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DOI: 10.2225/vol14-issue3-fulltext-1 *SHORT COMMUNICATION*

Influence of the pH of glutaraldehyde and the use of dextran aldehyde on the preparation of cross-linked enzyme aggregates (CLEAs) of lipase from *Burkholderia cepacia*

Eduardo Caballero Valdés[1] ✉ · **Lorena Wilson Soto**[1] · **Germán Aroca Arcaya**[1]

1 Facultad de Ingeniería, Escuela de Ingeniería Bioquímica, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile

✉ Corresponding author: eduardo.caballero.icb@gmail.com
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Abstract The preparation of cross-linked enzyme aggregates (CLEAs) of lipase has been a challenge due the low amount of lysine residues that lipases have on their surface. The results show that CLEAs prepared using dextran aldehyde (100-200KDa) have a higher hydrolysis activity and particle size (activities between 3186 ± 21 U/g of CLEA and 4800 ± 30 U/g of CLEA and particle sizes between 52.6 ± 18.7 µm and 126.2 ± 53.5 µm) than CLEAs prepared with glutaraldehyde (0.1 KDa) (activities between 894 ± 16 U/g of CLEA and 2874 ± 20 U/g of CLEA and particle sizes between 21.2 ± 5.1 µm and 83.4 ± 24.9 µm); Thermal stability assays of bioctalysts at 60ºC at pH 7.0 using phosphate buffer 25 mM showed that CLEAs prepared with dextran aldehyde have lower residual activity after 50 hrs (maximum residual activity of 46.8% in the CLEA) than CLEAs prepared with glutaraldehyde (maximum residual activity of 70.2% in CLEA). When considering hydrolysis activity, thermal stability and residual activity of CLEAs as a criteria for selecting the best preparation conditions, it has been found that the best condition for CLEAs preparation are to use glutaraldehyde as cross-linking reagent at pH 9.5, at a concentration of 3.5 g/l, and an enzyme/albumin ratio of 15.

Keywords: albumin, dextran aldehyde, glutaraldehyde, immobilisation, lipase

INTRODUCTION

Cross-linked Enzyme Aggregates (CLEAs) is an immobilisation method that uses the aggregated protein to be cross-linked, generating a solid biocatalist. These biocatalysts have proved to be a low-cost alternative as they do not require a carrier, they are easy to produce and have a high specific activity (Cao et al. 2000; Mateo et al. 2004; Schoevaart et al. 2004; Wilson et al. 2006; Majumder et al. 2008). Cross-linking takes place by the formation of bonds between the amino groups of lysine residues present on the enzyme surface and aldehyde groups of the cross-linking reagent. However, the preparation of CLEAs of lipases has some restrictions due to their low content of lysine residues on the lipase surface. Therefore, both the addition of polymers to favour cross-linking (Wilson et al. 2006), and the addition of albumin (Shah et al. 2006), have been proposed to improve the efficiency in the preparation of CLEAs. Although the latter strategy has shown good results, the optimum amount of albumin to prepare CLEAs has been determined only by measuring the increase of the hydrolysis activity of the biocatalyst, and not considering the evaluation of its stability (Shah et al. 2006). On the other hand, it has been observed that glutaraldehyde, a cross-linking reagent generally used for CLEA preparation (Cao et al. 2001; López-Serrano et al. 2002; Cabana et al. 2007; Illanes et al. 2007; Aytar and Bakir, 2008; Hara et al. 2008) has shown, in some cases, to have a negative effect over the enzymatic activity because of its small molecule weight (0.1 kDa), which could interfere with active site of the enzyme (Mateo et al. 2004). A possible solution is the use of polymers of glutaraldehyde, which are formed at alkaline pH, increasing the size of the cross-linking reagent (Kawahara et al. 1992; Wong, 1993). Another possibility is to consider the use of cross-linking reagents of greater size such as dextran aldehyde (100 a 200 kDa), which could improve the stability and increase the particle size of CLEAs (Mateo et al. 2004).

According to the aforementioned, the influence of the enzyme/albumin ratio, and the influence of dextran aldehyde and glutaraldehyde (at different pH) concentrations on both the hydrolytic activity and particle size, are reported. The residual activity of CLEAs after thermal stability testing was determined as an indirect measurement of the degree of enzyme cross-linking (Pedroche et al. 2007).

MATERIALS AND METHODS

Materials

Lipase PS from *Burkholderia cepacia* (Amano Enzymes), Glutaraldehyde grade II 25% aqueous solution, dextran from *Leuconostoc mesenteroides* average mol. wt. 100,000-200,000, *p*-nitrophenyl butyrate and Triton X-100, were supplied by Sigma-Aldrich. Acetone 99.5% and bovine serum albumin fraction V were supplied by Merck.

Preparation of CLEAs

Commercial lipase PS (200 mg powder containing 9 mg protein, determined according to bicinchoninic acid method, BCA) was suspended in phosphate buffer 25 mM pH 7.0 to obtain a concentration of 50 g/l, which has an hydrolysis activity of 2220 U/ml. Then, BSA (13 or 40 mg) was dissolved in 4 ml of enzyme solution, and 8 µl of Triton X-100 at 25% v/v were added. This solution was added dropwise in 16 ml of acetone to precipitate the lipase, keeping the suspension for 30 min at 4ºC, and a stirring rate of 200 rpm. Glutaraldehyde (pH 4.5 or 9.5) or dextran aldehyde were then added at different concentrations (1.5 or 3.5 g glutaraldehyde /l, and 1.7 or 2.7 g dextran aldehyde /l*)* in a 4ºC bath for 4 hrs, yielding CLEAs. Finally, the CLEAs formed were centrifuged once with distilled water, and twice with acetone. The stirring and centrifugation conditions were the same for all prepared biocatalysts to avoid their effect on the particle size of CLEA.

Dextran aldehyde preparation

Dextran 1.65 g (100 to 200 KDa) was dissolved in 50 ml of water, and 3.85 g sodium metaperiodate were added. The resulting solution was stirred at room temperature during 90 min. Subsequently, the solution was dialyzed five times, using a MW cutoff of 12 KDa against 5 l of water each time at room temperature during 2 hrs and under stirring. The final volume of the dextran polyaldehyde was 56 ml (Drobchenko et al. 1993).

Enzymatic hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB)

This assay was performed with a spectrophotometer (Perkin Elmer EZ 301), continuously measuring the increase in the absorbance at 348 nm produced by the released *p*-nitrophenol in the hydrolysis of 0.4 mM *p*-NPB in 25 mM sodium phosphate buffer at pH 7 and 30ºC. One molecule of *p*-NPB hydrolyzed produce 1 molecule of *p*-NP, which allow defining U in terms of *p*-NPB hydrolyzed. To initialize the reaction, 0.05 ml of lipase solution (0.1 g lipase /l) or suspension (10 g CLEA /l) were added to 2.5 ml of substrate solution. One unit of activity (U) was defined as the amount of enzyme necessary to hydrolyze 1 µmol of *p*-NPB per hour at the aforementioned conditions.

Thermal stability

CLEA suspensions were prepared in 25 mM phosphate buffer pH 7.0, at a concentration of 10 mg CLEA/ml. Samples were maintained at 60ºC, and the hydrolytic activity was quantified at 50 hrs. Percentage of the suspension activity at 50 hrs were calculated in relation to the initial suspension activity. These values are shown as suspension activity (%) in Table 1.

To quantify the residual activity of CLEAs, the suspension was centrifuged at the end of thermal stability (50 hrs), and the supernatant activity was measured. From the difference between the suspension and supernatant activities at 50 hrs it was obtained the residual activity of CLEAs. These values were calculated as percentage in regard to the activity of initial suspension at 60ºC.

Table 1. Residual activities of CLEAs prepared with dextran aldehyde and glutaraldehyde at pH 4.5 and 9.5, after 50 hrs in buffer phosphate 25 mM at pH 7.0 and 60ºC. The percentages are referred to the initial suspension activity (100%).

CLEA[3]				
Cross-linking reagent	Concentration (g cross-linking reagent /l)	Enzyme/albumin ratio (mg/mg)	**Suspension activity (%)[4]**	**Residual activity in CLEAs (%)**
Dextran aldehyde	1.7	5	121.5 ± 1.7	45.7 ± 0.7
	1.7	15	98.6 ± 1.1	46.8 ± 0.6
	2.2	5	82.9 ± 0.9	21 ± 0.6
	2.2	15	109.1 ± 1.3	34.1 ± 0.4
	2.7	5	72.5 ± 0.9	26.4 ± 0.5
	2.7	15	56.1 ± 1	5.5 ± 0.3
Glutaraldehyde pH 4.5	1.5	5	65.8 ± 0.9	37.8 ± 0.3
	1.5	15	40.9 ± 1.1	20 ± 0.4
	3.5	5	48.6 ± 1.2	2.8 ± 0.4
	3.5	15	116 ± 1.3	69.7 ± 0.7
Glutaraldehyde pH 9.5	1.5	5	61.7 ± 1.1	40.9 ± 0.6
	1.5	15	65 ± 1.4	32.7 ± 0.7
	3.5	5	65.3 ± 1.1	51 ± 0.7
	3.5	15	93.2 ± 1.5	70.2 ± 0.8

[1]Each individual CLEA was done twice and each measurement was done in duplicate. [2]The absolute values corresponding to 100% are shown on Table 2 and Table 3 for each CLEA as Hydrolysis activity (U/g).

Determination of particle size

The size of the particle was determined by means of optical microscopy (Nikon Eclipse 50i) of a 10 mg CLEA/ml suspension in anhydrous hexane. Images with a 100x magnification were obtained; they were processed by means of Image Tool 2.0 program to determine the size of the particles and their standard deviation.

RESULTS AND DISCUSSION

Effect of enzyme/albumin ratio and dextran aldehyde concentration on hydrolytic activity and particle size of CLEA

All CLEAs referred to in this study have been prepared considering different cross-linking reagent concentrations (both dextran aldehyde and glutaraldehyde), but equivalent regarding their ratio aldehyde groups/amino groups offered by both lipase and albumin.

It has been statistically determined that at enzyme/albumin ratio of 5 (Table 2), the hydrolysis activity of CLEAs from lipase PS at a dextran aldehyde concentration of 2.2 g/l have a significant difference with the hydrolysis activities at other concentrations. However, the particle sizes of these CLEAs at different dextran aldehyde concentrations don't have significant difference (p-value > 0.4282). These results show that an enzyme/albumin ratio of 5 (more presence of albumin into the CLEAs) allows reaching a maximum particle size. This maximum particle size is independent of cross-linking reagent concentration. The combined effect of high albumin concentration in CLEAs and the high molecular weight of the cross-linking reagent would favors the formation of more tangled net, producing CLEAs of great particle size, even with a low concentration of cross-linking reagent.

Regarding the results using an enzyme/albumin ratio of 15 (Table 2), the statistical analysis showed that it has significant difference in the activities of CLEAs prepared at dextran aldehyde concentrations of 1.7 and 2.2 g/l. There is not significant difference between activities at 2.2 and 2.7 g/l of dextran aldehyde, which is possibly due to the diffusional restrictions caused by the size increasing of the

biocatalyst. Despite CLEAs show big standard deviations, they present significant difference on particle size (p-value < 0.01), which is due to the elevated number of particles taken for this measurement (> 40). At this enzyme/albumin ratio, CLEAs have low amount of albumin into their structures. Particle sizes of CLEAs show an increase directly proportional to the dextran aldehyde concentration.

Table 2. Influence of the enzyme/albumin ratio and dextran aldehyde concentration on the size and hydrolytic activity of CLEAs.

CLEA[1]		Hydrolysis activity (U/g)	Particle size (μm)
Enzyme/albumin ratio (mg/mg)	Concentration (g dextran aldehyde /l)		
5	1.7	3204 ± 26	126.2 ± 53.5
15	1.7	4170 ± 27	52.6 ± 18.7
5	2.2	3852 ± 22	116.6 ± 54.3
15	2.2	4800 ± 30	65.9 ± 26.2
5	2.7	3186 ± 21	120.2 ± 56.3
15	2.7	4770 ± 28	86.9 ± 29.5

[1]Each individual CLEA was done twice and each measurement was done in triplícate.

On the other hand, it was analyzed the influence of enzyme/albumin ratio on the responses. It was determined that there are significant differences on the activities and particle size of CLEAs. When the enzyme/albumin ratio increases the CLEAs show lower particle size and higher catalytic potential. This behavior can be observed on all dextran aldehyde concentration studied.

Effect of enzyme/albumin ratio, glutaraldehyde concentration and pH of glutaraldehyde on hydrolytic activity and particle size of CLEA

Table 3 shows the results obtained with CLEAs cross-linked with glutaraldehyde. The variables studied in this case were pH and concentration of the glutaraldehyde solution, and enzyme/albumin ratio. Previous to CLEAs preparation, the stability of lipase PS in relation to the pH conditions at which the glutaraldehyde was used, was studied; there was no loss of hydrolytic activity during 5 hrs incubation.

The study of the effect of pH value of glutaraldehyde solution on particle size was determined. Significant difference between CLEAs prepared using glutaraldehyde at pH 4.5 and 9.5 was found (p-values < 0.002 in the four comparissons carried out), which indicate the effect of using polymer of glutaraldehyde over CLEA structure (Figure 1). The pH of glutaraldehyde has also a significant effect over activity, displaying that cross-linking of lipases with polymers of glutaraldehyde leading a decrease on the activity.

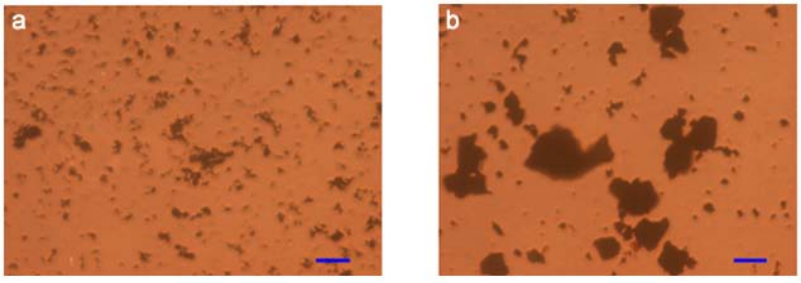

Fig. 1 Picture from optic microscopy (40 X). (a) CLEAs prepared with glutaraldehyde at pH 4.5, glutaraldehyde concentration of 35mM and enzyme/albumin ratio of 15. (b) CLEAs prepared with glutaraldehyde at pH 9.5, glutaraldehyde concentration of 35mM and enzyme/albumin ratio of 15. Regarding the analysis with software Image Tool, the particle size of each CLEA (a and b) is indicated in Table 2. Blue line on the right bottom of the figure indicates a distance of 100 μm.

The results in Table 3 show that CLEAs produced with glutaraldehyde at pH 4.5 have a size that is independent from both the amount of cross-linking reagent and the enzyme/albumin ratio. It has been determined that the predominant species of glutaraldehyde in an aqueous solution at acid pH are cyclic hemiacetals with molecular weight similar to that of glutaraldehyde monomer. According to the aforementioned, the intermolecular interactions that take place with this type of molecules in the cross-linking step would be of short-distance order. Thus, these bonds would not have impact on the size of CLEAs produced, but they would have an effect on their activity, depending on the variables in Table 3.

In this regard, we can see that at pH 4.5 and enzyme/albumin ratio of 15, the activity does not depend on the concentration of glutaraldehyde. However, at the enzyme/albumin ratio of 5, the activity decreases when the cross-linking reagent concentration increases.

If we evaluate the performance of biocatalysts produced with glutaraldehyde at pH 9.5, we can mention that for a enzyme/albumin ratio of 15, the size of CLEA does not depend on the cross-linking reagent concentration. However, when working at a ratio of 5, the particle size of CLEA becomes dependent on the cross-linking reagent concentration, increasing its particle size as the glutaraldehyde concentration increases. This is consistent with the use of dextran aldehyde, because in this case glutaraldehyde forms polymers.

When comparing CLEA9.5/3.5/15 and CLEA4.5/3.5/15 (Table 3), we can observe that the latter is 63% smaller than the former. However, the activity of CLEA9.5/3.5/15 is not significantly affected compared to CLEA4.5/3.5/15, indicating a low incidence of diffusional restrictions. The explanation is based on the fact that CLEAs prepared with glutaraldehyde at pH 4.5 would generate smaller CLEAs, and with less space between enzyme aggregates because the cross-linking reagent is a monomer. On the other hand, in CLEAs produced with glutaraldehyde at pH 9.5, the equilibrium shifts towards the formation of polymers of glutaraldehyde, which gives as a result greater CLEAs and more space between enzyme aggregates, thereby preventing the occurrence of diffusional restrictions.

Table 3. Influence of enzyme/albumin ratio, glutaraldehyde concentration, and pH on the size and hydrolytic activity of CLEAs.

CLEA[2]				
pH of glutaraldehyde	concentration (g glutaraldehyde /l)	Enzyme/albumin ratio (mg/mg)	**Hydrolysis activity (U/g)**	**Particle size (mm)**
4.5	1.5	5	2034 ± 17	25.8 ± 8.7
4.5	1.5	15	2874 ± 20	27.1 ± 7.9
4.5	3.5	5	1272 ± 19	26.9 ± 9.4
4.5	3.5	15	2670 ± 18	21.2 ± 5.1
9.5	1.5	5	894 ± 16	33.3 ± 9.4
9.5	1.5	15	1578 ± 17	57.9 ± 23.8
9.5	3.5	5	882 ± 17	83.4 ± 24.9
9.5	3.5	15	2214 ± 19	57.2 ± 21

[2]Each individual CLEA was done twice and each measurement was done in triplícate.

Effect of enzyme/albumin ratio and cross-linking reagent concentration on the residual activity of biocatalysts

In order to select more suitable CLEAs for future applications, the percentage of suspension activity and the percentage of residual activity in CLEAs after 50 hrs at 60ºC, have been evaluated. Thus, among the CLEAs that show the highest suspension activities, the ones that retain more activity after 50 hrs using dextran aldehyde as cross-linking reagent are CLEA1.7/15 with 46.8% of the initial activity (Table 1). This indicates that CLEAs prepared with dextran aldehyde are not well cross-linked; therefore, the enzyme would detach from the CLEA, showing a greater suspension activity. According to the aforementioned, it is known that CLEAs are formed mainly but not only by intermolecular cross-linking of enzyme aggregates, since intramolecular interactions may also take place (Wold, 1972).

Based on this, we can infer that dextran aldehyde is a lineal molecule of high molecular weight (average molecular weight 5 times higher than lipases, and 2.5 times higher than albumin), which could establish a low number of intramolecular bonds between its aldehyde groups and the amino groups of the enzyme. This effect is reflected in the low activity that is possible to retain in the biocatalyst in an experience of thermal stability after 50 hrs (Table 1).

In this regard, the CLEAs produced using glutaraldehyde (Table 1) show that CLEAs with the highest suspension activities, and the CLEAs with more residual activity are, in both cases, the ones produced with a highest amount of cross-linking reagent and low amount of albumin (CLEA4.5/3.5/15 and CLEA9.5/3.5/15), which can be explained by the fact that they have a great amount of cross-linking reagent per protein mass (taking into account lipase and albumin). From these two CLEAs, the one that has a greater retention of activity is CLEA9.5/3.5/15 with 70.2% of the initial activity.

According to the selection criteria mentioned at the beginning of this section, we can compare the data shown in Table 1, and determine that even though CLEAs cross-linked with dextran aldehyde have a higher activity than those prepared with glutaraldehyde, retain much less activity after 50 hrs (only 46.8% of residual activity in the best case scenario), showing little potential for industrial processes.

CONCLUDING REMARKS

Considering that the objective of this study was to identify the influence of using different types and sizes of cross-linking reagents, and the addition of albumin on the activity, particle size and stability of CLEAs of lipase PS from *Burkholderia cepacia,* we can mention that CLEAs produced with dextran aldehyde do not present an adequate potential to be applied in processes, mainly due to inadequate cross-linking of the biocatalyst.

It was determined that the size of CLEAs influences the activity of these biocatalysts only at high enzyme/albumin ratio in the case of CLEA prepared with dextran aldehyde, and in all cases of CLEAs prepared with glutaraldehyde solution at pH 9.5.

Instead, it was determined that the best way to obtain CLEAs of lipase with relatively high activity, stability, and adequate cross-linking is using glutaraldehyde at pH 9.5, with a glutaraldehyde concentration of 3.5 g/l, and an enzyme/albumin ratio of 15; this one has the highest potential for processes in which its recovery and reuse are required.

On the other hand, thermal stability assays determined that CLEAs prepared with glutaraldehyde are able to keep more residual activity than CLEAs prepared with dextran aldehyde, which means that glutaraldehyde is better as cross-linking reagent for lipases than dextran aldehyde.

As future prospects of study, it has been found that the enzyme/albumin ratio is a variable which may be optimized in respect to the hydrolysis activity or the ability of CLEAs to retain their activity.

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