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ORIGINAL PAPER

Characterization of Cross-Linked Lipase Aggregates

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Abstract Commercially available microbial lipases from different sources were immobilized as cross-linked enzyme aggregates (CLEAs) using different precipitants and glutaraldehyde as cross-linkers. These CLEAs were assayed based on esterification between lauric acid and *n*-propanol in solvent-free systems. Precipitants were found to have a profound influence on both specific activities and total activity recovery of CLEAs, as exemplified by *Candida antarctica* lipase B (CALB). 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. PEG200 precipitated CLEA gave the best specific activity (139% relative to acetone powder). The results of kinetic studies showed that V_{max}/K_m does not significantly change upon CLEA formation. This work presents a characterization of CLEAs based on an esterification activity assay, which is useful for exploring the synthetic application potential of CLEA technology with favorable perspectives.

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Introduction

Due to their ability to catalyze a diverse array of reactions, lipases (EC 3.1.1.3) are among the most broadly employed biocatalysts with applications in the detergent, oil and fats, organic synthesis and pharmaceutical industries [1, 2]. However, their widespread utilization is often limited by poor operability of free enzymes and the high cost of immobilization processing. The design of new enzyme immobilization techniques for the preparation of industrial biocatalysts has led to new opportunities in biocatalyst engineering [3, 4]. Very recently, cross-linked enzyme aggregates (CLEAs) have emerged as a novel and versatile approach to obtain immobilized enzymes without the use of any pre-existing carriers, in a form suited for both aqueous and non-aqueous environments [5–8]. The CLEA technique is attractive in both its simplicity and its robustness, combining purification with immobilization in one step.

There are several reports on the preparation of CLEAs of lipases with or without support, whereby many focused primarily on hydrolysis reactions; consequently, very few reported any evaluation of systematic esterification activity [5–12]. For mainstream applications of lipases, the most important and interesting industrial uses often involve esterification and transesterification reactions. The creation of useful enzyme preparations for these applications is therefore highly attractive.

In this study, CLEAs from a solution of *Candida antarctica* lipase B (CALB-L) were systematically investigated and evaluated for their esterification activity during

solvent-free biosynthesis of *n*-propyl laurate. Employing this approach, a wide range of precipitants was screened for their ability to produce more active CLEA preparations for ester synthesis with favorable perspectives for industrial uses. CLEAs of a wide variety of lipases were also prepared using a range of organic as well as aqueous precipitants for aggregation, followed by cross-linking with glutaraldehyde [6]. Finally, a kinetic study of the most active CLEA from CALB (prepared using PEG200 as precipitant) was also carried out based on esterification ability and was compared to both simple acetone powder of CALB-L and the commercially immobilized preparations (Novozym 435).

Materials and Methods

Materials

A solution of *C. antarctica* lipase B (CALB-L), Novozym 435 (*C. antarctica* lipase B immobilized on macroporous polyacrylate resin beads), and a solution of *Thermomyces lanuginosa* lipase (TL100L) were obtained from Novozymes A/S (Bagsvaerd, Denmark). Lipase from *C. rugosa* (Type VII) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Lipase AK "Amano" 20 (*Pseudomonas fluorescens*), Lipase PS "Amano" 30 (*Pseudomonas cepacia*), Lipase A "Amano" 12 (*Aspergillus niger*), Lipase M "Amano" 10 (*Mucor javanicus*), Lipase D "Amano" 350 (*Rhizopus delemar*) and Newlase F (*Rhizopus niveus*) were kindly donated by Amano Pharmaceutical Co. Ltd. (Tokyo, Japan). All other solvents and reagents were of analytical grade and obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Preparation of CLEAs of Lipases Using Organic Solvents

CLEAs of 10 enzymes were prepared using a modified literature procedure [6]. Lipase (1.0 mL enzyme stock solution or 100 mg enzyme powder) was dissolved in 2 mL sodium phosphate buffer (100 mM, pH 7) in a 25 mL conical flask and stirred gently at room temperature for 10 min. To the enzyme solution, 6 mL of precipitant (i.e., saturated $(NH_4)_2SO_4$ solution, acetone, butanone, 2-propanol, ethylene glycol, 1,3-propanediol, PEG200, PEG600 or *t*-butylmethyl ether) and 0.160 mL of glutaraldehyde solution (25%, w/v in water) were added, and the solution was stirred for 3 h at room temperature. Then 0.4 mL of sodium borohydride solution (100 mM) was added to reduce the Schiff's bases formed. Following a 15 min reaction period, the mixture was cooled down to 5 °C without stirring for 12 h to precipitate the CLEA. Precipitate was isolated by centrifugation at 4,000 rpm at 2 °C for 15 min. In the case of PEG200 and PEG600, no precipitation was observed even after cooling overnight at 5 °C; hence, 6 mL of 2-propanol was added to these preparations followed by cooling and centrifugation steps. The supernatant was decanted and the residue was washed with sodium phosphate buffer (100 mM, pH 7, 2 × 2 mL) with CLEA again recovered by centrifugation. The final preparation was washed with acetone (2 × 2 mL) and dried under a stream of nitrogen to obtain powdered CLEA. All preparations were carried out in duplicate, reported as the mean ± standard deviations.

Preparation of Acetone Powders of CALB-L and TL100L

The enzyme solution (5.0 mL) was dissolved in 10 mL sodium phosphate buffer (100 mM, pH 7) in a 25-mL conical flask and stirred gently at room temperature for 10 min. To this enzyme solution, 30 mL acetone was added. The solution was stirred for 3 h and then cooled to 5 °C after which the precipitated enzyme was isolated by centrifugation at 4,000 rpm and 2 °C for 15 min. The supernatant was decanted and the residue was washed with acetone (2 × 5 mL) followed by drying under a stream of nitrogen, resulting in an enzyme powder. All preparations were carried out in duplicate, reported as means ± standard deviations.

Synthetic Activity Assay

Lipase activity was assayed based on the number of propyl laurate units (PLU) formed per minute by unit enzyme preparation (mmol min^{-1} mg enzyme $preparation^{-1}$). Native lipase (acetone powder), prepared CLEAs and immobilized Novozym 435 lipase were all assayed for their synthetic activity based on the direct esterification reaction of lauric acid (LA) and *n*-propanol. In all cases, the initial reaction medium consisted of a stoichiometric mixture of substrates (9.98 mmol) and a fixed amount of added water (4 wt.% of lauric acid). Reactions were initiated by addition of biocatalyst and were carried out at 60 °C in 10 mL capped reaction vials stirred at 350 rpm. Progress of the esterification was monitored by determination of the residual acid content by titration with 0.1 M NaOH solution, using phenolphthalein as the end-point indicator and ethanol–chloroform (50/50, v/v) as quenching reagent. Conversion of lauric acid at given times were determined based on the relative reduction of the acidity index of the samples. All measurements were carried out in duplicate, and the average value of the two determinations was used for result evaluation.

Kinetic Studies

Kinetic constants for the esterification reaction of lauric acid with *n*-propanol by acetone powder, cross-linked aggregate and immobilized forms of CALB were determined using the classic Michaelis–Menton equation. Specifically, the concentration of lauric acid ([S]) was varied from 0.368 to 1.557 M while maintaining the other substrate (1-propanol) constant at 9 M. The concentration of enzyme employed was 1 mg/mL, water addition was fixed at 7.6 mg/mL and reaction temperature was 60 °C. A Lineweaver–Burk plot of 1/V versus 1/[S] was employed for V_{max} and K_m calculations.

Note that optimum temperature was determined following investigations from 40 to 80 °C (at 5 °C intervals) using the following reaction conditions: LA (0.5 g, 2.5 mmol); 1-propanol (0.185 mL, 2.5 mmol); water (0.02 mL, 4 wt.% of lauric acid); biocatalyst (10 mg, 2 wt.%); 300 rpm; 20 min reaction time.

Results and Discussion

Esterification Activity of CLEAs of CALB by Different Precipitants

Table 1 compares esterification activity and activity recovery of CLEAs of *Candida antarctica* lipase B (CALB) with its corresponding acetone powder. CLEAs of CALB-L obtained from four types of precipitants (i.e., acetone, PEG200, PEG600 and $(NH_4)_2SO_4$) showed elevated specific activities. PEG200 precipitated cross-linking aggregate had the highest specific activity, which exhibited 139% esterification activity of acetone powder under identical conditions. Interestingly, yields of CLEA preparations were also precipitant dependent. PEG600 butanone, and $(NH_4)_2SO_4$ as precipitants produced more than double the amount of CLEAs in comparison with the acetone powder. Precipitants such as acetone, 1,3-propanediol, and PEG200 also resulted in considerable CLEA yields. These results were very encouraging. In addition, 2-butanone precipitated CLEA obtained 157% activity recovery compared to the acetone powder.

Effects of precipitants on the activity of aggregates and CLEAs have been examined previously based on a hydrolytic assay. Despite the fact that the activity assay was based on a different reaction system, some similarities still exist with the reported results [6]. Certain generalizations were thus made possible. For example, use of either PEG or saturated $(NH_4)_2SO_4$ solution could yield improved activity recovery. In contrast, water-miscible lower alcohols such as methanol, ethanol, 1-propanol or 2-propanol, etc. [6], as well as 1,3-propanediol and ethylene glycol, led to a decreased specific activity. CLEA activity following precipitation by water-immiscible solvents like ethyl acetate and TMBE was also not promising. The yield of CLEAs from the dissolved enzyme solution is crucial to their total activity recovery. As depicted in Table 1, the cross-linking process resulted in a significant increase in weight of the lipase preparation. The contribution of the

Table 1 Activity evaluations with respect to esterification for the acetone powder and different CLEAs aggregated by different precipitating reagents of *Candida antarctica* lipase B

Lipase preparation[a]	Specific activity (1,000 × mmol min^{-1} mg^{-1})[b]	Preparation yield (g)	Total activity recovery (mmol min^{-1})[c]	Relative total activity recovery (%)[c]
CALB-powder	6.588 ± 0.329	0.405 ± 0.031	2.668 ± 0.187	100.0 ± 7.1
CLEA-Acetone	7.125 ± 0.356	0.755 ± 0.066	5.380 ± 0.380	201.6 ± 14.2
CLEA-2-butanone	5.138 ± 0.257	0.815 ± 0.072	4.187 ± 0.296	156.9 ± 11.1
CLEA-2-propanol	4.588 ± 0.229	0.345 ± 0.020	1.583 ± 0.112	59.3 ± 4.2
CLEA-E.G	3.975 ± 0.198	0.430 ± 0.034	1.709 ± 0.121	64.1 ± 4.5
CLEA-1,3-PD	3.713 ± 0.185	0.715 ± 0.053	2.654 ± 0.188	99.5 ± 6.9
CLEA-PEG200	9.188 ± 0.459	0.735 ± 0.078	6.753 ± 0.477	253.2 ± 17.9
CLEA-PEG600	7.138 ± 0.357	1.050 ± 0.112	7.494 ± 0.530	280.9 ± 19.9
CLEA-TBME	1.775 ± 0.089	0.495 ± 0.039	0.879 ± 0.062	32.9 ± 2.3
CLEA-$(NH_4)_2SO_4$	8.225 ± 0.412	0.880 ± 0.065	7.238 ± 0.512	271.3 ± 19.2

Reaction conditions: LA (0.5 g, 2.5 mmol); 1-propanol (0.185 mL, 2.5 mmol); water (0.02 mL, 4 wt.%); biocatalyst (10 mg, 2 wt.%); 60 °C; 350 rpm; 20 min

[a] All preparations were carried out using 5 mL *Candida antarctica* lipase B

[b] Specific activity (mmol min^{-1} mg^{-1}) was defined as mmol lauric acid converted into ester in 1 min, catalyzed by mg lipase preparation

[c] The total activity recovery was calculated as: specific activity × preparation weight. The total activity of acetone powder was defined as 100% and relative activities of CLEAs were calculated based on comparison with acetone powder. Data used for evaluation are mean ± standard deviations of two determinations

cross-linker (very low concentration) to the mass of the CLEA was very minimal, meaning that there was no explanation for the significant improvement in CLEA yield compared with the corresponding yield through simple precipitation. The real reason remains to be explored. For a more comprehensive assessment of CLEA preparations, the total activity recovery of CLEAs was calculated. PEG200-, PEG600-, and acetone-precipitated CLEAs showed better specific activities as well as preparation yields, producing higher total activity recovery (Table 1). Hence, they were selected for further investigations of other variables. While $(NH_4)_2SO_4$-precipitated CLEA also showed excellent results, it was not selected because it had been intensively studied before [6]. Acetone powder and acetone-precipitated CLEAs were included for comparison as many commercial enzyme powders are acetone precipitates.

CLEA Activities of Lipases from Different Sources

On the basis of the above study, precipitants such as acetone, PEG200 and PEG600 were employed in the preparation of CLEAs from other lipase sources. Synthetic activity, measured based on the esterification of lauric acid with 1-propanol, was thus assayed for a range of CLEAs in comparison with their activity in acetone powder (Fig. 1). Results show that esterification activities of CLEA preparations for lipases from different sources vary with precipitants and show different performances with respect to their corresponding acetone powders. Lipase from *Thermomyces lanuginosus* was an exception as the synthetic activity of its acetone powder was over three times higher than any of its CLEA forms. Lipase PS was another exception as acetone powder activity was also higher than all corresponding CLEA preparations. For the other lipases tested, on the contrary, CLEA preparations possessed elevated esterification activity versus their acetone powders, with PEG200 precipitated CLEAs generally exhibiting better activities. For instance, the CLEAs of *C. rugosa* lipase showed 270% activity relative to its original enzyme powder while for lipase D, the enhancement was even higher (Fig. 1). Different esterification activities observed for lipases originating from varied sources may be due to their intrinsic kinetics. The activity of CLEAs of lipases from the same source also varied according to precipitants (Fig. 1). The real reason is not clear. The hypothesis is likely that lipases from different sources have different glycosylated surfaces, corresponding to different lipo-hydrophilic properties [6]. This might result in different aggregating, packing and configuring behaviors of protein molecules when different precipitating reagents are applied.

Kinetic Properties of CLEAs

Reaction temperature is an important parameter that affects both the reaction rate and the solubilities of different substrates. The optimum temperature for both the acetone powder and the CLEA of CAL-B was found to be 60 °C, whereas Novozym 435 attains its maximum activity at 70 °C (Table 2). These results suggest that cross-linking by glutaraldehyde might give rise to a less rigid structure than immobilization, in which the latter itself provides a strong interacting microenvironment to restrain enzyme molecules and leads to a higher optimum temperature (Novozym 435). CLEA behavior, to a greater extent, resembles

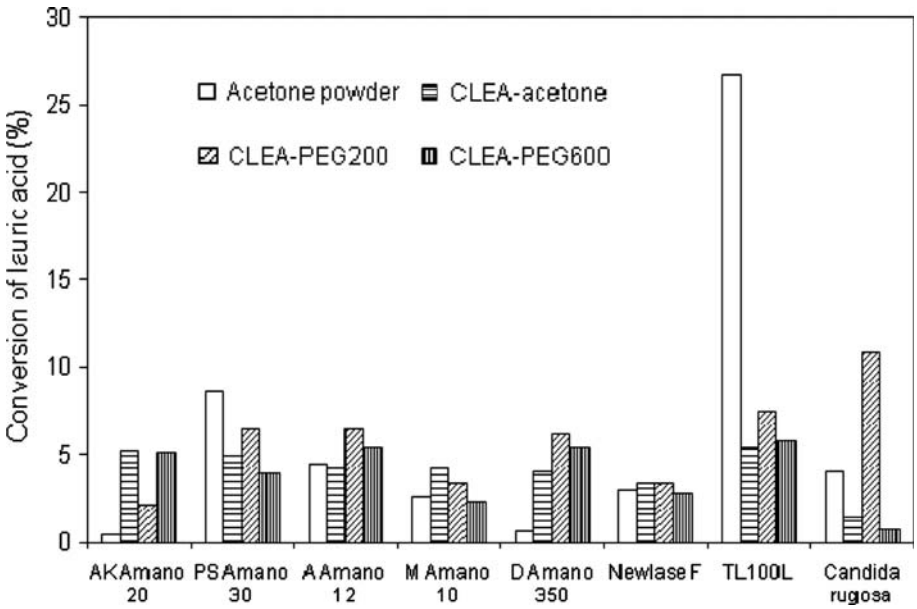

Fig. 1 Esterification activities of CLEAs of lipases from different sources. Reaction conditions: LA (0.5 g, 2.50 mmol); 1-propanol (0.185 mL, 2.50 mmol); water (0.02 mL, 4 wt.%); biocatalyst (10 mg, 2 wt.%); 50 °C; 300 rpm; 20 min. The mean of two determinations were used for evaluation. Standard deviations of resulting mean were lower than ±1%

Table 2 Comparison of kinetic parameters of acetone powder of CALB, CLEA-PEG200 of CALB and Novozym 435

Enzyme	Parameter			
	Optimum temperature (°C)	V_{max} (mmol mL^{-1} min^{-1})	K_m (mM)	V_{max}/K_m ($\times 10^3$ min^{-1})
CALB-acetone powder	60	51.55 ± 1.54	6.39 ± 0.17	8.07 ± 0.27
CLEA-CALB-PEG200	60	79.37 ± 2.22	11.23 ± 0.30	7.07 ± 0.31
Novozym 435	70	9.71 ± 0.28	4.83 ± 0.14	2.00 ± 0.07

Optimum temperature determination conditions: LA (0.5 g, 2.5 mmol); 1-propanol (0.185 mL, 2.5 mmol); water (0.02 mL, 4 wt.%); biocatalyst (10 mg, 2 wt.%); 300 rpm; 20 min, temperature range 40–80 °C with 5 °C intervals. For all three enzyme preparations, duplicate determinations gave the same optimum temperature value as indicated in the table. Kinetic study was conducted at 60 °C, with lauric acid concentration ([S]) varied from 0.368 to 1.557 M while the other (1-propanol) was kept constant at 9 M. Enzyme concentration was 1 mg/mL. Water addition was fixed at 7.6 mg/mL

that of free enzyme (acetone powder), allowing similar freedom, which corresponds to similar temperature optima.

Michaelis–Menton parameters provide a good insight into the kinetic behavior of enzymes. Table 2 shows V_{max}, K_m and V_{max}/K_m values for the three forms of CALB, namely acetone powder, CLEA obtained by cross-linking with glutaraldehyde using precipitant PEG200, and Novozym 435. Obviously, the CLEA preparation of CALB by PEG200 exhibits the highest reaction rate (V_{max}) for the synthesis of 1-propyl laurate, approximately 8 times higher than that of Novozym 435. The higher V_{max} associated with the PEG200-CLEA may be attributed to the high enzyme concentration by cross-linking of the proteins with glutaraldehyde, while the lower V_{max} of Novozym 435 may be the result of a dilution effect from the immobilization carrier. As another intrinsic kinetic parameter of enzymes describing catalytic efficiency, V_{max}/K_m shows only a marginal change from 8.07×10^3 (acetone powder) to 7.07×10^3 (CLEA by PEG 200), which agrees with a previous report [13]. This result, from another point, supports the already mentioned hypothesis in this context that the kinetic behavior of CLEA, to a greater extent, resembles free enzyme (acetone powder in this work) rather than a typical immobilized enzyme. In addition, the higher enzyme concentration of CLEAs may represent a leading reason for the higher specific activity of CLEA preparations.

In conclusion, preparation and characterization of cross-linking enzyme aggregates (CLEAs) of lipases from different sources was investigated based on an esterification activity assay using lauric acid reacted with 1-propanol. CLEA of CALB precipitated by PEG200 was found to retain the highest specific activity, whereas PEG600, ammonium sulfate, and acetone were also excellent precipitants. The results of kinetic studies indicate that the reaction behaviors of CLEAs, to a great extent, resemble a free enzyme (acetone powder), but with a higher V_{max}. Overall, CLEAs offers a facile and low-cost approach for lipase immobilization. The approach can provide mechanically stable and recyclable lipase preparations with high activity retention. It could provide a promising alternative for the current immobilization methodologies for synthetic applications.

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