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Analytical characterization and purification of a commercial extract of enzymes: A case study

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ABSTRACT

This paper presents a rational strategy to identify and quantify the components of a commercial extract of the lipase B of *Candida antarctica* that can be extended to the analytical investigation of other crude extracts of enzymes. These information provided the fundamental knowledge for the development of a methodology to obtain highly pure and catalytically active CALB enzyme.

The commercial extract Lipozyme® was subjected to a series of analytical techniques that allowed determining the presence of a non-soluble fraction; nucleic acids; benzoate and sorbate species and a mixture of three proteins. Particularly, it is worth noticing that the Bradford assay using CALB as standard instead of BSA proved to be a more reliable and accurate methodology to quantify the protein content of the assayed enzymatic samples. Size exclusion chromatography coupled with anionic exchange chromatography using a non-conventional, easy to remove buffer system such as ammonia–ammonium acetate afforded a sample that retains 47% of the proteins (being CALB the only enzymatic component of the purified sample) with a hydrolytic activity higher than the crude extract.

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1. Introduction

A cutting edge topic in bio-catalysis is the development of new materials based on immobilized enzymes. New biocatalysts facing further technological applications are synthesized with commercial extracts of enzymes which are far less expensive than pure enzymes. However, at this point new questions arise such as the nature of the components of such commercial extracts and how to determine its protein content. This is fundamental information in order to develop a reliable and reproducible synthesis of biocatalytic materials and a purification methodology. The complexity and variety of crude extracts of enzymes made necessary tailoring not only the analytical characterization but the purification strategies that sometimes are required to isolate an enzyme or mixtures of enzymes for further fundamental investigations on the relationships between biocatalytic activity-molecular structure and stability.

The lipase B of *Candida antarctica* immobilized onto polymethylmethacrylate is the most widely heterogeneous biocatalyst (known as Novozym® 435) used in industrial processes. Actually, the literature reports several methodologies to purify such enzyme that goes from single to multi steps purification methodologies depending on the particular sample and the impurities.

The lipase B of *C. antarctica* regularly possesses two other proteins with approximately 18 and 50 kDa. The 50 kDa have been removed on a diethylaminoethyl-cellulose column equilibrated with 25 mM Tris–HCl at pH 7.0 [1]. Further dialysis against distilled water for 24 h afforded 40–50% pure CALB.

The purification of *C. antarctica* lipase B fused to a cellulose-binding domain expressed in the yeast *Pichia pastoris* requires also two steps in order to afford a yield of 69% [2]. This particular culture medium was first purified through hydrophobic interaction chromatography using a butyl-Sepharose fast flow column. However, this procedure did not separate a 70 kDa protein which required a gel filtration step for further purification. A similar methodology was used by Magnusson et al. in order to purify CALB which active site (specifically the Thr40 amino-acid) was mutated in order to modify its enantioselectivity [3].

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To the best of our knowledge, the most complete investigation of a one-step purification strategy was reported by Pleiss and coworkers [4]. The authors developed a one-step method for purification of recombinant CALB from a culture and a crude extract (Chirazyme L-2) by ion-exchange chromatography adjusting the pH to 3 which enables bounding the enzyme to a cation-exchange resin. The enzyme was recovered from the column with a mixture of sodium formate, sodium citrate and sodium acetate at pH 5.5. Interestingly, they found that CALB shows isoelectric behavior in a broad pH range of pH going from 4 to 8.

More recently, Li and coworkers reported one-step purification based on biomimetic affinity chromatography. This methodology used synthetic ligands to bind and recover CALB from a protein extract [5]. The authors found that the ligands based on cyclohexylamine-propenylamine, cyclohexylamine-1-amantadine and M-aminophenylboronic acid-4 aminobenzamidine yield 73% recovery and 91% purification of CALB.

The literature demonstrates that each enzymatic system requires a specific purification methodology and necessarily the composition of the sample must be known in order to apply a rational design. Online with this observation, the present investigation gathers fundamental information about the nature and quantity of the components of the commercial extract known as CALB L from Novozymes. This information allowed tailoring a purification method in order to isolate the lipase B of *C. antarctica*. The strategy to develop such a purification methodology can be extended to other enzymatic systems.

2. Experimental

2.1. Enzymatic based materials

Highly pure bovine serum albumin BSA (lot 126H0255, 99%) and *C. antarctica* lipase recombinant from *Aspergillus oryzae* (Fluka, 7.2–10.8 U/mg) were purchased from Sigma Aldrich Argentina (10.9 U/mg). Additionally, the lipase B of *C. antarctica* CALB L (Lipozyme® LCN02102) provided by Novozymes Brazil (Paraná, Brazil) was used. This commercial extract of CALB (1.0 ml) was centrifuged at 9600 g for 30 min at 4 °C in a refrigerated Hermle centrifuge.

2.2. SDS-PAGE electrophoresis for proteins recognition

The samples for the SDS-PAGE were denaturalized by adding a buffer solution containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol followed by heating at 100 °C for 10 min. The 12% polyacrylamide gel was prepared with stacking in a BioRad Mini Protean® III equipment and a Tris–glycine running buffer at a pH 8.8 was used [6]. A volume of 5 μl of each simple was analyzed along with molecular weight markers of known molecular weight (from 14 to 90 kDa) LMW of GE-Healthcare. The electrophoresis was carried with a 30 mA current during stacking and 60 mA during resolution. After the electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (USB) allowing visualization of the separated proteins.

2.3. Agarose gel electrophoresis for nucleic acids recognition

The amount of agarose corresponding to 1% (w/v) loading was dissolved by heating in a microwave in 30.0 ml of Tris–acetate–EDTA buffer and then 3 μl of the specific dye GelRed™ (Biotium) was added. The samples (20–40 μl) were prepared with 2–4 μl of the buffer. The electrophoresis was performed at 70 V and the nucleic acids were revealed with UV light.

2.4. Procedure for the quantitative precipitation of proteins with ammonium sulfate

A quantitative precipitation was performed by adding 2.00 ml of saturated solution of $(NH_4)_2SO_4$ to 1.00 ml of sample and cooled at 4 °C overnight. The sample was centrifuged at 9600 g for 30 min maintaining the temperature at 4 °C in order to separate the precipitated solid that contains ammonium sulfate crystals. The solid was further dried for 5 days under vacuum until constant weight and redissolved in 3.00 ml of distilled water. A solution aliquot (250 μl) of the solution was taken and further diluted with 200 ml of distilled water and 2.00 ml of concentrated HCl. The sulfate species SO_4^{2-} were precipitated by adding 10.0 ml of a solution containing 5% (w/v) $BaCl_2$. The solid thus obtained was filtered, washed, dried, calcined at 800 °C and finally, weighted. The assay that was performed by triplicate possesses an accuracy in the ±3.3–4.8 mg range.

2.5. Determination of the protein content through the Bradford assay

The calibration curves were performed with pure bovine serum albumin (BSA) and *C. antarctica B* lipase both from Sigma Aldrich as indicated in Section 2.1. Standard solutions of about 1 mg/ml of BSA and CALB enzymes were prepared and their concentrations were determined through their absorbance at 280 nm. The extinction coefficient ε of BSA 43 820 M^{-1} cm^{-1} was obtained from the literature [7]. However, a reliable value for the CALB enzyme was not found. The literature indicates that the extinction coefficient ε can be calculated through the contributions of the aminoacids tyrosine (Tyr), tryptophan (Trp) and cystine (disulfide bonds) to the absorbance at 280 nm according to Eq. (1) [7]. In this context, the extinction coefficients of CALB results equals to 41 285 M^{-1} cm^{-1}.

$$\varepsilon_{280} \quad (M^{-1}\,cm^{-1}) = n^{\circ}\,Trp \times 5500 + n^{\circ}\,Tyr \times 1490 + n^{\circ}\,Cystine \times 125 \tag{1}$$

Then, the protein concentration C was calculated with the extinction coefficient ε, the molecular weight M (M_{BSA} = 66 296 [8] and M_{CALB} = 33 273 [9]), the optical path length b and the absorbance of the solution A at 280 nm with Eq. (2):

$$C \quad (mg/ml) = \frac{A \times M}{\varepsilon \times b} \tag{2}$$

The solution of CALB containing theoretically 1 mg/ml possesses an absorbance (at 280 nm) equals to 0.2797 indicating a real concentration of 0.2267 mg/ml of proteins. A new solution of 1.016 mg/ml was prepared considering that 1 mg of CALB possesses only 22.67% of protein.

In contrast, the absorbance of the starting solution of BSA was A_{280} = 0.6003 indicating that the concentration was 0.9080 mg/ml (close to the theoretical value).

The standard solutions of BSA and CALB were further diluted in 1/5, 2/5, 3/5 and 4/5 ratios. The absorbance at 595 nm of the mixtures between 50 ml of these dilutions and 2.5 ml of Bradford' reagent was determined for quadruplicate in an Agilent 8453 E spectrophotometer in order to perform a calibration curve [10].

2.6. UV–vis spectroscopy

The UV–vis spectra of all samples in the range between 200 and 400 nm were registered using an Agilent 8453 spectrophotometer. The samples were diluted properly so that their absorbances were within measure range of the equipment.

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