Solid-Phase Chemical Amination of a Lipase from *Bacillus thermocatenulatus* To Improve Its Stabilization via Covalent Immobilization on Highly Activated Glyoxyl-Agarose

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In this paper, the stabilization of a lipase from *Bacillus thermocatenulatus* (BTL2) by a new strategy is described. First, the lipase is selectively adsorbed on hydrophobic supports. Second, the carboxylic residues of the enzyme are modified with ethylenediamine, generating a new enzyme having 4-fold more amino groups than the native enzyme. The chemical amination did not present a significant effect on the enzyme activity and only reduced the enzyme half-life by a 3−4-fold factor in inactivations promoted by heat or organic solvents. Next, the aminated and purified enzyme is desorbed from the support using 0.2% Triton X-100. Then, the aminated enzyme was immobilized on glyoxyl-agarose by multipoint covalent attachment. The immobilized enzyme retained 65% of the starting activity. Because of the lower p*K* of the new amino groups in the enzyme surface, the immobilization could be performed at pH 9 (while the native enzyme was only immobilized at pH over 10). In fact, the immobilization rate was higher at this pH value for the aminated enzyme than that of the native enzyme at pH 10. The optimal stabilization protocol was the immobilization of aminated BTL2 at pH 9 and the further incubation for 24 h at 25 °C and pH 10. This preparation was 5-fold more stable than the optimal BTL2 immobilized on glyoxyl agarose and around 1200-fold more stable than the enzyme immobilized on CNBr and further aminated. The catalytic properties of BTL2 could be greatly modulated by the immobilization protocol. For example, from (*R*/*S*)-2-*O*-butyryl-2-phenylacetic acid, one preparation of BTL2 could be used to produce the *S*-isomer, while other preparation produced the *R*-isomer.

Introduction

Lipases are among the most used enzymes in biocatalysis.[1,2] However, the low stability of enzymes is hindering their implementation.[3,4] The use of lipases from thermophilic microorganisms may be a solution for this stability problem.[5−8]

Lipase from *Bacillus thermocatenulatus* (BTL2) is a stable[9−12] and very interesting enzyme.[13,14] However, considering the broad range of conditions where lipases may be used,[15−17] the preparation of immobilized biocatalysts with very high stability of this lipase seems to be interesting. The multipoint covalent attachment[18−20] of the enzyme, for example, using glyoxyl agarose beads,[21] will increase the enzyme stability. The enzymes are immobilized on this support at alkaline pH value only via several simultaneous attachments by its external surface containing the highest density of amino groups.[22,23] The intensity of the enzyme-support multipoint covalent reaction strongly depends on the amount of reactive groups in both the support[24,25] and the enzyme.[26] The enrichment, via different techniques, in reactive groups on the enzyme surface has been already successfully assayed to improve the multipoint covalent immobilization.[26,27] For example, the chemical amination of the protein surface via reaction of the carboxylic groups (e.g., Asp and Glu) with ethylenediamine after activation with carbodiimide is a well described and easy-to-control reaction.[28−32]

BTL2 presents 97% sequence homology and 94% identity with the lipase from *Bacillus stearothermophilus*.[33,34] This allows making a model of the tridimensional structure of the enzyme (see Methods). The number of aspartic and glutamic groups (42 residues mainly exposed to the medium) is much higher than the number of Lys moieties (12 residues). Figure 1 shows two faces of the enzyme surface where the enrichment in amino groups that can be obtained by this strategy seems clear. Moreover, the p*K* of these new groups is lower (around 9.2)[35] than that of the amino ε-group of Lys (around 10.7).

Many lipases present a specific catalytic mechanism of action, the so-called interfacial activation.[36−38] The open form of a lipase may be adsorbed and stabilized on any hydrophobic surface (drops of substrate, other lipases or proteins, hydrophobic supports[39−43]) through the large hydrophobic pocket formed by the areas surrounding their active center and the internal face of their lid even at low ionic strength.[43] This reversible immobilization may be used to simplify the chemical amination of lipases, by amination of the adsorbed enzyme in solid phase.

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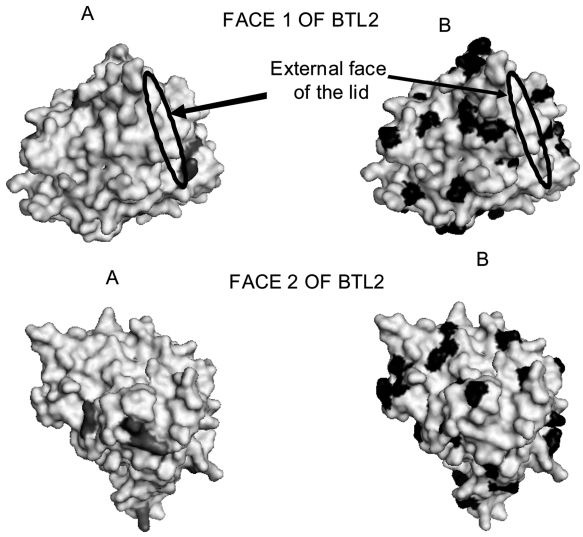


Figure 1. Distribution of Lys, Asp, and Glut on two faces of the surface of BTL2. Panels A show the Lys in light gray. Panels B show the Asp and Glut in dark gray. It is based in the closed form of the lipase from *B. stearothermophilus*, the only available form. The arrow in face 1 shows the position of the external face of the lid. In face 2, the active center is in the opposite side.

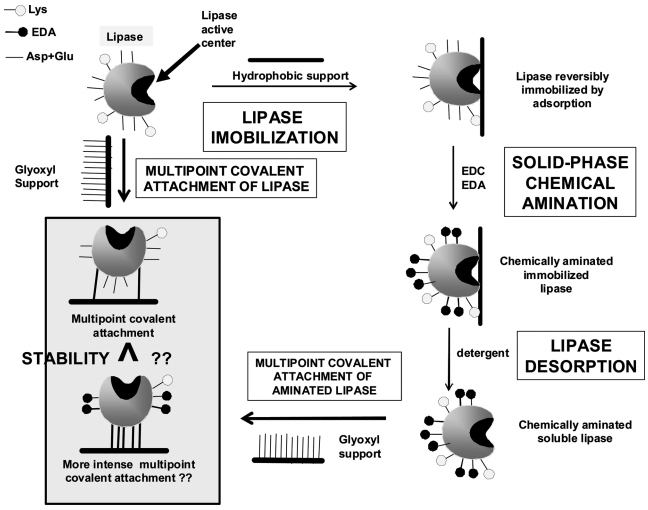


Figure 2. Scheme of the strategy that is proposed in this paper to improve the stabilization of lipases by solid-phase amination of the enzyme to improve their multipoint covalent immobilization on glyoxyl agarose: 1,2-ethylenediamine (EDA); 1-ethyl-3-(dimethylamino-propyl) carbodiimide (EDC).

After, the lipases may be desorbed by adding detergents to obtain a soluble enzyme that may be then immobilized on glyoxyl agarose (Figure 2). Considering that lipases tend to form bimolecular structures,[41,42] the immobilization in the presence of detergents far from being a problem is necessary to ensure the immobilization of individual enzyme molecules.

On the other hand, it has been shown that the catalytic properties of lipases may be very easily altered.[44−49] Thus, to select a final immobilization protocol of lipases, one should consider not only the enzyme stability and activity but also its selectivity in the target reaction. In this paper, the results obtained after applying this novel strategy to BTL2 are presented.

Materials and Methods

Materials. Ethanolamine hydrochloride, 1-ethyl-3-(dimethylamino-propyl) carbodiimide (EDC), dimethylaminopyridine (DMAP), and Triton X-100 were from Sigma. 1,4-Dioxane, *p*-nitrophenyl butyrate (p-NPB), 1,2-ethylenediamine (EDA), and benzamidine was from Fluka. Butyl sepharose CL-4B and cyanogen bromide activated Sepharose 4 B (CNBr; average diameter, 100 μm) were purchased from GE Healthcare (Uppsala, Sweden). Sintered glass funnel with a porosity of 20 μm was from Povel(Spain). Cross-linked agarose (10 BCL) was kindly donated by Hispanagar S.A. (Burgos, Spain) and its modification to glyoxyl agarose (activated with 200 micromols/g of support) was performed as described elsewhere.[50] (*R*/*S*)-2-*O*-Butyryl-2-phenylacetic acid (**1**) was synthesized as previously described.[49] 2,4,6-Trinitrobenzensulfonic acid (TNBS) was from Fluka. Other reagents and solvents used were of analytical or HPLC grade. BTL2 expressed in *E. coli* was produced as previously described.[13]

Methods. *Modeling of the Three-Dimensional Structure of Lipase from Bacillus thermocatenolatus (BTL2).* A three-dimensional model of BTL2 was constructed on the basis of the reported crystallographic structure of lipase from *Bacillus stearothermophilus* (PDB code 1KU0). The model was built by using the SWISS-MODEL automated homology modeling server of the Swiss Institute of Bioinformatics (http://swissmodel.expasy.org).[51]

Standard Enzymatic Activity Assay. This assay was performed by measuring the increase in the absorbance at 348 nm produced by the released *p*-nitrophenol in the hydrolysis of 0.4 mM pNPB in 25 mM sodium phosphate at pH 7 and 25 °C, using a themostatized spectrum with continuous magnetic stirring. To initialize the reaction, 0.05 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. The amount of enzyme used in the assays yielded a maximum increment of absorbance/min of 0.15. One international unit of pNPB activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol of pNPB/min (IU) under the conditions described above.

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, following a previously described procedure.[52] The crude extract from *E. coli* containing BTL2 was diluted four times with 10 mM sodium phosphate at pH 7.0 to a concentration of 5 mg/ml. Then, butyl-sepharose was added in a 1/10 (w/v) proportion, and gently stirred for twelve hours at 25 °C. Periodically, the activity of suspensions and supernatants was measured by using the pNPB assay. After enzyme adsorption, the lipase preparation was vacuum filtered using a sintered glass funnel and abundantly washed with distilled water. BTL2 was desorbed from butyl-sepharose by suspending the immobilized enzyme in a relation 1/10 (w/v) in the buffer indicated in each case (depending on the final use of the enzyme) containing 0.2% (v/v) of Triton X-100 during 1 h 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 that of the native lipase. Final purification yield was over 95%. The activity of the pure enzyme solution was 20 IU/mL, having a specific activity of 500 ± 25 IU.

Chemical Amination of Immobilized BTL2. A total of 1 g of immobilized lipase (covalently on CNBr agarose beads (see below) or via adsorption on butyl agarose beads) was added to 10 mL of 1 M EDA at pH 4.75 under continuous stirring. Solid EDC was added to the suspension to a final concentration of 10 mM, conditions that ensure the full modification of all exposed carboxylic groups of the protein.[28−30] After 90 min of gentle stirring at 25 °C, the immobilized-modified preparations were vacuum filtered using a sintered glass funnel and incubated for 4 h in 0.1 M hydroxylamine at pH 7 and 4 °C to recover the EDC-modified tyrosines.[53] The enzyme preparations were filtered and washed with 25 mM sodium phosphate at pH 7.5 and with an excess of distilled water. The aminated BTL2 preparations, immobilized on CNBr or butyl agarose (this will be used to obtain the soluble aminated enzyme) were stored at 4 °C. The aminated BTL2 was called BTL2-A.

Immobilization of Different BTL2 on Different Supports. In this work, two different enzyme loads were used. For standard experiments, to prevent diffusion problems, 5 pNPB units/g of support (low loading) were offered to the support. When the biocatalyst required being more active, the enzyme loading was increased to 500 pNPB units/g of support (high loading). In all cases, more than 95% of the lipase became immobilized on the different used supports.

Immobilization of BTL2 on CNBr-Activated Support. BTL2 was desorbed from butyl-sepharose using 50 mM sodium phosphate at pH 7 containing 0.2% (v/v) Triton X-100 as described in the purification section. The immobilization of BTL2 on CNBr-activated support was performed for 15 min at 4 °C to reduce the possibilities of getting a multipoint covalent attachment between the enzyme and the support.[22] During the immobilization and further blocking of the support the suspension was submitted to continuous gentle stirring. Highly loaded preparations were prepared by using 25 mL of the purified enzyme per g of support. Lowly loaded biocatalysts were prepared by diluting 0.25 mL of purified BTL2 to 25 mL using the same buffer. 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 vacuum filtered using a sintered glass funnel and washed with abundant water, to eliminate the detergent. This immobilized enzyme was called CNBr-BTL2.

Immobilization of BTL2 on Glyoxyl-Agarose Beads. BTL2 or BTL2-A was desorbed from butyl-sepharose using 100 mM sodium bicarbonate as immobilization buffer at pH 9 or 10.0 containing 0.2% (v/v) Triton X-100 as described in the purification section. Highly loaded derivatives were prepared using 1 g of glyoxyl-support and 25 mL of pure BTL2 or BTL2-A. Lowly loaded biocatalysts were prepared by using 0.25 mL of purified enzyme diluted with 9.75 mL of the corresponding immobilization buffer (pH 9 or 10). The mixture was maintained at the indicated temperatures during the desired times. Reduced glyoxyl-agarose was used in a reference suspension to discard unspecific adsorptions. As reaction end-point, solid sodium borohydride was added to a concentration of 1 mg/mL[21] 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.

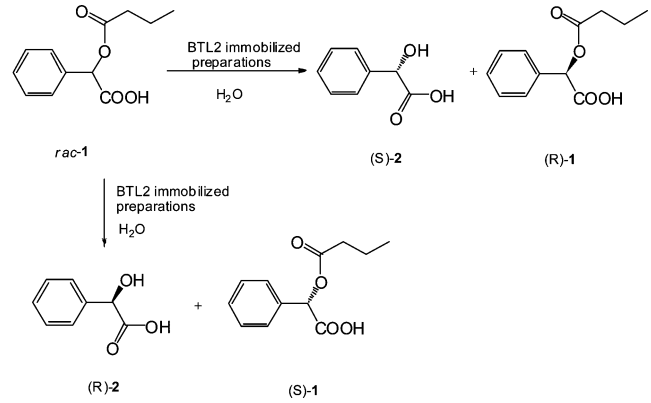
The glyoxyl biocatalysts prepared were the following: **G-BTL2** was prepared by immobilization of BTL2 during 24 h at 25 °C and pH 10. **G(9)-BTL2-A** was prepared by immobilization of BTL2-A during 24 h at 25 °C and pH 9. **G(10)-BTL2-A** was prepared by immobilization of BTL2-A during 24 h at 25 °C and pH 10. **G(9/10)-BTL2-A**. BTL2-A was first immobilized on glyoxyl-agarose at 25 °C and pH 9 (1 h) and further incubated at pH 10 and 25 °C for 24 h.

Thermal Inactivation of Different BTL2 Immobilized Preparations. The different BTL2 preparations were incubated in 25 mM sodium phosphate at pH 7.0 and 75 °C. Samples were withdrawn periodically using a pipet 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 by triplicate and the standard error was under 5%.

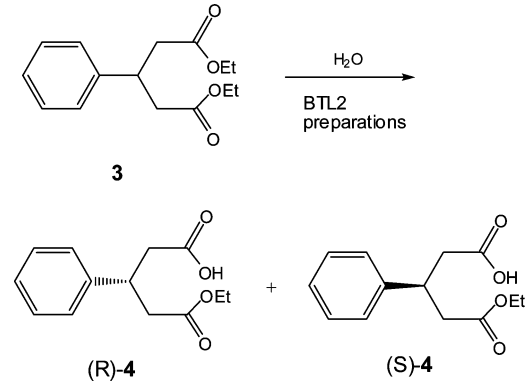
Inactivation of Different BTL2 Immobilized Preparations in the Presence of Organic Cosolvent. Enzyme derivatives were washed with the cosolvent/50 mM Tris-HCl aqueous solution at pH 7 and 4 °C. Subsequently, the enzyme derivatives were resuspended in such solution and incubated at 30 °C. Samples were withdrawn periodically, and the activity was checked following the above assay. Experiments were carried out by triplicate, and standard error was under 5%.

Enzymatic Hydrolysis of (R,S)-2-O-Butyryl-2-phenylacetic Acid (1). A total of 500 mg of immobilized preparation was added to 2.5 mL of 5 mM of **1**, in 25 mM sodium phosphate at 25 °C and pH 7, under continuous mild stirring (Scheme 1). A pH-stat Mettler Toledo DL50 graphic was used to maintain the pH value constant during the reactions. The conversion was analyzed by RP-HPLC (Spectra Physic SP 100 coupled with an UVdetector Spectra Physic SP 8450) using a Kromasil C18 (25 cm × 0.4 cm) column. Products were eluted using acetonitrile−10 mM ammonium phosphate (35:65, v/v) at pH 2.95 as

Scheme 1. Kinetic Resolution of **rac-1** by Immobilized BTL2 Preparations



Scheme 2. Asymmetric Hydrolysis of **3** by Immobilized BTL2 Preparations



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

The enzyme enantiospecificity was calculated directly from the ratio between the reaction rates of both isomers (using hydrolysis degrees between 10 and 20%).

Synthesis of Diethyl 3-phenylglutarate (3). A solution of ethanol (70 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (3.6 mmol) with DMAP (0.72 mmol) in diethyl ether (10 mL) was added dropwise over a stirred solution of 3-phenylglutaric acid (1.44 mmol) in diethyl ether (25 mL). The mixture was stirred at 25 °C for 5 h and the reaction was followed by HPLC. After that, the mixture was extracted with 100 mM NaCl solution. The aqueous phase was re-extracted with diethyl ether. The combined organic solvent phases were dried with $NaSO_4$ and the solvent was evaporated. The crude preparation was washed several times with cold ether (5 × 2 mL) and dried in vacuum. Yield: 98%. ^1H NMR (400 MHz $CDCl_3$): δ 7.33−7.20 (m, 5H, Ph); 4.52 (m, 1H, CH), 4.13 (m, 4H, 2 × CH_2), 2.54 (m, 4H, 2 × CH_2), 1.29 (t, 6H, 2 × CH_3). Purity: >99%

Enzymatic Hydrolysis of Diethyl 3-Phenylglutarate (3). A total of 0.5 g of immobilized preparation was added to 2.5 mL of 1 mM of **3** dissolved in 10 mM sodium phosphate at pH 7 and 25 °C (Scheme 2). During the reaction, the pH value was maintained constant using a pH-stat Mettler Toledo DL50 graphic. The enzymatic activity was defined as micromoles of substrate hydrolyzed per minute per g of catalyst. 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 (25 cm × 0.4 cm) column was used. The mobile phase was acetonitrile−10 mM ammonium

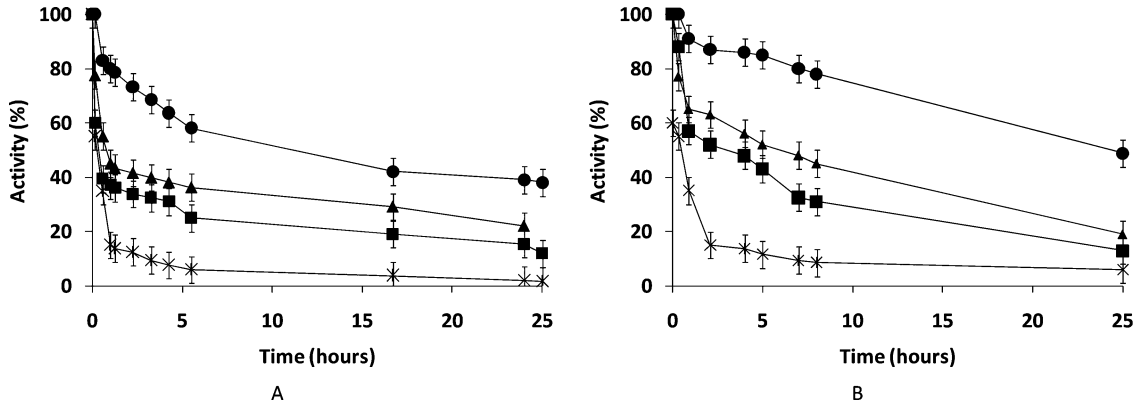


Figure 3. Effect of the immobilization protocol on the stability of BTL2. Experiments were carried out as described in methods using lowly loaded enzyme preparations. (A) Inactivation courses at 75 °C in 25 mM sodium phosphate pH 7. (B) Inactivation courses in the presence of 80% of dioxane at 30 °C and pH 7. Asterisk: CNBr-BTL2; squares: glyoxyl-BTL2 prepared at pH 10 during 1 h at 25 °C; circles: glyoxyl-BTL2 prepared at pH 10 during 24 h at 25 °C; triangles: glyoxyl-BTL2 prepared at pH 10 during 24 h at 4 °C.

phosphate (35:65, v/v) at pH 3. Detection of the compounds was performed by recording the absorbance at 225 nm. The enantiomeric excess (ee) of the formed monoester was analyzed by chiral reverse phase HPLC. The mobile phase was acetonitrile (25%) and 10 mM ammonium phosphate (75%) at pH 2.95 and the analyses were performed at a flow of 0.7 mL/min by recording the absorbance at 205 nm. The enantioselectivity was defined as the ratio between the concentrations of the *R*- and *S*-isomers of ethyl 3-phenylglutarate. The absolute configuration was assigned in agreement with the results of Ostaszewski and co-workers.[54]

Titration of the Amino Groups Using Pycryl Sulfonic Acid. Primary amino residues were titrated using TNBS.[55] A total of 500 mg of BTL2-agarose (having 3 mg of enzyme/g of support) were added to 30 mL of 0.1% (w/v) TNBS in 0.1 M sodium borate at pH 9, and the mixture was incubated for 30 min at room temperature. Then the immobilized enzyme was washed with distilled water and 0.1 M sodium borate at pH 9. A total of 100 mg of the colored preparations were then resuspended in 2 mL of sodium borate, pH 9, and their spectra were determined. As a reference, the support free of enzyme was treated in a similar way.

Results

1. Effect of the Immobilization of BTL2 on CNBr-Agarose Beads. These studies were performed offering 5 IU of enzyme per g of support to prevent diffusion problems that could alter the results.

Very mild conditions were used to produce this enzyme preparation that was used as a reference. After 15 min, 100% of lipase became immobilized on the support under the previously described conditions. The immobilized BTL2 kept 65% of its activity against pNPB compared to the soluble enzyme.

The stability of the soluble enzyme at 50 °C and pH 7 was similar to that of the CNBr-BTL2 preparation (half-life of 300 min for the soluble enzyme and 400 min for CNBr-BTL2). It may be assumed that the BTL2 immobilization on CNBr, under the immobilization conditions used (short reaction time and low temperature) did not have any significant effect on the BTL2 stability. Thus, this CNBr-BTL2 preparation seemed to be a good reference to study the stabilization achieved by immobilization of BTL2 on glyoxyl agarose, avoiding any intermolecular phenomena that could generate any artifact during the inactivation of the soluble enzyme (aggregation, interaction with interfaces).[56] In the case of lipases, the tendency of these enzymes to form bimolecular aggregates with different properties to those of the monomer makes even more convenient the use of an immobilized enzyme as a reference in these studies.[40,41]

2. Optimization of the Immobilization of BTL2 on Glyoxyl-Agarose. These studies were also performed offering 5 IU of enzyme per g of support to prevent diffusion problems that could alter the results.

The full immobilization of BTL2 on glyoxyl agarose at pH 10 and 25 °C takes 3.75 h. The immobilization rate was not very high, in agreement with the lack of regions in the BTL2, which were rich in Lys residues.[9,10,22] Different immobilized preparations of glyoxyl-BTL2 were prepared changing the immobilization time and temperature to alter the possibilities enzyme-support multireaction. All preparations expressed around 60−65% of the activity. However, the stabilities were quite different (Figure 3). Maximal stabilization was obtained under conditions that maximized the multipoint covalent attachment: long times (24 h) and moderate temperatures (25 °C).[25] This permitted to improve the half-life from 10 min at pH 7 and 75 °C for CNBr-BTL2 to 10 h for the optimal preparation (a stabilization factor of 60; Figure 3A). The stability of the different immobilized preparations of BTL2 in the presence of different dioxane concentrations was also studied. All of them retained 100% of their activity after 100 h of incubation in the presence of 50% of dioxane at 25 °C, showing the high stability of this enzyme. Using 80% of dioxane and 30 °C (Figure 3B), the optimal preparation (the one prepared at 25 °C after 24 h) was 75-fold more stable than the CNBr-BTL2 preparation. Considering that the stability increased under conditions where the multipoint covalent attachment was favored,[25] the most likely explanation for this stabilization should be the multipoint covalent attachment of the enzyme on the support.

The achieved stabilization by the optimal immobilization protocol of BTL2 on glyoxyl-agarose (G-BTL2) was quite significant but not as high as that reported in many other cases.[21] This could be a consequence of the relatively poor Lys content[9−12] on the surface of the enzyme and the lack of a region very rich in these groups.

3. Effect of the Chemical Amination on the CNBr-BTL2 Properties. A further improvement in the enzyme stability was intended by trying to increase the number of enzyme-support bonds using the strategy defined in Introduction (Figure 2): solid phase chemical amination of BTL2 and its immobilization on glyoxyl agarose beads. These studies were performed offering 5 IU of enzyme per g of support to prevent diffusion problems that could alter the results.

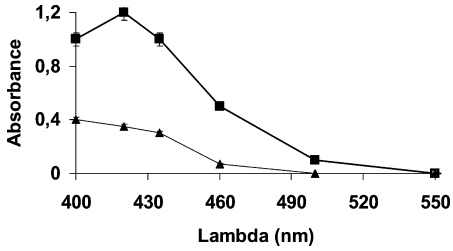


Figure 4. UV spectrum of different BTLA immobilized preparations modified with TNBS. Experiments were performed as described in Methods. Triangles: BTL2 immobilized on CNBr or butyl agarose and then titrated with TNBS; squares: BTL2-A immobilized on CNBr or butyl agarose and then titrated with TNBS.

CNBr-BTL2 was used to study the effects of this chemical modification on the enzyme properties because of its close stability compared to the soluble enzyme and the possibility of studying this preparation under any condition preventing any kind of artifacts (aggregation, precipitation, etc.).

The chemical amination of the already folded enzyme could have a moderate effect on the enzyme activity, but the effect on the enzyme stability may be difficult to predict: many ionic bridges are now transformed in repulsion interactions, and the amount of charged groups of the modified enzyme surface will be different from that of the native enzyme.

The carboxylic groups of BTL2 immobilized on CNBr-agarose (low loading, see Methods) were modified with EDA and EDC under previously described conditions to modify 100% of medium exposed carboxylic groups of proteins (to produce CNBr-BTL2-A).[27] The success in the modification was checked by titration of the primary amino groups by TNBS (Figure 4), where it is possible to observe a 3.5-fold increment in the adsorption of the modified enzyme at the wavelengths studied. This figure was quite similar to the expected increment in A if all the aspartic and glutamic moieties had been modified, although it may be possible that the ε of TNBs may depend on the nature of the primary amino group. The amination of the enzyme gave a CNBr-BTL2-A having 110% $\pm$ 10 of the activity of CNBr-BTL2 against pNPB. This full amination reduced the CNBr-BTL2 enzyme stability (Figure 5) by around a 2−4 factor (in inactivations caused by heat or organic solvents). Figure 6 shows the pH versus activity profile of the CNBr-BTL2 and CNBr-BTL2-A. The amination moves the optimal pH value for the enzyme from pH 9 to a broad flat in pH values 7−8, with higher activities at pH under 8 and lower activities at pH over this value.

4. Production of BTL2-A. Considering that the modification did not negatively affect the enzyme activity and reduce the stability merely by a 2−4-fold factor, it is possible to use this fully modified enzyme in subsequent studies, bearing in mind that a hyperstabilization may be expected. These effects, that are milder than those found for other enzymes, might be related to the high intrinsic rigidity of BTL2. For example, using penicillin G acylase and glutaryl acylase, only around 50% of the carboxylic groups on the enzyme surface could be aminated, because the full amination of these enzymes reduced the enzyme stability by more than a 100-fold factor.[27] In fact, even the partial amination of these enzymes presented a much higher impact on the enzyme stability than the full modification in the case of CNBr-BTL2, decreasing the enzyme half-life even 20-fold.[27,57]

Thus, the same modification described in the previous point was performed in BTL2 adsorbed on butyl agarose, with a similar nonsignificant effect on the enzyme activity. BTL2-A could be desorbed from the butyl agarose using 0.2% triton X-100 and utilized for immobilization on glyoxyl agarose. The full aminated enzyme should have 4-fold more amine groups than the native enzyme.[27−32] The titration with TNBS of the native and aminated adsorbed on butyl agarose presented a spectrum almost identical to that of the enzyme immobilized on CNBr, with a 3.5 increment in absorbance (Figure 4). In fact, it was identical to that obtained when the free enzyme was modified, then adsorbed on butyl agarose and then titrated with TNBS. This suggested that the adsorption on butyl-agarose did not produce serious steric hindrances to the chemical amination.

5. Effect of the Amination of BTL2 on the Immobilization on Glyoxyl Agarose Beads. To prevent diffusion problems, 5 IU of BTL2-A per g of support were offered in all the experiments (see Methods).

BTL2-A immobilization on glyoxyl-agarose at pH 10 proceeded much more rapidly than in the case of the native enzyme (Figure 3) and in less than 5 min no activity was observed in the supernatant.

The unmodified enzyme did not immobilize when it was incubated in the presence of glyoxyl agarose at pH 9, even after 16 h. However, BTL2-A was fully immobilized on the support after only 30 min (Figure 7), more slowly than BTL2-A at pH 10 but much more rapid than BTL2 at pH 10 (Figure 3). At pH 9, due to the high pK value of the Lys amino groups, the only reactive groups are those new groups introduced by chemical modification (with a pK of 9.2).[35] This result could be produced by the fact that there are 3-fold more new amino groups in BTL2-A than Lys in the BTL2.

This lower pH could select an area of the enzyme for its immobilization that may be different that the one involved in the immobilization at pH 10, because Lys reactivity should be extremely low at pH 9 and only the chemically introduced groups will be reactive. For example, Figure 2 shows two faces of BTL2 that are very rich in Asp and Glu moieties but very poor in Lys residues.

BTL2-A immobilized at pH 9 was in some instances incubated at pH 10 for 24 h to favor a more intense multipoint covalent attachment between the enzyme and the support (involving the new amino and the Lys groups; see Methods).[25,35] All these enzyme preparations retained around 60−65% of the BTL2-A initial activity.

6. Stability of the Different BTL2-A Preparations. Thermal inactivations of differently immobilized BTL2 preparations described above were performed at pH 7 and 75 °C (Figure 8A). The half-life of CNBr-BTL2-A under these conditions was around 3 min. BTL2-A immobilized at pH 10 (G(10)-BTL2-A) presented a half-life of 10 h, very similar to that of G-BTL2. BTL2-A immobilized at pH 9 (G(9)-BTL2-A) was slightly less stable (half-life around 8 h). However, the best results were obtained when the enzyme was first immobilized at pH 9 and further incubated at pH 10 for 24 h (G(9/10)-BTL2-A). This derivative was more than 2-fold more stable than G-BTL2 and over 400-fold more stable than the corresponding CNBr-BTL2-A. The optimal preparation retained 100% of its activity after 100 h of incubation at pH 7 and 65 °C.

Next, the stabilities of the BTL2 preparations in the presence of 80% dioxane were studied (Figure 8B). CNBr-BTL2-A presented a half-life of less than 4 min under these conditions. Again, G-BTL2 and G(10)-BTL2-A gave very similar stability, with a half-life of 10−15 h. G(9)-BTL2-A gave a stability slightly lower than these preparations. However, if this preparation was further incubated at pH 10, the stability in the presence of 80% dioxane reached the highest value, with a half-life of around 75 h. G(9/10)-BTL2-A was over 5-fold more stable than

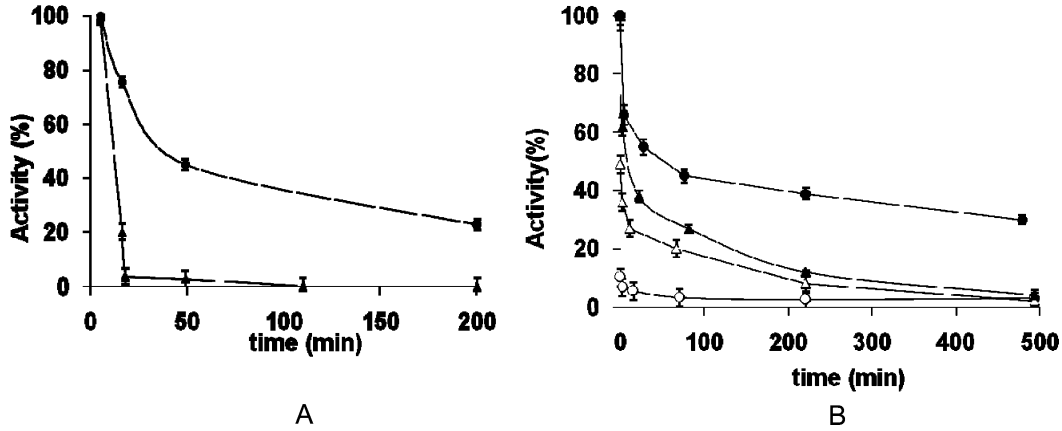


Figure 5. Effect of the chemical amination on the stability of CNBr-BTL2. Lowly loaded biocatalysts were used. Other specifications are described in Methods. (A) Inactivation courses at 75 °C in 25 mM sodium phosphate pH 7. (B) Inactivation curses in the presence of 75% of dioxane, at pH 7 and 30 °C. Circles: CNBr-BTL2; triangles: CNBr-BTL2-A.

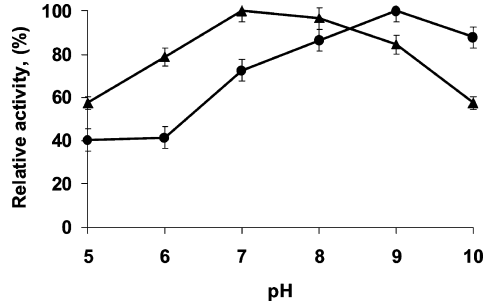


Figure 6. Effect of the chemical amination on the pH/activity profile of CNBr-BTL2. Experiments were performed at 25 °C using pNPB as substrate. A solution of 25 mM sodium acetate /25 mM sodium phosphate/25 mM sodium borate was used as buffer. Circles: CNBr-BTL2; triangles: CNBr-BTL2-A.

G-BTL2 and enabled a stabilization of more than 1200 compared to CNBr-BTL2-A. The optimal preparation retained 100% of its activity after 100 h of incubation in the presence of 60% dioxane at 30 °C and pH 7.

G(9/10)-BTL2-A presented a pH/activity profile very similar to CNBr-BTL2-A, with more activity expressed under drastic pH (e.g., 71 vs 57% at pH 10 or 67 vs 57% at pH 5), very likely as a reflection of the rigidity of the optimal preparation.

It is possible that the regions that were the richest ones in Lys groups may be different to the regions/s that was/were the richest areas in Asp + Glu. For example Figure 2 shows two sides of the BTL2 structure that are very rich in carboxylic groups (that should be modified with EDA in BTL2-A) and very poor in Lys residues. These areas could never be relevant in the immobilization of the enzyme on glyoxyl agarose before amination, while could be important in the immobilization of the aminated enzyme.

All the results suggest that BTL2-A immobilized at pH 9 on glyoxyl agarose might involve an area of the protein that was different to the one involved in the immobilization at pH 10, because BTL2-A immobilized at pH 9 and later incubated at pH 10 becomes significantly more stable than BTL2-A immobilized at pH 10. This occurs even though the immobilization at pH 10 should involve the area of the protein where there is the highest possibilities to get an intense multipoint covalent attachment,[22] in this case the richest areas in Lys + Glu + Asp. On the other hand, the immobilization at pH 9 should involve the richest areas in Glu + Asp. Apparently, the rigidification of this area could be more relevant for the stabilization of this enzyme, even though the intensity of the multiattachment should be lower that the one obtained by immobilization at pH 10.[22] This solid-phase chemical amination of the protein surface to improve their stabilization by multipoint covalent attachment on glyoxyl−agarose could be very easily extrapolated to any other lipase.

7. Hydrolytic Resolution of (*R*,*S*)-2-*O*-Butyryl-2-phenylacetic Acid (1) Catalyzed by Different Immobilized BTL2 Preparations. Mandelic acid is a precursor of several substances, as for example Plavix (clopidogrel bisulfate).[58] Highly loaded BTL2 preparations were employed due to the extremely low activity displayed by the low loaded derivatives (see methods). The activities of different BTL2 immobilized preparations against racemic **1** at pH 7 and 25 °C are shown in Table 1 (Scheme 1). This mandelic derivative is interesting by presenting a ionizable carboxylic group and the chiral center in the nucleophile.[44,45,49]

CNBr-BTL2 was the most active preparation, with an activity of 2.02×10^{-2} IU/g, G-BTL2 showed around 50% of that activity, while G(9)-BTL2-A was 18-fold less active than CNBr-BTL2. No product was detected using G(9/10)-BTL2-A after 60 h in the described conditions (see Methods). It should be considered that the activities of all these preparations were very similar (ranging between 60 and 65%) using pNPB as substrate. CNBr-BTL2 and G-BTL2 preparations exhibited enantiospecificity toward the *S*-isomer with *E* values of 1.8 and 4, respectively. Very interestingly, G(9)-BTL2-A displayed a very high enantiospecificity (with a value of 70) but preferring the opposite isomer, that is, it mainly hydrolyzed *R*-**1**. This permitted to get *S*-**1** with an ee of 99% and a yield higher than 47% after 500 h of reaction.

Table 2 shows the results obtained in this reaction at pH 5. The effect of the change in the reaction pH value was very significant, but it depended on the enzyme preparation studied. CNBr-BTL2, the most active preparation at pH 7, reduced its activity by almost a 20-fold factor (with a value of 0.12×10^{-2} IU/g), while all the glyoxyl preparations improved the activity (by a 10−20-fold factor) with this decrease in the pH value. Thus, G-BTL2 became almost 100-fold more active than CNBr-BTL2 (10.5×10^{-2} IU/g.). Remarkably, G(9/10)-BTL2-A presented an activity similar to that of the G(9)-BTL2-A, while at pH 7 the activity could not be even detected. This change in the pH value may alter the physical properties of the substrate which contains an ionizable carboxylic group.

In this case, all preparations preferred to hydrolyze the *S*-isomer, with CNBr-BTL2 and G(9/10)-BTL2-A showing enantiospecificities higher than 100. The use of G(9/10)-BTL2-A

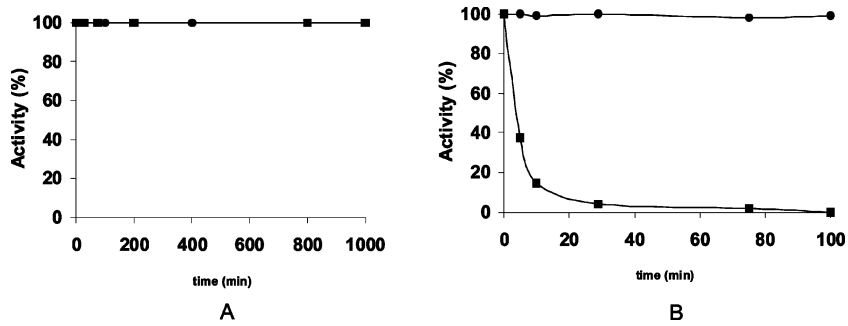


Figure 7. Effect of the chemical amination on the rate of immobilization of BTL2 on glyoxyl-agarose. Immobilizations were performed as described in Methods using 50 IU of BTL2 in 100 mL and 10 g of glyoxyl-agarose. Please note the change in axis X scale. (A) Immobilization course at pH 9 and 25 °C of BTL2. (B) Immobilization course at pH 9 and 25 °C of BTL2-A. Circles: supernatant of the reference suspension; squares: supernatant of the immobilization suspension.

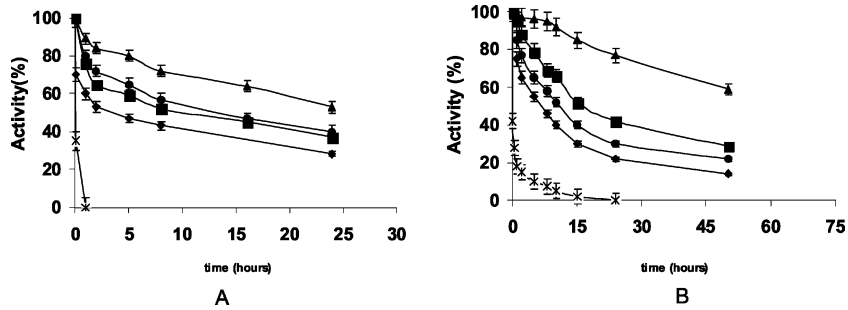


Figure 8. Effect of the chemical amination on the stabilization of BTL2 by immobilization on glyoxyl agarose. Experiments performed as described in Methods using lowly loaded enzyme preparations. (A) Inactivation courses in 25 mM sodium phosphate at 75 °C and pH 7. (B) Inactivation courses in the presence of 80% of dioxane at 30 °C and pH 7. Asterisk: CNBr-BTL2-A; rhombus: G(9)-BTL2-A; squares: G-BTL2; circles: G(10)-BTL2-A; triangles: G(9/10)-BTL2-A.

Table 1. Hydrolytic Resolution of **rac-1** at pH 7 and 25°C, Catalyzed by Different BTL2 Immobilized Preparations[a]

enzyme preparations	activity[b]	enantio-preference	enantiospecifity[c]
CNBr-BTL2	0.00202 ± 0 0.00025	*S*	1.8 ± 0.01
G-BTL2	0.0095 ± 0.0001	*S*	4 ± 0.3
G(9)-BTL2-A	0.0012 ± 0.0001	*R*	70 ± 5
G(9/10)-BTL2-A	$<1.5 \times 10^{-4}$	n.d.	n.d.

[a] Highly loaded BTL2 preparations were utilized. Experiments were performed as described in methods. [b] Activity: (micromol/min/g biocatalyst). [c] Enantiospecificity was calculated as described in methods, with a substrate hydrolysis in the range of 15−20%.

Table 2. Hydrolytic Resolution of **rac-1** at pH 5 and 25 °C Catalyzed by Different BTL2 Immobilized Preparations[a]

enzyme preparations	activity[b]	enantio-preference	enantiospecifity[c]
CNBr-BTL2	0.0012 ± 0.0002	*S*	>100
G-BTL2	0.105 ± 0.005	*S*	10 ± 1
G(9)-BTL2-A	0.023 ± 0.002	*S*	28 ± 3
G(9/10)-BTL2-A	0.017 ± 0.003	*S*	>100

[a] Highly loaded BTL2 preparations were used. Experiments were carried out as described in Methods. [b] Activity: micromol/min g biocatalyst. [c] Enantiospecificity was calculated as described in Methods, with a substrate hydrolysis in the range of 15−20%.

permitted to obtain around 50% *R*-**1** and 50% *S*-**2** acid with an ee higher than 99% after 50 h of reaction.

G(9)-BTL2-A presented a much lower enantiospecificity ($E = 28$). In this biocatalyst, the enzyme presented the same orientation in the support surface as G(9/10)-BTL2-A but was less stable (and it may be assumed due to a less intense multipoint covalent attachment)

These results are a clear example that fully agrees with previous reports[44−49] that state that the immobilization of a lipase on a support via different regions or with different rigidity may greatly alter the activity, specificity, enantiospecificity and the effect of the experimental conditions on those enzyme catalytic features. The specificity of BTL2 has been greatly altered when submitted to different immobilization protocols. While all of them presented similar specific activity against pNPB, the specific activities were very different using this more complex substrate. On the other hand, it has also been shown that the same immobilized lipase may change the enantiopreference by a slight change in the experimental conditions (e.g., G(9)-BTL2-A prefers **1**-*R* at pH 7 and **1**-*S* at pH 5). However, the effect of a variable may be very different when changing the immobilized preparation: the decrease of the pH from 7 to 5 decreased the activity of CNBr-BTL2 by a 20-fold factor, while that of G-BTL2 increased that by a 10-fold factor. Finally, the use of different immobilization protocols on the same lipase has permitted to prepare BTL2 preparations able to produce, from racemic **1**, **1**-*S* (G(9)-BTL2-A at pH 7) or **1**-*R* (e.g., CNBr-BTL2 and G(9/10)-BTL2-A) at pH 5) isomers of 2-*O*-butyryl-2-phenylacetic acid. Although the changes in the absolute values of the enantioselectivity have been previously reported in many examples,[44−49] this strong inversion in the enantiopreference is not common.[18]

8. Hydrolysis of Diethyl 3-Phenylglutarate (3) by Different Immobilized Preparations of BTL2. Table 3 shows the results obtained in the hydrolysis of **3** catalyzed by highly loaded (see methods) preparations of BTL2 (Scheme 2).

The biocatalysts prepared using BTL2 were less active (0.03 IU/g for CNBr-BTL2 and 0.027 IU/g for G-BTL2) than the ones prepared using BTL2-A (0.125 and 0.07 IU/g for G(9)-BTL2-A and G(9/10)-BTL2-A, respectively).

All the preparations produced mainly *S*-ethyl phenylglutarate (**4**), but the asymmetry in the reaction was quite different depending on the biocatalyst. The lowest enantioselectivity was

Table 3. Asymmetric Hydrolysis of 3 at pH 7 and 25 °C Catalyzed by Different BTL2 Immobilized Preparations[a]

enzyme preparations	activity[b]	enantio-preference	enantioselectivity[c]
CNBr-BTL2	0.03 ± 0.002	*S*	55 ± 4
G-BTL2	0.027 ± 0.001	*S*	13 ± 1
G(9)-BTL2-A	0.125 ± 0.01	*S*	40 ± 3
G(9/10)-BTL2-A	0.07 ± 0.006	*S*	10 ± 1

[a] Highly loaded BTL2 preparations were utilized. Experiments were performed as described in Methods. [b] Activity: micromol/min/g biocatalyst. [c] Enantioselectivity was calculated as described in Methods, with a substrate hydrolysis in the range of 15−20%.

obtained using G-BTL2 and G(9/10)-BTL2-A (around 10), while CNBr-BTL2 and G(9)-BTL2-A showed a value of 55 and 40, respectively.

Again all the enzyme properties could be altered by the immobilization. Now, the most active preparation was G(9)-BTL2-A and the most enantioselective was CNBr-BTL2. This last preparation permitted to get *S*-**4**, with an enantiomeric excess of 96% and a yield higher than 99% after 10 h of reaction (phenylglutaric acid was not detected after this reaction time).

Conclusion

The strategy presented in this paper has permitted to prepare a very stable and active immobilized biocatalyst of a lipase in a quite simple way by using tools that are initially very different. Thus, three different tools have been employed to obtain a very stable lipase biocatalyst: the use of an enzyme from thermophilic origin, the amination via solid phase chemical modification of the reversibly immobilized enzyme and the multipoint covalent immobilization of the chemically aminated enzyme.

The use of a lipase permits its reversible and selective adsorption on hydrophobic supports.[43] This selective adsorption is not only useful to purify the lipase but also permits to take advantage of the solid phase modification, improving the control of the reaction, saving dyalisis steps, and so on. This makes very simple and rapid the amination of the enzyme. The enzyme activity remained unaltered by the full chemical modification (perhaps due to the high intrinsic stability of the thermophilic enzyme employed) while its stability decreased by the amination (by only a 2−4-fold factor). However, a more intense multipoint covalent attachment compensates that initial decrease, giving immobilized biocatalysts more stable than the optimal preparation obtained on glyoxyl agarose using the native enzyme (by a 5-fold factor in the presence of cosolvents) and around 1200-fold more stable than the modified enzyme immobilized on CNBr.

The higher reactivity of the new amino groups (compared to that of the amino groups of the Lys residues) makes possible to immobilize the enzyme at pH 9 on glyoxyl-agarose. This point was important in the case of BTL2 to get an improved stability.

However, lipases that were not stable enough at pH 10 to permit its immobilization on glyoxyl agarose, could now be aminated and immobilized at milder pH values on glyoxyl agarose, permitting their stabilization by multipoint covalent attachment.[21]

The protocol that permitted the highest stability of the immobilized enzyme was the amination of BTL2; the further immobilization of BTL2-A at pH 9 and, finally, its incubation at pH 10 to improve the reactivity of all amino groups of the protein.[25] Other interesting fact is that BTL2-A immobilized much more rapidly at pH 9 than BTL2 at pH 10.

Moreover, the differently immobilized BTL2 preparations exhibited very different catalytic properties: specificity, enantiospecificity, and enantioselectivity have been greatly altered by the immobilization-stabilization protocol.

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