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# Purification of The Lipase B of *Candida antarctica* from a Commercial Enzymatic Extract

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**Abstract:** This paper presents a rational strategy to purify the lipase B of *Candida antarctica* of the commercial extract Lipozyme® through size exclusion coupled with anionic exchange chromatography using a non conventional, easy to remove buffer system such as ammonia-ammonium. In this context, each step of the purification was followed through the determination of the protein content, esterase activity measurements, SDS-PAGE, agarose electrophoresis, UV-spectroscopy and isoelectric focusing. The purification of the commercial extract afforded a sample that retains 47% of the proteins (being CALB the major enzymatic component of the purified sample) with a hydrolytic activity higher than the starting crude extract.

**Keywords:** Protein purification, CALB, UV absorbance, Bradford, size exclusion chromatography, Ion exchange chromatography.

## 1. INTRODUCTION

It is well known that the lipase B of *Candida antarctica* immobilized onto polymethylmethacrylate (called Novozym® 435) is the most widely heterogeneous biocatalyst 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 *Candida antarctica* (CALB) regularly possesses two other proteins with approximately 18 kDa 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 hs afforded 40-50 % pure CALB.

The purification of CALB 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 active site of which (specifically the Thr40 amino-acid) was mutated in order to modify its enantioselectivity [3].

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To the knowledge of the authors 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 4.0 to 8.0.

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 starting sample and the ones obtained in each step of the purification must be known in order to apply a rational design. Online with this observation, this investigation presents a tailored purification method in order to isolate the lipase B of *Candida antarctica* from a commercial crude extract.

## 2. EXPERIMENTAL

### 2.1. Enzymatic Based Materials

*Candida antarctica* lipase recombinant from *Aspergillus oryzae* (Fluka, 7.2-10.8 U/mg) was purchased from Sigma

2211-5455/14 $58.00+.00

Aldrich Argentina (10.9 U/mg). Additionally, the lipase B of *Candida antarctica* CALB L (Lipozyme® LCN02102) was provided by Novozymes Brazil (Paraná, Brazil). 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. Chromatographic Purification of CALB

*Size-exclusion chromatography.* The crude extract Lipozyme® was purified using size-exclusion chromatography. 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 1.5 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 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 pHs (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 ten CV. On the other hand, cation-exchange chromatographies (CEC) were performed using the same column filled with Source 15S (GE Healthcare). The elution pHs (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 by volatile salts. In this context, it is expected that these substances and water would be quickly removed upon liophilization.

### 2.3. SDS-PAGE Electrophoresis for Following Protein Purification

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. Electrophoresis gels (12% polyacrylamide for the resolving gel and 5% polyacrylamide for the stacking gel) was prepared ina BioRad Mini Protean® III equipment and Tris-glycine running buffer at 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.

### 2.4. 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 µl to 40 µl) were prepared with 2 µl to 4 µl of the buffer. The electrophoresis was performed at 70 V and the nucleic acids were revealed with UV light.

### 2.5. Determination of the Protein Content Through the Bradford Assay

The calibration curve was performed using pure *Candida antactica B* lipase from Sigma Aldrich as standard. Initially, a solution of about 1 mg/ml of CALB was prepared. Its concentration was determined through the absorbance at 280 nm and the extinction coefficient ε calculated according to the equation (1) [7]. In this context, the extinction coefficients of CALB results equals to 41285 $M^{-1}$ $cm^{-1}$.

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

Then, the protein concentration **C** is calculated with the extinction coefficient ε, the molecular weight **M** ($M_{CALB}$ = 33273 [9]), the optical path length **b** and the absorbance of the solution **A** at 280 nm with the equation (2).

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

In this context, 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.

The standard solution of CALB was 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's reagent was determined for quadruplicate in an Agilent E 8453 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 nm and 400 nm were registered using an Agilent E 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 polyacrilamide gels (5%) with pH immobilized gradients. Wide range ampholytes (Biolyte 3-10 carrier ampholytes, Bio-Rad) were used to prepare the gels [11]. 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 measure 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 measure the absorbance at 405 nm during 90 seconds. 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.

## 3. RESULTS AND DISCUSSION

Size-exclusion and ion exchange chromatography were screened with the aim of isolating the lipase B of *Candida antarctica* from Lipozyme®. In the first case, the chromatographic phases 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 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 [12]. 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, it 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 spectroscopy, 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 the section 2.8.

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

### 3.1. High Recovery of Proteins from the Crude Extract Through Size-Exclusion and Exchange Chromatography

The samples were purified using size exclusion chromatography media which allows the separation of macromolecules of different size. Sephacryl S-100 HR and S-200 HR allow proteins separation between 5 and 100 kDa, and between 5 and 250 kDa, respectively. However, the chromatographic runs are slow and only small volumes of sample can 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. The Fig. (**1a**) shows the profile of the fractions obtained when the crude extract without the non soluble portion removed through centrifugation (sample called **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). Three peaks (called P1, P2 and P3) were obtained and four fractions [P1, P2 P3(1) and P3(2)] were collected. The first peak P1 shows similar absorbance levels at 260 nm and 280 nm which would indicate the presence of nucleic acids which was further confirmed by agarose electrophoresis (data no shown). The second and third peaks absorb at 280 nm indicating the presence of proteins, whereas a minor contribution of the signal at 260 nm is observed. The last peak eluted after one column volume showing very intense signals of absorbance at 260 nm and slightly lower at 280 nm which evidences the presence of sorbate and benzoate species [13, 14]. SDS-PAGE analysis (see Fig. **2a**) indicates that both fractions of the third peak P3(1) and P3(2) possess the highest proportion of the lipase CALB along with minor contribution of a protein of 21.5 kDa and 66 kDa. Moreover, these fractions show a similar electrophoretic profile as the standard CALB provided by Sigma Aldrich (see lane 2 in Fig. **5a**). However, in the P3, the 66 kDa band is much less intense than in the commercial CALB since that protein eluted in the P2 fraction (see Fig. **2a**). The isoelectric focusing analysis of the third fraction further confirmed the presence of the lipase B of *Candida 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 kDa and 66 kDa according to the SDS-PAGE analysis discussed before (data not shown) [9].

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

Three pHs (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 Figs. (**1b** and **1c**). In fact, these results are in agreement with the

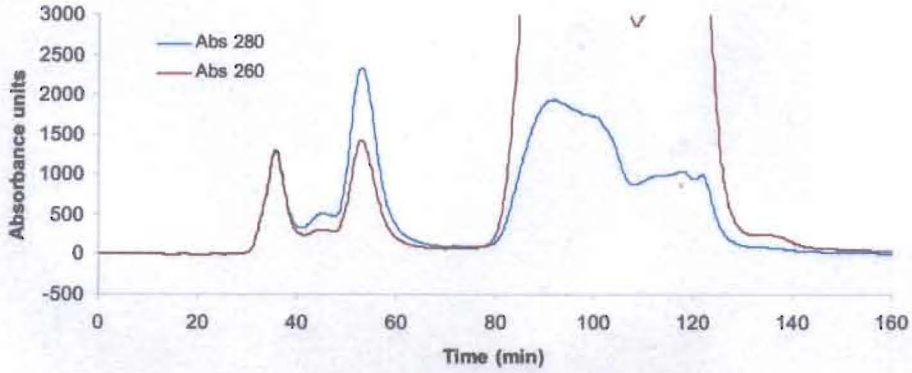
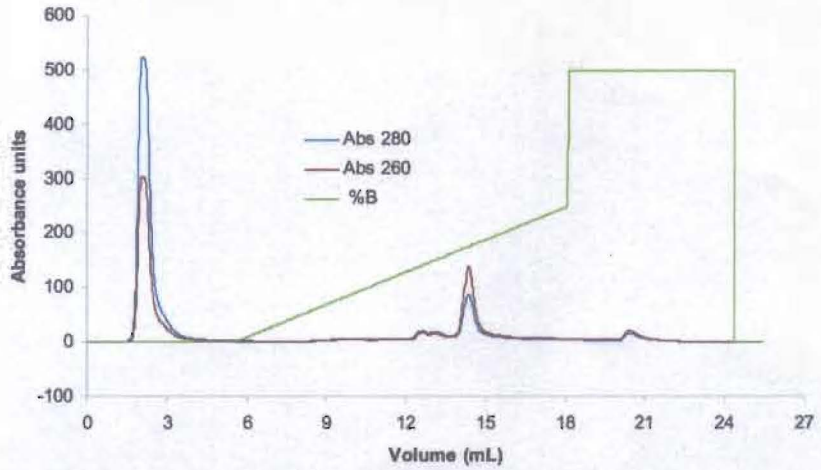
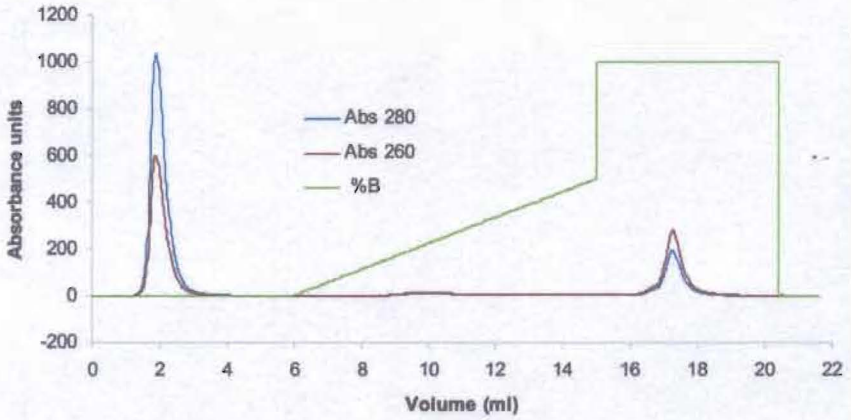



**Fig. (1).** Elution patterns of the crude extract after centrifugation CE subjected to size exclusion chromatography SEC (1a); AEC (1b) and CEC (1c) exchange chromatography.

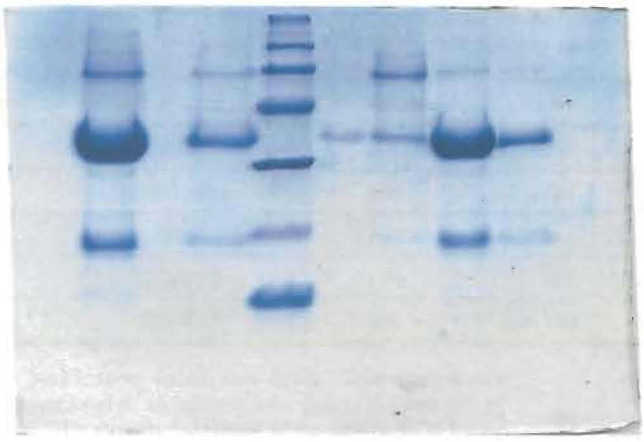
**(1a)** Sephacryl S-100 HR at pH = 8 with $NH_4Ac/NH_3$ 0.1M; 0.5 ml/min for sample injection and 1ml/min for elution; column volume: 50 ml, sample volume: 1 ml. The fractions P1, P2, P3 and P4 were collected for analysis.



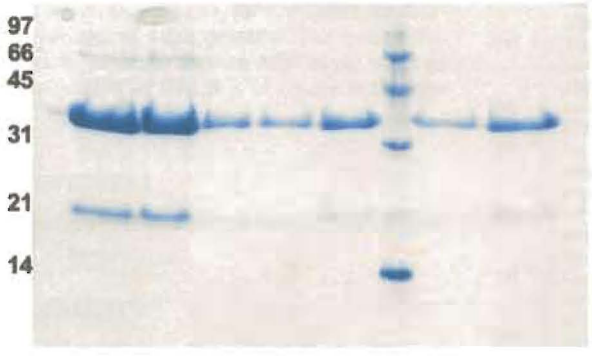

**(1b)** AEC (Source 15Q) at pH = 8.5 with two buffer systems A: $NH_4Ac/NH_3$ 0.05 M and B: $NH_4Ac/NH_3$ 0.05 M with NaCl 1 M, column volume: 2.5 ml, sample volume: 100 µl of the fraction P3 lyophilized and further diluted in $NH_4Ac/NH_3$ 0.05 M.




**(1c)** 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.




(a)




(b)

**Fig. (2).** SDS Page analysis of the crude extract after centrifugation **CE** and the various fractions obtained after purification through SEC, AEC and CEC. **(2a) CE**, commercial CALB (Sigma Aldrich) and the three peaks P1, P2 and P3 (two fractions P3(1) and P3(2)) eluted in the SEC. **(2b)** 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.

investigation reported by Trodler et al. [4]. Nevertheless further analysis of the non-retained fraction using AEC at pH 8.5 and CEC at pH 5.5 demonstrated further degree of purification. In this context, the SDS-PAGE of the non retained fraction in the AEC at pH 8.5 (called **NRa**) show practically only one protein band at about 33 kDa (see Fig. **(2b)**, lanes 4 and 7). In return, in the non retained fraction of CEC at pH 5.5 (called **NRc**) a weak band at 21.5 kDa was found (see Fig. **(2b)**, lanes 5 and 8). Additionally both chromatograms show signals of low intensity that elute within the NaCl gradient region or in the washing region. These fractions possess a more intense absorbance at 260 nm rather than at 280 nm which evidences the presence of nucleic acids in their composition (Fig. **1b** and **1c**).

## 4. CONCLUSIONS

In view of the results described above, a tailored made rational purification methodology was developed for this particular crude extract. In this context, the Table **1** summarizes the amount of proteins, yields, specific activity and the purification factor involved 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 retains 27% of the protein content of the crude extract being the step that leads to the highest removal of proteins of the present methodology (see the amount of proteins in Table **1**). The treatment of the centrifuged sample **CE** with the size exclusion chromatography SEC using a non conventional, easy to remove buffer system such as ammonia-ammonium acetate improved the purification process. In this context, SEC allowed to obtain an enzymatic sample without the benzoate and sorbate species and without most of the nucleic acids (see sample **P3** in Table **1**). Moreover, 82% of the protein was retained with a similar specific activity as the crude extract. This enzymatic sample possesses mainly CALB with minor amounts of two other proteins of 21.5 kDa and 66 kDa. These two proteins were retained in anionic and cationic exchange chromatography columns at pHs 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 was

**Table 1. 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).**

| 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 |

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 increased 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, conservants, 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.

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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