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CLEAs of *Candida antarctica* lipase B (CALB) with a bovine serum albumin (BSA) cofeeder core: Study of their catalytic activity

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ABSTRACT

Highly active CALB cross-linked enzyme aggregates (CLEAs) were synthesized using a layered methodology based on the synthesis of a cross-linked protein cofeeder core over which an external layer of lipase was later cross-linked. The layered CALB CLEAs were characterized in terms of their catalytic activity in three different test reactions: esterification of oleic acid and ethanol in absence of solvents, esterification of oleic acid and heptanol in organic medium, and hydrolysis of triolein in emulsioned medium. The impact of the cross-linker/protein mass ratio on CLEAs activity, and its evolution with storage time were evaluated in the solventless synthesis of ethyloleate. The amount of cross-linker used showed to be a key parameter for the evolution of the catalytic activity of CLEAs during storage. Under the best conditions found, hyperactivated CALB CLEAs with up to 188% of recovered activity in ethyl oleate synthesis were obtained. In terms of hydrolytic activity mature layered CALB CLEAs showed a retained activity of 68%. The assay of dried mature layered CALB CLEAs in heptyl oleate synthesis showed catalytic activities much higher than the one exhibited by free CALB, reaching 1 h-fatty acid conversions of 14% and 2%, respectively. The high catalytic activity shown by layered CALB CLEAs, suggests that they are an interesting alternative specially for the catalysis of fatty acid esterifications in both organic and solventless medium.

1. Introduction

In the last decade, cross-linked enzyme aggregates (CLEAs) have emerged as an improved, effective and low cost method of lipase immobilization [1]. CLEAs are obtained by cross-linking of physical enzyme aggregates that result from the addition of organic solvents, non-ionic polymers or salts to an aqueous solution of proteins. The cross-linking step gives a more stable superstructure to the aggregates by establishing covalent bonds between enzymatic protein molecules, which render them permanently insoluble [2]. Cross-linked enzyme aggregates present several interesting advantages over carrier-bound immobilized enzymes, such as highly concentrated enzymatic activity, high stability of the produced superstructure, and that no previous purification of the enzyme is needed.

In the last years, the CLEAs methodology has been applied to different enzymes including penicillin acylase, esterases, trypsin, oxynitrilases, lipases, nitrilases, galactosidase, catalase, lacasses, etc. [3]. In the field of lipases (EC 3.1.1.3), a family of enzymes able to catalyze fats hydrolysis, as well as esterification, transesterification, alcoholysis and aminolysis reactions; CLEAs methodology has demonstrated to lead to active and stable biocatalysts. In the early work of Lopez-Serrano and co-workers [4], authors synthesized CLEAs of seven commercial lipases with more than ten-fold increase in activity respect to native enzyme. Since then, a number of lipases have been made into active CLEAs, including others lipases such as those from *Candida rugosa*, *Burkholderia cepacia*, *C. antarctica* A, *C. antarctica* B, *Pseudomonas* sp., and *Thermomyces lanuginosa* [5–11].

C. antarctica B lipase (CALB), and in particular its commercial immobilized form Novozym 435, are well-recognized active biocatalysts for fatty acid esterifications and, in a lesser extent, ester hydrolysis [12–17]. CLEAs of *C. antarctica* B lipase obtained by aggregation followed by cross-linking have been synthesized

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previously. In 2007, Sheldon et al. [18] reported on the synthesis of CLEAs of CALB with higher activities in both aqueous and organic media than the ones exhibited by the commercial biocatalyst Novozym 435. The activity of CLEAs of CALB in supercritic carbon dioxide and ionic liquids has also been assayed [19].

As briefly introduced before, traditional CLEAs synthesis implies the formation of enzyme aggregates by use of an aggregating agent, followed by cross-linking of the precipitated enzymes. However, during the formation of covalent bonds, the cross-linker may bind amino acids associated with the active site of the catalytically active enzyme, and thus reduce the residual activity of CLEAs. The previous drawback can be prevented using molecules known as protein feeders/co-feeders which are characterized by the presence of a large amount of lysine residues in their surface, to which the cross-linker may bind and thus avoid the loss of activity of the catalytically active protein. Addition of bovine serum albumin (BSA) is known to facilitate CLEAs preparation in cases in which the protein concentration of the enzyme preparation is low and/or the enzyme activity is vulnerable to high concentrations of cross-linker [20,21]. A number of recent contributions have shown the benefits of using BSA as protein co-feeder in the synthesis of CLEAs of different lipases, which have been expressed in terms of increased retained activity and/or increased stability of the CLEAs by more effective cross-linking steps [11,20,22]. However, results of our group in terms of retained activity of CLEAs of *T. lanuginosa* (TLL) lipase showed that even if BSA addition increased

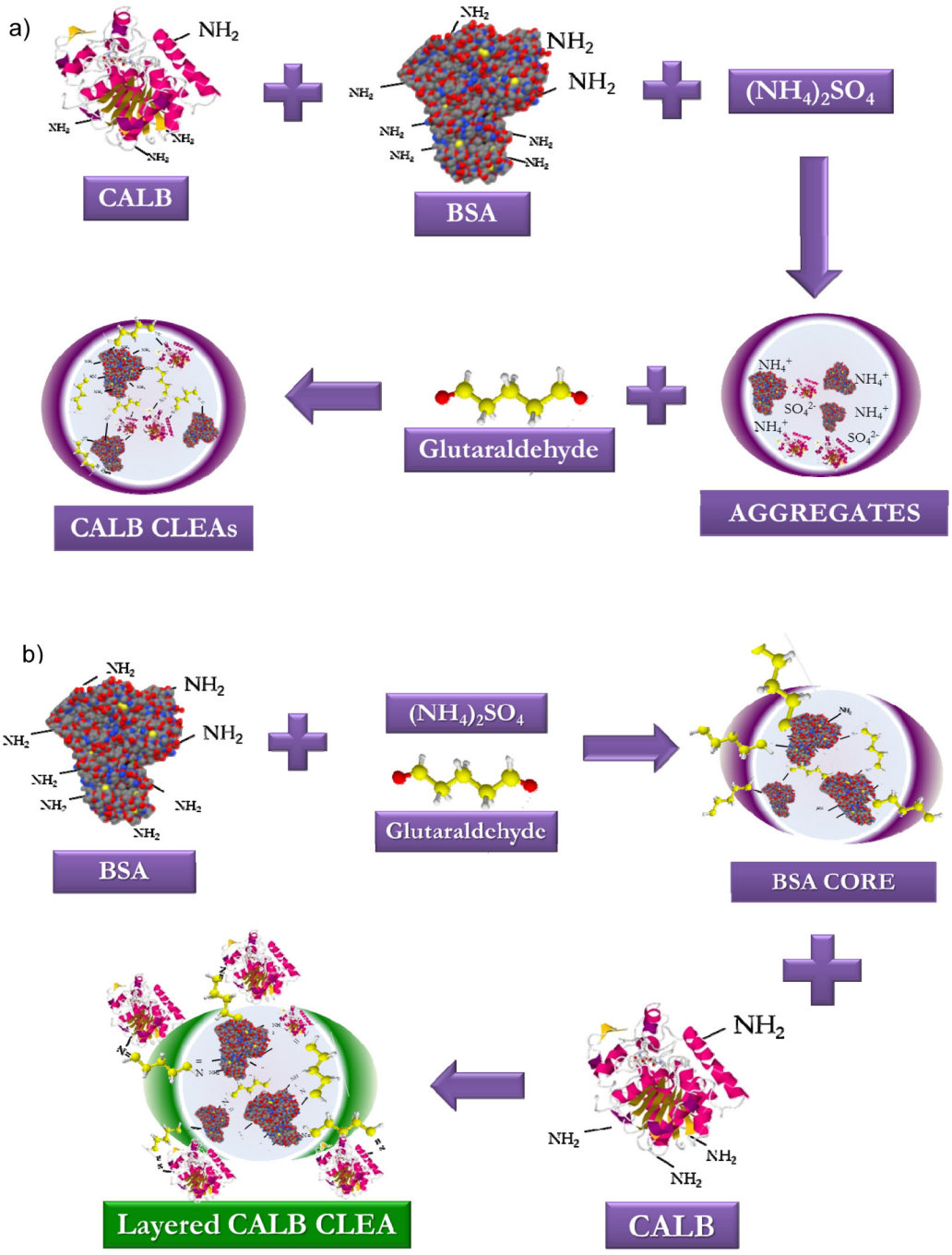


Fig. 1. Synthesis of CALB CLEAs. (a) Traditional approach; (b) layered approach. The images of BSA and CALB were obtained from the Protein Data Bank [23,24].

the retained activity of CLEAs, absolute specific activity values were far from those measured for free lipase (i.e. between 20% and 30% with respect to free lipase) [11]. The previous was explained in terms of the occlusion of lipase by BSA during CLEAs synthesis, which remained in the interior of the biocatalyst with evident mass transfer restrictions. Considering the much higher content of lysine residues of BSA with respect to TLL (60 vs 7), the lipase may be trapped in the interior of BSA aggregates, with reduced accessibility to substrate contact. To overcome the drawbacks of traditional BSA addition, a layered CLEAs synthesis approach was designed [11]. Layered CLEAs consist of a cross-linked protein cofeeder core (i.e. BSA) over which one or more layers of protein cofeeder/lipase are succesively cross-linked. The much higher retained activity of layered CLEAs of TLL when compared with their non-layered CLEAs counterparts, suggested that the addition of lipase only once BSA core has been formed provides the benefits of the use of a protein co-feeder but without occluding lipase [11].

In the current contribution, layered CLEAs of lipase from *C. antarctica* B are synthesized for the first time following the same procedure described for TLL. Since CALB is added to the synthesis medium only once BSA aggregates have been formed and cross-linked, CALB is expected to become slightly cross-linked over the BSA cores by the residual amount of cross-linker added. Fig. 1 schematizes a proposal of the synthesis of CALB CLEAs by the traditional versus the layered approach (Figs. 1a and b, respectively).

The catalytic activity of layered CALB CLEAs is characterized in three different test reactions. The first reaction chosen to assay CLEAs activity is the synthesis of ethyl oleate by esterification of oleic acid and ethanol in absence of solvents. Ethyl oleate is a fatty ester with wide application in cosmetic and food additives industries, in the production of tailored triglycerides, in diesel fuel additives, in the pharmaceutical industry as a solvent for lipophilic drugs such as esteroids, and as stationary phase in gaseous chromatography [25,26]. Solvent-free media assure highly concentrated systems in which even low conversions lead to high product recovery. The impact of the mass of cross-linker (glutaraldehyde) in the activity of layered CLEAs of CALB in the described reaction is studied. Maturation (storage time effect) of layered CALB CLEAs is also evaluated. Moreover, considering that sugars have previously been used as additives for lipases homologous with CALB [27], the capacity of dextrose to improve the stability and/or activity of layered CALB CLEAs is also investigated. Aiming to further characterize the catalytic activity of layered CALB CLEAs the ones with highest activity in ethyl oleate synthesis are further assayed in the synthesis of heptyl oleate in organic medium and in the hydrolysis of triolein in emulsioned medium, as representatives of reactions in which lipases are frequently employed.

2. Materials and methods

2.1. Materials

Commercial solution of *C. antarctica* lipase B (CALB 5000 U/mL; 300 mg precipitable protein (PP)/mL) was kindly donated by Novozymes, Brazil. Bovine serum albumin (BSA; 30%, w/v) was purchased from Wiener Lab (Argentina). Glutaraldehyde solution (25%, v/v) was obtained from Fluka. Oleic acid, phenolphthalein and potassium hydroxide (KOH) were bought to Anedra. Ethanol (99%), n-heptane (95%) and diethyl ether (99%) were from Dorwill and ammonium sulphate ($(NH_4)_2SO_4$) was purchased from Cicarelli. Dextrose was obtained from Anedra. Heptanol (98%), Triton X-100, Tris–HCl (1 M) buffer and triolein (65%) were purchased from Sigma Aldrich. All reactants used were analytical grade.

2.2. Determination of precipitable protein (PP) content in commercial enzyme solutions

The methodology used for determining the concentration of precipitable protein in commercial enzyme solutions has been reported before [28]. Briefly, ammonium sulphate solution (100% saturation, 4 °C) was poured into appropriate vessels containing 1 mL of the commercial enzymatic solution of CALB until complete precipitation took place. Variable volumes of the ammonium sulphate solution were used, from 1:1 to 1:6 enzyme solution:salt solution (v/v). Precipitation was performed in an ice bath with gentle magnetic stirring during 5 h. The obtained precipitates were recovered and dried in vacuum at ambient temperature until constant weight was verified. Minimal lipase activity measured in the remaining supernatant, as well as minimal coprecipitated salt presence in the precipitates (scanning electron microscopy observations), allowed finding a very good estimation of the concentration of PP in the CALB commercial enzyme preparation (i.e. 300 mgPP/mL [28]).

2.3. CLEAs synthesis

BSA (500 μL, 30%, w/v) was precipitated with a saturated solution of $(NH_4)_2SO_4$ (1.4 mL, 500 mg/mL) and cross-linked with variable quantities of glutaraldehyde. The synthesis was maintained for 2 h in an agitated ice bath, while a core of BSA was stabilized. Then, CALB (240 μL, containing 72 mg of PP) was slowly added and the conditions were maintained for additional 45 min. The mass of cross-linker used in the synthesis of the CLEAs accounted for the following cross-linker/PP mass relationships: 1.11, 1.39, and 1.67 (mg of cross-linker per mg of PP from CALB). Under the conditions chosen, the salt/PP mass ratio was in all cases 9.7 mg/mg. After this period, the suspension was centrifuged at 6000 rpm for 5 min at room temperature. The recovered one-layered CLEAs were washed three times with distilled water (500 μL) and the supernatant and washing solutions were conserved for subsequent activity determinations. The synthesized CALB CLEAs were stored as wet solids at 4 °C and their activities were measured at 0, 1 and 4 days from preparation for the different glutaraldehyde amounts used. For the cross-linker amount which led to the highest recovered activity, the synthesis protocol was scaled up to 360 μL of CALB (containing 108 mg of PP), in order to produce enough mass of CLEAs to allow activity determinations also after 8 and 17 days of maturation. The effect of the addition of dextrose on CLEAs activity/stability was evaluated by use of a protocol analogous to the procedure described previously. However, at the moment of lipase addition 80 μL of a dextrose solution (1.6 mg/μL) was also added. Cross-linking conditions were maintained for 45 min, and recovery operations were similar to those previously described.

2.4. Test reactions

2.4.1. Solvent-free ethyl oleate synthesis

The reaction mixture included oleic acid (3 g), ethanol (0.5 g) added in stoichiometric ratio, and water (20%, w/w considering the weight of oleic acid). Proper aliquots of CLEAs, supernatants or washing solutions were alternatively added to the reaction vial, which was kept at 45 °C with constant magnetic stirring (350 rpm) for 3 h. After that time, 10 mL of a mixture ethanol/ether (50%, v/v) was added in order to stop the reaction. The esterification was monitored every hour by titration of duplicate withdrawn samples with KOH using phenolphthalein as end-point indicator. The accurate

sampling from the biphasic system generated has been previously optimized by our group [29].

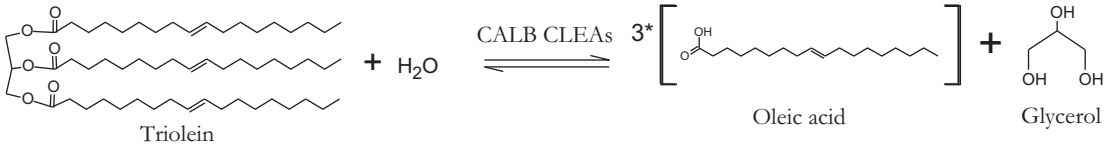
(1)

2.4.2. Heptyl oleate synthesis in organic medium

The assay conditions were adapted from those reported by Domínguez de María et al. [30], who proposed the synthesis of heptyl oleate in n-heptane as a proper test reaction for the assay of the esterification activity of lipases and their differentiation with esterases. The reaction mixture consisted of stoichiometric quantities (i.e. 210 mM) of heptanol (0.5 g) and oleic acid (1.2 g) in a total volume of 20 mL in n-heptane. Proper aliquots of CLEAs, supernatants or washing solutions were alternatively added to the reaction vial, which was kept at 30 °C with constant magnetic stirring (650 rpm) during 24 h. At selected times 10 mL of a mixture ethanol/ether (50%, v/v) was added in order to stop the reaction. The esterification level was determined by titration of duplicate withdrawn samples with KOH using phenolphthalein as end-point indicator.

(2)

Triolein hydrolysis in emulsioned medium: The hydrolysis of triolein used as test reaction was performed following a procedure adapted from Rocha et al. [31]. The reaction mixture consisted of triolein (0.5 g), triton X-100 (2.5 g), buffer Tris–HCl 1 M dilution 0.1 in water (5 g), and distilled water (2 g). The pH of this mixture was 5.5. The mixture was pre-incubated at 45 °C for 10 min with continuous stirring at 1000 rpm. Then, a proper amount of the target sample (commercial enzyme solution, synthesized CLEAs, residual supernatant, or recovered washing solutions) was added and the reaction started. After 5 min of reaction, hydrolysis was quenched by addition of ethanol (20 mL). Enzymatic activity was calculated by determination of liberated fatty acid through titration with 0.05 N KOH of duplicate samples and using phenolphthalein as end-point indicator.



(3)

2.5. Calculation of the recovered activity of CLEAs

The percentage of recovered activity retained in layered CALB CLEAs is herein defined as the ratio between specific activity of CALB CLEAs and the specific activity of free CALB (μmol/min × mg), both measured under identical reaction conditions. A rigorous protocol to determine specific activities in CLEAs has been published recently by our group, with particular focus on a proper estimation of the protein content of CLEAs [28]. Briefly, quantification of total precipitable protein present in CLEAs requires the use of a mass balance which explicitly considers the fraction of protein that remained in the supernatant of the CLEAs synthesis medium, as well as the fraction of protein that was washed away during the washing steps performed during CLEAs recovery. These values are obtained from activity determinations of target aliquots performed in linear regions of conversion-time and activity-protein mass/concentration data (which need to be determined ad hoc for each reaction test used) [28]. Considering the total volume of supernatant and washings, their PP content is calculated. Subtraction of this value from the total amount of PP used for CLEAs synthesis gives the amount of precipitable protein remaining in CLEAs. Once activity determinations of CLEAs and free lipase are performed using aliquots that fall within linear ranges of activity/mass of PP data, their specific activity – and thus CLEAs recovered activity – can be easily obtained. A value of specific activity of CLEAs 100% implies that the synthesized CLEAs have the same specific activity than free CALB.

3. Results and discussion

3.1. Activity of layered CLEAs of CALB in ethyl oleate synthesis in solventless medium

The activity retained by CALB CLEAs in ethyl oleate synthesis was calculated as described in Section 2.5. To do so, kinetic data from reactions catalyzed by increasing contents of free CALB were obtained and assay conditions were chosen based in the maximum values of the linear regions (i.e. 60 min of reaction, 18 mg of PP – data not shown). Fig. 2 shows the results of the recovered activity of fresh CALB CLEAs prepared with different amounts of glutaraldehyde, as a ratio of their specific activity to the specific activity of free CALB (1.7 μmol/min × mg) measured under identical assay conditions. The mass of cross-linker used for the synthesis of CLEAs accounted for cross-linker/PP from CALB mass ratios of 1.11, 1.39 and 1.67. As it is shown in Fig. 2, all CLEAs showed higher specific activity than free CALB, which is evidenced by retained activity values higher than 100%. The previous means that under the synthesis conditions chosen, the layered CALB CLEAs perform better than free lipase, which may be assigned to a more active conformation of lipase in the aggregates. The possibility of obtaining hyperactive CLEAs of lipases is well documented [4,32,33]. Hyperactivation shown by lipase CLEAs has generally been attributed to the lipase being induced to adopt a more active conformation [32,33]. Hyperactivation of CALB shown in the esterification of fatty acids in solventless medium upon formation of CLEAs has been reported previously. Prabhavathi et al. (2009) studied the synthetic activity retained by CALB CLEAs aggregated by different precipitating reagents. The reaction test chosen was the solvent-free esterification of lauric acid with n-propanol. Among the CLEAs of CALB studied, those obtained using PEG600, ammonium sulfate, PEG200 and acetone as precipitants were observed to attain over 200% total activity recovery in comparison with acetone powder directly precipitated from the liquid solution by acetone [34]. Activity yields of CALB CLEAs that exceeded that of native CALB in hydrolytic test reactions have also been reported [4,35]. CLEA preparations of CALB which performed better than Novozym 435 in both aqueous and organic systems have been reported [35].

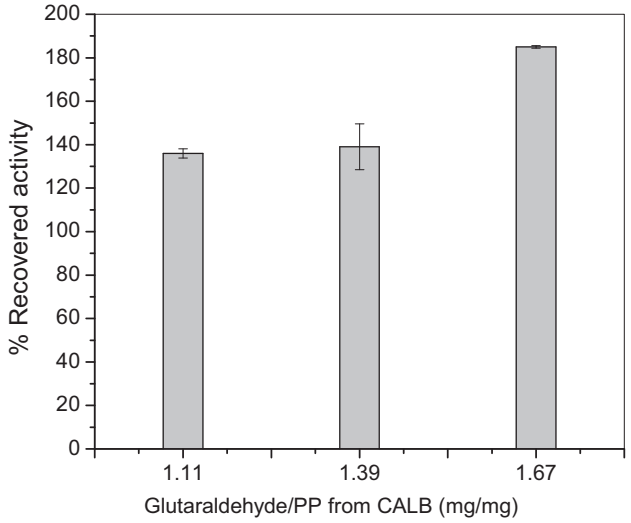


Fig. 2. Recovered activity of fresh CALB CLEAs prepared with different glutaraldehyde/PP ratios (w/w) when assayed in the solvent-free synthesis of ethyl oleate. Reaction conditions are detailed in Section 2.4.

Among the layered CALB CLEAs synthesized in the current contribution, the highest recovered activity was found for the aggregates cross-linked with 1.67 mg of glutaraldehyde per mg of PP from CALB, which retained 185% when compared with free CALB. In the case of CLEAs cross-linked with a cross-linker/PP mass ratio of 1.11 the amount of solid CLEAs recovered was much lower than expected, indicating that the mass of cross-linker used was not enough to render all aggregates permanently insoluble. In the case of using an even lower ratio (i.e. 0.55 mg of glutaraldehyde/mg of PP) in the synthesis, no solid could be recovered. These results can be explained in terms of an ineffective stabilization of the aggregates through covalent bonds at low cross-linker loadings.

The synthesized CALB CLEAs were stored wet at 4 °C without solution present. The recovered esterification activity of CLEAs synthesized with different glutaraldehyde amounts after 0, 24 and 96 h of storage was assayed in order to determine the stability of the high activity values measured in fresh CLEAs. Fig. 3 illustrates that although fresh CLEAs showed highest activity when 1.67 mg

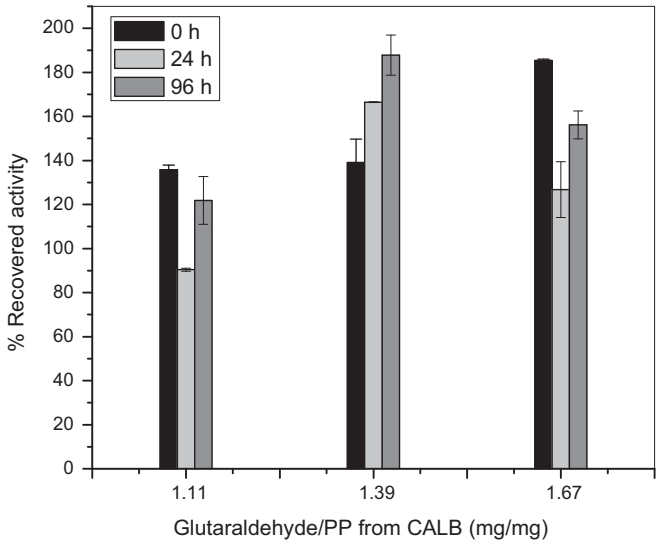


Fig. 3. Effect of storage time on the evolution of the recovered activity of CALB CLEAs prepared with different glutaraldehyde/PP ratios (w/w). Test reaction: solvent-free synthesis of ethyl oleate. Reaction conditions are detailed in Section 2.4.

of glutaraldehyde per mg of PP from CALB were used (Fig. 2), later activity data evidences that storage time induced conformational changes in lipase that impacted differently on the evolution of activity depending on the amount of cross-linker used. In the case of CLEAs synthesized with a ratio of 1.11 mg of glutaraldehyde/PP from CALB, the highest recovered activity (136%) was the one measured immediately after synthesis. After 24 h of storage at 4 °C, the recovered activity dropped to 90% of the specific activity measured for free CALB under identical conditions. However, after 96 h of maturation, CLEAs activity raised to 122%, exceeding once again that of free CALB. The same type of profile was observed for the retained activity of the CLEAs synthesized with a cross-linker/PP ratio of 1.67, although absolute values were higher than those obtained for a ratio of 1.11 mg/mg (fresh CLEA: 185%; 24 h: 156%). Finally, CLEAs obtained with a cross-linker/PP ratio of 1.39 showed a different behavior, in which maturation time continuously increased the specific activity of the prepared biocatalysts. Although fresh CLEAs showed 139% of retained activity, after 96 h of wet storage at 4 °C the retained activity raised to 188% with respect to free CALB, giving the highest measured recovered activity. Evidently, conformational changes induced during storage/maturation are dependent on the synthesis protocol, which in this case is exemplified by the effect of the amount of cross-linker used. Covalent links induced during aggregates cross-linking, have shown to play a key role in the evolution of biocatalyst activity as a function of conformational changes that occur during storage time. In view of the previous results, CLEAs synthesized with a cross-linker/PP mass ratio of 1.39 were assayed after 8 and 17 days of maturation, giving 130% and 125% of recovered activity, respectively. Similar values obtained after 8 and 17 days of maturation suggest that conformational changes during storage had stopped.

As shown in Fig. 3, storage time played a key role in the activity exhibited by CLEAs. Slow conformational changes suffered by CALB due to the cross-linking with glutaraldehyde/rearrangements that the lipase suffers during storage, may justify the variations found in CLEAs activity during storage time. Maturation efficiency also showed to be highly dependent on the amount of cross-linker used, which further suggests that conformational changes suffered by lipase are due to cross-linker presence. Fig. 4 schematizes a proposal of the reactions that may have taken place during CALB CLEAs storage. As schematized in the left side of Fig. 4, it is hypothesized that in the CLEAs surface there are remaining reactive glutaraldehyde molecules (dashed-dotted line). During storage time, the glutaraldehyde molecules on the CLEAs surface may react with amine residues of the lipase surface positioned close to the active site, and thus induce changes in its conformation that could modify (positively/negatively) lipase activity (dotted line, upper right-side scheme of CLEAs). Moreover, even if no activity-sensitive residues of the lipase are particularly affected by new links produced during storage, the generation of excessive glutaraldehyde-lipase linkage points could restrict lipase flexibility and thus affect its catalytic activity. Glutaraldehyde molecules on the CLEAs surface may also react with the amine residues of the albumin surface (full line, lower right-side scheme of CLEAs) without significantly affecting the activity retained by CLEAs.

The addition of dextrose did not show the beneficial results expected. After 17 days of maturation, optimum layered CLEAs synthesized without dextrose had 125% of recovered activity, whereas CLEAs which included dextrose retained only 60% of the specific activity of free lipase. Results may be explained in terms of a possible reduction of lipase activity due to hydrogen bonds established between the hydroxyl groups of dextrose and polar side chains of aminoacids of the oxyanion hole of CALB (Thr40; Asp134; Gln157). Somashekar et al. [36] found that lipases from *Rhizomucor miehei* and *C. rugosa* were inhibited by D-glucose, a phenomenon which

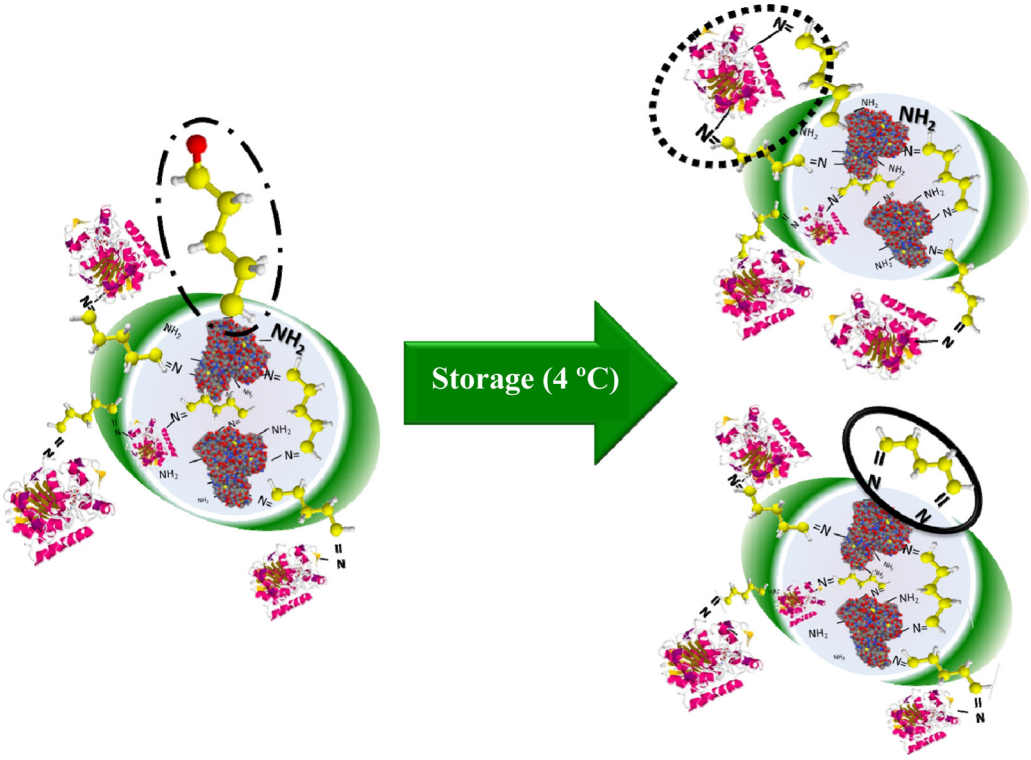


Fig. 4. Proposal of the reactions that may have occurred during CALB CLEAs storage.

they assigned to possible hydrogen bonding of the hydroxyl groups of the sugar with certain oxyanion residues of the lipases.

Solvent-free systems are highly concentrated media, economically and operationally interesting for industrial processes. In this kind of systems not only the cost of the solvent itself is avoided, but also its separation from un-reacted substrates and products. Moreover, smaller reactors can be used; and since reactants are present at high concentration, the mass/moles of product generated by reaction are usually high even if conversion is moderate. To illustrate the previous for the current system, the productivity expressed as total μmol of ester produced per mg of protein present in CLEAs, was calculated. Productivity values were calculated for reaction times of 60, 120 and 180 min; for the synthesis catalyzed by mature CLEAs (storage time = 96 h) prepared with different cross-linker/PP from CALB mass ratios and without dextrose addition. Results are shown in Fig. 5.

As expected for a linear function of conversion, the productivity of the reaction for CLEAs increases with reaction time, until constant values are achieved. Once again the best option also in terms of productivity is the mature CALB CLEA synthesized with a glutaraldehyde/PP ratio of 1.39 which leads to the maximum productivity measured in 120 min of reaction (268 $\mu mol/mg_{PP}$). Similar productivities are also achieved for CLEAs synthesized with the other cross-linker/PP ratios used, but 180 min of reaction are required.

3.2. Activity of layered CLEAs of CALB in heptyl oleate synthesis in organic solvent medium

Aiming to explore the synthetic activity of layered CLEAs of CALB in another fatty acid esterification in less concentrated medium, the biocatalyst was assayed in the synthesis of heptyl oleate in organic solvent. Compared with the first synthetic medium assayed (esterification of oleic acid and ethanol in solventless medium), heptyl oleate is herein synthesized in a medium with lower substrate concentration and much lower viscosity. Moreover, the use of an alcohol with higher molecular weight and in lower concentration is expected to induce a lower inhibitory effect on CALB. Mature layered CLEAs of CALB (cross-linked/mgPP mass ratio = 1.39) were assayed in heptyl oleate synthesis in both dried and wet state. Photographs of dried and wet layered CALB CLEAs are shown in Fig. 6. Dried CLEAs were obtained upon drying of wet CLEAs (synthesis is described in Section 2.3) in a conventional oven at 30 °C until 86–90% of the initial water content had been removed. Results of the evolution of heptyl oleate synthesis catalyzed by free CALB, mature humid layered CALB CLEAs, and mature dried layered CALB

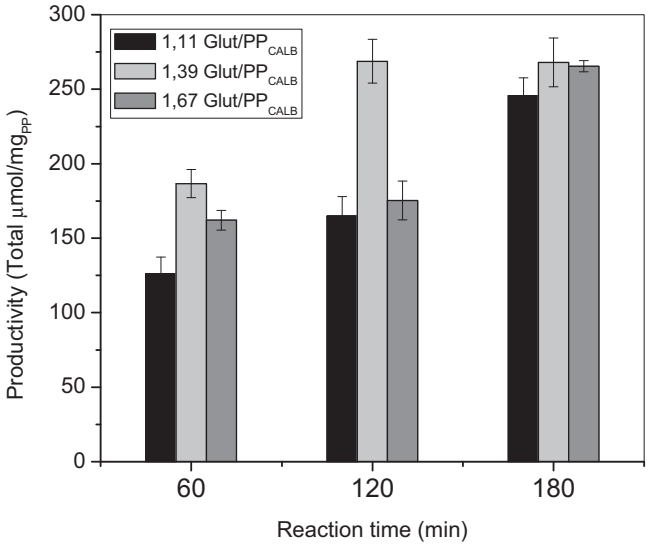


Fig. 5. Ethyl oleate synthesis productivity as a function of time catalyzed by mature CALB CLEAs prepared with different glutaraldehyde/PP ratios (w/w). Reaction conditions are detailed in Section 2.4.

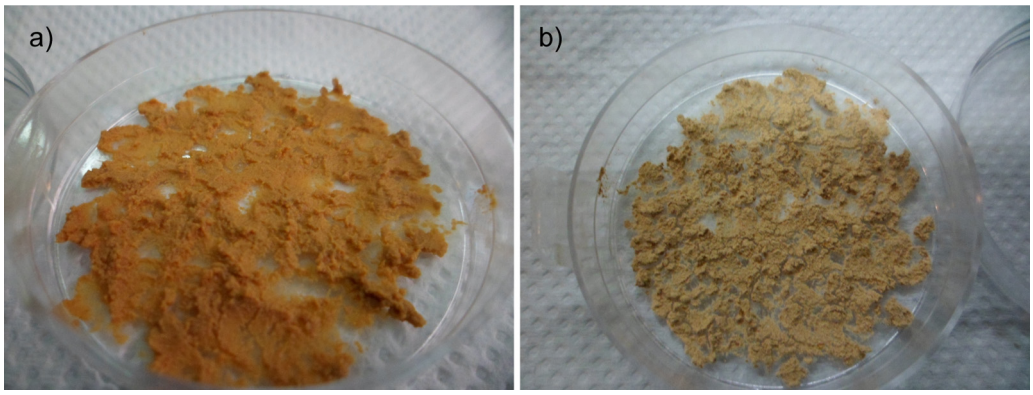


Fig. 6. Photographs of mature layered CALB CLEAs assayed in heptyl oleate synthesis. (a) Humid CLEAs, (b) dried CLEAs.

CLEAs – all containing equal PP contents (i.e. 18 mg) –, up to 24 h of reaction are shown in Fig. 7 in terms of oleic acid conversion.

Data evidences that under the conditions detailed in Section 2.4, in the first hours of reaction the evolution of reaction catalyzed by wet layered CALB CLEAs is similar to the one observed when using free CALB. On the other hand, dried CALB CLEAs show much higher synthetic activity than free CALB, with 1 h and 5 h-conversions of 14% and 50%, versus 2% and 3% for free CALB, respectively. After 24 h of reaction the conversion of oleic acid achieved with free CALB, wet CLEAs and dried CLEAs account for 34%, 16% and 60%, respectively. The noticeable difference of activity between wet and dried layered CALB CLEAs may be explained in terms of the effect of water retained by the biocatalyst in esterification reactions. Water plays multiple roles on lipase-catalyzed esterifications. First of all, water is absolutely necessary for the catalytic function of enzymes because it participates, directly or indirectly, in all non-covalent interactions that maintain the conformation of the catalytic site of enzymes [37–39]. On the other hand, in esterification/hydrolysis reactions it is well-known that the water content affects the equilibrium conversion as well as the distribution of products in the system [39–41]. Based on the two effects of water mentioned above, optimum water contents in the range of 0.2–3% are frequently reported [39,42–44]. Svensson et al. [45] reported that for this type of reversible reactions the activating effect of water dominates at water contents below the optimum, while at higher water contents the net esterification rates decrease, which may be a result of water acting as a substrate in hydrolysis of the acyl-enzyme intermediate. In this context, the higher activity expressed by dried CALB CLEAs may be explained in terms of their lower water content with respect to wet CALB CLEAs and free CALB for aliquots with equal precipitable protein content. Water retained by dried layered CALB CLEAs is hypothesized to be enough for giving the lipase the proper flexibility to display high activity, but without promoting ester hydrolysis.

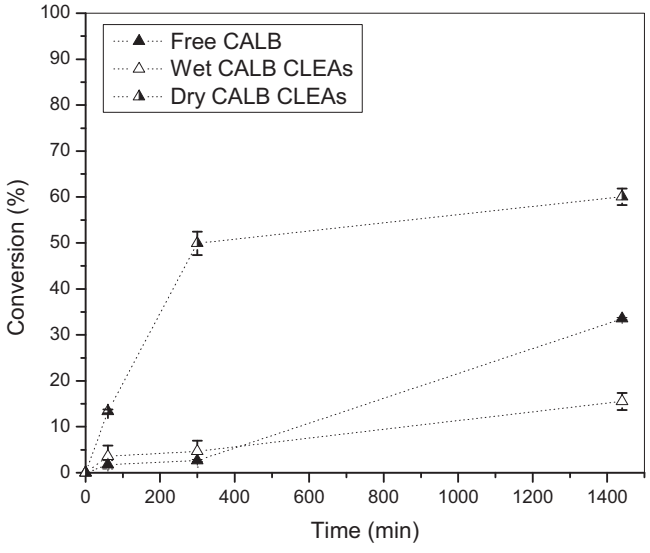


Fig. 7. Evolution of heptyl oleate synthesis catalyzed by free CALB, and mature layered CALB CLEAs (in wet and dried state). Reaction conditions are detailed in Section 2.4.

3.3. *Activity of layered CLEAs of CALB in triolein hydrolysis in emulsioned medium*

The activity of mature layered CLEAs of CALB (cross-linked/mgPP mass ratio = 1.39) was further assayed in a triglyceride hydrolysis reaction. The test reaction chosen was the hydrolysis of triolein in an emulsioned medium, which was selected as a model of industrial reactions of oils and fats hydrolysis. Retained activity values were determined within linear conversion-time and activity-amount of PP intervals (i.e. 5 min, 0.5 mg of PP). In the case of mature layered CLEAs of CALB the activity retained in triolein hydrolysis achieved 68% of the one found for free lipase (i.e. specific activity of CALB in the current system: 63.4 μmol/min × mg). Evidently, the conformation of CALB obtained in layered CLEAs, – which showed to be so convenient for the catalysis of the esterification reactions assayed –, was not equally suitable for triolein hydrolysis. Moreover, given the size of the triglyceride, access of this substrate to the internal lipase molecules might have been partially hindered even in the layered methodology.

4. Conclusion

Layered CLEAs of lipase B from *C. antarctica* with a BSA core have demonstrated to be an interesting alternative for the catalysis of chosen fatty acid esterifications in organic and solventless medium. Under controlled synthesis conditions, the layered CLEAs of CALB showed higher specific activity than free CALB when tested in the direct esterification of oleic acid and ethanol in solventless medium. Under the best conditions found in terms of cross-liker/PP from CALB ratio and storage time, CALB CLEAs with up to 188% of recovered activity were obtained. The key role of the amount of glutaraldehyde used and its effect on the evolution of the activity retained by CALB CLEAs with storage time was illustrated. Layered CLEAs of CALB with highest activity were further assayed in a fatty acid esterification in organic medium. In this medium, layered CLEAs of CALB also showed hyperactivation with respect to free CALB, especially if used with previous drying. The assay of the target biocatalyst in the hydrolysis of triolein in emulsioned

medium, showed a retained catalytic activity of 68% with respect to free CALB.

The results herein summarized suggest that layered CALB CLEAs appear as an attractive option for the catalysis of fatty acid esterifications in both organic and solventless medium. Further research on the use of layered CALB CLEAs in other fatty acid esterifications is currently going on.

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