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Optimized preparation of CALB-CLEAs by response surface methodology: The necessity to employ a feeder to have an effective crosslinking

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ABSTRACT

Lipase B from *Candida antarctica* (CALB) has been immobilized using the CLEA technique. Due to the low content of surface Lys on the enzyme and the purity of the commercial preparation, CALB crosslinking did not work properly, and it was always possible to find some CALB (as molecules or soluble aggregates) when analyzing the CLEA using SDS-PAGE. To improve the crosslinking, bovine serum albumin was used as a feeder, and after optimization using response surface methodology, the glutaraldehyde crosslinking step was effective, and permitted to greatly stabilize the enzyme (no activity decrease was observed after a time where the free enzyme was almost fully inactivated). After two experimental designs, the best conditions for preparation of CALB–BSA-CLEA were: protein concentration (3 mg/mL), *tert*-butylalcohol as precipitant, precipitation for 60 min; precipitant concentration, 50% v/v; and glutaraldehyde concentration (1.5% w/v).

1. Introduction

Lipases are among the most relevant enzymes in organic chemistry because they combine a broad range of substrates with a high regio- and/or enantio specificity and/or selectivity [1–5]. They are used from energy to food industries, to fine and pharmaceutical chemistry [1–7]. The use and manipulation of lipases requires bearing in mind that these enzymes suffer drastic changes in their structure during catalysis, involving the displacement of a polypeptide called lid and the change between an open and a closed structure [8,9]. This flexibility has allowed the lipase properties to be strongly modulated by strategies that permit to alter those changes, for example, via medium engineering, immobilization and genetic or chemical modifications [6,7,10–14]. Lipase B from *Pseudozyma antarctica* (formerly *Candida antarctica*) is the most used lipase in biocatalysis [15,16]. As the enzyme is worldwide known as CALB, we will keep that nomenclature throughout the paper. The 3D-protein structure of this lipase has been resolved [17]. It has a very small lid and does not suffer a large increase in activity by interfacial activation, but may still become adsorbed on hydrophobic surfaces through the surface areas near its active center and allowing the lid to be displaced [18–20].

A critical step in the preparation of most industrial enzyme biocatalysts is its immobilization. This may allow enzyme reusability (if the enzyme is stable enough) and also, if properly carried out, it may improve various enzyme features (mainly stability, but also activity, selectivity, specificity, or inhibitions problems) [21–25]. There are many different immobilization techniques, and each of them has advantages and disadvantages, as it has been recently reviewed [26]. The technique of enzyme immobilization by the preparation of crosslinked enzyme aggregates (CLEAs), designed by Prof. Sheldon's group [27–29] has revealed itself as a very interesting one, mainly when a very high volumetric loading is desired (e.g., if the substrate did not permit to reach very high activity values). The preparation of CLEAs involves the precipitation of the enzyme and the further chemical crosslinking of the protein aggregate to prevent enzyme re-dissolution when the precipitating agent is removed [26–29]. In the absence of a proper crosslinking, the "CLEA" could be just used in organic media (where the enzyme will be insoluble), but if the crosslinking is effective, the particle will remain insoluble under any reaction conditions [26]. Since no pre-existing support is required, production costs are reduced and a

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high activity per gram of biocatalyst can be obtained since most of the biocatalyst mass will be protein [26–29]. Among the problems of this strategy, the low mechanical resistance and severe diffusional limitations may be mentioned [26–29]. The preparation of lipase CLEAs using different conditions has been already used to alter enzyme properties [30], and CALB has been used in several instances to prepare CLEAs [31–33].

The crosslinking step is a critical step in the preparation of CLEAs. Some enzymes that are very poor in superficial Lys residues cannot be properly crosslinked and in those cases the use of a polymer rich in amino residues (usually polyethylenimine) has been proposed [34–38]. The poly-ionic nature of these polymers alters the enzyme environment, producing a partition of organic solvents or dissolved gases from the enzyme surroundings. In some instances this has permitted enzyme stabilization against these reagents [39,40]. In other cases, the use of other proteins like "feeders" has been proposed, e.g., bovine serum albumin (BSA) [41–45]. The use of polymers such as polyethylenimine may give better possibilities to involve the protein in the crosslinking, owing to the very high density of amino groups in the polymer, the use of a protein may have some advantages. Among them, it may be remarked a likely lower interaction between the protein feeder and the target enzyme, a lower alteration of the enzyme environment, and the possibility of getting better values on enzyme stabilization due to the higher protein rigidity compared to that of the polymers.

It is worth noting that, the preparation of CLEAs is a quite complex process. Any factor that may alter the protein precipitation or the aggregate crosslinking may affect the particle size, and activity recovery, in many instances co-interacting among them [46,47]. This makes the optimization of the preparation of these biocatalysts quite complex, mainly if varying one factor at a time, while keeping the other factors constant [48], a method that may be inefficient when there are interactions among several variables. Moreover, this is a time-consuming procedure and often does not bring about the co-interaction effects between various parameters, while this may be achieved using statistical methods [48]. The use of response surface methodology (RSM) for optimizing this process may be a potent tool to optimize these complex processes.

In this paper, we have used RSM to optimize the preparation of CLEAs of CALB, because as it has been previously commented the CLEAs preparation may be a quite complex process. Moreover, the effects of BSA as a feeder on the promotion of an intense crosslinking on the aggregated proteins have been analyzed. The physical stability of the CLEA particles will be evaluated by the release of enzyme molecules under drastic conditions; moreover the stability of the immobilized enzyme will be assessed.

2. Materials and methods

2.1. Materials

Glutaraldehyde (25% v/v stabilized in methanol), p-nitrophenyl butyrate (pNPB), bovin serum albumin (BSA) were purchased from Sigma. CALB was kindly supplied by Novozymes (a protein concentration of 5 mg/mL and a specific activity of 27.8 U/mg in pNPB hydrolysis, see below). All other reagents and solvents were of analytical degree.

2.2. Standard measurement of CALB activity

This assay was performed by measuring the increase in absorbance at 348 nm produced by the released p-nitrophenol in the hydrolysis of 0.4 mM p-nitrophenyl butyrate (p-NPB) in 25 mM sodium phosphate at pH 7.0 and 25 °C. To start the reaction, 50–100 μL of lipase solution or suspension was added to 2.5 mL of substrate solution. One international unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1 μmol of p-NPB per minute under the conditions described previously. Protein concentration was determined using Bradford's method with BSA as standard protein [49].

Table 1
Variables and their levels used in the full factorial design for precipitant agent optimization.

Variable	Name	Coded level		
		-1	0	1
X	Precipitant agent	Ammonium sulfate	*tert*-Butylalcohol	Dimethyl ether
Y	Precipitant concentration (% v/v)	50	70	90
Z	Reaction time (min)	15	30	60

2.3. Preparation of CALB-CLEAs

A commercial CALB preparation was diluted using 5 mM sodium phosphate at the desired pH value, to reach the desired protein concentration. In some instances BSA was added to this solution. 5 mL of this solution was added to 30 mL of precipitant reagent under a stirring of 500 rpm for 12 h. Then, the desired amount of glutaraldehyde was added. Finally, the CLEAs were recovered by centrifugation. The CLEAs were resuspended in 10 volumes of sodium phosphate 5 mM sodium phosphate at the desired pH value and centrifuged 4 times to ensure the elimination of all non-immobilized enzyme. Then, the CLEAs were stored at 4 °C. Table 1 shows the main studied variables in the optimization.

2.4. Experimental design

Two sequential full factorial designs (3^3) with three variables varying at three levels were carried out in order to obtain the optimal conditions for CLEAs preparation: first optimizing the precipitant agent and second the protein and crosslinking reagent concentrations. The variables and their coded and uncoded values are presented in Table 1 for the first design and Table 2 for the second design. Tables 3 and 4 show the 27 experiments of each design. The runs were performed in a random order, but are presented in standard order in the tables.

2.5. SDS-PAGE experiments

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [50] using a Miniprotean tetra-cell (Biorad), 15% running gel in a separation zone of 9 cm × 6 cm, and a concentration zone of 5% polyacrylamide. One hundred milligrams of the immobilized enzyme samples were re-suspended in 1 mL of rupture buffer (2% SDS and 10% mercaptoethanol), boiled for 5 min and a 20 μL aliquot of the supernatant was used in the experiments. Gels were stained with Coomassie brilliant blue. Low molecular weight markers from Fermentas were used (10–200 kDa).

Table 2
Variables and their levels used in the full factorial design for CLEA-BSA–CALB preparation optimization.

Variable	Name	Coded level		
		−1	0	1
X	Protein concentration (mg/mL)	3	5	7
Y	Precipitant concentration (% v/v)	50	70	90
Z	Glutaraldehyde concentration (% w/v)	0.5	1	1.5