

Article

Multi-Comobilipases: Co-Immobilizing Lipases with Very Different Stabilities Combining Immobilization via Interfacial Activation and Ion Exchange. The Reuse of the Most Stable Co-Immobilized Enzymes after Inactivation of the Least Stable Ones

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Abstract: The lipases A and B from *Candida antarctica* (CALA and CALB), *Thermomyces lanuginosus* (TLL) or *Rhizomucor miehei* (RML), and the commercial and artificial phospholipase Lecitase ultra (LEU) may be co-immobilized on octyl agarose beads. However, LEU and RML became almost fully inactivated under conditions where CALA, CALB and TLL retained full activity. This means that, to have a five components co-immobilized combi-lipase, we should discard 3 fully active and immobilized enzymes when the other two enzymes are inactivated. To solve this situation, CALA, CALB and TLL have been co-immobilized on octyl-vinyl sulfone agarose beads, coated with polyethylenimine (PEI) and the least stable enzymes, RML and LEU have been co-immobilized over these immobilized enzymes. The coating with PEI is even favorable for the activity of the immobilized enzymes. It was checked that RML and LEU could be released from the enzyme-PEI coated biocatalyst, although this also produced some release of the PEI. That way, a protocol was developed to co-immobilize the five enzymes, in a way that the most stable could be reused after the inactivation of the least stable ones. After RML and LEU inactivation, the combi-biocatalysts were incubated in 0.5 M of ammonium sulfate to release the inactivated enzymes, incubated again with PEI and a new RML and LEU batch could be immobilized, maintaining the activity of the three most stable enzymes for at least five cycles of incubation at pH 7.0 and 60 °C for 3 h, incubation on ammonium sulfate, incubation in PEI and co-immobilization of new enzymes. The effect of the order of co-immobilization of the different enzymes on the co-immobilized biocatalyst activity was also investigated using different substrates, finding that when the most active enzyme versus one substrate was immobilized first (nearer to the surface of the particle), the activity was higher than when this enzyme was co-immobilized last (nearer to the particle core).

Keywords: combilipase; enzyme co-immobilization; reuse of the most stable co-immobilized enzyme; interfacial activation; PEI in enzyme immobilization

1. Introduction

Lipases find applications in many different industrial areas, such as energy, fine chemicals, food and polymer chemistry or as component of detergents [1–8]. This is because they do not require cofactors, are very stable in a variety of reaction media (including organic solvents [9–11] and new media [12–19]) and can recognize a great diversity of substrates (they are quite unspecific enzymes) and simultaneously exhibit a great regio- and enantio-selectivity and/or specificity [20–24], being able to catalyze a huge diversity of reactions, including promiscuous ones [25]. These features place lipases among the most used enzymes in biocatalysis [26–29].

The mechanism of action of lipases, the so-called interfacial activation, is different to that of other esterases [30–32]. The lipase active center is usually isolated from the external medium by a polypeptide chain called lid [33–37]. This lipase “closed” form is in equilibrium with a form where the lid is shifted, exposing a large hydrophobic pocket to the medium, allowing some lipase activity. Via this hydrophobic pocket, the “open” form of the lipases becomes strongly adsorbed on drops of the natural substrates (oils or fats), shifting the conformational equilibrium towards the lipase open form and greatly increasing its activity (the reason for the name of this mechanism) [30–32].

Among the many uses of lipases, we can remark some cascade reactions, like regioselective step by step modification of multifunctional compounds, or full modification of oils. This one includes some of the most important applications of lipases, like the production of free fatty acids via oil and fats hydrolysis [38–40] and of biodiesel via transesterification of those substrates [41,42]. A specific oil actually is a mixture of many different substrates and, during its full transformation, di- and mono-glycerides are formed. Under these circumstances, it is highly unlikely that an individual lipase may be the optimal one for the full transformation of these heterogeneous substrates. That way, it has been shown that the combined use of different lipases, combining different enzyme specificities, selectivities or different sensibility to changes in the reaction conditions, produces a faster reaction and a higher conversion yield, mainly when using these heterofunctional substrates [43–56]. Even the use of the same lipase immobilized following different strategies may permit to get this positive effect [57]. The simultaneous use of several lipases has also proved to be useful in interesterification reactions [58]. Thus, the interest of combilipases is clear. Moreover, in some cases, co-immobilized lipases have been used in these processes [59–64]. However, if the objective of the lipases is a single selective or specific modification of a substrate, the mixture of different lipases, with different selectivities and specificities, will decrease the performance, in these cases a lipase as pure as possible will be recommended. Lipases co-immobilization raised some kinetic advantages in cascade reactions, as all the enzymes are exposed to high concentrations of the intermediate products from the first time of the reaction, eliminating the usual lag time in these processes [65,66]. However, conventional co-immobilization has some problems, like the fact that all enzymes must be immobilized on the same support surface using the same chemistry, and that, if the stabilities of the enzymes greatly differ under operational conditions, all immobilized enzymes must be discarded after the inactivation of the least stable enzyme even when the other enzymes maintain their full initial activities [65,67]. This seems just a technological problem, but it can prevent the industrial implementation of co-immobilized lipases. Usually, different long term enzyme stability problem on the design of co-immobilized enzymes is ignored [65,67].

The hyperactivation of lipases by stabilizing the open form of the enzyme is an objective of many researchers. That way, it may be obtained by preparing crosslinked enzyme aggregates [68] or lyophilizing [69] the enzyme in the presence of detergents, by immobilizing the enzymes in the presence of detergents [70,71], by crosslinking immobilized enzymes in the presence of detergents [72,73], etc. However, the immobilization of lipases on hydrophobic supports is a straightforward way of achieving this stabilization of the open form of lipases [74].

Using lipases, immobilization via interfacial activation on hydrophobic supports is a good solution, as it stabilizes lipases even more than multipoint covalent attachment [75,76], and it also purifies and hyperactivates the lipases [74]. However, even using this immobilization strategy, the problem of the

different enzyme stabilities remained. The support may be reused after inactivation of the least stable enzyme by desorbing all the lipases from the support, but the most stable enzymes will also be desorbed, and only after their further purification, they may be reused in a new co-immobilized biocatalyst batch. This purification will be time consuming and may, in some cases, become very difficult. To solve this problem, the use of acyl-glyoxyl agarose [77,78] and the step by step immobilization of the lipases on them, [79,80] have been proposed. The most stable lipases were covalently immobilized on the octyl-glyoxyl agarose beads after their interfacial activation on the octyl layer [81]. Next, the least stable lipases were immobilized on the reduced biocatalyst (that no longer exhibited chemical reactivity), and these least stable enzymes could be released after their inactivation by incubation of the co-immobilized biocatalyst in detergent solutions after their inactivation. This permitted the reuse of the most stable among the co-immobilized enzymes, but it has two problems: the loading of one specific lipase could be only increased if reducing the loading of the other enzymes, and the use of detergents raised a new problem, as the elimination of the detergent required exhaustive washings [79].

One alternative solution is the immobilization of the most and the least stable lipases using different immobilization strategies (being the last one reversible). For example, the most stable lipases could be immobilized on hydrophobic supports via interfacial activation [81], coated with polyethylenimine (PEI), and then the least stable lipases could be immobilized over this composite via ion exchange. This strategy has been successfully used to co-immobilize a lipase and a β -galactosidase [82]. PEI has many positive effects on enzyme stability [73,83–89], and in the case of lipases, the PEI coating even tends to increase the enzyme activity versus some substrates. Moreover, it may prevent enzyme release of the just physically adsorbed lipase molecules [86,90]. Finally, it has been successfully employed as an adhesive agent in the production of lipase multilayers [91–93].

The release of the least stable enzyme after its inactivation will be performed using concentrated salt solutions, that will not release the lipases immobilized via interfacial activation, simplifying the recovery of the most stable immobilized enzyme biocatalysts [82].

In this paper, we have used different lipases to prepare a multi (five lipase component)-co-immobilized biocatalyst using octyl-vinyl sulfone agarose as immobilization support [94] and PEI as adhesive agent between the two enzyme layers (the ones in contact with the support surface, that will be the most stable enzymes, and the ones in contact with the polymer, that will be the least stable enzymes) [82,91–93]. Octyl-vinyl sulfone (octyl-VS) has been used to prevent enzyme release from the support under any kind of operation conditions. Moreover, the remaining vinyl sulfone groups may be blocked with aspartic acid [94]. This way, we can ensure that a strongly adsorbed layer of PEI on the biocatalysts surface is achieved even if not all the support surface is coated with enzyme molecules. Moreover, this should reinforce the PEI ionic adsorption on the biocatalysts (to reduce PEI release during the incubation in high salt concentration utilized to release the least stable and inactivated enzyme from the biocatalyst) [95]. As enzymes, we have selected some of the most used ones, such as lipases A and B from *Candida antarctica* (CALA and CALB) [96–99], *Thermomyces lanuginosus* (TLL) [100] or *Rhizomucor miehei* (RML) [101,102], and the commercial and artificial phospholipase Lecitase ultra (LEU) [103].

The objective of this new communication is to build co-immobilized biocatalysts where the most stable enzymes can be reused after the least stable enzymes inactivation. For this goal, in a first step, the stabilities of the five immobilized enzymes were compared, and then the most stable ones were immobilized on octyl-vinyl sulfone agarose, blocked with aspartic acid and finally coated with PEI. Then, the least stable enzymes were immobilized via ion exchange on this composite. After the inactivation of the least stable enzymes, it was checked if it was possible to release the inactivated enzymes by incubation in highly concentrated salt solutions without affecting the activity of the most stable and immobilized enzymes. Finally, new batches of the least stable enzymes were immobilized to recover a combi-biocatalyst similar to the initial one.

2. Results and Discussion

2.1. Immobilization and Stabilities of the Different Enzymes on Octyl Agarose

Figure 1 shows the immobilization courses of the 5 enzymes on octyl agarose. Immobilization is complete and very quick in all cases.

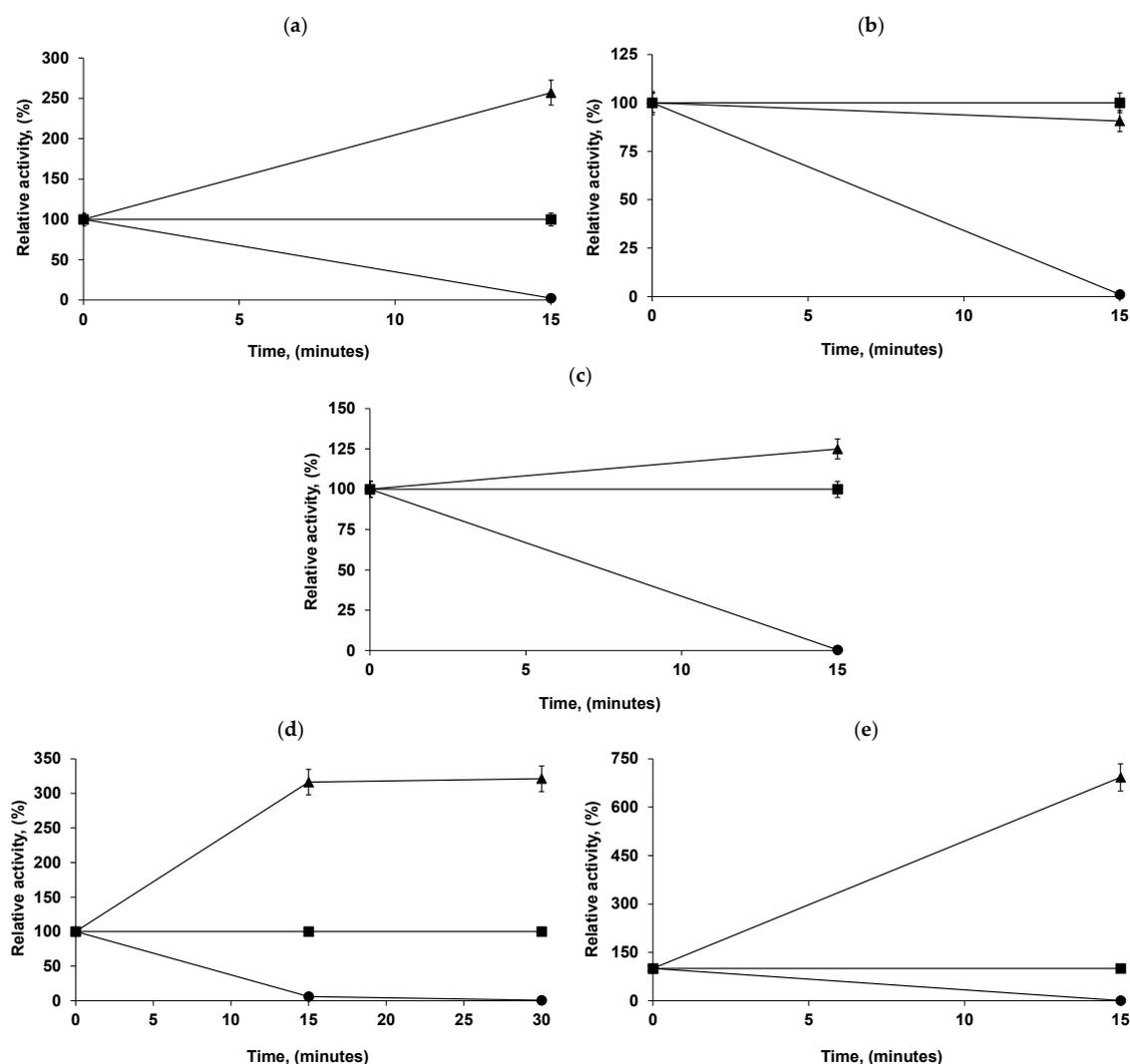


Figure 1. Immobilization courses of different lipases on octyl agarose beads. (a) *Candida antarctica* lipase A (CALA), (b) *Candida antarctica* lipase B (CALB), (c) *Thermomyces lanuginosus* (TLL), (d) Lecitase ultra (LEU) and (e) *Rhizomucor miehei* (RML). Solid squares: reference; solid triangles: suspension and solid circles: supernatant. Other specifications are described in Methods.

As expected, only CALB did not experiment an increase in its *p*-nitrophenyl butyrate (*p*-NPB) activity upon immobilization, but the activity slightly decreased (to 90%). CALA increased its activity upon immobilization by over 2.5-fold, TLL by 1.3, LEU by 3.2, but it is RML the lipase that suffered the highest hyperactivation, reaching an expressed activity of 700% compared to the activity of the free enzyme. The increase in enzyme activity has been related to the mechanism of lipase immobilization on hydrophobic supports: interfacial activation that stabilized the open form of the lipases [74,81]. That way, it was confirmed that it may be possible to build an immobilized combilipase of the 5 lipases using octyl agarose (results not shown). However, this will have interest only in the case that all immobilized enzymes will have similar stabilities. For this purpose, the enzymes were inactivated under several conditions.

Figure 2 shows that at pH 7.0 and 60 °C, octyl-CALA, octyl-CALB and octyl-TLL retained their full activity after 3 h. In that time, octyl-LEU was fully inactivated while octyl-RML retained only around 5% of the initial activity.

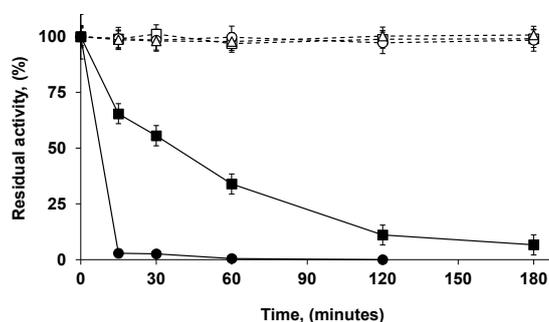


Figure 2. Inactivation courses of the octyl agarose immobilized enzymes at pH 7.0 and 60 °C. Dotted line, empty squares: octyl-CALA; dotted line, empty circles: octyl-CALB; dotted line, empty triangles: octyl-TLL; solid line, solid squares: octyl-RML and solid line, solid circles: octyl-LEU. Other specifications are described in Methods.

That is, we can classify the 5 immobilized enzymes in 2 different groups, enzymes that remain fully active under the inactivation condition (CALA, CALB and TLL), and enzymes that are fully inactivated under those conditions (RML, LEU). This perfectly exemplified the problem of the different enzymes stabilities raised in the Introduction section. An immobilized combilipase of these 5 prepared using octyl agarose will have two enzymes fully inactivated when the other 3 remained fully stable, but the 5 should be discarded if we need to have the 5 component combilipase. That way, we initialized the studies to check if the proposed strategy may be feasible for preparing an immobilized combilipase of the 5 enzymes enabling the reuse of the 3 most stable enzymes when the 2 least stable enzymes are inactivated. Obviously, a co-immobilized combilipase biocatalyst formed by TLL, CALA and CALB and others formed by RML and LEU could be feasible, the problem is just the 5-component biocatalyst.

2.2. Immobilization and Stability of LEU and RML in a Support Coated with PEI (Octyl-VS-PEI Agarose Beads)

The first step of the proposed strategy requires that the two least stable enzymes, in this case LEU and RML, can be immobilized on PEI, exhibiting at least similar properties to those obtained using octyl agarose in terms of stability and activity.

Figure 3 shows the immobilization courses of both enzymes on a support coated with PEI. Some hyperactivation upon immobilization (by 2.5 folds using LEU, by 5 using RML) was observed. These positive effects on enzyme activity remained with a similar intensity after 24 h. These results could be related to the positive effects that the coating with PEI presented when used to coat immobilized LEU or RML [92,93,104].

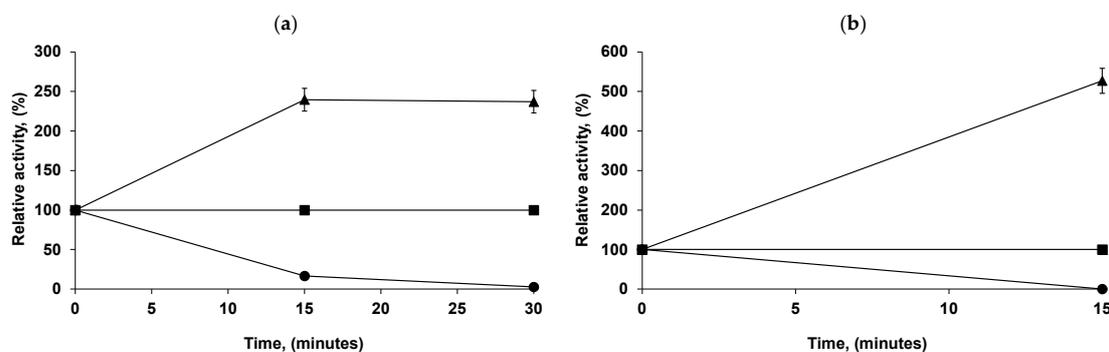


Figure 3. Immobilization courses of different enzymes in octyl-VS-PEI agarose beads. (a) LEU and (b) RML. Solid squares: reference; solid triangles: suspension and solid circles: supernatant. Other specifications are described in Methods.

Next, we checked the stability of the PEI-immobilized enzymes. Figure 4 shows that both enzymes immobilized on PEI coated supports presented a stability even slightly higher than when immobilized on octyl agarose, at least at pH 7.0 and 60 °C. That is, the immobilization of these enzymes on a layer of PEI could be a good alternative for their immobilization considering both, activity and stability of the immobilized enzymes.

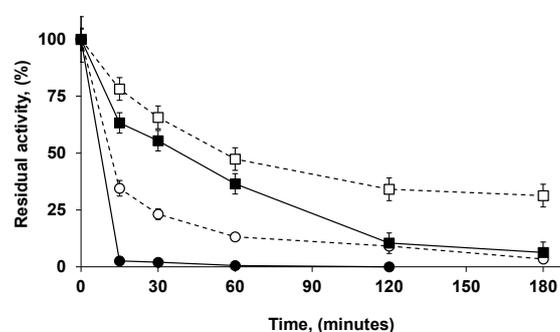


Figure 4. Inactivation courses of different lipase biocatalysts at pH 7.0 and 60 °C. Dotted line, empty squares: octyl-VS-PEI-RML; dotted line, empty circles: octyl-VS-PEI-LEU; solid line, solid squares: octyl-RML and solid line, solid circles: octyl-LEU. Other specifications are described in Methods.

2.3. Covalent Immobilization of CALA, CALB and TLL on Octyl-VS

As explained in the introduction, we intend to use octyl-VS blocked with aspartic acid as supports to build the final 5 immobilized combi-enzymes biocatalysts. To this goal, the enzymes were immobilized at pH 5.0 on octyl-VS (to ensure the immobilization via interfacial activation), and were later incubated at pH 8.0 to get some covalent bonds, following the strategy described in [94] with some modifications (see Methods section). Finally, the biocatalysts were modified with aspartic acid as described in Methods. As shown in Figure 5, the immobilized biocatalysts did not release any enzyme molecule to the medium after this treatment. Thus, when the biocatalysts were boiled in SDS, the SDS-PAGE analysis of the supernatant did not show any enzyme lane, in the 3 cases.

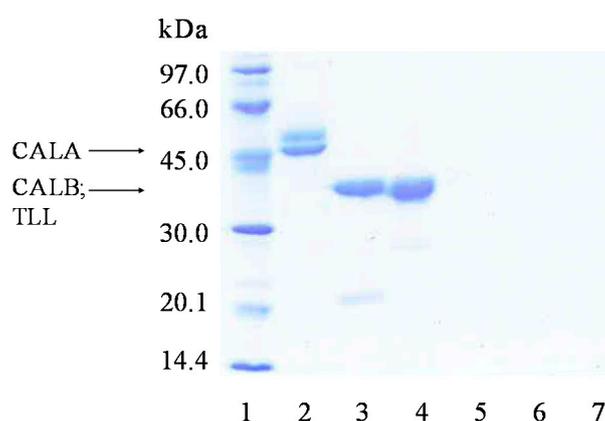


Figure 5. SDS-PAGE analysis of the irreversibly immobilized enzymes. Lane 1: Low weight molecular markers; Lane 2: octyl-CALA; Lane 3: octyl-CALB; Lane 4: octyl-TLL; Lane 5: octyl-VS-CALA; Lane 6: octyl-VS-CALB and Lane 7: octyl-VS-TLL. Other specifications are described in Methods.

Moreover, the 3 enzymes covalently immobilized in octyl-VS remained fully stable at pH 7.0 and 60 °C (not shown results). The coating of the 3 immobilized enzymes with PEI did not affect the activity retention when incubating the biocatalysts at 60 °C and pH 7.0 (the 3 enzymes remained fully active after 3 h) (not shown results). An analysis of the enzyme activity may be found later.

2.4. Immobilization and Desorption of LEU and RML from Octyl-VS-CALA-PEI

Before making the final combilipases, another compulsory point was to confirm that the release LEU and RML from a PEI layer build over an immobilized lipase in a support modified with aspartic acid groups was feasible. To reach this goal, CALA was immobilized on octyl-VS, the biocatalyst was blocked with aspartic acid and finally coated with PEI. Later, LEU and RML were independently immobilized on the support, incubated in growing concentrations of ammonium sulfate and the biocatalysts were analyzed via SDS-PAGE. Figures 6 and 7 show that in both cases, using 0.25 M ammonium sulfate neither LEU nor RML (the only enzymes that can be released to the medium as CALA is covalently attached to the support) could be found in the SDS-PAGE of the biocatalysts.

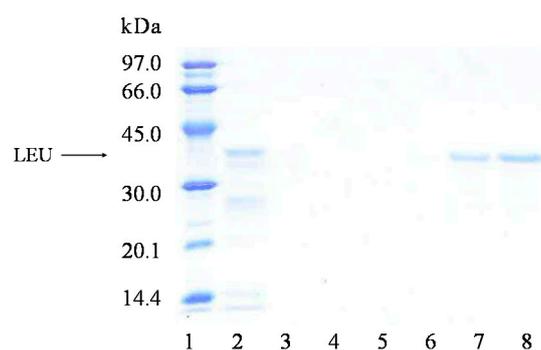


Figure 6. SDS-PAGE analysis of octyl-VS-CALA-PEI-LEU after incubation and washing with solutions having different concentrations of ammonium sulfate (AS). Lane 1: Low weight molecular markers; Lane 2: initial preparation; Lane 3: after incubation in 0.1 M AS; Lane 4: after incubation in 0.25 M AS; Lane 5: after incubation in 0.5 M AS; Lane 6: after incubation in 1.0 M AS; Lane 7: after incubation in 2.0 M AS and Lane 8: after incubation in 4.0 M AS. Other specifications are described in Methods.

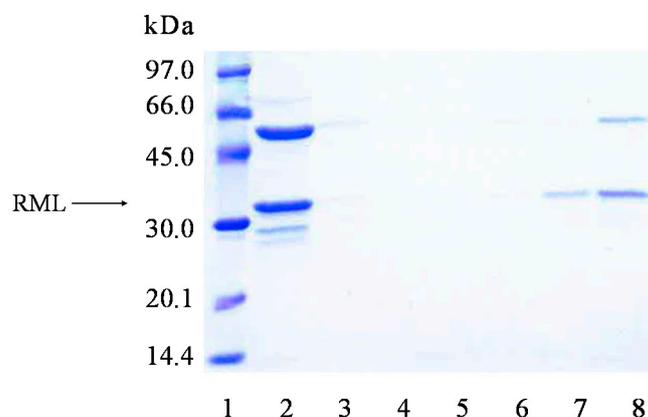


Figure 7. SDS-PAGE analysis of octyl-VS-CALA-PEI-RML after incubation and washing with solutions having different concentrations of ammonium sulfate (AS). Lane 1: Low weight molecular markers; Lane 2: initial preparation; Lane 3: after incubation in 0.1 M AS; Lane 4: after incubation in 0.25 M AS; Lane 5: after incubation in 0.5 M AS; Lane 6: after incubation in 1.0 M AS; Lane 7: after incubation in 2.0 M AS and Lane 8: after incubation in 4.0 M AS. Other specifications are described in Methods.

However, using 1, 2 and 4 M of this compound, it was possible to find some enzyme molecules that remained adsorbed on the support (for LEU the tiny bands make to visualize them difficult). The effects increased when the concentration of ammonium sulfate increased, and this was not described using galactosidase [95]. Therefore, it should be related to the specific biocatalyst or lipases features. One possibility is that some PEI could be released to the medium as occurred using lipases coated with PEI [82,95], even with the presence of aspartic acid in the support, when using very high concentrations of ammonium sulfate, exposing some support surface that could immobilize released

enzyme molecules by interfacial activation. In fact, Table 1 shows that some PEI was released to the medium after the incubation on ammonium sulfate solutions.

Table 1. Effect of the incubation in solutions presenting different concentrations of ammonium sulfate on the PEI that remained attached to the octyl-VS-CALA biocatalyst, determined by titration of the primary amino groups using 2,4,6-trinitrobenzene sulfonic acid (TNBSA) (see Material and Methods).

Ammonium Sulfate, (M)	PEI on the Support, (%)
0.1	98
0.25	86
0.5	83
1	84
2	79
4	78

Another possibility is that at these high ionic strengths, PEI may become more hydrophobic than the medium and permit lipase interfacial activation (some authors state the hydrophobic character of the PEI bone). Whatever the reason (this is under study in our laboratory), we selected 0.5 M of ammonium sulfate to release the least stable enzymes from the biocatalysts.

2.5. Building of the Combi-lipases

After checking the different pieces of the puzzle to confirm the feasibility of the proposed strategy of building a biocatalyst in a way that permits the reuse of the most stable enzymes after inactivation of the least stable ones, we prepared two different combi-biocatalysts, based on a different order in the co-immobilization of the enzymes. As immobilization on octyl agarose and on PEI is very rapid, we expected that the order of the enzyme immobilization may be related to the order of the enzymes in the support, forming different enzyme crowns, and therefore, considering the different activities of the enzymes versus different compounds, may give different final activities depending on the immobilization order, by altering the diffusional limitations problems [105–111]. The first immobilized enzyme will be on the external areas of the support pores, the last will be nearer to the core of the support particle [112].

Figure 8 shows the preparation of the two biocatalysts. In all cases, the enzymes immobilization was completed in a very short time. A detailed effect on the biocatalysts' activity will be discussed later (Tables 2–4).

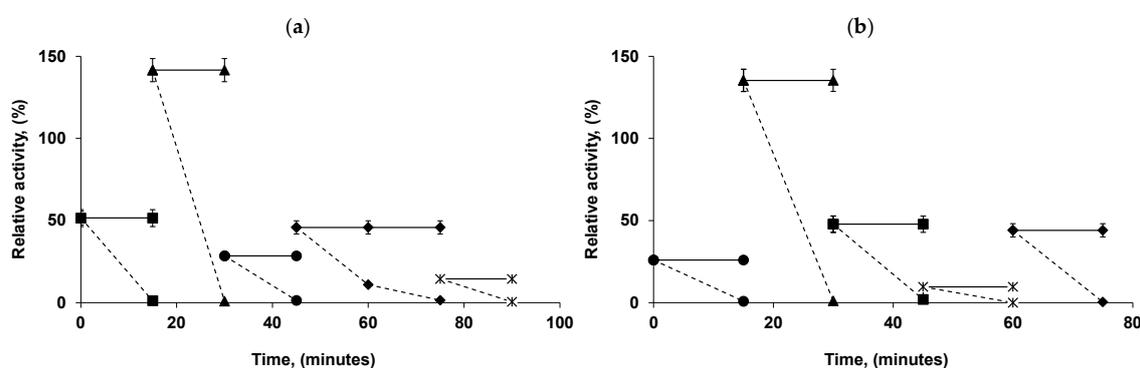


Figure 8. Activities evolution of the supernatants of the immobilization suspensions during the preparation of multi-combi-lipases. (a) Octyl-VS-CALA-TLL-CALB-PEI-LEU-RML and (b) octyl-VS-CALB-TLL-CALA-PEI-RML-LEU. Solid line: reference lipase solution; dotted line: supernatant. Squares: CALA; triangles: TLL; circles: CALB; rhombus: LEU and X: RML. Other specifications are described in Methods.

Table 2 shows the activities of the different individual biocatalysts.

Table 2. Activities of the different individual lipase immobilized biocatalysts versus different substrates. Activities are given as U/g. Activities were determined as stated in Methods.

Biocatalyst	Substrate			
	<i>p</i> -NPB	Triacetin	(<i>R</i>)-Methyl Mandelate	(<i>S</i>)-Methyl Mandelate
Octyl-CALA	102.67 ± 5.14	0.90 ± 0.04	0.255 ± 0.013	0.251 ± 0.013
Octyl-VS-CALA	106.00 ± 5.35	1.29 ± 0.07	0.092 ± 0.005	0.064 ± 0.003
Octyl-CALB	20.57 ± 1.03	8.60 ± 0.43	35.797 ± 1.790	4.680 ± 0.234
Octyl-VS-CALB	22.79 ± 1.15	8.16 ± 0.41	34.708 ± 1.738	4.215 ± 0.211
Octyl-TLL	148.31 ± 7.55	34.27 ± 1.72	0.041 ± 0.002	0.045 ± 0.002
Octyl-VS-TLL	114.16 ± 5.72	32.13 ± 1.71	0.016 ± 0.001	0.025 ± 0.001
Octyl-LEU	97.22 ± 4.82	6.47 ± 0.31	0.026 ± 0.001	0.041 ± 0.020
Octyl-VS-PEI-LEU	114.67 ± 5.75	20.97 ± 1.08	0.126 ± 0.006	0.135 ± 0.006
Octyl-RML	69.36 ± 3.52	21.49 ± 1.20	0.038 ± 0.002	0.038 ± 0.002
Octyl-VS-PEI-RML	65.68 ± 3.28	27.94 ± 1.35	0.292 ± 0.015	0.261 ± 0.013

Table 3. Activities of the different lipase immobilized or co-immobilized biocatalysts during the construction of Octyl-VS-CALA-TLL-CALB-PEI-LEU-RML versus different substrates. Activities are given as U/g. Activities were determined as stated in Methods.

Biocatalyst	Substrate			
	<i>p</i> -NPB	Triacetin	(<i>R</i>)-Methyl Mandelate	(<i>S</i>)-Methyl Mandelate
Octyl-VS-CALA	108.82 ± 5.10	1.21 ± 0.06	0.094 ± 0.004	0.061 ± 0.003
Octyl-VS-CALA-TLL	136.37 ± 6.82	37.21 ± 1.86	0.109 ± 0.005	0.099 ± 0.005
Octyl-VS-CALA-TLL-CALB	134.49 ± 5.53	40.22 ± 1.90	31.447 ± 1.472	4.080 ± 0.215
Octyl-VS-CALA-TLL-CALB-PEI	138.36 ± 6.81	40.92 ± 2.12	39.901 ± 2.015	4.253 ± 0.223
Octyl-VS-CALA-TLL-CALB-PEI-LEU	153.64 ± 7.70	47.54 ± 2.23	36.643 ± 1.865	4.320 ± 0.224
Octyl-VS-CALA-TLL-CALB-PEI-LEU-RML	176.52 ± 8.83	60.74 ± 3.10	37.078 ± 1.855	4.602 ± 0.235

Table 4. Activities of the different lipase immobilized or co-immobilized biocatalysts during the construction of Octyl-VS-CALB-TLL-CALA-PEI-RML-LEU versus different substrates. Activities are given as U/g. Activities were determined as stated in Methods.

Biocatalyst	Substrate			
	<i>p</i> -NPB	Triacetin	(<i>R</i>)-Methyl Mandelate	(<i>S</i>)-Methyl Mandelate
Octyl-VS-CALB	21.26 ± 1.08	7.88 ± 0.45	34.708 ± 1.738	4.104 ± 0.224
Octyl-VS-CALB-TLL	93.83 ± 4.72	34.36 ± 1.79	35.605 ± 1.753	3.928 ± 0.195
Octyl-VS-CALB-TLL-CALA	115.45 ± 5.83	34.09 ± 1.35	34.942 ± 1.497	3.909 ± 0.190
Octyl-VS-CALB-TLL-CALA-PEI	118.75 ± 5.95	30.75 ± 1.40	35.331 ± 1.698	4.144 ± 0.210
Octyl-VS-CALB-TLL-CALA-PEI-RML	126.23 ± 6.32	39.54 ± 2.89	33.978 ± 1.707	4.131 ± 0.205
Octyl-VS-CALB-TLL-CALA-PEI-RML-LEU	166.73 ± 8.50	56.46 ± 3.05	34.790 ± 2.005	4.310 ± 0.221

Using *p*-NPB, the most active enzyme when immobilized on octyl agarose was TLL, shortly followed by CALA and CALB was clearly the least active enzyme (7 folds less active than TLL). Using triacetin, TLL remained the most active, but CALA became the least active (by more than 30 folds). Finally, using both methyl mandelate esters, CALB was the most active preparation while TLL become the least active one, with a very low activity compared to CALB (more than 1000-fold less active than CALB with (*R*)-methyl mandelate). CALB prefers to hydrolyze the (*R*)-isomer while the other two enzymes have scarcer enantiospecificity.

Changing the support by octyl-VS and blocking the biocatalysts with aspartic acid, CALA activity versus *p*-NPB remains almost unaltered, but it increased by more than 40% using triacetin and decreased to around 25% using (*S*)-methyl mandelate and to 36% using the (*R*)-isomer. CALB almost did not alter the activity versus any of the substrates while TLL decreased the activity versus *p*-NPB (to less than 80%) and both isomers of methyl mandelate (to 40% using the (*R*)-isomer and to 55% using the (*S*)-isomer). That is, the covalent immobilization effects on enzyme activity were different depending on the substrate and enzyme, as it has been described in many different papers [66,113–118].

The immobilization of LEU on PEI coated support even gave slightly higher activity values than the enzyme immobilized on octyl using *p*-NPB, the difference was more relevant using triacetin (LEU immobilized in PEI agarose was 3.2 folds more active than when immobilized on octyl agarose) and became very important using both enantiomers of methyl mandelate. In fact, using the (*R*)-isomer the activity increased by 4-fold. RML immobilized on PEI gave similar activity values to that when immobilized on octyl agarose using *p*-NPB, increased using triacetin (by a 30%) and both enantiomers of methyl mandelate (by almost 7 folds).

That way, the immobilizations following the proposed strategies were in general positive for the enzyme activity even compared with the use of octyl agarose.

Next, we will discuss in detail the activity evolution during the building of the combi-biocatalyst octyl-VS-CALA-TLL-CALB (Table 3).

When co-immobilizing CALA and TLL, using *p*-NPB, the final activity is below the expected value, very likely because of the high activity of both enzymes using this substrate, and the addition of CALB did not increase the observed activity. Using triacetin, the immobilization of TLL on the immobilized CALA biocatalyst produced an increase of activity similar to the expected value, as CALA activity versus this substrate is very poor. The immobilization of CALB on these two-component combi-biocatalysts has scarce effect on the biocatalysts' activity versus triacetin, very likely once again due to substrate diffusional limitations. Using both isomers of methyl mandelate, the co-immobilization of CALA and TLL produced activities similar at the expected ones, as the activity with these substrates is very poor for both enzymes and diffusional problems will be irrelevant. When co-immobilizing CALB, the activity was slightly under the values observed in the only CALB preparations, perhaps due to the increase in the diffusional problems, as in this instance CALB is in the core of the biocatalyst, and this increases the diffusional limitations of the substrate to reach this enzyme. In any case the discrepancies regarding the expected activity are small, and mainly relevant using the (*R*)-isomer as CALB activity with this enantiomer is much higher and diffusional limitations may become more relevant. The difference in activity with the CALB biocatalysts immobilized alone is only around 10%.

The coating of this three-component co-immobilized combi-pase with PEI has also diverse effects depending on the substrate, in agreement with previous positive effects reported of this treatment for the activity of these immobilized enzymes in octyl agarose [91–93]. PEI modification of many immobilized enzymes is a usual tool to improve enzyme performance, as stated in the introduction [83]. Using *p*-NPB as substrate, the enzyme activity remains almost constant, using triacetin the activity increased by around a 6%, using (*S*)-methyl mandelate activity almost remains constant, but using the (*R*)-isomer, the activity increased by more than a 25% (Table 3). That way, the enzyme PEI coating is not only not negative for enzyme activity, but it produced a general activity increase, even if it should increase the diffusional limitations, suggesting an improvement of the enzymes' specific activity. Next, LEU was immobilized. This produced an increase of the hydrolytic activity versus *p*-NPB and triacetin, although lower than the expected value by the activity of PEI-LEU (Table 2). This could be caused by an increase in the diffusional problems of the substrate to reach the enzymes under the PEI layer, as LEU will reduce the mobility of the polymeric bead at least in the area where the enzyme is immobilized increasing the tortuosity of the substrate path towards the immobilized enzyme active center [105–111]. Moreover, the pore diameter will be also reduced [119]. If the substrate was any of the methyl mandelate esters, the activity of the final biocatalysts was slightly reduced: LEU was not very active versus this substrate and their immobilization will make CALB less accessible to this substrate, the most active enzyme with it.

The immobilization of RML on the octyl-VS-CALA-TLL-CALB-PEI-LEU biocatalyst promoted a relevant increase in the activity of the biocatalysts versus *p*-NPB and triacetin (Table 3), although the biocatalysts showed an activity similar versus these substrates to the addition of the activities of the activity of LEU and RML immobilized on PEI polymeric bed (Table 2), becoming far from the expected value of the activity that the five enzymes should express versus this substrate. This may be explained by the increase of the substrate diffusional limitations: the biocatalyst pore diameter will be smaller,

the tortuosity of the substrate path to reach the enzymes under the PEI layer will be higher [105–111]. Activity versus both methyl mandelate esters slightly increased after RML immobilization on the four components combilipase immobilized biocatalyst.

Table 4 shows the building of the alternative 5 component combi-biocatalyst. In this, CALB is the first immobilized enzyme, and later TLL is immobilized.

TLL and CALB co-immobilization increased the activity versus *p*-NPB of the immobilized combi-enzyme, but not to the levels that even the individual TLL offered. Again, the reason for this result may be due to the promotion of diffusional problems, larger in the case of this substrate due to the solubility problems that reduced the concentrations that it is possible to use. Using triacetin, the value of activity also clearly increases way, almost to the expected value (only 15% less than the addition of the activity of immobilized CALB and TLL). Using both isomers of methyl mandelate, the co-immobilization of TLL had not relevant effects, as CALB is by far more active than TLL with these substrates.

Next, CALA was co-immobilized on octyl-VS-CALB-TLL. Using *p*-NPB, the biocatalysts activity increased by 20%, much less than the value expected from the activity of CALA with this substrate (again, this may be attributed to an increase in diffusional limitations with the enzyme being immobilized near the core of the agarose particles) [105–111]. The activity versus triacetin and methyl mandelate esters was just maintained after CALA co-immobilization. The coating with PEI of the octyl-VS-CALB-TLL-CALA had almost no effect on enzyme activity versus any of the substrates.

Comparing the two co-immobilized combilipases activities, octyl-VS-CALB-TLL-CALA was less active using *p*-NPB and triacetin as substrate, and more active using (*R*)-methyl mandelate when compared to octyl-VS-CALA-TLL-CALB. This could fit with the location of the enzyme exhibiting the highest activity versus each substrate. When it is at the mouth of the pore, it will suffer lower substrate diffusional limitations than if the enzyme is in the core of the particle [105–111]. Differences are not so large as to confirm the existence of concentric enzyme crowns, but results suggested this possibility.

The immobilization of RML on octyl-VS-CALB-TLL-CALA-PEI increased the activity versus triacetin or *p*-NPB (as usual, not to the expected levels), but slightly decreased the activity with the methyl mandelate esters, perhaps because the substrate had more difficulties in reaching the most active enzyme versus this substrate (CALB). This is clearer with (*R*)-methyl mandelate, where CALB activity is higher. The immobilization of LEU increased the activity versus all the substrates (Table 4), but except for the methyl mandelate substrates, in a lower value that the expected one (Table 2). Octyl-VS-CALB-TLL-CALA-PEI-RML-LEU was slightly less active than octyl-VS-CALA-TLL-CALB-PEI-LEU-RML versus all substrates (differences did not exceed 12%), suggesting that the order of the enzyme immobilization really affects the activity of the biocatalysts with the different substrates, but not in a very relevant way.

2.6. Reuse of the Most Stable Immobilized Enzymes after the Inactivation of the Least Stable Co-Immobilized Enzymes

Figure 9 shows the inactivation courses of octyl-VS-CALA-TLL-CALB-PEI, octyl-VS-CALB-TLL-CALA-PEI, octyl-VS-PEI-RML-LEU and octyl-VS-PEI-LEU-RML at 60 °C and pH 7.0.

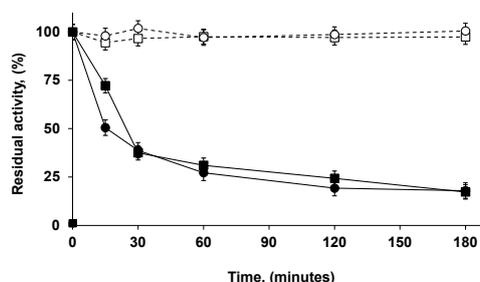


Figure 9. Inactivation courses of some co-immobilized biocatalysts at pH 7.0 and 60 °C. Dotted line, empty squares: octyl-VS-CALA-TLL-CALB-PEI; dotted line, empty circles: octyl-VS-CALB-TLL-CALA-PEI; solid line, solid squares: octyl-VS-PEI-RML-LEU and solid line, solid circles: octyl-VS-PEI-LEU-RML. Other specifications are described in Methods.

It may be confirmed that the activity of the combi-biocatalyst formed by the co-immobilization of the 3 most stable enzymes is fully maintained after 3 h, while the less stable enzymes become quickly inactivated and after 30 min 50% of the activity is left. There are some differences in the inactivation between both less stable enzyme combi-biocatalyst, but not relevant enough to be able to extract some conclusions on the importance of the order of immobilization in the apparent inactivation kinetics. Figure 9 shows that after all the treatments, the enzymes stability differences were maintained. That way, we performed the inactivation of the 2 combilipases co-immobilized enzymes (Figure 10) by incubating the biocatalysts for 180 min at 60 °C and pH 7.0, conditions where LEU and RML were almost fully inactivated. Then, the combi-biocatalysts were washed with 0.5 M of ammonium sulfate at pH 7.0 to release the inactivated LEU and RML. Following the results from Table 1, that indicated some PEI release, the biocatalysts were incubated again in PEI to ensure a similar coating of the biocatalysts with PEI, and then fresh LEU and RML were added.

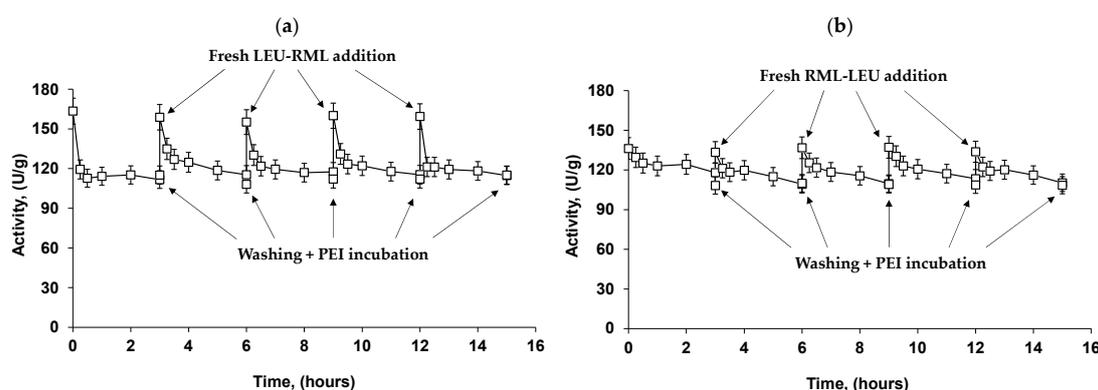


Figure 10. Cycles of inactivation, incubation and washing with ammonium sulfate, incubation with PEI and reloading of the least stable lipases of the combilipases. The steps are indicated by the arrows. (a) Octyl-VS-CALA-TLL-CALB-PEI-LEU-RML and (b) octyl-VS-CALB-TLL-CALA-PEI-RML-LEU. Inactivations have been performed by incubation at pH 7.0 and 60 °C. Other specifications are described in Methods.

As it can be visualized in the figures, this process could be repeated for at least 5 cycles with similar values of activity after thermal enzyme inactivation, washing and PEI coating of the 3 component biocatalysts and reloading with fresh LEU and RML. That way, the strategy may be considered successful, as the most stable enzymes could be reused after inactivation of the least stable enzymes.

3. Materials and Methods

3.1. Materials

Different commercial liquid lipase formulations were used in this study: NovoCor[®] ADL (lipase A from *Candida antarctica*, 18.75 mg of protein/mL); Lipozyme[®] CALB L (lipase B from *Candida antarctica*, 12 mg of protein/mL); Lipozyme[®] TL 100 L (lipase from *Thermomyces lanuginosus*, 30.4 mg of protein/mL); Palatase[®] 20,000 L (lipase from *Rhizomucor miehei*, 3.77 mg of protein/mL) and Lecitase[®] Ultra, an artificial chimeric phospholipase [103] (18.75 mg protein/mL) were kindly donated by Novozymes (Alcobendas, Spain). Bradford's method was used to quantify the protein concentration [120], employing bovine serum albumin as reference. Octyl-Sepharose[®] CL-4B beads and low molecular weight (LMW) calibration kit for SDS electrophoresis (14.4–97 kDa) were acquired from GE Healthcare (Alcobendas, Spain). Branched polyethylenimine (Mw 10,000) was from Polysciences Europe (Warrington, UK). *p*-Nitrophenyl butyrate, triacetin and L-aspartic acid was purchased from Sigma Aldrich Spain (Madrid, Spain). Ammonium sulfate, divinyl sulfone, (*R*)-(-) and (*S*)-(+)-methyl mandelate and 2,4,6-trinitrobenzene sulfonic acid (TNBSA) solution were from Thermo Fisher scientific Spain, (Madrid, Spain). All other reagents and solvents were of analytical grade.

3.2. Methods

All experiments were performed by triplicate and the results are reported as their mean values and the standard deviation.

3.2.1. Preparation of Octyl-Vinyl Sulfone Support (Octyl-VS)

A modification of the protocol described by Albuquerque et. al. [94] was performed. 15 mL of divinyl sulfone (final concentration of 0.35 M) was added to 400 mL of 333 mM sodium carbonate at pH 11.5 and stirred with a magnetic stirring bar until the solution turned homogeneous. Then, 20 g of octyl agarose beads was added and left under gentle agitation for 2 h. Finally, the support was vacuum filtered using a sintered glass funnel, washed with an excess of distilled water and stored at 6–8 °C.

3.2.2. Immobilization of Lipases on Octyl Agarose Beads

The immobilization of lipases on octyl agarose support was performed by interfacial activation [121], using 1 mg of enzyme per gram of wet support. The lipase liquid commercial solutions were diluted in 5 mM sodium phosphate at pH 7.0 and 25 °C (10 mL) and subsequently added to the support (1 g). The immobilization was conducted under gentle mechanical stirring and the immobilization course was measured using *p*-NPB as substrate by taking samples of the immobilization suspension, immobilization supernatant and a reference solution (an enzyme solution prepared under identical conditions) at different time intervals to calculate immobilization yield and expressed activity [122]. After immobilization, the biocatalysts were vacuum filtered using a sintered glass funnel, washed with distilled water and stored at 6–8 °C.

3.2.3. Immobilization of the Least Stable Enzymes on Octyl-VS-PEI Support

First, the PEI support was prepared. Octyl-VS support was blocked with a solution of 2 M aspartic acid at pH 8.0 overnight at room temperature (10 mL of acid aspartic solution per 1 g of support), vacuum filtered using a sintered glass funnel and washed with abundant distilled water. Then the aspartic blocked support was incubated with a solution of 10% (*w/v*) of PEI at pH 7.0 and 4 °C in a proportion of 10 mL of PEI solution per 1 g of support during 18 h under gently stirring, obtaining octyl-VS-PEI support. Finally, the support was vacuum filtered using a sintered glass funnel, washed with an excess of distilled water and stored at 6–8 °C. The least stable lipases were immobilized on octyl-VS-PEI support by ion exchange, using 1.5 mg of each enzyme per gram of wet biocatalyst. The lipase liquid commercial solutions were diluted in 5 mM sodium phosphate at pH 7.0 and 25 °C (10 mL) and subsequently added to the support (1 g). For the sequential immobilization, the immobilization suspensions after first enzyme immobilization were vacuum filtered and washed with 5 mM sodium phosphate at pH 7.0, and then the second enzyme was offered. The immobilization was conducted under gently mechanical stirring and the immobilization course was measured using *p*-NPB. After immobilization, the biocatalysts were vacuum filtered using a sintered glass funnel, washed with distilled water and stored at 6–8 °C.

3.2.4. Co-Immobilized Biocatalysts Preparation

Two different immobilized lipase combi-biocatalysts were prepared by varying the order of the sequential immobilization of the most stable enzymes on octyl-VS support. After the coating of the co-immobilized enzymes with PEI, the least stable lipases were sequentially immobilized varying the order in each combi-biocatalyst. The enzyme order in the name of the biocatalyst is the order in which they had been immobilized.

3.2.4.1. Immobilization of the Most Stable Lipases on Octyl-VS Support

The most stable lipases were immobilized on octyl-VS support by interfacial activation, using 1 mg of each enzyme per g of wet support. The lipase liquid commercial solutions were diluted in 5 mM

sodium acetate at pH 5.0 and 25 °C (10 mL) and subsequently added to the support (1 g). For the sequential immobilization, the suspensions were vacuum filtered using a sintered glass funnel and washed with 5 mM sodium acetate at pH 5.0 after the immobilization of each enzyme, before offering the next one. The immobilization was conducted under gentle mechanical stirring and the immobilization course was followed using *p*-NPB as substrate. After the immobilization, the biocatalysts were vacuum filtered using a sintered glass funnel, washed with distilled water and resuspended in 50 mM sodium bicarbonate at pH 8.0 and 25 °C for 4 h, to favor the enzyme-support covalent reaction (maintaining the relation 10 mL of buffer solution by 1 g of support). Finally, the octyl-VS biocatalysts were blocked by incubating them in 2 M aspartic acid at pH 8.0 and 25 °C for 16 h (10 mL of blocking solution per 1 g of biocatalysts). The covalently immobilized and blocked biocatalysts were vacuum filtered using a sintered glass funnel, washed with abundant distilled water and stored at 6–8 °C.

3.2.4.2. Coating of Immobilized Enzymes with PEI

The lipase biocatalysts prepared as described in Section 3.2.4.1 were treated with a solution of 10% (*w/v*) of PEI at pH 7.0 and 4 °C in a proportion of 1 g per 10 mL of PEI solution during 18 h, producing octyl-VS-enzyme-PEI biocatalysts. Afterwards, the biocatalysts were vacuum filtered using a sintered glass funnel, washed with abundant distilled water and stored at 6–8 °C.

3.2.4.3. Immobilization of the Least Stable Lipases on Octyl-VS-Enzyme-PEI Biocatalysts

The least stable lipases were immobilized on octyl-VS-enzyme-PEI biocatalyst by ion exchange, using 1.5 mg of each enzyme per gram of wet biocatalyst. The lipase liquid commercial solutions were diluted in 5 mM sodium phosphate at pH 7.0 and 25 °C (10 mL) and subsequently added to the support (1 g). The immobilization suspensions were vacuum filtered using a sintered glass funnel and washed with 5 mM sodium phosphate pH 7.0 after the immobilization of the first immobilized enzyme, and the second immobilized enzyme was offered. The immobilization was conducted under gently mechanical stirring and the immobilization course was measured using *p*-NPB. After immobilization, the biocatalysts were vacuum filtered using a sintered glass funnel, washed with distilled water and stored at 6–8 °C.

3.2.5. Determination of Enzyme Activity

One enzyme activity unit (U) was defined as μmol of substrate hydrolyzed per minute under the described conditions.

3.2.5.1. Hydrolysis of *p*-NPB

The enzymatic activity was determined by measuring the increase in the absorbance at 348 nm caused by the *p*-nitrophenol released during the hydrolysis of *p*-NPB (isosbestic point, ϵ under these conditions is $5150 \text{ M}^{-1} \text{ cm}^{-1}$ [123]) using a Jasco spectrophotometer (V-730) (Jasco, Madrid, Spain). 50 μL of 50 mM *p*-NPB (dissolved in acetonitrile) was added into 2.5 mL of 25 mM sodium phosphate at pH 7.0 and 25 °C. The reaction was initialized by adding 50 μL of sample (free or immobilized enzyme) under magnetic stirring and thermostatization.

3.2.5.2. Hydrolysis of Triacetin

A mass of 0.05 g of wet biocatalysts were added to 1–5 mL of 50 mM of triacetin in 50 mM sodium phosphate at pH 7.0 and 25 °C, under continuous gently stirring using a roller mixer (Tube Roller MXT6S, Scilogex, CT, USA). Under these reaction conditions, the enzyme product, 1,2 diacetin, suffers acyl migration and a mixture with 1,3 diacetin is obtained [124]. The different reaction products were determined by HPLC, Jasco UV 15–75 (Jasco, Madrid, Spain) under conditions where both diacetins co-eluted. The column was a HPLC Kromasil C18 (15 cm \times 0.46 cm) (Análisis Vinicos, Tomelloso, Spain) and a solution of 15% acetonitrile/85% Milli-Q water at 25 °C was used as mobile phase with a

flow rate of 1 mL/min. The compounds detection was performed at 230 nm, injecting samples of 20 μ L and the retention times were about 4 min for diacetins and 18 min for triacetin. Conversions between 15–20% were used to calculate the initial reaction rates.

3.2.5.3. Hydrolysis of (R)- or (S)-Methyl Mandelate

A mass of 0.05 g of wet biocatalyst was added to 0.5–10 mL of 50 mM of (R)- or (S)-methyl mandelate in 50 mM of sodium phosphate at pH 7.0 and 25 °C, under gently stirring using a roller mixer (Tube Roller MXT6S, Scilogex, CT, USA). The products of the reaction were determined by HPLC Jasco UV 15–75 (Jasco, Madrid, Spain). The column was a HPLC Kromasil C18 (15 cm \times 0.46 cm) (Analisis Vinicos, Tomelloso, Spain) and a solution of 35% acetonitrile/65% Milli-Q water with 10 mM of ammonium acetate at pH 2.8 at 25 °C was used as mobile phase with a flow rate of 1 mL/min. The compounds detection was performed at 230 nm by injecting reaction samples of 20 μ L. The retention times were about 2.4 min for mandelic acid and 4.2 min for methyl mandelate. Conversions between 15–20% were used to calculate the initial reaction rates.

3.2.6. Lipase Biocatalysts Thermal Inactivations

Biocatalysts were incubated in 50 mM Tris HCL at pH 7.0 and 60 °C. The pH was adjusted at 25 °C, thus some changes in the real pH value may be expected at 60 °C, although this should not be relevant for our purposes. Tris HCL was used as buffer to avoid the deleterious effects of phosphate buffer on lipase stability [125,126]. Periodically, samples were withdrawn and their residual activities were measured using the *p*-NPB assay described above. Residual activities were calculated as the percentage of the biocatalysts' initial activity.

3.2.7. Desorption of the Least Stable Lipases from the Supports

One g of the octyl-VS-enzyme-PEI-enzyme biocatalysts was incubated in 10 mL solutions of ammonium sulfate at different concentrations (0.1 M, 0.25 M, 0.5 M, 1.0 M, 2.0 M and 4.0 M) in 50 mM Tris HCL at pH 7.0 and 25 °C for 2 h. Afterwards, the biocatalysts were vacuum filtered using a sintered glass funnel, washed 4 times using 20 mL of ammonium sulfate solution per gram of biocatalysts and finally washed with abundant distilled water.

3.2.8. Analysis of the Immobilized Enzymes by SDS-PAGE

SDS-polyacrylamide gel electrophoresis (PAGE) analyses were performed according to Laemmli [127], using a 5% polyacrylamide gel as concentration gel and a 12% polyacrylamide as resolution gel. Before the SDS-PAGE, the immobilized enzymes were diluted in rupture buffer (8% (*w/v*) SDS and 10% mercaptoethanol (*v/v*)), calculating a maximum final protein concentration of 0.3–0.5 mg of protein per mL solution, the samples were boiled for 8 min and centrifuged. This treatment released all enzyme molecules not covalently attached to the support [77]. Finally, 15 μ L aliquots of the obtained supernatants and 8 μ L of LMW marker were loaded in the gel. The current was 100 V. The gels were stained with Coomassie brilliant blue.

3.2.9. Titration of Primary Amino Groups in the Biocatalysts

A quantity of 0.5 g of octyl-VS-Enzyme-PEI biocatalyst was incubated in 5 mL of 17.5 mM TNBSA solution on 100 mM sodium carbonate for one hour at 25 °C and pH 8.0 and under gently mechanical stirring. Then the biocatalysts were vacuum filtered using a sintered glass funnel, washed with distilled water and finally washed with 100 mM sodium carbonate pH 8.0. 0.25 g of samples was resuspended on 2.5 mL of 100 mM sodium carbonate at pH 8.0. The biocatalysts absorbances were measured at 590 nm under magnetic stirring. The respective samples not incubated with TNBSA were used as blanks.

3.2.10. Reuses of the Multi-Combilipases

After the inactivation of the least stable lipases, the biocatalysts were incubated with 0.5 M ammonium sulfate as described in Section 3.2.7. Then, the washed biocatalysts were treated with a solution of PEI as explained in Section 3.2.4.2 and finally, a new batch of the least stable lipases was immobilized as described in Section 3.2.4.3.

4. Conclusions

This paper shows the production of combilipases co-immobilized in the same particle. We have clearly demonstrated the problem of the different enzymes' stabilities when co-immobilizing enzymes, even using enzymes traditionally considered very stable ones such as lipases. Using traditional co-immobilization protocols, this difference will make necessary to discard immobilized and fully active enzymes because the least stable ones have been inactivated. Octyl, octyl-VS or even PEI coated supports may be very interesting methods to co-immobilize several enzymes, and if grouped by stability, they may be good co-immobilization lipase strategies (in the case of our enzymes, LEU and RML could be co-immobilized in one particle, CALB, CALA and TLL in other). However, we have shown how combining different immobilization strategies, the most and least stable enzymes may be co-immobilized and the most stable enzymes may be reused after the inactivation of the least stable ones. Thus, this difference in stability of the co-immobilized enzymes is an actual problem that should be considered in the sign of co-immobilized biocatalysts, not only focused on the reuse of the most stable enzyme, but even in the activity relation between the most and the least stable enzymes. The paper shows, as many others [66,113–118], how different immobilization protocols may alter enzyme specificity. Moreover, it gives some clues, which need to be confirmed, on how the order of the co-immobilization of enzymes may give biocatalysts with different activity properties, mainly when diffusional limitations may be raised and the activity of the co-immobilized enzymes differ each other with different substrates [112,128]. Another interesting question that is opened in this communication is to make a deeper analysis on the reasons that cause that the use of a very high ionic strength on these biocatalysts is not convenient to release the enzymes immobilized following the presented strategy, problem that did not exist using galactosidase.

We have used 5 different lipasic enzymes, but it may be considered that this strategy could be extended to any number of enzymes, as long as the enzymes may be classified in two stability ranges. If the range of enzyme stabilities may be divided in three clearly different groups, this strategy will not be enough to avoid the problem of the discarding of fully active enzymes, as some enzymes will remain active with the other two groups have been inactivated. That is, the development of strategies that permits to have several levels of selective enzyme release seem still necessary.

Author Contributions: S.A.-P. and D.C. performed the experiments; R.F.-L. supervised the research and developed the concepts; V.C.C. helped in the support characterization; V.C.C. and R.F.-L. wrote the first draft of the paper, all authors helped in the final editing of the paper. All authors have read and agree to the published version of the manuscript.

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