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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.

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}.

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

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):

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

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.

2.7. Isoelectric focusing analysis

Isoelectric focusing was performed in polyacrylamide gels (5%) with pH immobilized gradients. Wide range ampholytes (Biolyte 3-10 carrier ampholytes, Bio-Rad) were used to prepare the gels [11]. After the runs, the gels were fixed and colored with the dye Coomassie Brilliant Blue R-250.

2.8. Determination of the esterase activity of the CALB

The esterase activity of the various samples obtained from the crude extract was determined using *p*-nitrophenyl dodecanoate (Sigma) as substrate. The reaction mixtures contained 2.70 ml of buffer Tris–HCl 0.1 M pH 8.0 with 0.0075% (v/v) of Triton X-100; 100 μl of sample and 200 μl of *p*-nitrophenyl dodecanoate 2 mM in acetonitrile: 2-propanol 20:80. Measures were performed in a thermostatized cell in an Agilent E 8453 spectrophotometer. The sample and the buffer were incubated at 37 °C prior the addition of the substrate. When the substrate was added to the mixture, the reaction began and the kinetic curves corresponding to the release of *p*-nitrophenol due to enzymatic hydrolysis were obtained through the measurement of the absorbance at 405 nm during 90 s. The activity was calculated using the initial rates in saturating substrate condition (0.133 mM in the reaction mixture) and the amount of *p*-nitrophenol released was estimated by performing a calibration curve of this substance in the same conditions used for the activity measures. The enzymatic activity was expressed in International units (IU), being one unit the amount of enzyme that releases 1 μmol of *p*-nitrophenol/min under the assayed conditions.

2.9. Chromatographic purification of CALB

Size-exclusion chromatography. The crude extract Lipozyme® and the commercial lipase B of *C. antarctica* from Sigma were purified using size-exclusion chromatography. A Tricorn 10/50 (GE Healthcare) column filled with Sephadex G-50 (GE Healthcare) was equilibrated with a mixture of Tris–HCl 0.1 M with NaCl 25 mM at pH 8.0. A sample volume of 500 μl of the samples (previously filtered using a nylon membrane Osmonic 0.45 μm) was loaded onto the column at a flow rate of 0.5 ml/min.

Alternatively, a XK 16/40 column (GE Healthcare) filled with Sephacryl S-200 HR (GE Healthcare) and Sephacryl S-100 (GE Healthcare) were used to further purify the crude extract after removal of the non-soluble fraction and a fraction eluted from the size exclusion chromatography previously mentioned. In each case 1.0 ml of the samples were loaded onto the column at a volumetric flow rate of 0.5 ml/min and eluted with one column volume (CV) of the buffer at 0.5 ml/min. In this particular case, a mixture of ammonium acetate (0.1 M)–ammonia $NH_4CH_3CO_2$–NH_3 at pH 8.4 was used as buffer solution.

Ion exchange chromatography. As the last step of the purification, the partially purified fraction of CALB obtained by size exclusion

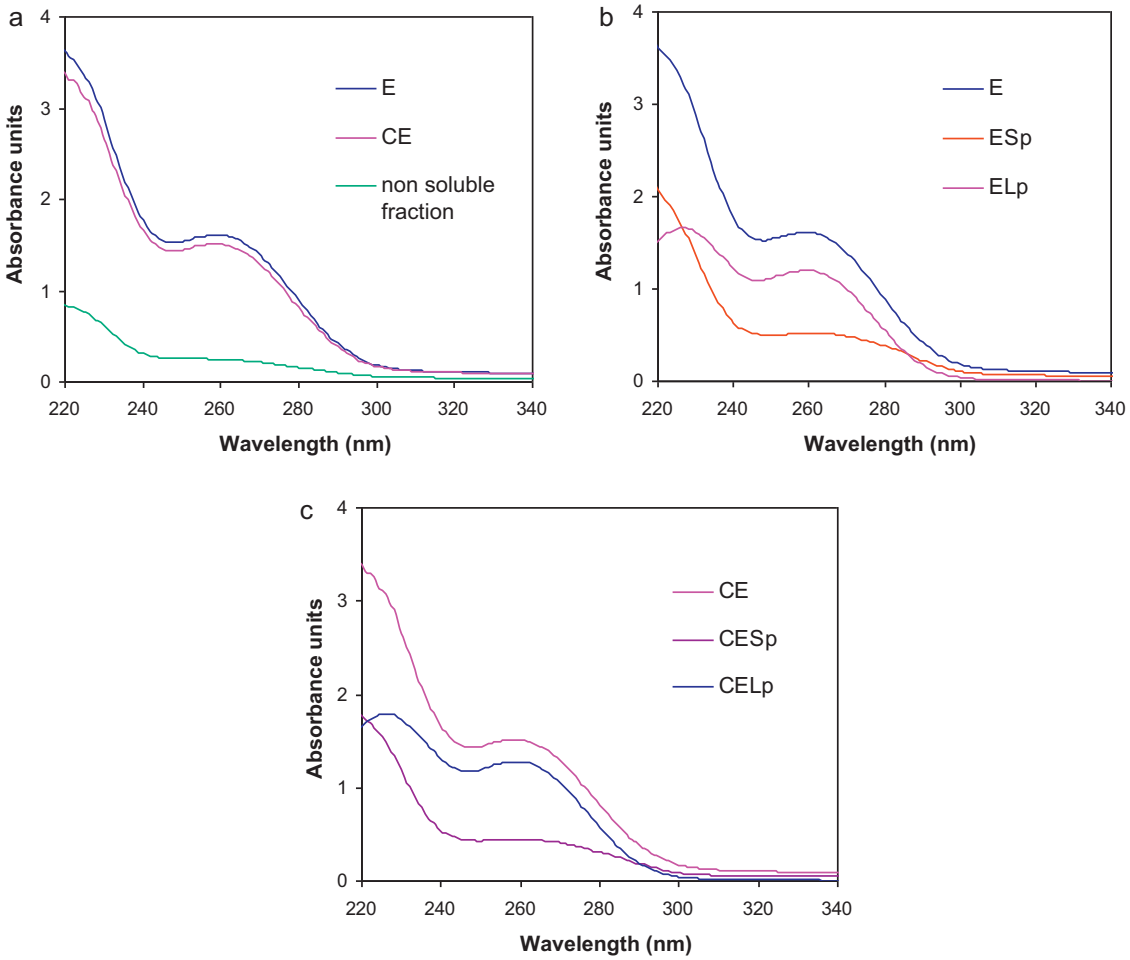


Fig. 1. Comparison of the UV–vis spectra of the various fractions isolated from the crude extract Lipozyme® CALB L: (a) crude extract before **E** and after centrifugation **CE** and the non-soluble fraction; (b) solid precipitated from **E** with ammonium sulfate (**ESp**) and supernatant solution (**ELp**) and (c) the solid precipitated from **CE** with ammonium sulfate (**CESp**) and supernatant solution (**CELp**) after separation of the non-soluble fraction.

chromatography was loaded onto anionic or cationic exchange columns using different elution conditions, depending on the particular case. On the one hand, anion-exchange chromatographies (AEC) were performed using a Tricorn 10/50 column filled with Source 15Q (GE Healthcare) at different pH conditions. The selected pH value (7.7, 8.5 and 9.5) were fixed with ammonium acetate [$NH_4CH_3CO_2$ (0.1 M)-NH_3] buffer. After loading the sample onto the column, the unbound material was eluted by three column volumes (CV) of the buffer without NaCl. After that, a gradient of NaCl 1 M was applied on the following 10 CV. On the other hand, cation-exchange chromatographies (CEC) were performed using the same column filled with Source 15S (GE Healthcare). The pH values of the elution (3.7 and 5.5), in these cases, were fixed with mixtures of ammonium acetate ($NH_4CH_3CO_2$) 0.1 M and acetic acid (HCH_3CO_2). Although such solutions were not commonly used in the chromatography separations, they were selected because they are composed of volatile salts. In this context, it is expected that these substances and water would be quickly removed upon lyophilization.

3. Results and discussion

3.1. Investigation of the components of a commercial extract of CALB

This section describes a series of procedures performed on the commercial extract of CALB (called **E** from now on) in order to establish the nature and quantity of its major components. Initially, the commercial extract of CALB was centrifuged according to the procedure described in Section 2.1 in order to separate a bottom layer composed of a non-soluble material of jelly, yellowish appearance and a clear supernatant layer (called **CE**). In a second step, saturated solution of ammonium sulfate was added to **E** and the fraction **CE** in order to assess the precipitable materials (see Section 2.4 for details). The precipitated solids (the solid fractions recovered from **E** and **CE** were called **ESp** and **CESp**, respectively) were separated from the clear supernatant solutions (the liquid fractions recovered from **E** and **CE** were called **ELp** and **CELp**, respectively). The samples **E**, **CE**, **ESp**, **ELp**, **CESp**, **CELp** and also the non-soluble fraction of the commercial extract of CALB were analyzed through UV–vis spectroscopy. Fig. 1a shows the UV–vis spectra of the samples **E**, **CE** and also the non-soluble fraction; Fig. 1b compares the spectra of the commercial extract **E** with the fractions **ESp** and **ELp** obtained after precipitation with ammonium sulfate and Fig. 1c shows the clear supernatant fraction **CE** after centrifugation and the fractions **CESp** and **CELp** obtained after precipitation.

The commercial extract possesses intense signals at 230 and 260 nm that dominate the spectra even after separation of the non-soluble materials. The signal at 230 nm could be attributed to the presence of sodium benzoate and the signal at 260 nm might be attributed to either potassium sorbate or nucleic acids [12,13]. The commercial extract known as Lipozyme® CALB L possesses total organic solids (TOS) (4%), glycerol (25%), sorbitol (25%), water (46%), sodium benzoate (0.2%) and potassium sorbate (0.1%) according to a technical document provided by Novozymes [14]. The separation of benzoate and sorbate of the enzymatic extract is necessary since these substances have absorbance maxima that interfere with some measurements such as the quantification of proteins by absorbance at 280 nm. Moreover they interfere in reactions catalyzed by the CALB extract [15]. Sodium benzoate, potassium sorbate and the nucleic acids remained in the clear supernatant layer **CE** after centrifugation and even after precipitation although in a lesser extent (see the spectra of **ELp** and **CELp**). The solids obtained after precipitation (see the spectra of **ESp** and **CESp**) show an absorbance maxima shifted toward higher wavelengths than the corresponding supernatants that might indicate the presence

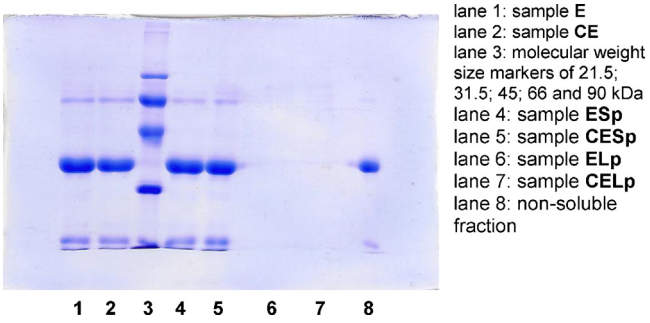


Fig. 2. SDS-PAGE analysis of the crude extract of enzyme Lipozyme® CALB L before **E** and after centrifugation **CE**; non-soluble fraction; solid precipitated from **E** with ammonium sulfate (**ESp**) and supernatant solution (**ELp**) and the solid precipitated from **CE** with ammonium sulfate (**CESp**) and supernatant solution (**CELp**) after separation of the non-soluble fraction. Additionally the molecular weight size markers of 21.5, 31.5, 45, 66 and 90 kDa are presented.

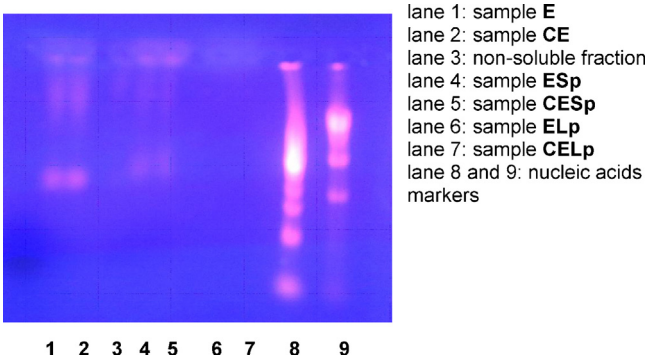


Fig. 3. Agarose gel electrophoresis of the crude extract of enzyme Lipozyme® CALB L before **E** and after centrifugation **CE**; non-soluble fraction; solid precipitated from **E** with ammonium sulfate (**ESp**) and supernatant solution (**ELp**) and the solid precipitated from **CE** with ammonium sulfate (**CESp**) and supernatant solution (**CELp**) after separation of the non-soluble fraction.

of proteins that typically absorb at 280 nm. Additionally, the lower intensity of the signals at 230 and 260 nm would indicate a less content of sodium benzoate and potassium sorbate. The SDS-PAGE and agarose electrophoresis provided further evidences of the nature of the species that composes the fractions (see Figs. 2 and 3, respectively). Fig. 2 shows that the commercial extract with and without the non-soluble fraction (lanes 1 and 2), the solids precipitated with ammonium sulfate (lanes 4 and 5) and the non-soluble species (lane

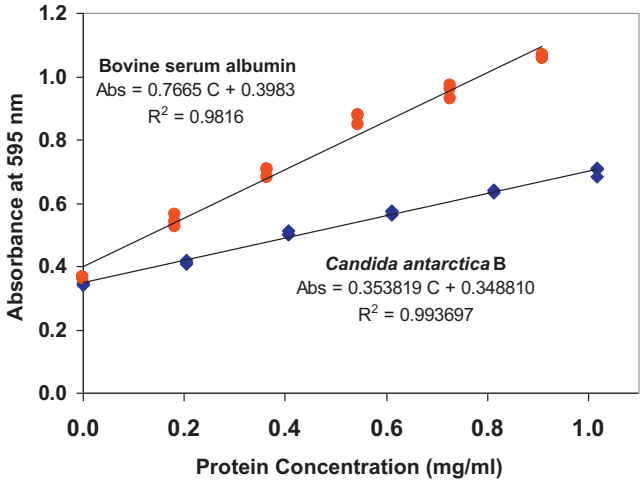


Fig. 4. Absorbance at 595 nm versus protein concentration in mg/ml of various aqueous solutions of bovine serum albumin and the lipase B of *Candida antarctica* subjected to the Bradford methodology.

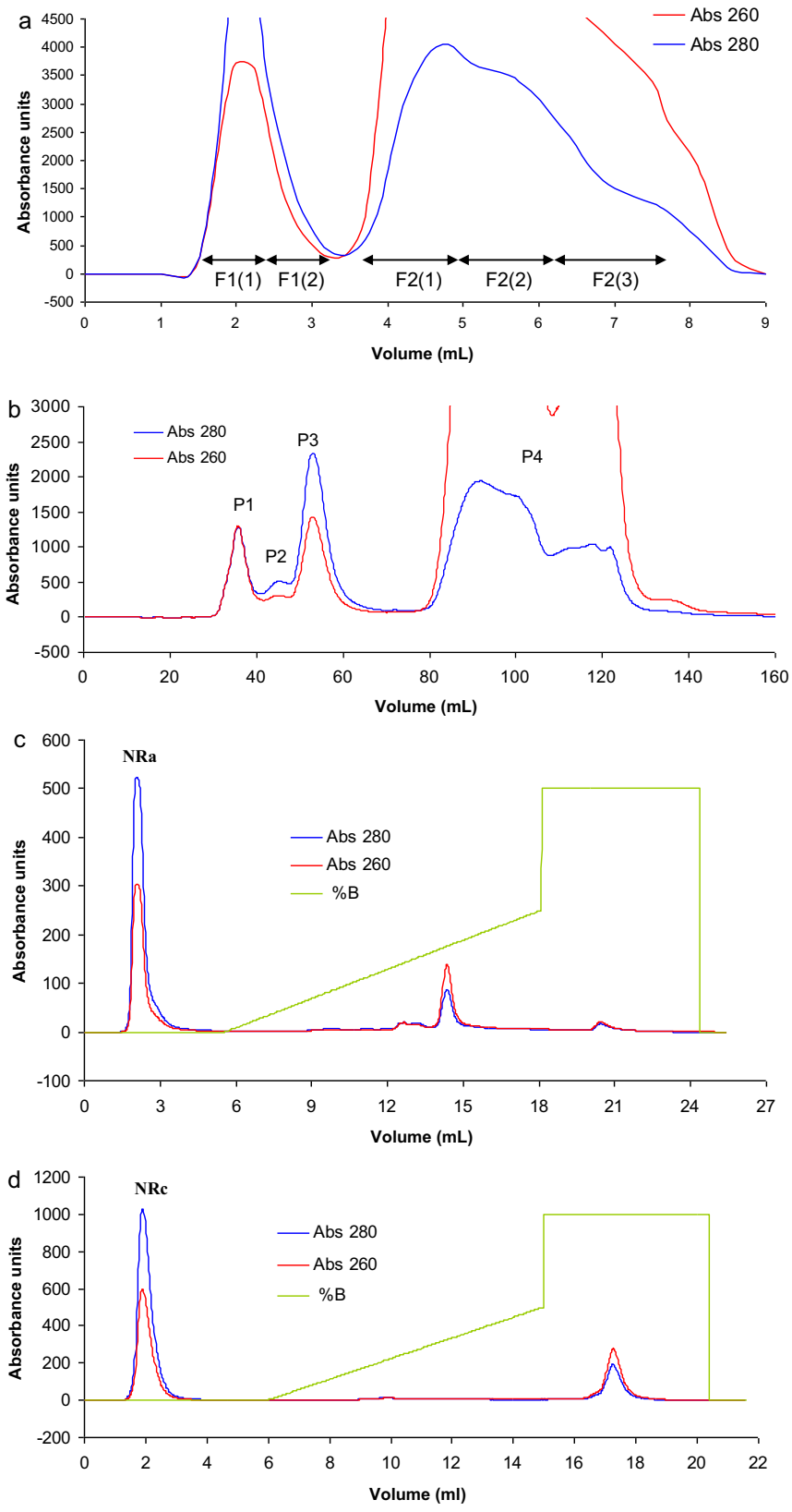


Fig. 5. Elution patterns of the crude extract after centrifugation CE subjected to size exclusion chromatography SEC (a and b); AEC (c) and CEC (d) exchange chromatography. (a) Sephadex G-50 at pH 8 with Tris–HCl 0.1 M NaCl 25 mM, flow rate: 0.5 ml/min, column volume: 3.9 ml, sample volume: 500 μl. The fractions F1(1), F1(2), F2(1), F2(2) and F3(3) were collected for analysis. (b) Sephacryl S-100 HR at pH 8 with NH_4Ac/NH_3 0.1 M NaCl 25 mM; 0.5 ml/min for sample injection and 1 ml/min for elution; column volume: 50 ml, sample volume: 1 ml. The fractions P1, P2, P3 and P4 were collected for analysis. (c) AEC (Source 15Q) at pH 8.5 with two buffer systems A: NH_4Ac/NH_3 0.05 M and B: NH_4Ac/NH_3 0.05 M with NaCl 1 M, column volume: 2.5 ml, sample volume: 100 μl of the fraction P3 lyophilized and further diluted in NH_4Ac/NH_3 0.05 M. (d) CAC at pH 5.5 with two buffer systems A: NH_4Ac/HAc 0.05 M and B: NH_4Ac/HAc 0.05 M with NaCl 1 M; column volume: 1.82 ml, sample volume: 100 μl of the fraction P3 lyophilized and further diluted in NH_4Ac/HAc 0.05 M.

8) possess a similar profile composed by a major band between the 31.5 and 45 kDa which might corresponds to the CALB enzyme (typically, 33.5 kDa), as well as two other bands of less intensity below 21.5 kDa and at 66 kDa. Instead, the clear supernatant solutions after precipitation (lanes 6 and 7) do not contain proteins which indicate that the precipitation is quantitative. Additionally, as shown in Fig. 3, the commercial extract before and after precipitation (lanes 1 and 2) and the solids precipitated from these samples (lanes 4 and 5) contain a certain amount (low according to the intensity of the bands) of nucleic acids. The supernatant solutions remaining after precipitation (lanes 6 and 7) do not contain nucleic acids while the non-soluble fraction (lane 3) shows a band of low intensity at the starting point of the electrophoresis.

These analyses demonstrated that both the proteins and the nucleic acids are precipitated all together with ammonium sulfate. This precipitation method was not selected as a strategy of the purification of CALB, due to the co-precipitation of other substances as nucleic acids and the precipitation agent, ammonium sulfate. Instead, various chromatographic techniques were assayed in order to develop a methodology to recover the most pure CALB as possible. This investigation required an accurate and reliable technique to quantify the proteins in the crude extract before and after the purification steps. In this context, the following sections address the quantification of proteins considering various methodologies. Moreover, the use of both bovine serum albumin BSA and the lipase B of *Candida antarctica* CALB as standards in the protein quantification is compared.

3.2. Comparison of CALB and BSA as standards in the spectrophotometric determination of the protein content: direct absorbance measurement

It is well known that the aqueous solutions of bovine serum albumin BSA and the lipase B of *Candida antarctica* CALB, as most of proteins, possess the maximum of absorbance at 280 nm regardless of the concentration. The UV absorbance at 280 nm is attributed to the aromatic amino acids, mainly Trp, Tyr and Phe in a lesser extent. In this context, it is interesting to compare the contribution of such residues to the sequence of amino acids of the proteins. BSA possesses two Trp residues and twenty Tyr residues in its sequence of 583 amino acids [16].

The contribution of Tyr residues is less abundant (only nine residues) in the sequence of 317 amino acid of the lipase B of *C. antarctica* compared with BSA, however, it possesses five Trp residues [9]. The contribution of these residues to the UV absorbance at 280 nm allows the calculation of the extinction coefficient ε through the equations provided in Section 2.5. While the extinction coefficients of both proteins are similar (43 820 $M^{-1}\,cm^{-1}$ for BSA and 41 285 $M^{-1}\,cm^{-1}$ for CALB), their molecular weights are very different (66 296 for BSA and 33 273 for CALB). It comes clear that the quantification of a protein using one or the other standard will be rather different. Nevertheless, most if not all the quantification methodologies use BSA for calibration purposes regardless of the nature of the protein to be quantified. Now, how is the impact (if there is one) of using BSA or CALB in a specific colorimetric method for protein's quantification? This question is addressed in the following sections through the investigation of the Bradford assay.

3.2.1. Comparison of CALB and BSA as standards in the spectrophotometric determination of the protein content through the Bradford assay

Fig. 4 shows the absorbance at 595 nm (Bradford assay) versus the concentration of various solutions of BSA and CALB in aqueous media (prepared according to Sections 2.5 and 2.6).

Table 1
Quantification of protein in the various fractions of the crude extract CALB L using bovine serum albumin and the lipase B of *Candida antarctica* as standards in the Bradford assay. **E**, crude extract; **CE**, centrifuged extract clear layer; **ELp** and **ESp**, clear fraction and solid fraction obtain after precipitation of the crude extract with ammonium sulfate, respectively; **CELp** and **CESp**, same as before with the crude extract without the non-soluble fraction.

Sample	Bradford assay	
	BSA standard (mg/ml)	CALB standard (mg/ml)
E	5.93	15.19
CE	5.05	13.03
Non-soluble fraction	1.72	4.87
ELp	0.16	0.60
ESp	5.41	13.90
CELp	0.26	0.85
CESp	4.70	12.15

The results clearly demonstrate that there is an underestimation of the concentration of CALB when BSA is used as a standard. For example a 0.60 mg/ml of CALB possesses an absorbance equal to 0.56 that would corresponds to a concentration equal to 0.21 mg/ml when using BSA calibration curve.

At this point, it is important to remark Bradford' method bases. Bradford assay relies on the binding of Coomassie Brilliant Blue G-250 to protein, especially to the arginyl and, in a lesser extent, to lysyl, and histidyl residues. This electrostatic interaction is mainly due to the negative charge of the dye in its more anionic form, which has an absorbance maximum at 595 nm; secondarily other aromatic residues, as Trp, Phe and Tyr participate in the binding of anionic species to the protein [17]. Furthermore, the neutral species of Coomassie Brilliant Blue G-250 also bind to proteins, indicating the participation of hydrophobic interaction and Van der Waals forces between proteins and the dye [18]. This finding confirmed the fact that both hydrophobic and electrostatic interactions stabilize the anionic form of the dye [19]. Bearing this in mind, it is clear that the binding of the protein to the dye would be different, according to the amino acid composition of the protein. Proteins poorly binded to Coomassie Brilliant Blue G250 result in an underestimation of protein content when Bradford method is used for quantification [20]. In this sense, it is interesting to remark that CALB possesses 317 amino acids with 18 positive residues (9 Lys, 8 Arg and 1 His). In contrast, BSA has 583 amino acids with 98 positive residues (59 Lys, 22 Arg and 17 His). Therefore, BSA presents a considerable higher proportion of its amino acids with a positive charge than CALB (16.8% versus 5.7% for CALB). This observation clearly shows that the interaction between the dye and each protein would be considerably different for both proteins, explaining why the underestimation takes place when BSA is used as standard.

The present results provide evidences of the non-accurate results that might be obtained with the protein taken as a standard (BSA in this case) is not the one to be quantified. In this context, Noble and Bailey reported a comparison between the concentration of model proteins obtained both through a highly accurate quantitation of specific amino acids (AAA assay) and a dye-based assay using BSA as standard [21]. The authors observed that the variation between the concentrations of protein derived using BSA standard when compared to the true value using AAA ranged from 2% to 8%. Actually, this observation is further proved when the amounts of protein in the various fractions of the crude extract (as previously described in Section 3.1) using BSA and CALB in the Bradford assay are compared. Table 1 shows that the content of protein is lower when BSA is used as standard compared to CALB. In this particular case, the lipase B of *C. antarctica* is the standard of choice considering that is the major component of the crude extract as discussed before. Besides this observation, the results show that the crude extract after the removal of the non-soluble fraction possesses

13.0 mg/ml of protein that is quantitatively precipitated (93%) with ammonium sulfate (the fraction **CESp** has 12.15 mg/ml). Surprisingly, the weight of the fraction **CESp** was much higher than the one expected if only proteins where involved in the precipitation. This observation that will be further discussed in the following sections provided evidence that not only proteins but also nucleic acids and other substances are precipitated with ammonium sulfate.

3.3. Tailoring a purification strategy to isolate CALB from a crude extract

Size-exclusion and ion exchange chromatography were screened with the aim of isolating the lipase B of *C. antarctica* from Lipozyme®. In the first case, three chromatographic phases such as Sephadex G-50, Sephacryl S-200 HR and Sephacryl S-100 HR were investigated. For the ion exchange chromatography, Source 15Q and Source 15S were used. Regularly, Tris–HCl is the buffer of choice when the working pH is in the range comprised between 7.5 and 9.5. Nevertheless, volatile buffers such as NH_4Ac–NH_3 or NH_4Ac–HAc (depending on the pH of the assay) were also used in this investigation [22]. In this context, it is worth noticing that the salts that compose the buffer system Tris–HCl interfere with some specific analyses, such as infrared analysis of proteins in the Amide I region. Therefore, is desirable to use an alternative buffer such as the ammonia–ammonium acetate which is quickly eliminated by a simple lyophilization process.

In each case, the components of the resultant fractions were analyzed through UV–vis, SDS-PAGE, agarose gel electrophoresis and isoelectric focusing. The amount of proteins was determined through the Bradford methodology and the esterase activity was assessed through the hidrolysis of *p*-nitrophenyl dodecanoate as substrate as described in Section 2.8.

The results presented in the previous sections demonstrated that the crude extract is composed of both small molecules (glycerol, sorbitol, potassium benzoate, sodium sorbate) and macromolecules such as nucleic acids. Additionally, the crude extract possesses not only CALB enzyme (33.5 kDa) but two other ones with 66 and 21.5 kDa. Despite the general recommendations for protein purification, which indicate size exclusion chromatography as a polishing step at the end of the entire process, knowledge of the composition of the crude sample led to the conclusion that this separation method would be more effective as a first step for the proposed purification.

In order to discuss the ability of each methodology as building blocks for the tailoring of the more suitable method for CALB purification from Lipozyme® CALB L, the results of the various purification strategies are presented in the following sections.

3.3.1. High recovery of proteins from the crude extract through size-exclusion and exchange chromatography

Initially Sephadex G-50 was used to purify CALB since allows the separation of large macromolecules heavier than 30 kDa that eluted in the non-retained fraction. Moreover the separation is achieved in short times and large sample volumes can be used. The purification of both the commercial CALB and the crude extract Lipozyme® CALB L through size exclusion chromatography using Sephadex G-50 provided two fractions as observed in Fig. 5a. The first eluted fraction shows an UV signal at 280 nm and a second one of less intensity at 260 nm. The second eluted fraction predominantly possesses an UV absorbance at 260 nm. Fig. 5a shows five fractions that were collected for further analysis through UV–vis spectroscopy. The spectra of the fractions F1(1) and F1(2) (both correspond to the first elution) along with the spectra of the centrifuged crude extract are presented in Fig. 6a. Fig. 6b shows the UV–vis spectra of the fractions F2(1), F2(2) and F2(3) (correspond to the second elution) along with the centrifuged and filtered crude extract.

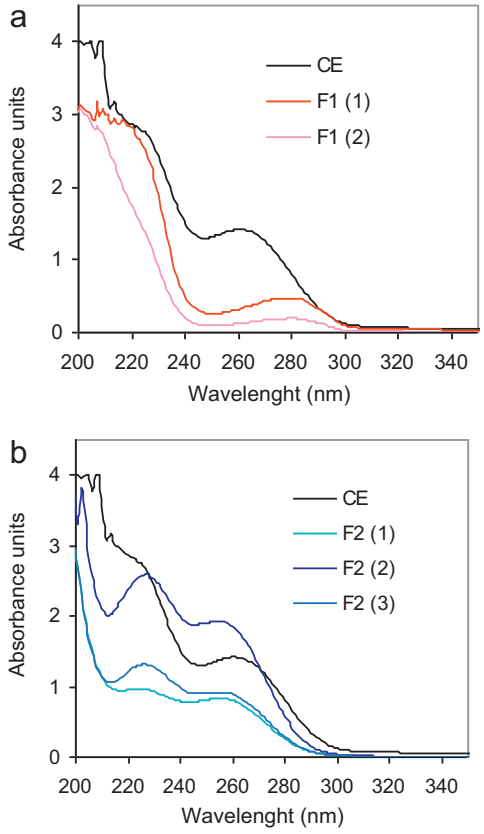


Fig. 6. UV–vis spectra of the crude extract **CE** after centrifugation along with the fractions F1(1) and F1(2) that belongs to the first peak eluted from Sephadex G-50 (a). (b) Compares **CE** with the fractions F2(1), F2(2) and F2(3) belonging to the second peak eluted from the same chromatography.

The results show that the first eluate possesses an absorbance peak at 280 nm indicating that it is composed mainly of proteins, while the second one is composed of species with absorbance peaks at 230 nm (benzoate) and 260 nm (sorbate and nucleic acids). The Bradford assay further confirmed the presence of 1.2 mg/ml of proteins in the first eluate while proteinaceous components are not detectable in the second peak. Interestingly, SDS-PAGE analysis (see Fig. 7a) demonstrates that the protein profile of the commercial lipase and the first eluate are similar and both possess the most intense band of about 33 kDa that corresponds to the lipase B of *C. antarctica.* Additionally, two other bands of about 66 and 21 kDa are observed. A weak band at 33 kDa is observed in the second eluate providing further evidences of the negligible amount of proteins. The observation that this fraction did not possess nucleic acids according to the agarose gel electrophoresis proved that it is composed of sorbate and benzoate species (see Fig. 8, lane 4). In contrast, the purification with Sephadex G-50 was not able to separate the nucleic acids that were observed in the sample **E**, **CE** filtered (called **CFE** from now on) and the first retentate (see Fig. 8, lane 3).

Additionally, the steps involved in the purification such as removal of the non-soluble fraction, filtration, removal of sorbate and benzoate did not alter the esterase activity of the lipase (data not shown).

In a second attempt, the samples were purified using size exclusion chromatography media which allows the separation of macromolecules of different sizes. Sephacryl S-100 HR and S-200 HR allow proteins separation between 5 kDa and 100 kDa, and between 5 kDa and 250 kDa, respectively. However, the chromatographic runs are slow and only small volumes of sample can

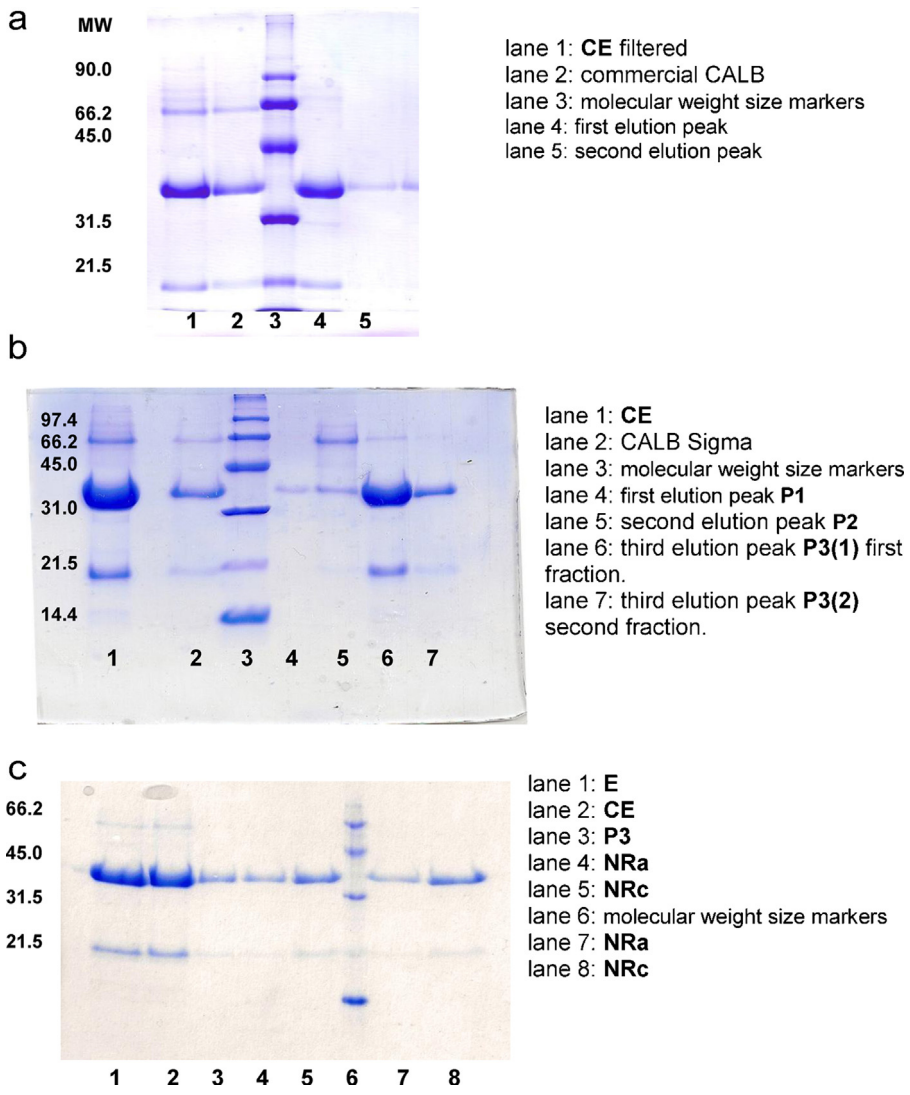


Fig. 7. SDS-PAGE analysis of the crude extract after centrifugation **CE** and the various fractions obtained after purification through SEC, AEC and CEC. (a) **CE**, commercial CALB (Sigma Aldrich) and the two peaks F1 and F2 eluted from Sephadex G-50. (b) **CE**, commercial CALB (Sigma Aldrich) and the three peaks P1, P2 and P3 (two fractions P3(1) and P3(2)) eluted in the SEC. (c) Crude extract before and after centrifugation (**E** and **CE**, respectively), peak **P3** eluted in the SEC, and the fractions not retained **NRa** and **NRc** in the AEC and CEC chromatography, respectively.

be loaded. In this particular case, both the buffers Tris–HCl and ammonia–ammonium acetate were assayed with similar results therefore, this last buffer system was chosen since its removal is straightforward.

In this context, the purification was improved through Sephacryl S-100 HR and Sephacryl S-200 HR, allowing the separation of a major fraction of the nucleic acids. Fig. 5b shows the profile of the fractions obtained when the sample **CE** was loaded onto Sephacryl S-100 HR using the buffer NH_4Ac–NH_3 0.1 M at pH 8.4. The profile obtained with Sephacryl S-200 HR was similar (data not shown). In both cases, three peaks (called P1, P2 and P3) were obtained: P3 was collected in two fractions [P3(1) and P3(2)]. The first peak P1 showed similar absorbance levels at 260 and 280 nm which would indicate the presence of nucleic acids which was further confirmed by agarose electrophoresis (see Fig. 8b, lane 1). The second and third peaks absorbed at 280 nm indicating the presence of proteins, whereas a minor contribution of the signal at 260 nm and an intense absorption at 230 nm was observed. The last peak eluted after one column volume showing intense signals of absorbance at 230 and 260 nm which evidenced the presence of sorbate and benzoate species. SDS-PAGE analysis (see Fig. 7b) indicated that two fractions of the third peak P3 had the highest proportion of the lipase CALB along with minor contribution of a protein of 21.5 and 66 kDa. Moreover, these fractions showed a similar electrophoretic profile as the commercial CALB provided by Sigma Aldrich. However, in the P3, the 66 kDa band was much less intense than in the commercial CALB since that protein eluted in the P2 fraction (see Fig. 7b). The isoelectric focusing analysis of the third fraction further confirmed the presence of the lipase B of *C. antarctica* with p*I* around 6.5 with a minor contribution of another proteins of p*I* around 3.7 and 8.2 that might have 21.5 and 66 kDa according to the SDS-PAGE analysis discussed before (data not shown) [9].

This third fraction had 8.18 mg/ml of proteins that constitutes 82% recovery of the proteins present in the crude extract after removal of the non-soluble fraction (9.96 mg/ml proteins). The analysis through the Bradford method showed that the quantity of proteins in the samples P1 and P2 was much less than in P3. Moreover, the enzymatic activity assays showed practically null levels of activity for samples P1 and P2 (data not shown).

Further attempts to isolate the three proteins described before were performed by purifying the third fraction P3 eluted from

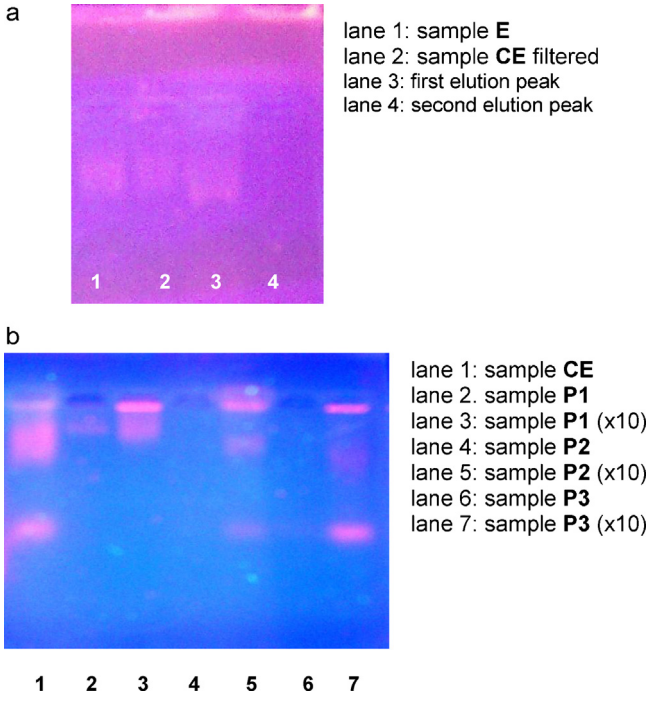


Fig. 8. Agarose gel electrophoresis of the crude extract after centrifugation **CE** and the various fractions obtained after purification through SEC, AEC and CEC: (a) crude extract before and after centrifugation (**E** and **CE**, respectively) and the two peaks F1 and F2 eluted from Sephadex G-50; (b) sample **CE** and the three peaks **P1**, **P2** and **P3** as eluted in the SEC and further concentrated 10 times (×10).

the size-exclusion chromatography (using Sephacryl S-100 HR) through anion and cation-exchange chromatography.

Three pH values (7.7, 8.5 and 9.5) were screened in the particular case of the anion-exchange (Source 15Q) and pH 3.7 and 5.5 for the cation-exchange (Source 15S) chromatography. Surprisingly, no binding of CALB on the column was observed regardless the pH values as observed in the Fig. 5c and d. In fact, these results are in agreement with the investigation reported by Trodler et al. [4]. The authors found that the lipase B of *Candida antarctica* is neutral with only a slight decrease of total charge from pH 5.0 to 9.0. At pH values lower than 5.0, CALB is positively charged and at pH values higher than 8.0, is negatively charged. This broad isoelectric region of CALB prevents its binding to the ion exchange columns due to the absence of a net charge of the enzyme. However, in this report, the p*I* value about 6.5 was determined by an IEF assay. The CALB is electrically charged in the pH range comprised between 5.0 and 8.0 (positive charge below pH 6.5 and negative charge above pH 6.5). Despite this finding, the protein was not retained by the tested ion exchange media. This observation could be explained considering that CALB possesses only 5% of basic amino acids and the same proportion of acidic amino acids. Therefore, the scarce charge that the CALB possesses in the pH values near its p*I* might be insufficient for an effective adsorption to the chromatographic phase.

Nevertheless, the analysis of the non-retained fraction using AEC at pH 8.5 and CEC at pH 5.5 demonstrated an improved degree of purification. The SDS-PAGE of the non-retained fraction in the AEC at pH 8.5 (**NRa**) showed practically, only one protein band at about 33 kDa (see Fig. 7c, lanes 4 and 7). In return, in the non-retained fraction of CEC at pH 5.5 (**NRc**) a weak band at 21.5 kDa was found (see Fig. 7c, lanes 5 and 8). Additionally both chromatograms showed signals of low intensity that elute within the NaCl gradient region or in the wash region. These fractions presented a more intense absorbance at 260 nm rather than at 280 nm which evidenced the presence of nucleic acids in their composition (see Fig. 5c and d).

Table 2
Amount of proteins, yield %, specific activity (IU/mg) and purification factor obtained in the crude extract before and after centrifugation (samples **E** and **CE**, respectively) and further purified through size exclusion chromatography SEC (sample **P3**), anion AEC and cation CEC exchange chromatography (samples **NRa** and **NRc**, respectively). The amount of proteins calculated in each step is referred to the original sample volume (1 ml).

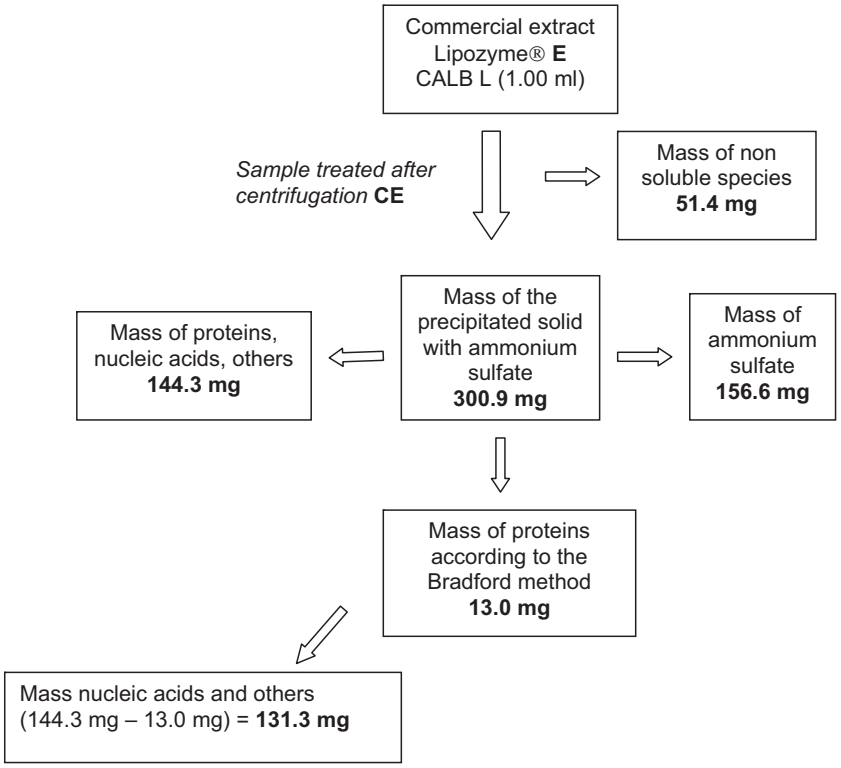
Sample	Procedure	Proteins (mg)	Yield (%)	Specific activity (IU/mg)	Purification factor
E	None	13.72	100	2.1	–
CE	Centrifugation	9.95	72.5	1.8	0.84
P3	SEC	8.18	59.6	2.5	1.19
NRa	AEC pH 8.5	6.49	47.3	2.9	1.38
NRc	CEC pH 5.5	6.89	50.2	1.9	0.87

4. Conclusions

Scheme 1 summarizes the procedures applied on the commercial extract Lipozyme® CALB L and the quantification of its various components. The addition of a saturated solution of ammonium sulfate on 1.00 ml of the starting extract (after elimination of the non-soluble species) afforded 144.3 mg/ml of a solid mass containing proteins, nucleic acids and other soluble species. On the other hand, the centrifugation of the extract allowed determining that the amount of the non-soluble species is equal to 51.4 mg/ml. Moreover, the specific quantification of proteins through the Bradford methodology indicated that the protein content equals to 13.0 mg/ml after the removal of the non-soluble fraction. Therefore subtracting the amount of proteins and non-soluble species from the precipitated solid is possible to estimate that the commercial extract Lipozyme® CALB L possesses 131.3 mg of nucleic acids and other non-identified substances (benzoate and sorbate species within them).

In view of the results described above, a tailored made rational purification methodology was developed for this particular crude extract. In this context, Table 2 summarizes the amount of proteins, yields, specific activity and the purification factor involve in the successive steps of such methodology. As a first step, the non-soluble fraction of the crude extract was removed through centrifugation. The non-soluble fraction retained 27% of the protein content of the crude extract being the step that afforded to the highest removal of proteins of the present methodology (see the amount of proteins in Table 2). The treatment of the centrifuged sample **CE** with Sephadex G-50 medium with a conventional Tris–HCl buffer allowed the removal of small molecules such as, benzoate and sorbate species. However, the size exclusion chromatography SEC using a non conventional, easy to remove buffer system such as ammonia–ammonium acetate further improved the purification process. It is worth noticing, that the liophilization of 5 ml of purified sample with Sephacryl S-100 HR using both Tris–HCl 0.1 M and NH_4Ac–NH_3 0.1 M buffers afforded 72 mg and 7 mg of solid residue, respectively. This simple experiment demonstrated that ammonia–ammonium acetate buffer provides a cleaner, residue free sample. Additionally, the specific activity of the sample obtained with the NH_4Ac–NH_3 buffer before and after the liophilization showed similar values as the one already shown in Table 2 (see entry corresponding to P3 sample).

SEC allowed to obtain an enzymatic sample free of nucleic acids, benzoate and sorbate species (see sample **P3** in Table 2). Moreover, 82% of the protein was retained with a similar specific activity as the crude extract. This enzymatic sample possesses mainly CALB with minor amount of two other proteins of 21.5 and 66 kDa. These two proteins were retained in anionic and cationic exchange chromatography columns at pH values equal to 8.5 and 5.5, respectively. The specific activity increased slightly with AEC at pH 8.5 (showing a purification factor of 1.38) and decreases when CEC at pH 5.5



Scheme 1. Procedures applied on the commercial extract Lipozyme® CALB L and quantification of its various components such as non-soluble fraction, proteins, nucleic acids between others.

was used (the purification factor was 0.87). This observation might be associated to an activity loss due to the low pH of the solution. The slightly increase in the purification factor for the AEC could be explained due to the fact that the main protein present in the crude extract is CALB since the different purification steps allowed the separation of others components as nucleic acids, preservatives, salts, cellular constituents, and small quantities of other proteins. The results discussed above allow to conclude that the AEC at pH 8.5 as the last step of purification of CALB is of choice in the present methodology.

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