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Medium engineering on modified *Geobacillus thermocatenulatus* lipase to prepare highly active catalysts

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ABSTRACT

The influence of additives on the activity of different covalently and site-specific chemically modified immobilized preparations of a lipase from *Geobacillus thermocatenulatus* (BTL2) was investigated with a view to obtain a very high active biocatalyst. Non-ionic surfactant and co-solvents at different concentration range were applied. The CNBr-BTL2 immobilized preparation, a very mild immobilized enzyme with similar properties to the soluble enzyme, exhibited an increase in activity of 3 fold in the presence of 20% (v/v) co-solvent (e.g., 1,4-dioxane) and 2.6 fold when Triton X-100 (v/v) was added in the hydrolysis of *p*-nitrophenylbutyrate. This immobilized preparation was hyper-activated in the presence of both additives although without a synergistic effect. The CNBr-BTL2 modified with polymers showed mild hyperactivation in the presence of each additives and even a synergy in the presence of both.

In the best of cases, the HOOC-PEG1500-CNBr-BTL2 preparation showed up to 11 fold higher activity in the presence of additives combination than in absence of them.

The glyoxyl-BTL2 preparation was hyper-activated in a similar way than CNBr-BTL2 in the presence of detergents but much less with co-solvents. However, the modified glyoxyl-BTL2 preparations were hyper-activated with solvent (2 fold) but not with detergent. An increase of 3 fold in activity for the modified glyoxyl-BTL2 preparations was observed in the presence of both additives.

1. Introduction

The improvement of the catalytic activity of enzymes is important for their different industrial applications, in particular, processes where the enzyme is quite selective but exhibits very low activity towards non-natural substrates [1,2]. Different methodologies have been applied for this propose such as genetic engineering by modification of key aminoacids [3,4], the use of different immobilization techniques [5,6] or different chemical modification reactions [7–9].

The lipase from *Geobacillus thermocatenulatus* (BTL2) is a very interesting biocatalyst. This is a thermostable enzyme with interesting biotechnological applications [10–12]. This enzyme is the first crystallized lipase with two lids, which implies a more complex catalytic mechanism [13]. Indeed, the hyper-activation of this enzyme towards hydrophobic supports by interfacial activation mechanism was of 2 fold [11], lower than the hyper-activation observed with other lipases [14]. Recently, a 2–3 fold increase of BTL2 activity against different esters was achieved after site-specific chemical modifications [15]. It was argued that modification with polymers with different charges on a flexible immobilized enzyme could generate different salt bridges or repulsion of different domains of the enzyme. This mechanism affects in a different way the movement of both lids when the enzyme switches from closed to open conformation.

The presence of different additives such as biopolymers (polypeptides or polysaccharides) as synthetic polymers (polyethylene glycols or polypropylene glycols) has shown a positive influence on the stability and also on the enzymatic activity. Others additives such as detergents enhance the activity of different lipases [16–19], probably due to the stabilization of the enzyme open conformation by coating their hydrophobic pockets [20].

The addition of co-solvents usually caused a negative effect on the enzymatic activity [21]. However, an improvement on the lipase activity was obtained in several cases by operating within the concentration range [22,23].

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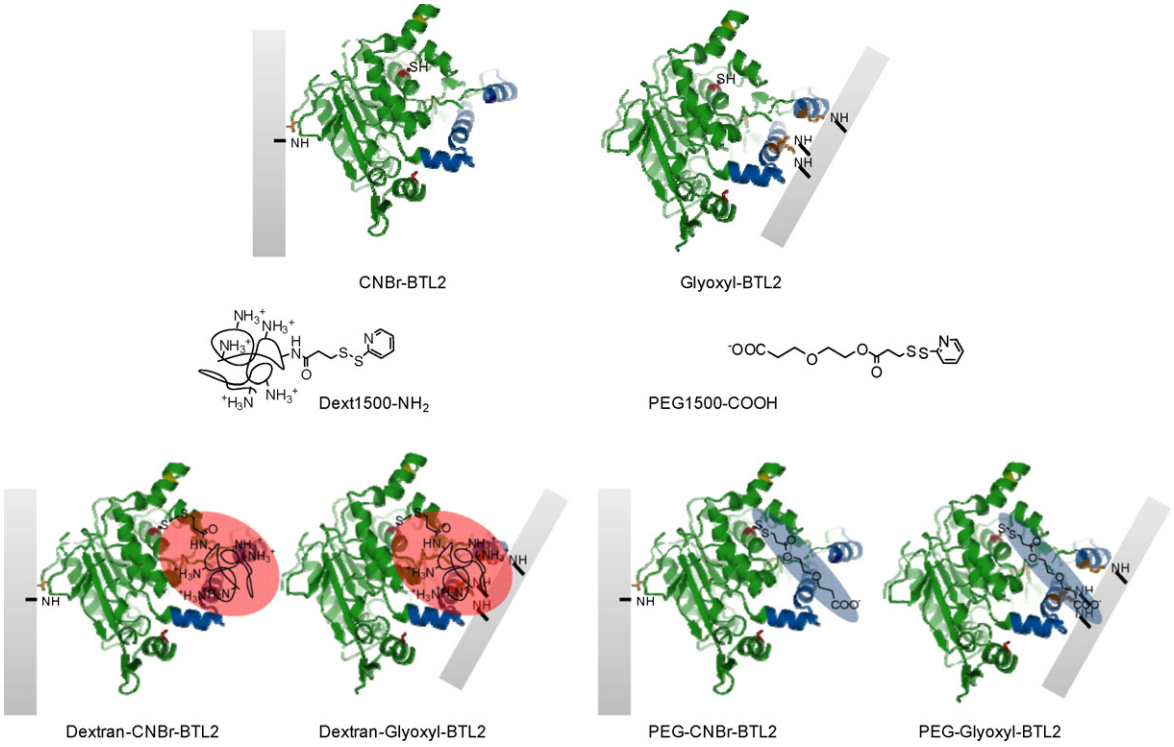

Fig. 1. Scheme of different BTL2 immobilized preparations used. Double lid (residues 169–239) (blue). The structure of BTL2 was obtained from the Protein Data Bank (pdb code: 2w22) [31] and the picture was created using Pymol vs. 0.99.

Very likely, the exposition of the lipase hydrophobic pocket to the reaction medium may be less disfavored in the presence of low concentrations of co-solvents.

Herein we have studied how these additives affect the catalytic activity of BTL2 as well as site-specific modified BTL2 molecules in order to obtain a very high active biocatalyst. BTL2 was previously immobilized on two different supports: (a) CNBr activated agarose beads – to obtain immobilized but non-rigidified lipase derivatives immobilized *via* one-point covalent attachment, and (b) highly activated glyoxyl-agarose beads – to promote its stabilization/rigidification by multipoint covalent immobilization. Two different chemical modifications were performed on these two BTL2 preparations by a thiol-disufide exchange on solid phase (Fig. 1).

2. Material and methods

2.1. General

Glyoxyl-agarose 6BCL was from Biotica S.A. Cyanogen bromide agarose (CNBr-activated Sepharose 4BCL) was purchased from Pharmacia Biotech (Uppsala, Sweden). Lipase from *Geobacillus thermocatenulatus* BTL2 was produced as previously described [24].

3-(2-Pyridyldithio) propionyl-aminated Dextran (Mr 1500 kDa) (PDP-Dextran1500-NH_2), 3-(2-Pyridyldithio)-propionyl-monocarboxylated polyethylene glycol (PEG) (Mr 1500 kDa) (PDP-PEG1500-COOH) were prepared as previously described [21]. Dithiothreitol (DTT), sodium borohydride, Triton® X-100, dioxane, 2-propanol and p-nitrophenyl butyrate (pNPB) were from Sigma. Other reagents and solvents used were of analytical or HPLC grade.

2.2. Enzymatic activity assay

The activities of the soluble lipase, supernatant and enzyme suspension (during the immobilization process) and their immobilized preparations were analyzed spectrophotometrically measuring the increase in absorbance at 348 nm produced by the release of *p*-nitrophenol (*p*NP) ($\epsilon = 5.150\ M^{-1}\ cm^{-1}$) in the hydrolysis of 0.4 mM *p*NPB in 25 mM sodium phosphate in different conditions (aqueous phase or in the presence of different concentrations of Triton X-100 or co-solvents) at pH 7 and 25 °C. To start the reaction, 0.05–0.2 mL of lipase solution or suspension were added to 2.5 mL of substrate solution under magnetic stirring. Enzymatic activity is given as μmol of hydrolyzed *p*NPB per minute per mg of enzyme (IU) under the above described conditions. In the case of assays in the presence of co-solvents, the enzyme suspension was previously equilibrated with equivalent co-solvents solutions.

2.3. Purification of BTL2

To purify the lipase from any other contaminant proteins (e.g., esterases), the enzyme preparation was incubated under continuous stirring in 10 mM sodium phosphate at pH 7.0 and butyl-agarose beads was added. The crude extract from *Escherichia. coli* containing BTL2 was diluted four times with 25 mM sodium phosphate at pH 7.0 to a concentration of 5 mg/ml. Then, butyl-agarose was added in a 1/10 (v/v) proportion and gently stirred for three hours at 25 °C. Periodically, activity of suspensions and supernatants were measured by the *p*NPB assay. After that, adsorbed lipase preparation was abundantly washed with distilled water. BTL2 was desorbed from the support by suspending the immobilized enzyme in a 1/10 (w/v) ratio in 25 mM sodium phosphate buffer at pH 7.0 containing 0.5% (v/v) of Triton X-100 during 1 hour at room temperature.

Following these protocols, a quantitative immobilization of lipase activity was observed and the SDS–PAGE analysis of the adsorbed protein preparation showed a single band with a molecular weight corresponding to the native lipase (Fig. S1, see supporting information). Final purification overall yield was 95%. A solution of 0.5 mg lipase purified/mL was obtained.

2.4. Covalent immobilization of BTL2

A solution of 0.5 mg pure lipase/mL in 25 mM phosphate buffer pH 7 with 0.5% Triton X-100 obtained as described in Section 2.3 was used in each case. The immobilization process was followed by the enzymatic assay previously described.

2.4.1. Immobilization on CNBr-activated agarose

The immobilization of BTL2 on CNBr-activated support was performed for 15 min at 4 °C to reduce the possibilities of a multipoint covalent attachment between the enzyme and the support. 10 mL of lipase solution were added to one gram of support and the reaction was maintained for 1 h. Periodically, activity of suspensions and supernatants was measured by the *p*NPB assay. The enzyme-support immobilization was ended by incubating the support with 1 M ethanolamine at pH 8 for 2 h. Finally, the immobilized preparation was washed with abundant water, to eliminate the detergent. The immobilization yield was >95% obtaining a biocatalyst of 5 $mg_{lipase}/g_{support}$.

2.4.2. Immobilization on glyoxyl-agarose

10 mL of lipase solution were added to 250 mM sodium bicarbonate buffer at pH 10.3 and the pH of the final solution was adjusted to pH 10.1. Then one gram of glyoxyl-agarose (aldehyde activated support) was added and the reaction was maintained for 24 h. Periodically, activity of suspensions and supernatants was measured by using the *p*NPB assay. When the immobilization was finished, 20 mg of $NaBH_4$ were added during 30 min and then the suspension was filtered and abundantly washed with distilled water (200 mL × 5). The immobilization yield was >95% obtaining a biocatalyst of 5 $mg_{lipase}/g_{support}$.

2.5. Chemical modification of BTL2 immobilized preparations

The BTL2 immobilized preparation (CNBr-BTL2 or glyoxyl-BTL2) was treated with 50 mM DTT solution in 25 mM sodium phosphate at pH 8.0 for 30 min. 0.345 mL of PDP-activated polymer (PDP-Dextran1500-NH_2 or PDP-PEG1500-COOH) solution were dissolved in 2.7 mL of 500 mM sodium phosphate at pH 8.3. The final pH was adjusted at 8.0. Then, 0.2 g of reduced BTL2 immobilized preparation were added. The modification was followed by adsorption analysis of supernatant at 343 nm. Modification of the BTL2 immobilized preparation with 2-PDS (0.1 mM) was used as control experiment. SDS–PAGE analysis confirmed the modification [15].

3. Results and discussion

3.1. Effect of different additives on the activity of BTL2 immobilized preparations

The effect of the concentration of dioxane or 2-propanol (from 0 to 50% (v/v)) on the hydrolytic activity of the CNBr-BTL2 and glyoxyl-BTL2 immobilized preparations towards hydrolysis of *p*NPB is shown in Fig. 2.

The activity of the CNBr-BTL2 preparation increased 2 fold in the presence of 10% (v/v) concentration of both co-solvents. The maximum activity was achieved when 20% (v/v) co-solvent concentration was used, 3.3 fold with dioxane and 2.6 fold with 2-propanol (Fig. 2A). However, the enzymatic activity profile was quite different for both solvents at 30% (v/v) concentration. The biocatalyst activity increased 2.6 fold at this concentration of dioxane but decreased 30% in the presence of 30% (v/v) 2-propanol compared to the enzyme activity without co-solvent (Fig. 2A).

In the case of glyoxyl-BTL2, a smaller increase on the activity of the immobilized enzyme was observed and the profile in the presence of co-solvents was quite similar. The highest hyperactivation was also achieved at 20% co-solvent concentration (1.5 fold). At 30% concentration, almost 50% activity of the enzyme was lost with both co-solvents (Fig. 2B).

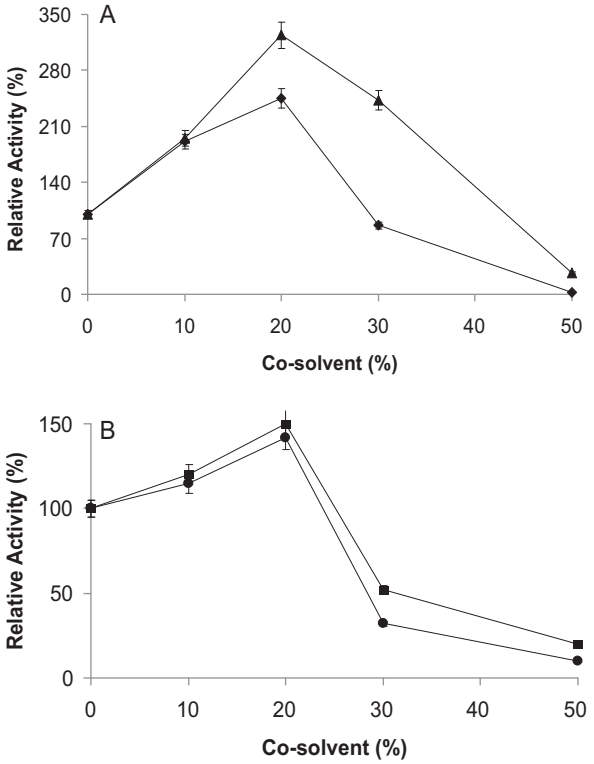

Fig. 2. (A) Effect of the co-solvent on the hydrolytic activity of CNBr-BTL2 at pH 7. Dioxane (triangles), 2-propanol (rhombus). (B) Effect of the co-solvent on the hydrolytic activity of glyoxyl-BTL2 at pH 7. Dioxane (squares), 2-propanol (circles).

Therefore, the immobilization procedure has important influence on the behavior of the hyperactivation of this enzyme with co-solvents although the accessibility of the substrate to the active site was similar for both BTL2 immobilized preparations (Fig. S2, see supporting information).

The effect of different concentrations of Triton X-100 (from 0 to 1% (v/v)) on the hydrolytic activity of the CNBr-BTL2 and glyoxyl-BTL2 immobilized preparations is shown in Table 1.

The CNBr-BTL2 preparation exhibited the highest activity value in the presence of 0.01% (v/v) of Triton X-100 (2.75 fold activation). The glyoxyl-BTL2 preparation showed the maximum activity value when 0.1% (v/v) detergent was used in the hydrolysis of *p*NPB (2.9 fold) (Table 1).

The irreversible inhibition of the enzyme with dpNPP revealed that the active site of BTL2 immobilized on glyoxyl-agarose is a little more accessible than in BTL2 immobilized on CNBr-agarose. However, in the presence of detergent, the inhibition was faster for glyoxyl-BTL2 (60% activity was lost in 5 min) than for CNBr-BTL2 (20% activity loss) (Fig. S3, see supporting information). This result seems to show that the detergent caused increased opening of the

Table 1
Effect of Triton X-100 concentration (w/v) in the activity of BTL2 immobilized preparations. Relative activity was based on the activity of the different BTL2 immobilized preparations at pH 7 in the hydrolysis of pNPB without additive was used.

Biocatalyst	Triton X-100 (%)				
	0	0.001	0.01	0.1	1
CNBr-BTL2	100	119	275	235	77
Glyoxyl-BTL2	100	110	220	290	81

Table 2
Effect of 20% (v/v) co-solvent on the activity of modified BTL2 immobilized preparations. Relative activity was based on the activity of the different BTL2 immobilized preparations at pH 7 in the hydrolysis of pNPB without additive was used.

Biocatalyst	Dioxane (%)	Propanol (%)
NH_2-Dextran-CNBr-BTL2	257	210
COOH-PEG-CNBr-BTL2	312	198
NH_2-Dextran-glyoxyl-BTL2	279	300
COOH-PEG-glyoxyl-BTL2	300	250

lids in the glyoxyl-BTL2 (an immobilized preparation with the more rigidified lipase) than CNBr-BTL2.

For both BTL2 immobilized preparations, the addition of higher concentrations of Triton (e.g., 1%) caused a decrease in the activity (around 10%) compared to that without detergent, probably because of the inhibitor effect of detergent at these concentrations, caused by interactions with areas near to the active site of the lipase [13].

3.2. Effect of different additives on the activity of chemically modified BTL2 immobilized preparations

The effect of co-solvents on the activity of a site-specific modified BTL2 on Cys64 with two different tailor-made activated polymers (PEG1500-COOH and Dextran1500-NH_2) was also evaluated (Table 2).

The chemical modifications introduced on CNBr-BTL2 did not improve the enhancement of the activity observed with this non-modified catalyst in the presence of 20% concentration of both co-solvents (Table 2). However, both modifications on glyoxyl-BTL2 permitted to increase up to two fold (from 1.5 to 3 fold) the hyperactivation achieved with the non-modified biocatalysts in both co-solvents (Table 2).

The effect of the detergent on the activity of chemically modified BTL2 immobilized preparation was studied at optimal conditions (Table 3).

In the presence of 0.01% Triton (v/v), the CNBr-BTL2 modified with Dextran1500-NH_2 showed lower hyperactivation (2 fold) whereas the HOOC-PEG-CNBr-BTL2 exhibited a slight higher hyperactivation (2.9 fold) when they were compared to non-modified BTL2 (Table 3). This result shows that the enzyme modified with a polymer with positive charges (Dextran1500-NH_2) seems to be in a more open conformation that the enzyme modified with a polymer with a negative charge (PEG1500-COOH). Indeed, the activity of BTL2 by Dextran modification was slightly higher than the enzyme activity by PEG modification [15]. Also, the chemical modifications of BTL2 provide better accessibility of substrates to the active site -studied by the irreversible inhibition with dpNPP- like the non-modified immobilized BTL2 in the presence of detergent [15].

However, the modified glyoxyl-BTL2 preparations did not undergo any hyper-activation with the addition of detergent (0.1% concentration v/v), opposite to the results obtained with glyoxyl-BTL2 (Table 2). The presence of the polymer which increased the activity of the enzyme after modification (e.g., 2.2 fold with dextran1500-NH_2 [15] may affect the movement of the lids or blocking the incorporation of the detergent on the protein surface (Fig. S4, see supporting information)

Table 3
Effect of Triton X-100 on the hydrolytic activity of chemically modified BTL2 immobilized preparations. 0.01% (w/v) for CNBr-BTL2 preparations and 0.1% (w/v) for glyoxyl-BTL2 preparations were used. Relative activity was based on the activity of the different BTL2 immobilized preparations at pH 7 in the hydrolysis of pNPB without additive was used.

Biocatalyst	Relative activity (%)
NH_2-Dextran-CNBr-BTL2	188
COOH-PEG-CNBr-BTL2	290
NH_2-Dextran-glyoxyl-BTL2	100
COOH-PEG-glyoxyl-BTL2	100

Table 4
Effect of additive combination in the activity of different BTL2 immobilized preparations. The activity of CNBr-BTL2 preparations were measured in the presence of 20% (v/v) dioxane and 0.01% (w/v) Triton X-100. The activity of glyoxyl-BTL2 preparations were measured in the presence of 20% (v/v) dioxane and 0.1% (w/v) Triton X-100. Relative activity was based on the activity of the different BTL2 immobilized preparations at pH 7 in the hydrolysis of pNPB in the without additive was used.

Biocatalyst	Relative activity (%)
CNBr-BTL2	320
NH_2-Dextran-CNBr-BTL2	500
COOH-PEG-CNBr-BTL2	1100
Glyoxyl-BTL2	150
NH_2-Dextran-glyoxyl-BTL2	350
COOH-PEG-glyoxyl-BTL2	300

3.3. Effect of the combination of different additives on the activity of the different BTL2 immobilized preparations

The improvement of the activity of the different BTL2 immobilized preparations was evaluated when both additives were used simultaneously. 20% concentration of dioxane and 0.01% Triton X-100 for CNBr-BTL2 or 0.1% Triton X-100 for glyoxyl-BTL2 were used (Table 4).

In these conditions, the CNBr-BTL2 preparation achieved three times more activity that without any additive. This result is similar than the obtained using both additives separately, namely, the combination of both effectors did not cause any synergistic effect (Table 4). However, the CNBr-BTL2 preparation modified with Dextran1500-NH_2 showed higher activity (5 fold) in these conditions. The modified CNBr-BTL2 with PEG1500-COOH was the most active catalyst, showing 11 times more activity in these conditions that without additives and more than three times higher than non-modified CNBr-BTL2 in the presence of both additives (Table 4). Therefore, it seems that a synergistic effect exists of both additives towards the site-directed modified BTL2 preparations.

The glyoxyl-BTL2 preparation exhibited 1.5 times more activity in the presence of both additives that without them (Table 4), similar to the value achieved in the presence of one co-solvent (Fig. 2). Thus, the combination of both effectors did not cause any positive effect.

The modified glyoxyl-BTL2 preparations showed 3–3.5 higher activity in these conditions than without additives, thus it was obtained a slight improvement in comparison with the non-modified counterpart.

The results for all glyoxyl-BTL2 preparations in the presence of both effectors were similar to those using co-solvent as additives, since Triton X-100 did not have any influence on the activity. However, the glyoxyl-BTL2 was hyper-activated in the presence of detergent (Table 1).

4. Conclusions

The influence of different additives to improve the activity of BTL2 was different depending on the immobilization protocol and the site-specific modification on the enzyme. The CNBr-BTL2 preparation was two times more hyper-activated than glyoxyl-BTL2 in the presence of 20% co-solvent.

The site-specific chemical modifications did not influence the hyperactivation profile of CNBr-BTL2 with co-solvents but modified glyoxyl-BTL2 preparations showed twice as much hyperactivation than glyoxyl-BTL2. In the presence of detergent, all CNBr-BTL2

preparations and glyoxyl-BTL2 improved the activity around three times whereas no hyper-activation was observed on the chemical modified glyoxyl-BTL2.

When the combined effect of both additives was evaluated, no synergistic effect was observed on the activity of CNBr-BTL2 or glyoxyl-BTL2. The irreversible inhibition using dpNPP at these conditions was similar for both immobilized BTL2 preparations (around 70% loss of activity in 7 min) (Fig. S5, see supporting information) but faster when it was compared with the inhibition in the presence of only one additive (Figs. S2 and S3). However, synergy was observed on the activity improvement of modified BTL2 preparations. The effect was higher on CNBr-BTL2 where the Dextran1500-NH_2 modified preparation exhibited five times more activity and the PEG1500-COOH showed an 11 fold increase in activity (the highest activity value ever achieved with this enzyme). The maximum activity increase achieved on modified glyoxyl-BTL2 was of 3.5 times.

Therefore, this study proposes a methodology for the generation of highly active catalysts with an important scientific and technological value, such as biodiesel production, synthesis of chiral drugs intermediates or synthesis of ω-3 or ω-6 fatty acids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.03.001.

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