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Preparation and characterization of combi-CLEAs catalyzing multiple non-cascade reactions

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Abstract

A novel cross-linked enzyme aggregates (CLEA) concept called combi-CLEA has been described. It is based upon the fact that CLEA can be made from heterogeneous populations of proteins/enzymes. Porcine pancreatic acetone powder crude extract was used for preparing CLEA in such a way that lipase, α-amylase, phospholipase A_2 activities were retained upto 100%. The lipase present in the CLEA showed greater thermal stability at 50 °C as compared to free enzyme. For lipase and phospholipase A_2, V_{max}/K_m showed no significant change upon combi-CLEA formation but decreased significantly for α-amylase activity from 190 to 114 min^{-1}. The lipase activity and α-amylase activity in CLEA were completely retained upto three cycles of use. The scanning electron microscopic (SEM) studies showed that morphology of CLEA changed upon inactivation by reuses.
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Keywords: α-Amylase; Glutaraldehyde cross-linking; Lipase; Combi-CLEA; Phospholipase A_2; Thermal stability

1. Introduction

Immobilized enzymes have found tremendous applications in life sciences [1–3]. Cross-linked enzyme aggregates (CLEA) have emerged as a novel and versatile biocatalyst design for obtaining immobilized enzymes for both aqueous and non-aqueous environments [4–6]. CLEA is obtained by cross-linking the precipitated enzyme by a bifunctional reagent. There are several advantages associated with this design [7]. An important feature is that one does not have to start with a purified enzyme preparation. The present work is based upon this particular feature and extends the concept of combi-CLEA [5,8]. If multiple enzyme activities can be simultaneously captured in a single CLEA, then this CLEA can be used for a variety of biotransformations. While combi-CLEAs have been mostly described for achieving two sequential steps for a single bioconversion/biotransformation, the approach of combi-CLEA in the present work is aimed at turning CLEA into a multipurpose biocatalyst capable of catalyzing non-cascade reactions. The design is for one biocatalyst preparation catalyzing various predetermined and unrelated biotransformations/bioconversions. It may be mentioned that utility of multi-enzyme complexes for multistep conversions for cascade catalysis has been reviewed recently [9].

As a "proof of the concept", pancreatic acetone powder extract was taken as the starting material. This extract is reported to be rich in several enzymatic activities such as lipase, α-amylase and phospholipase A_2 [10–12]. The challenge was to (a) develop a method for simultaneous precipitation of these three enzymes and (b) identify cross-linking conditions which allow retention of significant level of activities for all the three enzymes. The present work describes the results with this system and includes the preliminary characterization of "combi-CLEA".

2. Experimental

2.1. Materials

Pancreatic acetone powder and *p*-nitrophenylpalmitate (*p*-NPP) were purchased from Sigma Chemical Co., St. Louis, USA. Soybean lecithin was obtained from Fluka, England. Glutaraldehyde (25%, v/v) and starch were obtained from Merck, Germany. All other reagents used were of analytical grade.

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2.2. Extraction of enzymes from pancreatic acetone powder [12]

The extraction of enzymes from pancreatic acetone powder was carried out by suspending the powder (10 mg/ml) in 0.05 M Tris–HCl buffer, pH 6.8 and then vortexing the mixture for 5 min at 30 °C. The mixture was then centrifuged at 10,000 × *g* for 5 min. Supernatant (crude enzymatic extract) was used for preparation of combi-CLEA.

2.3. Precipitation of enzymes by organic solvents

Chilled organic solvents (acetone, dimethoxyethane and *t*-butanol, 5 ml each) were added dropwise separately to samples of crude enzymatic extract (1 ml) with shaking and kept for 15 min at 4 °C for complete precipitation of enzymes and then centrifuged for 5 min at 10,000 × *g*. The supernatant was discarded and precipitate was redissolved in 0.05 M Tris–HCl buffer, pH 6.8. Lipase, α-amylase and phospholipase A_2 activities were then checked.

2.4. Preparation of combi-CLEA

Chilled dimethoxyethane (DME, 5 ml) was added to crude enzymatic extract (1 ml) in capped centrifuge tubes. After keeping the mixture for 15 min at 4 °C for complete precipitation of enzymes, varying amounts of glutaraldehyde were added. The tubes were shaken continuously during this addition. The mixture was kept at 4 °C for 4 h with constant shaking at 300 rpm. At the end of the reaction time, suspension was centrifuged at 10,000 × *g* for 5 min. The supernatant was decanted and the pellets were washed three times with 0.05 M Tris–HCl buffer, pH 6.8 to remove unreacted glutaraldehyde. The final enzyme preparation was kept in the same buffer (1 ml) at 4 °C.

2.5. Assay of lipase

The hydrolytic activity of lipase was monitored by measuring hydrolysis of *p*-NPP at 410 nm [13]. One unit (U) of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of *p*-nitrophenol per minute at 37 °C and pH 7.0.

2.6. Assay of α-amylase

α-Amylase activity was estimated using starch (1%, w/v) as a substrate [14]. The reducing sugar generated was measured using 3,5-dinitrosalicylic acid [15]. One unit (U) of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of reducing sugar (estimated as maltose) per minute at 37 °C.

2.7. Assay of phospholipase A_2

Phospholipase A_2 activity was estimated using soybean lecithin (2%, w/v) as a substrate [16]. The fatty acids liberated during the reaction were titrated immediately with 0.02N NaOH using an automatic titrator (Metrohm, Switzerland). One unit (U) of enzyme activity is defined as the amount of enzyme that liberates 1 μmole of titratable fatty acids per minute from lecithin emulsion at pH 8.9 and 25 °C.

2.8. Protein estimation

Protein concentration was determined according to the procedure described by Bradford [17] using bovine serum albumin as the standard protein.

2.9. Determination of kinetic parameters

The kinetic parameters of free enzymes and CLEA were determined by measuring the initial rates of enzymes with varying amounts of respective substrate solutions under the assay conditions. The data were fitted in Hanes–Woolf equation using Leonora software to calculate the kinetic parameters [18].

2.10. Thermal stability study

Thermal stability was determined for lipase, α-amylase and phospholipase A_2 present in combi-CLEA. Thermal stability of lipase was studied at 50 °C for 30 min and that of α-amylase and phospholipase A_2 was studied at 60 °C for 30 min. In all the three cases, the activity at 0 min was taken as 100%.

2.11. pH and temperature optima of lipase, α-amylase and phospholipase A_2 in combi-CLEA

pH optima of lipase, α-amylase and phospholipase A_2 in CLEA were studied in the pH range of 3.5–9.5. Temperature optima of lipase, α-amylase and phospholipase A_2 in CLEA were studied in the temperature range of 25–65 °C.

2.12. Reusability of lipase and α-amylase in combi-CLEA

To evaluate the reusability of lipase and α-amylase in combi-CLEA, the CLEA in each case was washed with buffer after each use and then suspended again in a fresh reaction mixture to measure enzyme activity. The residual activity was calculated by taking the enzyme activity of the first cycle as 100%.

3. Results and discussion

In order to capture multiple activities in the ultimate product CLEA, it is necessary that all these activities are simultaneously precipitated to a significant level. The three key enzyme activities monitored in the present work were lipase, α-amylase and phospholipase A_2 activities. The well-known proteases of pancreatic origin are present as zymogens and hence were not looked at. Fig. 1 shows that of the three precipitants (acetone, DME and *t*-butanol) tried, DME worked best for precipitating all the three enzyme activities completely. Higher than 100% activities observed in the precipitates were due to traces of organic solvents [7]. Fig. 2 shows that at 20 mM glutaraldehyde, all the three enzyme activities were completely retained in the CLEA

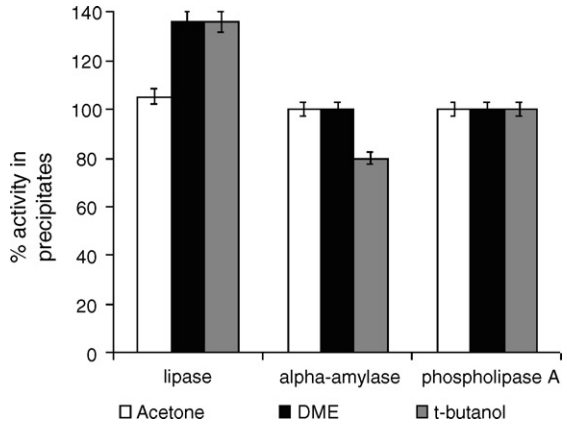

Fig. 1. Precipitation of enzymes with organic solvents. Three different precipitants (acetone, DME and *t*-butanol, 5 ml) were added to crude enzymatic extract (1 ml) for complete precipitation of enzymes at 4 °C as described in Section 2. The percentage activity of the enzyme in the precipitate was calculated by taking the initial activity as 100%. The experiments were done in triplicate and the error bar represents the percentage error in each set of readings.

product. The data, incidentally also show the differences in vulnerability which different enzyme activities have with respect to cross-linking with glutaraldehyde. The CLEA obtained with cross-linking of precipitates (obtained with DME) with 20 mM glutaraldehyde was further studied as combi-CLEA.

The Michaelis–Menten parameters provide a good insight into the kinetic behavior of enzymes. This information is critical with respect to immobilized enzyme in order to assess the effect of immobilization on kinetic performance of the enzymes. Table 1 provides V_{max}, K_m and V_{max}/K_m values for all the three activities as these are present in free form (in crude extract) and in the combi-CLEA. It should be kept in mind that precipitation as such may lead to partial purification of proteins and removal of low molecular weight impurities. This may also affect these kinetic parameters. Nevertheless, this data show that different enzyme activities have shown significant differences in the way their K_m and V_{max} changed upon CLEA formation. For lipase and phospholipase A_2, V_{max} and K_m did

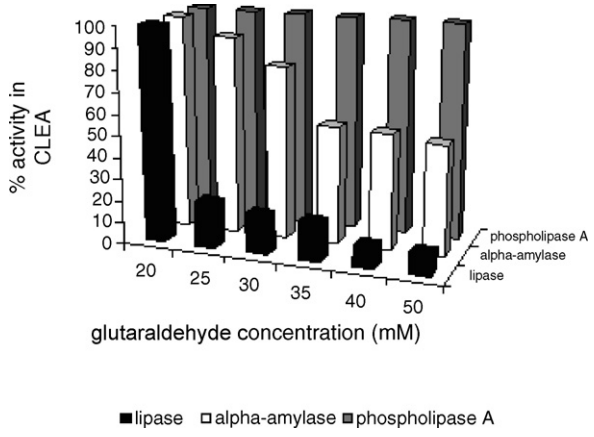

Fig. 2. Effect of varying amount of glutaraldehyde on CLEA activity. Combi-CLEA was prepared using varying concentration of glutaraldehyde (20–50 mM) as described in Section 2. Experiments were carried out in triplicate and percentage error in each set of readings was within 3%.

Table 1
Comparison of kinetic parameters of lipase, α-amylase and phospholipase A_2 in combi-CLEA

Enzyme	Parameter	Free	CLEAs
Lipase	V_{max} (μmol ml^{-1} min^{-1})	62.7	63.3
	K_m (mM)	0.2	0.2
	V_{max}/K_m (min^{-1})	313	316
α-Amylase	V_{max} (mg ml^{-1} min^{-1})	666	1195
	K_m (mg ml^{-1})	3.5	10.5
	V_{max}/K_m (min^{-1})	190	114
Phospholipase A_2	V_{max} (mg ml^{-1} min^{-1})	3.4	3.2
	K_m (mg ml^{-1})	9.3	8
	V_{max}/K_m (min^{-1})	0.3	0.4

Kinetic parameters of lipase, α-amylase and phospholipase A_2 were calculated with free enzymes and combi-CLEA.

not change drastically. Thus, the catalytic efficiency V_{max}/K_m changed only marginally upon CLEA formation for lipase from 313 to 316 and phospholipase A_2 from 0.3 to 0.4. On the other hand, V_{max} (upon the CLEA formation) improved for α-amylase activity but K_m worsened drastically. The later is not surprising in view of macromolecular nature of the substrate (starch) for α-amylase activity. The enzyme active site in CLEA would have an accessibility problem for the macromolecular substrate due to mass transfer constraints. This, of course, gets reflected in lowered catalytic efficiency of CLEA for α-amylase activity.

Another important parameter is the cross-linking time. The lipase-CLEA activity was found to decrease from 100 to 85 to 50% upon increasing cross-linking times of 4, 6 and 8 h, respectively. This decrease in activity, however, is by far compensated by a substantially higher stability of the lipase-CLEAs prepared by higher cross-linking times (Fig. 3). This phenomenon is in

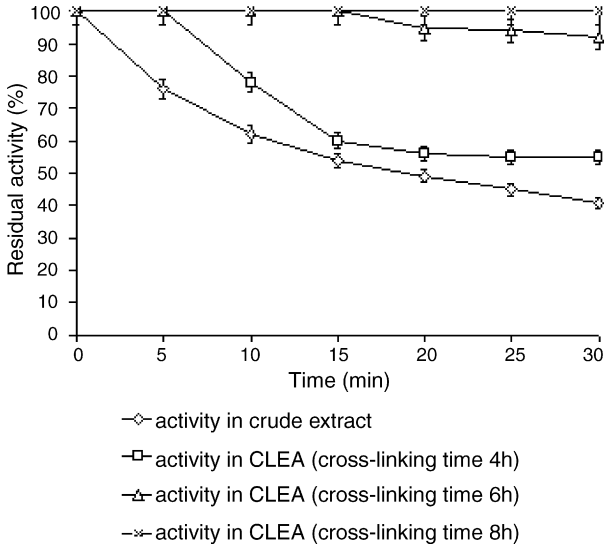

Fig. 3. Thermal stability of lipase in combi-CLEA. Thermal stability of lipase in free enzyme and CLEA was checked at 50 °C, using *p*-NPP as a substrate. An appropriate aliquot of free enzyme and CLEA (0.0038 U) was withdrawn at different time intervals, cooled to 37 °C (assay temperature) and assayed to measure their activities. The experiments were done in triplicate and the error bar represents the percentage error in each set of readings.

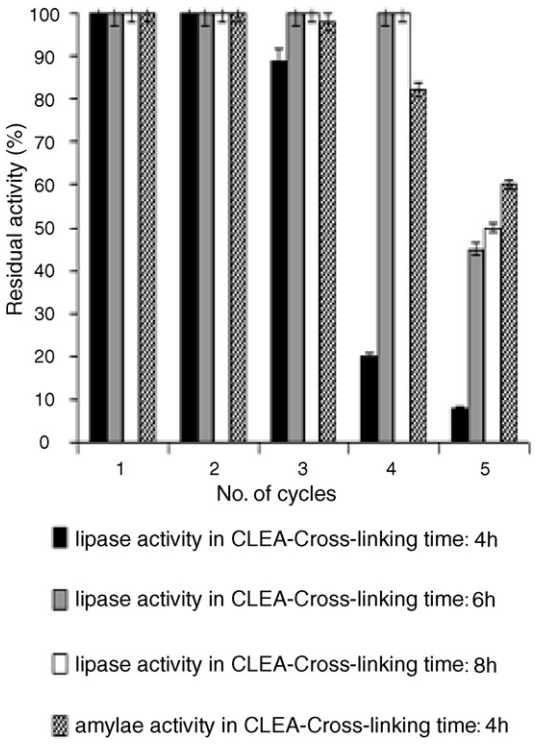

Fig. 4. Reusability of lipase and α-amylase in combi-CLEA. Reusability of lipase in combi-CLEA was studied by carrying out the hydrolysis of *p*-NPP at 37 °C for 30 min up to five cycles. After each cycle, the reaction mixture was centrifuged at 10,000 × *g* and the supernatant was read immediately at 410 nm. The CLEA recovered after centrifugation was used for next cycle of hydrolysis. The experiments were done in triplicate and error bars represents the percentage error in each set of readings. Reusability of α-amylase in combi-CLEA was studied by carrying out the hydrolysis of starch at 37 °C for 10 min up to five cycles. After each cycle, the reaction mixture was centrifuged at 10, 000 × *g* and the reducing sugar in the supernatant was measured by 3,5-dinitrosalicylic acid. The CLEA recovered after centrifugation was used for next cycle of hydrolysis. The experiments were done in triplicate and error bars represents the percentage error in each set of readings.

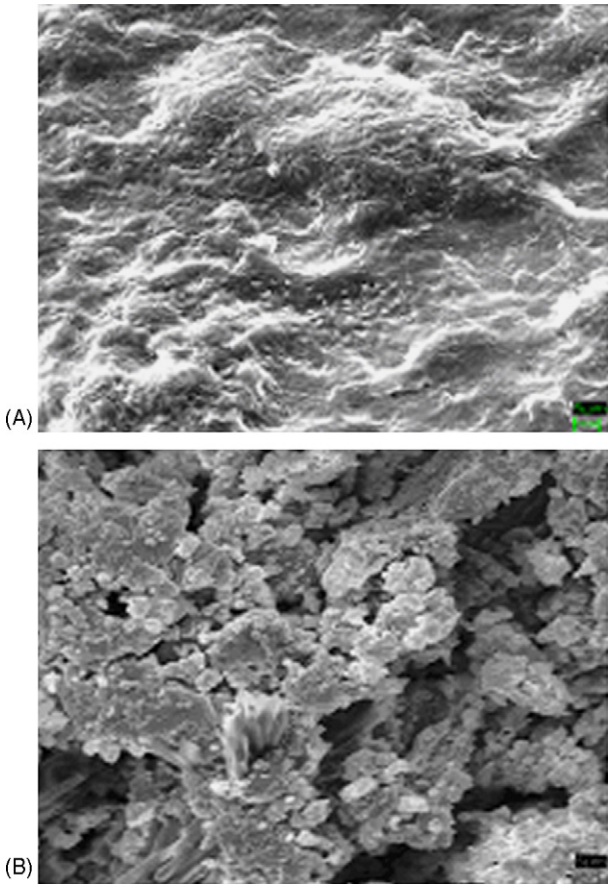

Fig. 5. (A) SEM image of combi-CLEA and (B) SEM image of combi-CLEA after five cycles of use (inactivated CLEA) (Magnification 1500×).

agreement with earlier observations of cross-linked enzymes [19,20].

Both the temperature optima and pH optima of lipase-CLEA's were almost the same as those for free lipase, i.e. 30–50 °C and pH 8.0, respectively. The same holds for both α-amylase (40 °C, pH 7.0) and phospholipase A_2 (60 °C, pH 8.0).

One main advantage of any immobilization method is that it leads to a reusable enzyme preparation. Repeated use of lipase-CLEA's and amylase-CLEA's up to three to four times was possible without loss of activity, in particular for those prepared using longer cross-linking times (Fig. 4). The logistics of phospholipase A_2 reusability study was difficult and hence this was not carried out. Soybean lecithin (2%, w/v) was in a thick emulsion form and hence it was not possible to recover CLEA after centrifugation for subsequent study since both soybean lecithin and CLEA were found to settle down together.

Leaching of protein and lipase activity was studied during reusability experiment of lipase-CLEA. After each cycle of use, lipase activity was checked in washings. These results suggest that loss in activity in fourth and fifth cycles might be due to inactivation of CLEA upon reuse rather than leaching of enzyme activity. Not much data are available in the literature regarding reusability of CLEAs except reusability of CLEAs of oxynitrilase [21]. Experience with wider range of system is desirable. The inactivation due to reuse is likely to be due to conformational changes which distort the active site. It may be noted that these combi-CLEAs have been prepared by utilizing low concentration of glutaraldehyde (20 mM) and hence may not have high density of cross-linking. The correlation between the extent of cross-linking and resultant protein stability is well known. The molecular level changes in conformation are also reflected at the macroscopic level. Comparison of scanning electron microscopic (SEM) pictures of the combi-CLEA before and after five times use in the lipase catalyzed hydrolysis of *p*-nitrophenylpalmitate shows that the inactivation is accompanied by a substantial change in morphology (Fig. 5).

4. Conclusions

The combi-CLEA prepared by cross-linking a crude protein/enzyme source, containing lipase, α-amylase and phospholipase A_2, showed good multi-enzyme activities as well as stability for repeated use. Such multi-enzyme-CLEA's are considered as useful preparations from both a laboratory and industrial point of view. For example, wheat germ is known to contain several enzyme activities such as acid phosphatase,

lipase and amylase [22]. Similarly, broth from fermentors growing microbes are often rich in several enzymes. In some cases, it is possible that such combi-CLEAs can be used for different biotransformations in either aqueous or non-aqueous media.

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