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Short communication

Effect of different parameters on the hydrolytic activity of cross-linked enzyme aggregates (CLEAs) of lipase from *Thermomyces lanuginosa*

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ABSTRACT

Cross-linked enzyme aggregates (CLEAs) of lipase from *Thermomyces lanuginosa* (TLL) were synthesized using $(NH_4)_2SO_4$ as precipitant and glutaraldehyde as cross-linking agent. CLEAs were assayed for their hydrolytic activity in a reaction performed in an emulsioned medium. The effects of the amount of precipitant, cross-linker, and different additives such as protein cofeeder, oleic acid, n-heptane, sodium dodecyl sulfate (SDS), polyethylenglicol (PEG) and ethylendiamine were studied at selected ratios with respect to TLL mass. Traditional non-layered CLEAs of TLL showed recovered activities between 3 and 31% when compared with native lipase. Novel TLL layered CLEAs consisting of a protein cofeeder core and successive layers of target lipase showed an important increase in their retained activity. The highest recovered activity was found for the one-layered non-additivated CLEAs of TLL which showed a recovered activity of 75%.

1. Introduction

Cross-linked enzyme aggregates (CLEAs) have a number of interesting features such as highly concentrated volumetric activity, high stability of the produced superstructure, low production cost, easiness of synthesis, facile recovery/reuse due to their heterogeneous character and the fact that no purified enzymes are needed [1].

CLEAs synthesis implies the formation of enzyme aggregates by use of an aggregating agent followed by cross-linking of precipitated enzymes. CLEAs of a number of enzymes have been synthesized in the last decade. In 2000, Cao et al. synthesized CLEAs of *Penicillin acylase* [2]. In 2002, López-Serrano et al. synthesized CLEAs of seven commercial lipases and CLEAs with more than tenfold increase in activity respect to native enzyme was reported [3]. Sheldon et al. [4] prepared CLEAs of *Candida antarctica* B lipase (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 tested [5]. Other reports on cross-linked enzymes, different than lipases, and stabilization strategies have been reported [6,7].

In the current manuscript CLEAs of *Thermomyces lanuginosa* lipase (TLL), were synthesized using ammonium sulphate as aggregating agent and glutaraldehyde as cross-linker. The test reaction used was the hydrolysis of triolein in an emulsioned medium, which was chosen as a model of industrial reactions of oils and fats hydrolysis. The feasibility of increasing CLEAs hydrolytic activity through bio-imprinting, and/or by providing a biphasic synthesis medium was also assayed. The effect of different additives such as cofeeder proteins and stabilizers of TLL in CLEAs was also analyzed. Finally, a novel layered CLEAs approach which implied the synthesis of a cross-linked core of BSA over which layers of BSA/TLL were precipitated and cross-linked, was developed with the aim of favoring the contact between lipase and substrates. At the best of the authors's knowledge, the preparation of layered CLEAs with a core of BSA and cross-linked TLL has not been reported before.

2. Materials and methods

2.1. Materials

Lipozyme TLL (5000 U/mL) from *T. lanuginosa* was kindly donated by Novozymes (Bagsvaerd, Denmark). Bovine serum albumin (BSA) 30% (w/v) was purchased from Wiener Lab (Argentina). Triton X-100, Tris–HCl (1 M) buffer and triolein (65%) were purchased from Sigma Aldrich. Ethanol (99%) and n-heptane were obtained from Dorwill and ammonium sulphate was purchased from Cicarelli. Glutaraldehyde solution (25%, v/v) and

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polyethylenglicol (PEG, M_W = 35 kDa) were obtained from Fluka and used as received. Oleic acid and sodium dodecyl sulfate (SDS) were obtained from Anedra.

2.2. CLEAs synthesis

2.2.1. Traditional (non-layered) approach

Commercial TLL solution (120 μL with 200 mg of precipitable protein (PP) per mL) and BSA (170 μL, 30%, w/v) were stirred for 10 min at 500 rpm in an ice bath. Then, saturated ammonium sulphate solution (700–1400 μL; 500 mg/mL) was added. After 2 h, glutaraldehyde solution (40–320 μL; 25%, v/v) was introduced and cross-linking was allowed to proceed for the following 2 h. Then, the suspension was centrifuged at 6000 rpm for 5 min at room temperature, and the precipitate was recovered and washed with distilled water three times. The obtained CLEAs were vacuum-dried at room temperature for 16 h.

2.2.2. Layered approach

For the synthesis of layered CLEAs of TLL, the protocol was similar to the one described in the previous section, but lipase addition was performed only after BSA cores were formed and cross-linked. BSA solution (250 μL; 30%, w/v) was precipitated by addition of saturated ammonium sulphate solution (700 μL, 500 mg/mL) in an agitated ice bath, and crosslinked with glutaraldehyde (200 μL, 25% v/v aqueous solution) during 2 h. Next, TLL was added (120 μL, containing 25 mg of PP) and the system was kept on stirring for 2 h more. Suspension was then centrifuged at 6000 rpm for 5 min at room temperature. The supernatant was decanted and the precipitate was washed with distilled water three times and stored at 4 °C for later activity assay. CLEAs with up to three "layers" (BSA/precipitant/cross-linker followed by TLL addition) were synthesized. The protocol was similar to the one just described for one "layer", but only one third of the commercial preparation of enzyme, precipitant and cross-linker were used for generating each layer. Contact time for BSA core formation and for later lipase crosslinking with remaining glutaraldehyde was reduced to 40 min for each layer. The effect of the use of PEG (10 mg) and the anionic surfactant sodium dodecyl sulfate (SDS, 42 mM) dissolved in distilled water, in the recovered activity of CLEAs was also assayed. Both PEG and SDS were added together with lipase solution when used.

2.3. Use of additives

The effect on the recovered hydrolytic activity of CLEAs of different additives acting as co-feeder (BSA, 25–75 mg), as well as for inducing bioimprinting (oleic acid, 75–225 mg), or interfacial activation of CLEAs (n-heptane, 25–75% (v/v) respect to total synthesis medium), was assayed by use of an experimental design of 3 factors and 3 levels. The software used was STATGRAPHICS Centurion, Versión. XV. 1, Computer software StatPoint, 2006. The synthesized CLEAs were washed with water twice, and the third washing was done with a 50/50 (v/v) heptane/water mixture in order to guarantee oleic acid removal. The effect of the treatment of synthesized CLEAs with ethylenediamine was also studied, using a mass ratio TLL (mgPP): ethylenediamine of 0.38.

2.4. Test reaction: hydrolysis of triolein

The hydrolysis of triolein was performed following a procedure adapted from Rocha et al. [8]. Experiments were performed twice showing an average relative error in the recovered activity measured of 2%. The reaction is a standard test and the reaction time used for activity tests is 5 min. No kinetic studies were explored.

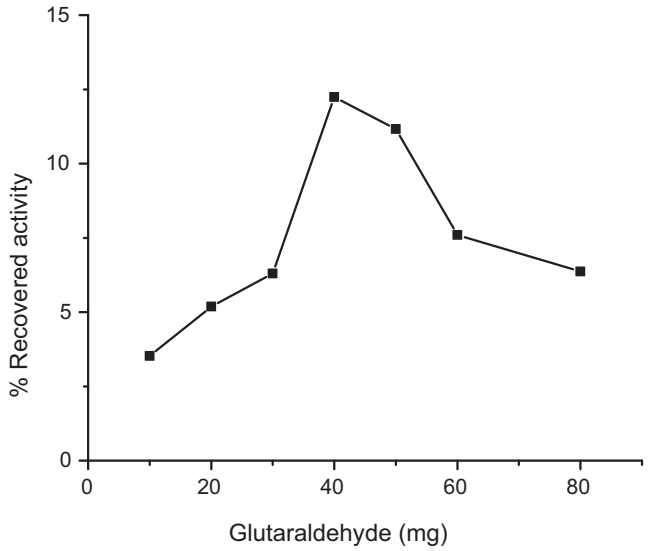

Fig. 1. Effect of the amount of glutaraldehyde used for crosslinking on the recovered hydrolytic activity of CLEAs of TLL. Mass of TLL: 24 mg; mass of $(NH_4)_2SO_4$: 350 mg. Mass of BSA: 50 mg.

2.5. Calculation of recovered activity of CLEAs

Recovered activity of CLEAs of TLL was calculated as the ratio of specific activity of CLEAs to specific activity of native TLL. Conditions for activity measurements (time and biocatalyst amount) were carefully chosen in order to guarantee linear activity responses. Precipitable protein (PP) content (determined by precipitation with ammonium sulphate) was used for calculating specific activities of native TLL. PP content in CLEAs was determined by mass balance considering PP lost in supernatant and washings [9].

2.6. Scanning electron microscopy

Scanning electron images (SEM) of TLL CLEAs were obtained using a JEOL 35CF microscope operated at 7 kV and equipped with a secondary electron detector and energy dispersive X-ray microanalysis (EDX).

3. Results and discussion

3.1. Effect of precipitant and cross-linker

The assay of different TLL/$(NH_4)_2SO4$ mass relationships (0.04–0.07) showed no significant differences in CLEAs recovered activity (results not shown). Then, the lowest amount of salt explored, (i.e. the highest TLL (PP/$(NH_4)_2SO4$ mass ratio = 0.07) – was chosen for further syntheses. The amounts of precipitating salt assayed were chosen aiming to guarantee that no $(NH_4)_2SO_4$ remained in the recovered CLEAs, which takes place at salt fractions higher than the ones assayed. On the other hand, with salt fractions lower than the ones used much enzymatic activity is lost in the supernatant of CLEAs synthesis medium, due to unsuccessful TLL precipitation.

The bifunctional agent used to cross-link TLL aggregates was glutaraldehyde, a dialdehyde of preponderant use in CLEAs synthesis [10]. Fig. 1 shows that for the synthesis conditions herein used highest recovered activity is obtained when 40–50 mg of cross-linker are used. Although widely used for the crosslinking of proteins, yet there is no agreement about the main reactive species that participate in the glutaraldehyde-mediated crosslinking process because monomeric and polymeric forms are in equilibrium

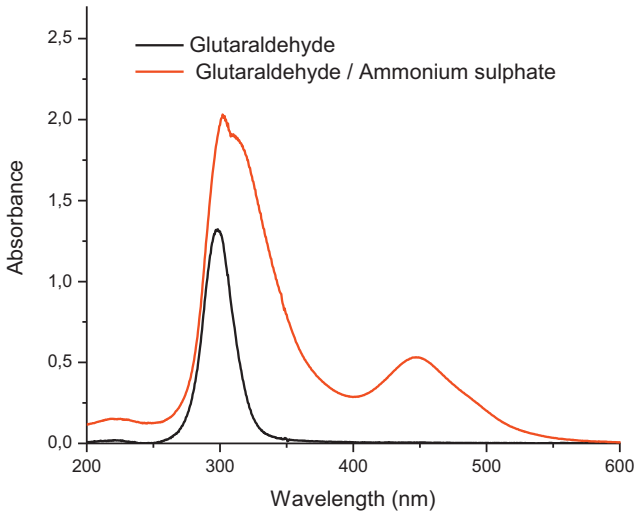

Fig. 2. UV/Vis spectra of glutaraldehyde and glutaraldehyde reacted with $(NH_4)_2SO_4$ 30 min, diluted.

[11]. Migneault et al. [11] reported the existence of up to 13 different structures of glutaraldehyde molecule in the cross-linker commercial aqueous solution (25%, w/w). Monsan et al. [12] considered glutaraldehyde reactions in acidic pH that lead to the production of imines. Hardy et al. [13] argued that the reaction of glutaraldehyde with proteins involves the formation of quaternary pyridinium compounds. Russo et al. [14] reported that the formation of piperidine or 5-hydroxy-pentanyl derivatives is the typical reaction product of amines with aldehyde under reductive conditions. Authors reported that the reaction is highly efficient and all amines of proteins and peptides can be completely modified with glutaraldehyde in less than 15 min at room temperature. In this contribution, the CLEAs synthesis medium in which crosslinking takes place is acidic due to the dissociation of the salt in water according to:

$$(NH_4)_2SO_4(s) + nH_2O \rightarrow 2NH_4^+(ac) + SO_4^{2-}(ac) \quad (1)$$

$$NH_4^+(ac) \Leftrightarrow H^+(ac) + NH_3(ac) \quad K_a = 1.8 \times 10^{-5} \quad (2)$$

$$SO_4^{-2}(ac) + H_2O(l) \Leftrightarrow HSO_4^-(ac) + OH^-(ac) \quad K_b = 8.3 \times 10^{-13} \quad (3)$$

Since CLEAs preparation solution is acidic when tested (pH 3–4), it follows that the NH_4^+ as acid is stronger than the sulphate ion as a base. The previous has at least two important consequences: (i) protons present in the acidic solution catalyze the oligomerization of glutaraldehyde, and (b) besides NH_2 from proteins ammonia may also react with glutaraldehyde. Therefore, glutaraldehyde reacts with the proteins (BSA and TLL) and with the protons/NH_3 to generate oligomers different from the original ones, displacing also the equilibrium of the ammonium ion to the right. In this context, UV/Visible studies were performed to check for evidence of the reaction between the ammonium sulphate and glutaraldehyde. Results are shown in Fig. 2 in comparison with glutaraldehyde solution spectra. After 30 min of contact, a new band centered at 450–460 nm appears in the glutaraldehyde-ammonium sulphate spectra. The band is assignable to oligomers of glutaraldehyde with pyridinic moieties. Polypyridine imide has been reported to present a clear band in the 450–460 nm, especially in acidic media [15].

3.2. Effect of additives

The effect on CLEAs recovered activity of the addition of variable amounts of BSA (proteic co-feeder), n-heptane and oleic acid to the synthesis medium, was explored by means of a factorial design. Addition of bovine serum albumin (BSA) as a proteic feeder 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 glutaraldehyde required to obtain aggregates [16,17] Glutaraldehyde reacts with free amino groups of the aminoacids present in the proteic structure, such as lysine. However, in some cases, glutaraldehyde can bind amino acids associated with the active site, reducing the activity of the obtained CLEAs. Moreover, in excess of glutaraldehyde the formation of clusters with mass transfer limitations is also possible. These drawbacks can be prevented by use of molecules with a large amount of lysine residues in the surface, such as BSA. Shah et al. prepared CLEAs of *Pseudomonas cepacia* lipase (PCL) with BSA addition. The recovered activity relative to that of free enzyme was 100% in contrast with the same preparation without BSA, which retained only 0.4% of activity [16]. More recently, the synthesis of CLEAs of lipase B from *C. antarctica* (CALB) showed difficulties in the crosslinking step due to the low content of surface Lys on CALB [18]. Addition of BSA as a feeder allowed an effective cross-linking step, and permitted to greatly stabilize the CLEAs. In reference to the other additives tested in the factorial design, previous works demonstrated activation of lipases when contacted with hydrocarbon/water interfaces as well as when bio-imprinted with fatty acids [19]. Table 1 summarizes the results obtained in terms of both recovered activity (%) and relative recovered activity (%) using a screening experimental design. The relative recovered activity assigned to the standard protocol was 100%. Under the current conditions, the addition of increasing quantities of BSA was found to linearly enhance the total mass of CLEAs recovered (data not shown). The regression model that accurately fitted experimental data showed a linear relationship between the mass of BSA added and the recovered activity of CLEAs (correlation coefficient = 0.88), according to a model that explains 77.9% of the variability of experimental data (ANOVA results are detailed in the Supplementary material section). The positive effect of BSA addition is explained by the fact that this protein provides lysine residues which glutaraldehyde can bind and avoid the denaturing of the target catalytically active protein. Considering that the approximate ratio of lysine residues in BSA vs TLL is 60/7, an effective "sequestration" of the TLL is supposed to take place. That is, TLL, without being cross-linked, is supposed to get trapped in the BSA precipitated aggregates and later cross-linked to generate the CLEAs.

Aqueous buffered medium traditionally used for CLEAs synthesis was partially replaced by n-heptane at different ratios. Interfacial activation in presence of water/hydrocarbon liquid/liquid surfaces, as well as bioimprinting with fatty acids, have shown good results for other lipases even in presence of high water contents [18,19]. In the present contribution, however, *p*-values higher than 0.05 for both n-heptane and oleic acid strategies indicated that the activation strategies detailed did not show a statistically significant impact on the recovered activity of TLL CLEAs.

3.3. Layered CLEAs

A modification of the traditional CLEAs synthesis protocol with co-feeder synthesis was designed. The approach implied the addition of lipase only after cofeeder aggregates were formed and cross-linked. Lipase was expected to form a slightly cross-linked layer over BSA cores, which might lead to enhanced lipase-substrate contact. CLEAs with up to three "layers" were prepared by successive precipitation and crosslinking of layers of lipase alternated with cross-linked BSA, resembling an onion-like structure. Fig. 3 schematizes layered CLEAs in comparison with traditional cross-linked aggregates with cofeeder addition.

Table 1
Experimental factors settings and measured response variables for the recovered hydrolytic activity of CLEAs of TLL with BSA, oleic acid and n-heptane.

Run number (randomized)	Experimental factors			Response variables	
	BSA (mg)	Oleic acid (mg)	N-heptane (%, v/v)	Recovered activity (%)	Relative recovered activity (%) [a]
5	25	75	75	9.7	87
7	75	75	75	27.6	247
10	75	75	25	26.8	240
6	25	225	25	6.1	55
8	25	75	25	3.3	30
9	75	225	25	16.7	150
4	50	150	50	22.6	203
3	75	225	75	31.3	280
1	50	150	50	22.8	204
2	25	225	75	6.4	57

[a] Relativized to standard conditions: lipase (25 mg), BSA (50 mg), ammonium sulphate solution (700 μL) and glutaraldehyde (50 mg).

As shown in Fig. 4a, the layers methodology led to a very high increase in retained activity when compared with non-layered TLL CLEAs (75% – one layer – versus 8% respectively). The much higher activity recovered by the layered approach (9 times the one obtained for non-layered CLEAs) suggests that the addition of lipase once BSA core has been formed provides the benefits of the use of a protein co-feeder but without occluding lipase. However, results with more lipase layers suggest that only the last lipase layer is effectively available for substrate contact. In that case, specific activities -calculated for total lipase content of the CLEAs- are significantly reduced.

Anionic surfactant sodium dodecyl sulfate (SDS) was added to CLEAs synthesis medium aiming to induce the precipitation of lipase in a stable conformation [20]. The use of BSA as co-feeder has been recently reported for CALB-CLEAs but with other test reaction and procedure [21]. Moreover, PEG was added with the aim of producing highly porous CLEAs, which may result in enhanced substrate access. Alternating BSA layers may hinder substrate access to internal lipase layers and introduce mass resistances that reduce catalytic activity recovery. Based on the water-soluble character of PEG molecules, the effect of this additive was assayed expecting it to be washed away during CLEAs washings, leaving pores behind. Results of recovered activities measured for layered CLEAs of TLL with BSA as proteic cofeeder and with/without addition of SDS and PEG are summarized in Fig. 4b. CLEAs were assayed without previous drying since recovered activities of fresh non-layered CLEAs were found to be similar to that of CLEAs vacuum dried for 16 h (data not shown). In the case of traditional non-layered CLEAs of TLL, addition of SDS and PEG had the expected positive effect. On the other hand, the negative effect

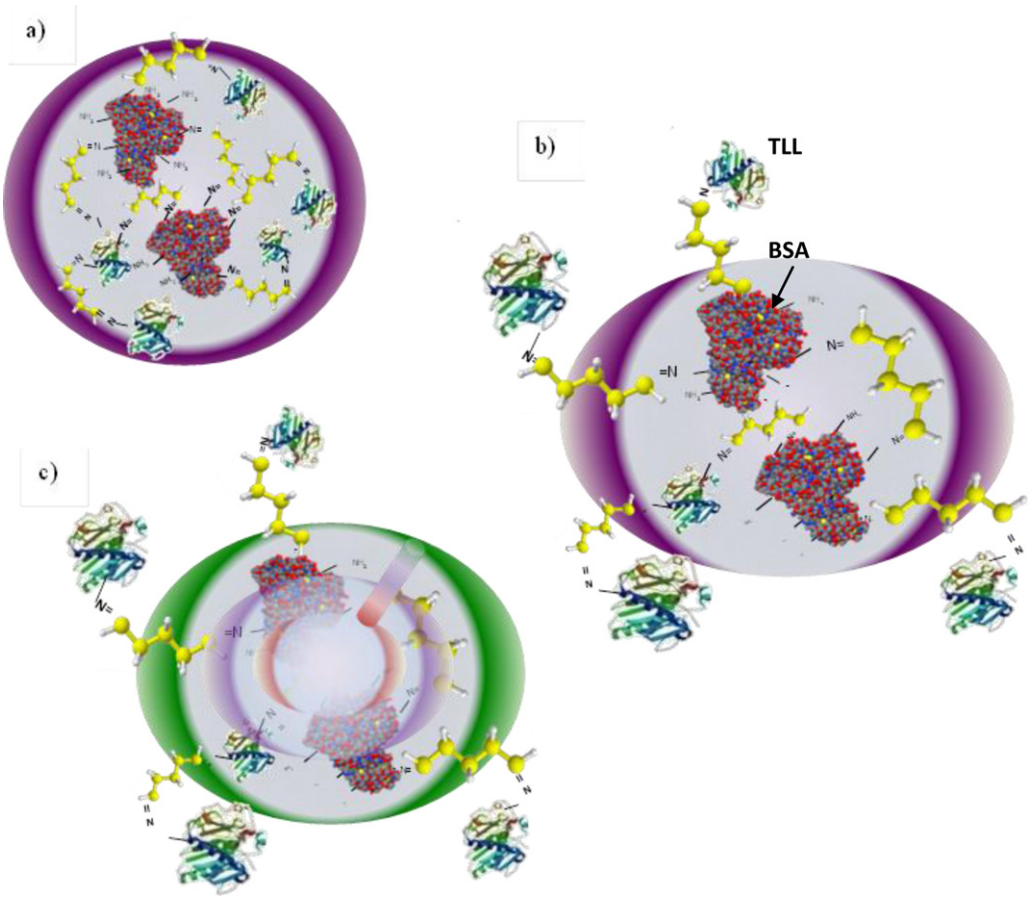

Fig. 3. Scheme of CLEAs (a) Traditional CLEAs with BSA used as protein cofeeder; (b) one lipase layer, (c) three lipase layers. (BSA 3V03) [22] (TLL 1DTE) [23].

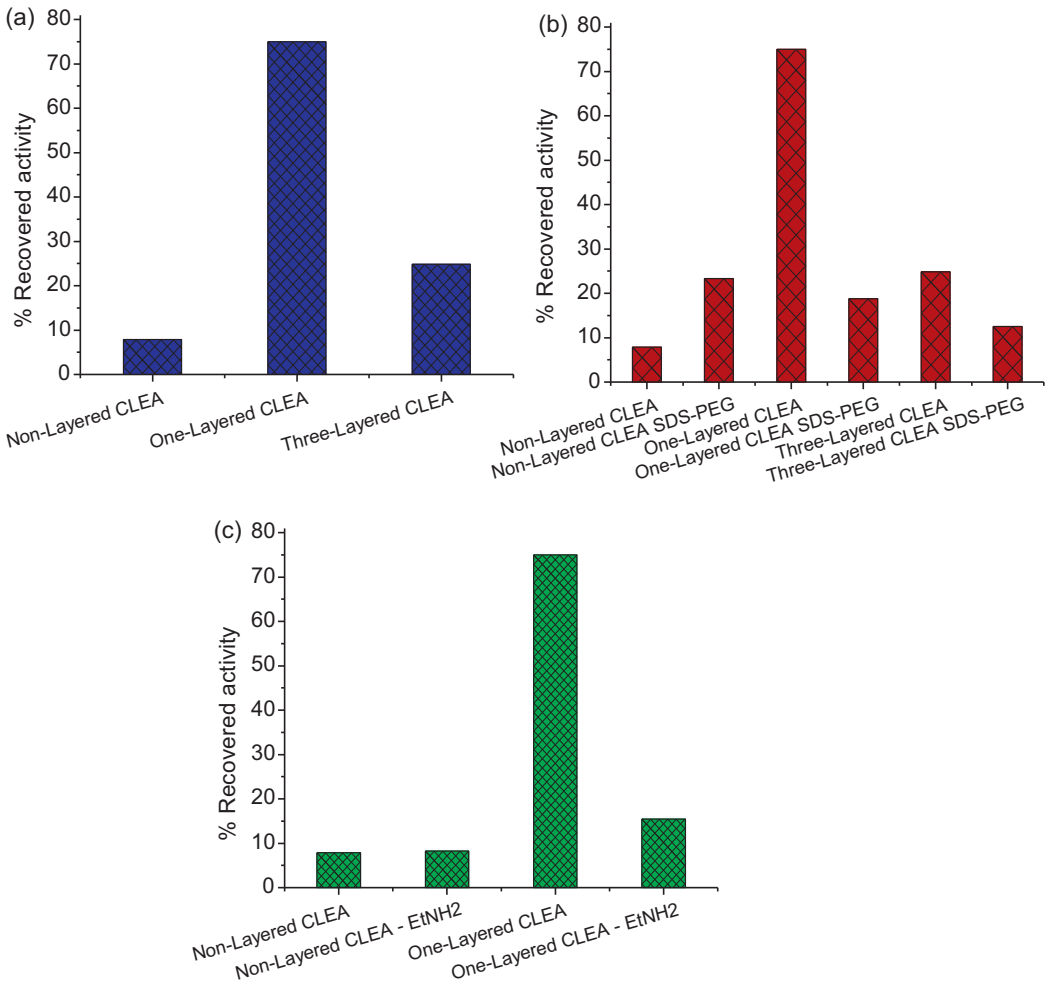

Fig. 4. Recovered activity of non-layered and layered CLEAs of TLL. Effect of additives. (a) Effect of the number of layers (0, 1, 3); (b) Effect of the addition of SDS and PEG for layered and non-layered CLEAs; (c) Effect of the use of amines ($EtNH_2$) for non layered and one-layered CLEAs.

of SDS and PEG on one and three-layered CLEAs is assignable to an undesirable impact on TLL conformation, denaturing of the enzyme and potential inhibition. The addition of amines did not improve the recovered activity of non layered CLEAs, which showed similar values than the ones achieved without amine addition (Fig. 4c). In the case of layered TLL CLEAs, addition of ethylenediamine resulted in a fivefold decrease in recovered activity.

Scanning electron micrographies of layered and non-layered CLEAs showed a distribution of sizes with diameters in the 100–600 nm range. In terms of surface morphology, layered and non-layered CLEAs look quite similar (please refer to Supplementary material). The previous was somehow expected due to the massive presence of BSA in both approaches, with its characteristic globular structure.

4. Conclusion

CLEAs of TLL were synthesized using ammonium sulphate as precipitant and glutaraldehyde as cross-linker. Interferences of ammonium sulphate as well as feasible reactions among glutaraldehyde, protein residues and the salt were discussed. Lipase/precipitant mass ratio and glutaraldehyde amount were explored and values of these parameters that led to highest recovered activities were used in the following assays. Results of the use of different additives during CLEAs synthesis showed the positive effect of the protein cofeeder BSA, whereas the use of templates, amines, and the use of a biphasic liquid synthesis medium did not lead to significant increases in CLEAs recovered activity. On the other hand, the one-layered protocol developed effectively led to CLEAs with significantly higher activity than their non-layered counterparts. Layered CLEAs appear as an attractive alternative with the benefits of cross-linked aggregates but without some of their main drawbacks.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2012.12.010.

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[23] TLL Image from the RCSB PDB ID 1DTE available: http://www.rcsb.org/pdb/explore/explore.do?structureId=1DTE