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Rational design of a new one-step purification strategy for *Candida antarctica* lipase B by ion-exchange chromatography

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Abstract

A fast and efficient one-step method for purification of lipase B from *Candida antarctica* by ion-exchange chromatography was developed by rational design. The electrostatic properties of the enzyme were calculated and validated by isoelectric focusing and measurement of the titration curve. *C. antarctica* lipase B shows an unusual pH profile with a broad isoelectric region from pH 4 to 8. At pH 3 *C. antarctica* lipase B can be bound to a cation-exchange chromatography column and was purified to homogeneity with a purification factor of 2.4. It was stable at pH 3, the residual activity was still 80% after 6 days incubation at 20 °C. The broad isoelectric region of *C. antarctica* lipase B is unique as compared to almost all other α/β-hydrolases which have a well-defined isoelectric point. A search in the lipase engineering database resulted in only one further α/β-hydrolase, the *Fusarium solani* cutinase, which also has a broad isoelectric region.

Keywords: *Candida antarctica* lipase B; Purification; Electrostatic potential; Chromatography; Titration curve; Molecular modelling

1. Introduction

Candida antarctica lipase B (CALB) (EC 3.1.1.3) is one of the most widely used lipases in biocatalysis [1]. CALB belongs to the family of serine hydrolases with an α/β-hydrolase fold and a catalytic triad consisting of Ser, His, and Asp [2] and is a highly versatile catalyst with activity towards a wide range of substrates [3,4]. CALB is stable in organic solvents without significant loss of activity and catalyzes reactions also at higher temperatures [1,5]. In contrast to many other lipases CALB has no lid covering the entrance to the active site [2] and shows no interfacial activation [6].

In most commercial preparations lipases are immobilized on different carriers or they are lyophilized as crude products that often contain different amounts of impurities. Although for many industrial processes crude enzyme preparations are sufficient, a general purification strategy for lipases is desirable for biochemical characterization of the wild type or an engineered mutant and for preventing side reactions in the biocatalytic process. Generally, lipases can be easily purified by ion-exchange chromatography (IEC) [7,8] and, in some cases, by additional chromatographic steps. However CALB has been shown not to bind to any chromatographic material and was found in the effluent [9]. Therefore, a more tedious purification method has been developed, which is based on gradually extracting impurities by multiple purification steps, including dialysis, ion-exchange chromatography, and hydrophobic interaction chromatography [9–11]. Until now no other purification method of CALB has been published to our knowledge.

What makes the purification of CALB difficult compared to other lipases and how to develop a one-step purification strategy for this enzyme? In this case study electrostatic properties of CALB were characterized by isoelectric focusing and titration curve in a fast theoretical analysis. Conditions were predicted under which CALB is expected to bind to an IEC column. Based on electrostatic calculations a one-step purification method was

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developed by rational design to purify CALB from a commercial enzyme preparation ($CALB_{com}$) and from the supernatant from expression in *Pichia pastoris* ($CALB_{rec}$).

2. Experimental

2.1. Electrostatic potential of CALB

The crystal structure of CALB (resolution of 1.55 Å) was taken from the Protein Data Bank (entry 1TCA) [2]. The program TITRA [12] was used to calculate pK_a values and protonation states of titratable groups. The titration curve was calculated using default parameters and acc_run [13] was used to calculate the solvent accessible surface area of each residue. Protonation states were calculated from pH 0 to 14 and were supplied to DELPHI [14] to calculate the respective electrostatic potentials of the protein. In the DELPHI calculations, the PARSE set [15] of partial atomic charges and atomic radii was used with the following parameters: interior dielectric constant 4, exterior dielectric constant 78.5, solvent probe radius 1.4 Å, ionic strength 0.145 mol/L. Water molecules were excluded from the electrostatic potential calculations. Electrostatic potentials were mapped on the solvent accessible surface area (SASA) using Pymol [16] to locate the charge distribution on the surface of CALB.

2.2. Experimental characterization of CALB

2.2.1. Enzymes and chemicals

Chirazyme L-2, lyo ($CALB_{com}$) was purchased from Roche Diagnostics (Mannheim, Germany) as a crude, lyophilized material. All other chemicals used were of analytical grade and obtained from Fluka (Buchs, Switzerland) or Riedel-de Haen (Seelze, Germany).

2.2.2. Expression of recombinant CALB

P. pastoris X-33 (Invitrogen, Karlsruhe, Germany) was transformed with vector pPICZαA (Invitrogen, Karlsruhe, Germany) bearing the CALB gene as described previously [17]. A preculture was prepared in a 50 mL shake flask with 5 mL buffered yeast extract medium (BMGY) with glycerol [containing 1% (w/v) yeast extract, 2% (w/v) peptone, 1% (v/v) glycerol, 5 mL (100 mg/mL) Zeocin, 100 mM potassium phosphate, pH 6.8] was inoculated with 50 mL stock seed. The flask was incubated for 48 h at 30 °C on an orbital shaker at 140 rpm. Afterwards 2 L shake flasks with 200 mL of BMGY medium were inoculated with the preculture and incubated for 48 h at 30 °C on a orbital shaker at 160 rpm until the cell density reached $A_{600} > 30$. The bioreactor cultivation was seeded with the content of the shake flask cultures up to an A_{600} of 0.5. Cultivation was performed in a 5 L reactor (Infors, Bottmingen, Switzerland) containing 5 L BMMY-medium [containing 1% (w/v) yeast extract, 2% (w/v) peptone, 5 mL methanol, 5 mL (100 mg/mL) Zeocin, 100 mM potassium phosphate, pH 6.8] with 186.25 g $MgSO_4 \cdot 7H_2O$, 125 mL MeOH, 1 mL antifoam 286 (Sigma, Deisenhof, Germany) and 1.25% PTM1 trace salts [18]. Temperature was controlled at 30 °C, and pH was maintained at 6.8 using NH_4OH (28%) and H_3PO_4 (10%). The airflow was maintained at 15 L/min and the rpm was maintained at 650. When the initial methanol 0.5% (v/v) in the culture broth was depleted, as indicated by an increase in dissolved oxygen, 25 mL of 100% methanol solution containing 1.2% (v/v) PTM1 was automatically fed. During the fermentation 1 mL antifoam Triton-X-100 (Sigma–Aldrich, Taufkirchen, Germany) was added. Methanol consumption was monitored using a balance (BP 4100, Sartorius, Goettingen, Germany) that was interfaced with the IRIS process control system (Infors, Bottmingen, Switzerland). Upon induction by methanol, $CALB_{rec}$ was secreted into the medium.

2.2.3. Biochemical characterization

The protein concentration was determined photometrically using a Bio-Rad protein assay kit (Bio-Rad, Munich, Germany), with bovine serum albumin as a standard protein [19]. The effect of temperature and pH on lipase activity and stability was determined photometrically at room temperature. A stock solution (10 mM) of *p*-nitrophenyl acetate was prepared in dimethylsulfoxide. 100 μL of this stock solution were mixed with 850 μL 100 mM Tris/HCl, pH 8.1. The assay was started by adding 50 μL of enzyme solution. The increase in absorption was followed at 412 nm on an Ultrospec 3000 photometer (Amersham Biosciences, Freiburg, Germany). The molar extinction coefficient used for the liberated *p*-nitrophenol is 15,000 L mol^{-1} cm^{-1}. One unit of enzyme activity is defined as the amount that liberated 1 μmol of *p*-nitrophenol/min under the above conditions. All activity measurements were carried out threefold. For determination of storage stability 100 μg/mL of $CALB_{rec}$ were incubated for 8 days in different buffers, 20 mM sodium formate (pH 2.5) and 20 mM sodium acetate at pH 3–6, at 4 and 20 °C. Remaining activity at different times was assayed as described above.

2.2.4. Purification of CALB by ion-exchange chromatography

Culture supernatant containing $CALB_{rec}$ was obtained by centrifugation of the culture broth at 10,800 × *g* for 15 min in a Sorvall RC5C Plus centrifuge. The supernatant was concentrated 14.4-fold by cross-flow ultrafiltration using a Millipore set-up with a silicone membrane (Pall, Germany) of 10 kDa cut-off value. The crude solutions of $CALB_{rec}$ and $CALB_{com}$ were purified using cation-exchange chromatography. A XK 16/20 column (Amersham Biosciences) filled with 7 mL Source 15 S (Amersham Biosciences) was equilibrated with 10 mM sodium formate, 10 mM sodium citrate, 10 mM sodium acetate, pH 3 (buffer A). A sample volume of 2 mL of the retentate was loaded onto the column at a volumetric flow rate of 5 mL/min. Afterwards, the column was washed with a threefold column volume of buffer A. CALB was eluted with five column volumes of 10 mM sodium formate, 10 mM sodium citrate, 10 mM sodium acetate, pH 5.5. Chromatography columns and resins were purchased from Amersham Biosciences. All chromatography experiments were carried out at room temperature (20 °C) using an ÄKTA explorer chromatography system (Amersham Biosciences) controlled