobilized enzymes were incubated in the presence of 0.5% of each of the three detergents and the residual activity determined after 1 h (Table 1). At a concentration of 0.5% (v/v) Triton X-100 produced a decrease in the activity of CNBr-SWL, while there was a small increment of enzyme activity for Gx-SWL. CTAB and SDS produced a decrease in the activity of CNBr-SWL. These results suggest that the ionic detergents produced the inactivation of the enzymes. In the case of Triton X-100, it seems a more complex effect: for Gx-SWL, there is a positive conformational change, while this is negative for CNBr-SWL. This is suggesting that Triton X-100 could produce the "opening" of the lipase, but also some other negative effects, which are less important in a more stable derivative as Gx-SWL.

The higher stability of Gx-SWL during thermal inactivation at different pH in the presence of organic solvents and detergents, suggested that the glyoxyl preparations were stabilized via multipoint covalent attachment, which produces more rigid enzyme structures, thus with less tendency to conformational changes [3].

3.6. Effect of the immobilization protocol on the enantiospecificity of SWL

In order to test properties of the lipase systems after different protocols of immobilization, they were used in the hydrolysis

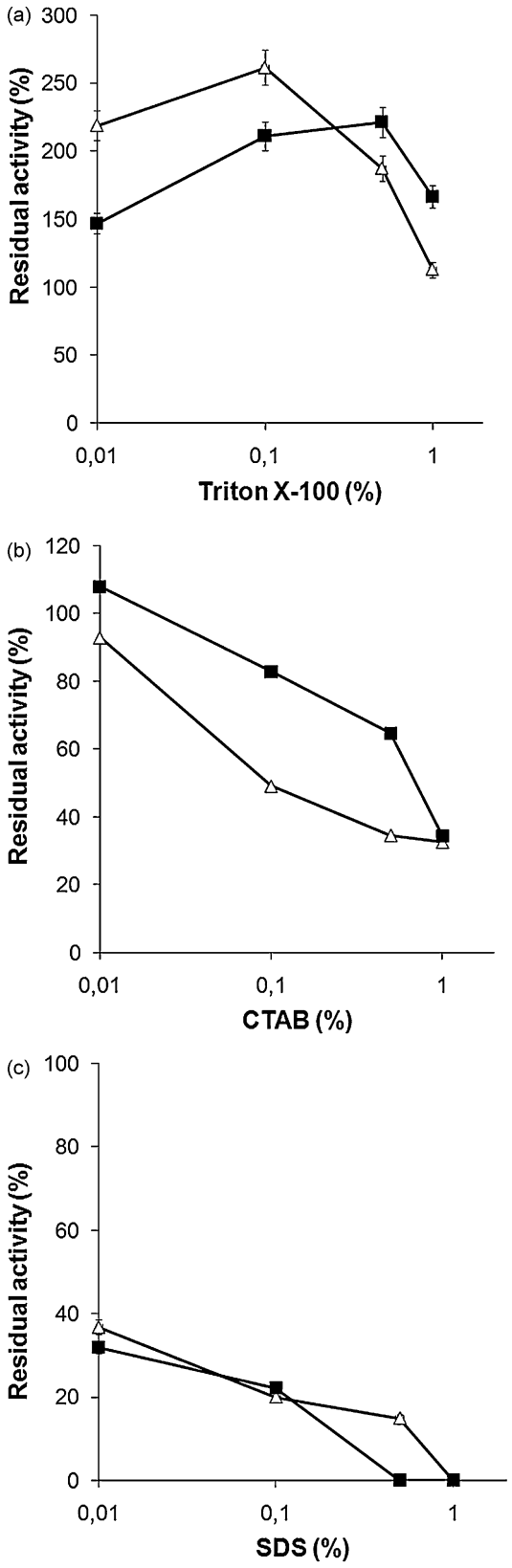

Fig. 6. Effects of detergents on SWL activity. (a) Triton X-100; (b) CTAB; (c) SDS. (△) CNBr-SWL; (■) Gx-SWL. All other conditions are described in Section 2.2.

Table 2
Performance of different SWL preparations for enzymatic hydrolysis of (±)-2-*O*-butyryl-2-phenylacetic acid.

Preparation	pH	Specific activity[a]	Stereochemical preference	*E*
Octyl-SWL	7	0.063 ± 0.004	R	56. ± 2
	5	0.016 ± 0.003	R	2.6 ± 0.1
CNBr-SWL	7	0.012 ± 0.002	R	2.4 ± 0.1
	5	0.015 ± 0.002	R	3.1 ± 0.2
Gx-SWL	7	0.013 ± 0.002	R	1.5 ± 0.1
	5	0.015 ± 0.002	S	3.9 ± 0.2

Experiments were performed at 25 °C.
[a] Specific activity in U h^{-1} $mg_{prot.}{}^{-1}$.

Table 3
Performance of different SWL preparations for enzymatic hydrolysis of HPBEt.

Preparation	Specific activity[a]	Stereochemical preference	*E*
Octyl-SWL	0.067 ± 0.004	R	1.2 ± 0.2
CNBr-SWL	0.072 ± 0.004	S	18.8 ± 1
Gx-SWL	0.044 ± 0.003	S	1.7 ± 0.2

Experiments were performed at 25 °C and pH 7.
[a] Specific activity in U h^{-1} $mg_{prot.}{}^{-1}$.

of three different chiral ester, (±)-2-*O*-butyryl-2-phenylacetic acid, (±)-methyl mandelate, and (±)-HPBEt. Table 2 shows the results for the hydrolysis of (±)-2-*O*-butyryl-2-phenylacetic acid. At pH 7, the most active enzyme was the immobilized on octyl-agarose, about five times more active than the other two immobilized SWL, while at pH 5 there was a decrease in the activity of this preparation, while the other two preparations suffered even a slight increase in the activity. Regarding the enantiomeric ratio, the highest value was obtained for the octyl preparation ($E = 56$) favoring the formation of the R-isomer. The other preparations presented a low but significant enantiospecificity for the same isomer, except the Gx-SWL at pH 5, showing higher activity for the S isomer ($E = 4$).

For the hydrolysis of racemic methyl mandelate, only octyl-SWL produced significant amounts of product (specific activity was 0.195) allowing determining enantiomeric ratio (enantioselectivity favored the S isomer with an *E* value of 3.5). Surprisingly, this biocatalyst was more active against this compound, almost twice as much as for (±)-2-*O*-butyryl-2-phenylacetic acid under similar conditions, while the other preparations were almost fully inactive against racemic methyl mandelate and quiet active against (±)-2-*O*-butyryl-2-phenylacetic acid.

For the hydrolysis of HPBEt (Table 3) the most active preparations were CNBr-SWL, followed by octyl-SWL, while the most enantioselective was octyl-SWL ($E = 19$), with the other two preparations showing almost no selectivity in this reaction.

The results presented in this section show that this lipase has a broad range of substrates and its properties might be greatly modulated by different immobilization protocols. Thus, the use of these different immobilization strategies may be used in order to increase the applicability of this novel and interesting enzyme.

All preparations were reused at least three reaction cycles without detecting a significant decrease in enzyme activity, suggesting that the enzyme was stable enough under that conditions and that the enzyme cannot desorbed from any of the supports.

4. Conclusions

Lipase from *S. warneri* EX17 could be immobilized following three different protocols (adsorption on hydrophobic supports, mild and multipoint covalent attachments). The low stability of the enzyme at pH 10, which is a condition required to immobilize the enzyme on glyoxyl-agarose, could be improved by the addition of some polyols such as 25% of glycerol. This allowed immobilizing the enzyme in glyoxyl-agarose at pH 10 retaining about 80% of activity. Immobilizations on CNBr did not have effect on the enzyme activity, while immobilization on octyl-sepharose increased the enzyme activity 1.6 times.

Gx-SWL showed the highest stability during thermal inactivation at all pH values, or in the presence of cosolvents, when compared to the other immobilized preparations. This suggested the promotion of a fairly intense support-enzyme covalent multipoint attachment. Moreover, all covalently immobilized SWL showed a hyperactivation in the presence of moderate concentrations of Triton X-100. Ionic detergents showed a more impact in activities. Incubation of the enzyme in the presence of these detergents suggested that they could inactivate immobilized SWL, although Gx-SWL system was somehow a little more resistant to this inactivation, perhaps due to the higher stability of this preparation.

SWL was able to hydrolyze different chiral esters, showing the broad specificity of this enzyme, granting some possible applications of the enzyme in fine chemistry. The specificity of the lipase to different substrates was strongly modulated by the immobilization protocol as many other lipases, suggesting that this may be a good option to further improve its performance in target reactions [3]. Using just three immobilization protocols, very significant differences were found among the properties of the immobilized enzyme. CNBr-SWL was the most active preparation against HPBEt, while octyl-SWL was the most active against the other two substrates. Moreover, the experimental conditions affected in a very different way the activity of the different preparations. Concerning SWL enantioselectivity, it was also possible to greatly modulate it through the immobilization technique employed. For example, using HPBEt as substrate, octyl-SWL exhibited an opposite enantiospecificity to the other two biocatalysts and was the most enantioselective in the hydrolysis of (±)-2-*O*-butyryl-2-phenylacetic acid. With this substrate, Gx-SWL at pH 5 again showed an inverse enantiopreference compared to the other preparations. All these results show that this lipase may be greatly improved via the so-called "conformational engineering" [3].

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