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New Tailor-Made Alkyl-Aldehyde Bifunctional Supports for Lipase Immobilization

Robson Carlos Alnoch [1,2], Ricardo Rodrigues de Melo [1,3], Jose M. Palomo [1], Emanuel Maltempi de Souza [2], Nadia Krieger [4] and Cesar Mateo [1,*]

1 Departamento de Biocatálisis, Instituto de Catálisis y Petroleoquímica (CSIC), Marie Curie 2. Cantoblanco, Campus UAM, 28049 Madrid, Spain; robsonalnoch@hotmail.com (R.C.A.); ricardorodriguesmelo@gmail.com (R.R.d.M.); josempalomo@icp.csic.es (J.M.P.)

2 Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Cx. P. 19081 Centro Politécnico, 81531-980 Curitiba, Paraná, Brazil; souzaem@ufpr.br

3 Departamento de Ciência de Alimentos, Faculdade de Engenharia de Alimentos (FEA), Universidade Estadual de Campinas, 13083-862 Campinas, São Paulo, Brazil

4 Departamento de Química, Universidade Federal do Paraná, Cx. P. 19081 Centro Politécnico, 81531-980 Curitiba, Paraná, Brazil; nkrieger@ufpr.br

* Correspondence: ce.mateo@icp.csic.es; Tel.: +34-915854768; Fax: +34-915854860

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Abstract: Immobilized and stabilized lipases are important biocatalytic tools. In this paper, different tailor-made bifunctional supports were prepared for the immobilization of a new metagenomic lipase (LipC12). The new supports contained hydrophobic groups (different alkyl groups) to promote interfacial adsorption of the lipase and aldehyde groups to react covalently with the amino groups of side chains of the adsorbed lipase. The best catalyst was 3.5-fold more active and 5000-fold more stable than the soluble enzyme. It was successfully used in the regioselective deacetylation of peracetylated D-glucal. The PEGylated immobilized lipase showed high regioselectivity, producing high yields of the C-3 monodeacetylated product at pH 5.0 and 4 °C.

Keywords: regioselective hydrolysis; biocatalysis; lipase; interfacial activation; covalent immobilization; tailor-made supports; enzyme stabilization

1. Introduction

Lipases (EC 3.1.1.3) normally catalyze the hydrolysis of carboxylic esters in aqueous media, but they can also be used to synthesize carboxylic esters in water-restricted media, exhibiting high regio-, chemo-, and enantioselectivity. Due to these properties, lipases have been used in different reactions, standing out among the most widely used enzymes in biotechnology [1,2].

Recently, a new lipase, LipC12, was identified in a metagenomic library constructed from soil samples contaminated with fat [3]. LipC12 had a specific activity against long-chain triglycerides (e.g., olive oil 1722 U·mg^{-1}) that is comparable to the specific activities of several well-known commercial lipases [3,4]. Furthermore, LipC12 was stable at moderate temperatures and in the presence of co-solvents such as methanol, propanol, or acetone [3]. These features suggest that LipC12 might be suitable for use in biocatalysis.

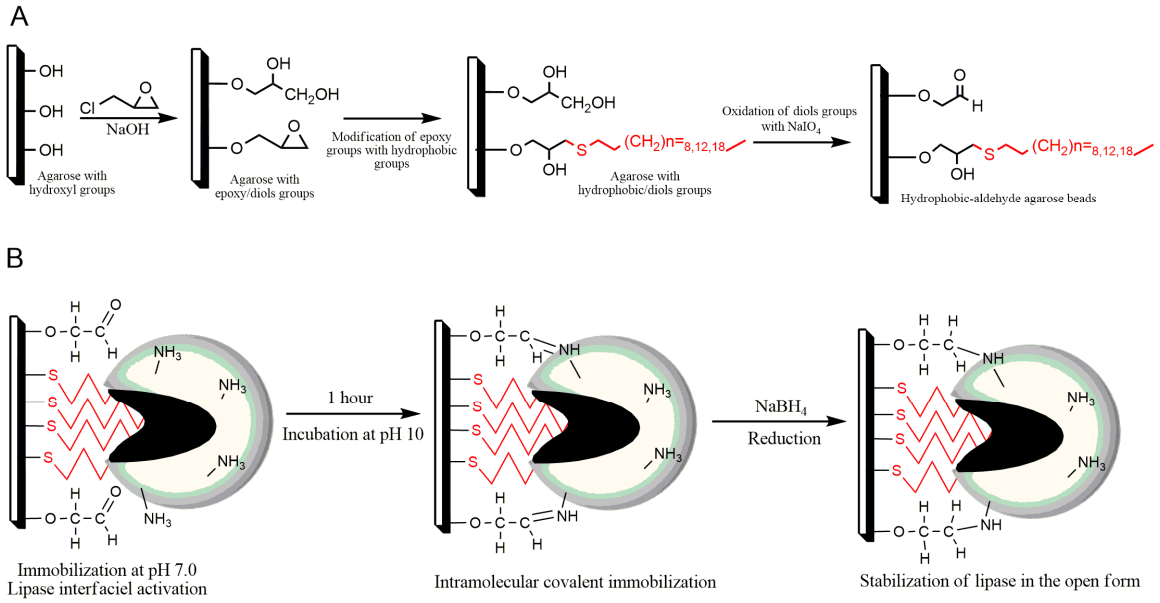
Typically, industrial biocatalytic processes require that the lipases be immobilized since that immobilization facilitates reutilization of the enzyme, reducing process costs [5]. The immobilization of different lipases has been performed using different immobilization methods such as covalent linkage with different reactive groups, electrostatic or hydrophobic adsorptions, entrapment, encapsulation, or cross-linked enzyme aggregates (CLEAs); and using different materials such as nanomagnetic particles, microspheres, organic or inorganic materials, porous and/or macroporous gel beads, graphene oxides, exfoliated bentonite, and many others [6–19]. The different immobilization methods have enabled the procurement of lipase catalysts with different properties in terms of activity, stability, and selectivity [5].

The most successful strategy used for lipase immobilization is adsorption on hydrophobic supports [5,20]. This strategy has permitted the purification and immobilization of various lipases in a single step [9,11,21,22]. These protocols are based on the special characteristics and mechanisms of lipases. In aqueous media, lipases are in equilibrium between closed and open forms. In the closed form, the lid, which is formed by a short alpha helix, secludes the catalytic site from the medium, making it inaccessible to the substrate, such that the lipase is in an inactive state. In the open and active form, the internal side of the lid and the surroundings of the active site form a hydrophobic pocket that is exposed to the medium. The open form is stabilized upon contact of the lipase with a hydrophobic surface, as occurs at the oil-water interface when lipases are used to hydrolyze triacylglycerides in oil-in-water emulsions [9,11]. The adsorption of lipases in the open form at this interface leads to high activity in a phenomenon that is called interfacial activation. Immobilization by adsorption on hydrophobic surfaces takes advantage of this phenomenon by fixing the lipase predominantly in its open conformation. This gives this method a significant advantage over other methods that immobilize the lipase by other regions and which therefore allow the immobilized lipase to equilibrate between the open and closed conformations. This method is specific and yields more active and selective catalysts [23,24], this being especially important for the catalysis of complex reactions, such as regioselective deprotection reactions with carbohydrates [25]. However, physical adsorption also has a significant disadvantage: the association between the protein and the support is reversible, meaning that the lipase can leach from the solid support, especially in the presence of low concentrations of detergents or solvents [9].

One strategy for preventing the leaching of lipases from hydrophobic supports would be to create covalent bonds between the adsorbed enzyme and the support. In fact, covalent immobilization of enzymes using aldehyde-activated supports is a widely used technique [26]. However, a heterofunctional support that combines hydrophobic and aldehyde groups in the same matrix has not previously been described.

In the present work, novel tailor-made alkyl-aldehyde supports were prepared (Scheme 1). The novel supports contain: (i) a very dense layer of different hydrophobic moieties (different alkyl groups) that are able to absorb lipases at neutral pH; and (ii) a high concentration of aldehyde groups that are able to react covalently with the enzyme, especially at alkaline pH. The presence of different groups with different functions on the surface of the support should permit better control of the immobilization, which occurs through a two-step mechanism: first the enzyme adsorbs onto the hydrophobic groups and then the aldehyde groups react with it, immobilizing it covalently.

These novel functionalized supports were used to immobilize the novel lipase LipC12, and the stability, activity, and regioselectivity of the new heterogeneous biocatalyst were tested. The best heterogeneous biocatalyst that was obtained was used in the regioselective hydrolysis of per-*O*-acetylated D-glucal, an interesting building block for the synthesis of various tailor-made di- and trisaccharides.



Scheme 1. (**A**) Preparation of new tailor-made alkyl-aldehyde supports; (**B**) Mechanism of immobilization-stabilization of lipases in the open form on new alkyl-aldehyde supports. *n* = C8 (1-octanethiol); C12 (1-dodecanethiol) and C18 (1-octadecanethiol).

2. Results and Discussion

2.1. Preparation of New Alkyl-Aldehyde Supports

Agarose beads were utilized as the base matrix for the construction of different bifunctional supports. The surface of the support, which is rich in primary hydroxyl groups, was activated in alkaline conditions, with epiclorohydrin, forming epoxy groups and diol groups (Scheme 1A). The total amount of activated primary hydroxyl groups was around 65 μmol·g^{-1}, with epoxy groups accounting for 23 μmol·g^{-1} and diol groups accounting for 42 μmol·g^{-1} (Table 1). The epoxy groups were functionalized with different bifunctional hydrophobic agents (octane-, dodecane-, and octadecane-thiol) in order to have supports containing groups with different degrees of hydrophobicity for interfacial adsorption of the lipase (Scheme 1A). The diol groups were then oxidized with sodium periodate, producing aldehyde groups. These aldehyde groups are capable of reacting covalently with different amine groups of the protein. Immobilization of the lipase on this support occurs in two steps: first, the enzyme adsorbs hydrophobically in an orientation that favors the open form; second, the aldehyde groups react covalently with the side chains of lysine that are exposed at the surface of the enzyme, fixing it covalently o the support (Scheme 1B).

Table 1. Quantification of groups on the new alkyl-aldehyde supports.

Support	Ligands (μmol·g^{-1})	Diol Groups (μmol·g^{-1})
Agarose-Epoxy	23 ± 0.4	43 ± 0.4
C8-aldehyde	23 ± 1	43 ± 1
C12-aldehyde	21 ± 1.6	41 ± 1.6
C18-aldehyde	19 ± 1.1	38 ± 1.1

The number of epoxy/ligands groups was calculated from the difference in periodate consumption between the hydrolyzed support and the initial epoxy support as described in the methods section. Results are expressed as the average of triplicate assays ± the standard error of the mean.

2.2. Immobilization of LipC12 on New Alkyl-Aldehyde Supports

Figure 1 shows the immobilization of LipC12 by adsorption onto the new alkyl-aldehyde supports. LipC12 was quite rapidly immobilized at pH 7.0 on all bifunctionalized supports, with complete immobilization (i.e., >95% removal of activity from the supernatant) occurring in less than 2 h.

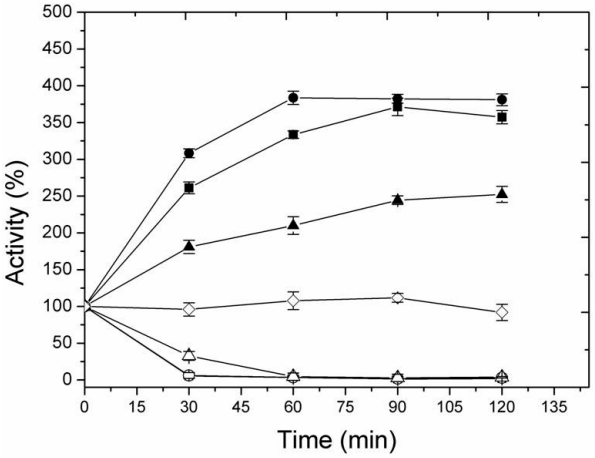


Figure 1. Immobilization courses of LipC12 on new alkyl-aldehyde supports. (□) C8-aldehyde; (○) C12-aldehyde; (△) C18-aldehyde. (◇) Control. Symbols: Black (suspension); Hollow (supernatant). Results are expressed as the average of triplicate assays ± the standard error of the mean.

LipC12 was activated by adsorption onto the support, with the activities measured for the suspension being significantly higher than that of the original supernatant (Figure 1). The highest value of recovered activity, 380%, was obtained with the preparation C12-aldehyde/LipC12. The preparations C8-aldehyde/LipC12 and C18-aldehyde/LipC12 also showed high values of recovered activity (>200%), showing the hyperactivation of lipase LipC12 immobilized these supports (Table 2). The results show that these new tailor-made supports allowed the immobilization of this lipase in its open conformation via interfacial activation [9,11].

Table 2. Principal parameters for immobilization of the lipase LipC12 on new alkyl-aldehyde supports.

Support	Immobilization Efficiency (%) [a]	Recovered Activity [b] (%)	Recovered Activity after Reduction [c]
C8-aldehyde	>95	357	346
C12-aldehyde	>95	380	370
C18-aldehyde	>95	252	256

[a] Calculated as the difference between the initial and final activities in the supernatant after 2 h of immobilization; [b] Recovered activity (%), measured as the ratio between the real activity ($U \cdot g^{-1}$ support) of immobilized LipC12 and theoretical activity of the immobilized LipC12 ($U \cdot g^{-1}$ support); [c] Recovered activity (%) after incubation at pH 10 for 1 h and reduction with $NaBH_4$.

In order to fix LipC12 covalently to the support, the immobilized preparations were incubated at different pH values (7.0, 8.5, and 10) for 1 h. After the incubation, the imine bonds formed between the enzyme and the support were then reduced by adding sodium borohydride. This reduction did not affect the activity of the immobilized enzyme (Table 2). No leaching of lipase was found after incubation in surfactants.

2.3. Thermal Inactivation of Different Immobilized LipC12 Preparations

The various immobilized LipC12 preparations previously incubated at different pH values were incubated in phosphate buffer 25 mM at 55 °C. In all cases, the thermal stability of the derivatives incubated at pH 10 was higher than that incubated at pH 8.5 and 7.0 or the only adsorbed preparations

(Figure S1). At pH 7.0, the reactivity of the amino groups of the enzymes was not high enough to produce a covalent attachment with the aldehyde groups; at pH 10, the increase in the reactivity of the amine groups of side chains close to the lid that promote the rigidification on this region resulting in a high stabilization. At 55 °C, C8-aldehyde/LipC12, C12-aldehyde/LipC12, C18-aldehyde/LipC12 conserved more than 80% of their activity after 24 h (Figure 2A) while the half-life of the soluble enzyme was 37 min.

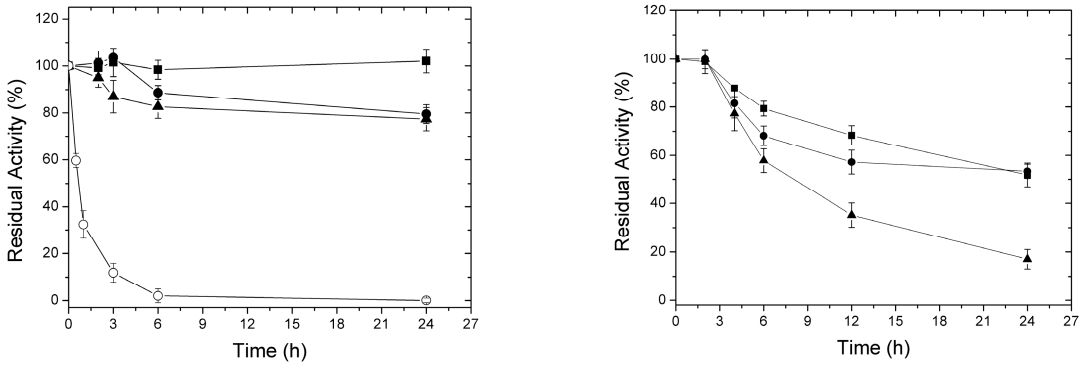


Figure 2. Thermal inactivation of LipC12 immobilized on different alkyl-aldehyde supports. (**A**) Inactivation was performed at pH 7.0, 55 °C after incubation at pH 10 for 1 h; (**B**) Inactivation was performed at pH 7.0, 80 °C after incubation at pH 10 for 1 h. (■) C12-aldehyde/LipC12; (●) C8-aldehyde/LipC12; (▲) C18-aldehyde/LipC12 and (○) Soluble enzyme. Results are expressed as the average of triplicate assays ± the standard error of the mean.

After 24 h incubation at 80 °C, C8-aldehyde/LipC12 and C12-aldehyde/LipC12 still had residual activities above 50%, while the residual activity of C18-aldehyde/LipC12 was only 20% (Figure 2B). Intermediary spacer arms (C8 and C12) supports produced a slight increment in the stability effect achieved when compared with C18. The half-lives were 22 h for C8-aldehyde/LipC12 and 21 h for C12-aldehyde/LipC12, while the soluble lipase lost 50% of the activity after only 15 s. This means that the alkyl-aldehyde-lipase preparations were from 2000- to 5000-fold more stable than the soluble enzyme (Table 3). Considering the retention of activity (Table 2) and stability, the C12-aldehyde/LipC12 preparation was chosen for the remaining studies.

Table 3. Half-lives (in hours) of the different immobilized preparations at 80 °C.

Preparations [a]	Half-Life ($t\frac{1}{2}$) at 80 °C	Stability Factor
Soluble enzyme	0.004	-
C8-aldehyde/LipC12	22	5500
C12-aldehyde/LipC12	21	5250
C18-aldehyde/LipC12	8	2000

[a] Preparations were incubated at 80 °C. Aliquots were withdrawn periodically for quantification of residual enzymatic activity to estimate the half-life according to Henley and Sadana [27].

2.4. Effect of Temperature and pH on Activity of Free and Immobilized LipC12

The optimum temperatures for the activity of free and immobilized LipC12 were determined over the temperature range of 20–90 °C. The maximum activity of the free enzyme was obtained at 30 °C while the optimal temperature for C12-aldehyde/LipC12 was 70 °C (Figure 3).

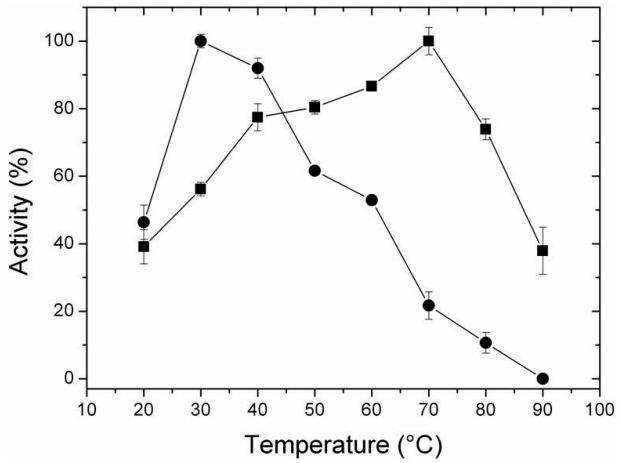


Figure 3. Effect of temperature on free and C12-aldehyde/LipC12 activity. (●) Soluble LipC12; (■) C12-aldehyde/LipC12. The activity was determined using p-nitrophenyl proprionate (pNPP) as the substrate, at pH 7.0. Results are expressed as the average of triplicate assays ± the standard error of the mean.

This shift in the optimal temperature was related to the improvement of the stability of the obtained preparation. The high improvement after adsorption and covalent linkage is important because it permits the transformation of a mesophilic enzyme into an enzyme with properties that are similar to, or even better than, those of enzymes from thermophile organisms, such as Bacillus thermocatenolatus lipase (BTL) and Thermus thermophilus lipase (TTL) [28,29].

In relation to the effects of pH on activity, the maximum activity was obtained at around pH 7.0 for both free LipC12 and C12-aldehyde/LipC12 (Figure 4).

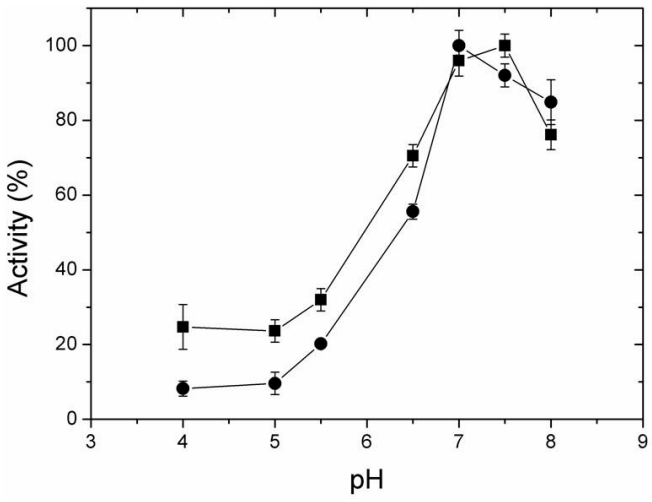


Figure 4. Effect of pH on free and C12-aldehyde/LipC12 activity. (●) Soluble LipC12; (■) C12-aldehyde/LipC12. The activity was determined using p-nitrophenyl proprionate (pNPP) as the substrate. Results are expressed as the average of triplicate assays ± the standard error of the mean.

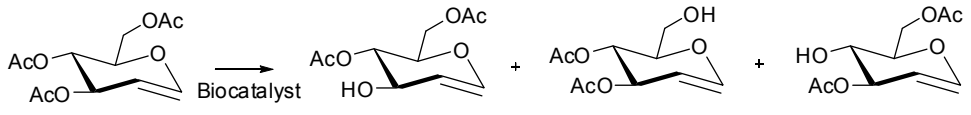
2.5. Regioselective Hydrolysis of 3,4,6-tri-O-acetyl-D-glucal by Immobilized LipC12

C12-aldehyde/LipC12 was used to catalyze the hydrolytic deacetylation of per-*O*-acetylated-D-glucal (**1**). The yield of this reaction depends strongly on the reaction conditions. The principal variables assayed were the pH and temperature. Additionally, the recovering of the optimal catalyst with PEG was performed. This treatment has demonstrated that it is able to improve the activity and stability [30].

The activity of the soluble enzyme was also assayed. However, at 25 °C, its activity was extremely low, so no attempt was made to assay it at 4 °C (data not shown).

At 25 °C, low regioselectivity was C12-aldehyde/LipC12 at both pH 7.0 and pH 5.0, producing only around 10% yield of monodeacetylated products at 100% conversion (Table 4). The PEGylated preparation, C12-aldehyde/LipC12-PEG, had a slightly improved regioselectivity at pH 5.0 and 25 °C, although the yield of 3-OH product (**2**) was only 22%.

Table 4. Regioselective hydrolysis of 3,4,6-tri-*O*-acetyl-D-glucal (**1**) using C12-aldehyde/LipC12.



1 **2** **3** **4**

Preparation	pH	T °C	Specific Activity ($U \cdot mg^{-1}$) *	Time (h)	Total Conversion [a] (%)	Yield 2 (%)	Yield 3 (%)	Yield 4 (%)	Other Products [b] (%)
C12-aldehyde /LipC12	7.0	4	18	96	77	52	1	7	17
C12-aldehyde /LipC12	5.0	4	4	96	34	26	1	1	6
C12-aldehyde /LipC12-PEG	5.0	4	15	96	81	69	0	3	9
C12-aldehyde /LipC12	7.0	25	140	24	100	5	4	0	91
C12-aldehyde /LipC12	5.0	25	140	24	100	11	0	0	89
C12-aldehyde /LipC12-PEG	5.0	25	110	24	100	22	2	2	74

(**1**)- 3,4,6-tri-*O*-acetyl-D-glucal; (**2**)- 4,6-di-*O*-acetyl-D-glucal; (**3**)- 3,4-di-*O*-acetyl-D-glucal; and (**4**)- 3,6-di-*O*-acetyl-D-glucal; * $\times 10^{-3}$; [a] Total conversion of substrate (**1**) with different products; [b] D-glucal and dideacetylated products.

The regioselectivity was higher at 4 °C than at 25 °C (Table 4). At pH 7.0, 52% of C-3-OH product (**2**) was obtained at 77% conversion, with slight conversion into 4-OH product (**4**) (7%) and 6-OH product (**3**) (1%), reducing the undesired product in 17% (Table 4). The PGEylation of this catalyst (C12-aldehyde/LipC12-PEG) allowed an improvement of the regioselectivity. This catalyst produced 69% of 3-OH product (**2**) at 81% conversion, and only 3% of 4-OH product (**4**) (Table 4).

The PEGylated catalyst was reused in three reaction cycles at 4 °C and similar reaction yields were obtained, demonstrating its reusability (Figure S2). However, the recycle of the catalysts in this reaction are not reported, these data are similar to others obtained by different authors for the hydrolysis of esters as reported by Macario et al. [31], where the catalyst (lipase of *Rhizomucor miehei* immobilized on zeolites) was used in the hydrolysis of methyl myristate for four cycles or Cao et al. [12] that recycled the catalyst (nanohybrids of *Yarrawia* lipolytica lipase) for 12 reaction cycles using *p*NPP as substrate.

3. Materials and Methods

3.1. Materials

The strains *E. coli* TOP10 (Invitrogen, Carlsbad, CA, USA) and BL21(DE3) (Novagen, Madison, MI, USA) and the vector pET-28a(+) (Novagen, Madison, MI, USA) were used as the recombinant protein expression system. Agarose 4 BCL was purchased from Agarose Bead Technologies (Madrid, Spain). Epichlorhydrine, iminodiacetic acid, triethylamine, sodium borohydride, sodium periodate, 1-octanothiol, 1-dodecanothiol, 1-octadecanethiol, tri-*O*-acetyl-D-glucal, polyethylene glycol (1.500), nickel(II) chloride hexahydrate, and high molecular weight protein (Sigma Marker™) were purchased

from Sigma (Sigma-Aldrich, St. Louis, MO, USA). The substrate *p*-nitrophenyl proprionate (*p*NPP) was synthesized according to Ghosh et al. [32]. All other chemicals used were of analytical grade.

3.2. Overexpression of Recombinant LipC12

E. coli BL21(DE3) cells carrying the pET28a(+)/lipC12 plasmid were grown in 500 mL of LB medium at 37 °C until an OD_{600} of 0.5 and induced by the addition of Iso-propyl β-D thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The induced culture was incubated for a further 16 h at 20 °C before harvesting the cells by centrifugation (10,000 rpm for 5 min) at 4 °C. The cell pellet was re-suspended in 30 mL of lysis buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM β-mercaptoethanol, 1% (*v*/*v*) Triton X-100 and 10% (*v*/*v*) glycerol) and disrupted by ultrasonication in an ice bath (15 cycles of 20-s pulses, 90 W, with 30-s intervals), using a SONICATOR® XL 2020 (Heat Systems-Ultrasonics Inc., New Highway, Farmingdale, NY, USA). The crude extract was then centrifuged at 15,000 rpm 30 min at 4 °C to pellet the cell debris.

3.3. Protein Content Determination and Electrophoresis Analysis

Protein content was determined by the Bradford method [33] using a Coomassie Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) with bovine serum albumin as the standard. Electrophoresis of protein samples was done with 12% (*w*/*v*) SDS-PAGE [34] and the gel was stained with Coomassie Brilliant Blue R-250 and destained with methanol/acetic-acid/water (5/1/4 *v*/*v*/*v*). A mixture of high molecular weight proteins (Sigma Marker™, Sigma-Aldrich®) was used as the molecular weight standard.

3.4. Lipase Activity Assay

Lipase activity was determined using *p*-nitrophenyl proprionate (*p*-NPP) as the substrate. Free or immobilized enzyme was added to the reaction mixture (0.4 mM *p*NPP, mM NaH_2PO_4 pH 7.0) and the increase of absorbance was monitored at 348 nm (at pH 7, $\varepsilon_{348\,nm}$ = 5150 $M^{-1}{\cdot}cm^{-1}$) [35]. One unit of activity (U) was defined as the production of 1 μmoL of *p*-nitrophenol per minute, under the assay conditions.

3.5. Preparation of Supports

3.5.1. Epoxy-Agarose

The epoxy-agarose support was prepared according to Mateo et al. [26]. Briefly, 10 g of agarose BLC (cross-linked 4% agarose beads) was mixed with 44 mL of distilled water, 3.2 g of NaOH, 200 mg of $NaBH_4$, 16 mL of acetone, and 11 mL of epichlorohydrin. The suspension was stirred for 16 h at 25 °C. The epoxy-agarose was washed with an excess of water, filtered through a glass filter, and stored at 4 °C.

3.5.2. Epoxy-Agarose-IDA-Ni^{2+}

The epoxy-agarose support was treated with 0.5 M iminodiacetic acid in solution at pH 11 for different durations (1, 3, 5, and 24 h), 25 °C. The support was then chelated with a $NiCl_2$ solution (30 $mg{\cdot}mL^{-1}$) for 1 h. Finally, the support was washed, filtered under using a glass filter, and stored at 4 °C.

3.5.3. Alkyl-Agarose-Aldehyde

The epoxy-agarose support was treated with 100 mM of different alkyl thiols (1-octanothiol; 1-dodecanothiol and 1-octadecanethiol) in a 25 mM $NaHCO_3$ solution at pH 10 for 24 h, 25 °C. For the treatment with 1-octadecanethiol, 50% (*v*/*v*) acetone was used as a co-solvent. The reagent was solubilized using a 50:50 (*v*/*v*) mixture of acetone and $NaHCO_3$ solution. After that, the supports were oxidized with $NaIO_4$ (100 mM), washed, filtered through a glass filter, and stored at 4 °C.

The number of epoxy/ligand groups was calculated from the difference in periodate consumption between the hydrolyzed support and the initial epoxy support. Periodate consumption was quantified using potassium iodide, as previously described [36].

3.6. Purification of Recombinant LipC12

The purification was performed using the IDA-Ni^{2+} supports prepared from agarose gel beads and activated with different amounts of metal chelate groups [37]. The optimal support was that obtained after 3 h of activation with IDA (data not shown). For the purification, 4 mL of crude extract (3.2 mg·mL^{-1}) was offered for 1 g of support and the residual activity of the supernatant was monitored over time. After that, the support was washed three times with 25 mM NaH_2PO_4 pH 7.0 and resuspended in the same buffer at increasing concentrations of imidazole. Figure S3 shows the protein band corresponding to the molecular mass of LipC12 (32 kDa) after SDS-PAGE of the eluate from IDA-Ni^{2+} support at 50 mM of imidazole. Table S1 summarizes the results of the purification step, showing an activity yield of 58%. The specific hydrolytic activity against *p*NPP was 6.2 U·mg^{-1}. This preparation was used in further experiments of immobilization.

3.7. Enzyme Immobilization

A standard protocol was established for the immobilization of LipC12 on all supports. One gram of support was suspended in 4 mL of enzyme solution (containing 0.6 mg of protein) in 25 mM NaH_2PO_4 at pH 7, 25 °C and left under mild stirring. The time course of immobilization was evaluated by determining the activity (Section 3.4) in aliquots of the supernatant and suspension removed over time. After the immobilization, the preparations were washed with 25 mM NaH_2PO_4 pH 7.0 and incubated in 4 mL of 25 mM $NaHCO_3$ at different pH values (7.0, 8.5, 10) at 25 °C for 1 h. Finally, the preparations were reduced by adding $NaBH_4$ (1 mg·mL^{-1}) at pH 10 and leaving the mixture under stirring for 30 min.

The immobilization efficiency (IE, %) was calculated as:

$$EI = \frac{A_i - A_f}{A_i} \times 100\% \quad (1)$$

where A_i is the hydrolytic activity (U) of the enzyme solution before immobilization and A_f is the hydrolytic activity (U) remaining in the supernatant at the end of the immobilization procedure.

The recovered activity (R, %) was calculated as:

$$R = \frac{A_o}{A_T} \times 100\% \quad (2)$$

where A_0 is the observed hydrolytic activity the immobilized preparation (U·g^{-1} of support) and A_T is the theoretical activity of the immobilized preparation (U·g^{-1} of support), calculated based on the amount of activity removed from the supernatant during the immobilization procedure.

In some assays, immobilized preparations were treated after reduction with PEG (polyethylene glycol). PEG was used as an additive due to its protective effect on the enzymes described in the literature [30,38]. To assay, 1 g of immobilized preparation was added to 10 mL phosphate buffer pH 7.0 25 mM containing 40% PEG1500 (*w*/*v*). The suspension was stirred for 2 h at 25 °C. After that, the preparation was washed, filtered under using a glass filter, and stored at 4 °C.

3.8. Thermal Stability

The thermal stabilities of free and immobilized LipC12 were assessed by incubation in sodium phosphate buffer (25 mM, pH 7.0) in a water bath at 55 and 80 °C. Inactivation was modeled based on the deactivation theory proposed by Henley and Sadana [19]. Inactivation parameters were determined from the best-fit model of the experimental data which was the one based on a two-stage

series inactivation mechanism with residual activity. Half-life was used to compare the stability of the different preparations, being determined by interpolation from the respective models described in [39].

3.9. Effect of pH and Temperature on the Activity of Free and Immobilized LipC12

The optimum temperature for the activity of free and immobilized LipC12 was determined over the temperature range of 20–90 °C. The effect of pH on the activity was determined over a range of pH 4.0–8.0, at 25 °C, using citrate (pH 4.0–6.0) and phosphate (pH 6.0–8.0) buffers at 25 mM. The activity was determined using *p*-nitrophenyl proprionate (*p*NPP) as substrate (Section 3.4). The activities were calculated in relation to controls that were treated identically, but without enzyme to control of spontaneous hydrolysis of the substrate.

3.10. Hydrolysis of 3,4,6-tri-O-acetyl-D-glucal

For the hydrolysis of peracetylated 3,4,6-tri-*O*-acetyl-D-glucal, 200 mg of immobilized Lipc12 was added to a solution (1.5 mL) of substrate-**1** (1 mM) in 25 mM of phosphate (pH 7.0) or acetate (pH 5.0) buffer. The reaction was carried out at 25 or 4°C, 50 rpm. Samples were removed and analyzed by reverse phase HPLC (Spectra Physic SP 100, Thermo Fisher-Scientific, Waltham, MA, USA) using a Kromasil C18 column (25 cm × 0.4 cm, 5 μm·Ø) and a UV detector (Spectra Physic SP 8450, Thermo Fisher-Scientific, Waltham, MA, USA) set at 220 nm. The mobile phase utilized was acetonitrile (20%) in milli-Q water. The products were characterized and identified as previously described in [24]. Retention times were: 3,4,6-tri-*O*-acetyl-D-glucal **1**-24.6 min, C-3 monodeacetylated **2**-6.3 min, C-6 monodeacetylated **3**-6.6 min and C-4 monodeacetylated **4**-8.1 min. One unit of activity (U) was defined as the hydrolysis of 1 μmol of substrate per hour. Activities were expressed as specific activities (U per mg of immobilized protein). The reutilization of immobilized preparations was studied using the same reaction conditions as described above.

4. Conclusions

Bifunctional supports with aldehyde and different hydrophobic groups have been synthesized. The main advantage of the immobilization protocol developed in the current work is the ease with which the amounts of aldehyde and hydrophobic groups on the surface of the support can be controlled. This enables modulation of immobilization conditions which may be adapted to the immobilization/stabilization of proteins which may be limited in commercial supports. This versatile strategy could also be applied to synthesize supports with other hydrophobic groups to immobilize different lipases, producing catalysts with different properties. These modulated lipase biocatalysts could be used to produce products that are difficult synthesize by traditional methods.

The use of different supports allowed us to obtain immobilized preparations of LipC12 with different activities and stabilities. The best catalyst was 3.5-fold more active and 5000-fold more stable than the soluble enzyme. Thus, the immobilization procedure converted a mesophilic enzyme into an enzyme that can operate at high temperature, with a maximal activity obtained at 70 °C.

The optimal catalyst was used for the regioselective hydrolysis of peracetylated-D-Glucal. The highest yield of the C-3 monodeacetylated product was 69% with a conversion of 81%, at pH 5 and 4 °C using the PEGylated preparation.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4344/6/12/191/s1, Figure S1: SDS-PAGE analyses of the LipC12 purification; Figure S2: Thermal stability of different preparations of LipC12; Figure S3: Hydrolysis of 3,4,6-tri-*O*-acetyl-D-glucal during successive reaction cycles; Table S1: Summary of the purification of LipC12.

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