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One-step, inexpensive high yield strategy for *Candida antarctica* lipase A isolation using hydroxyapatite

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ABSTRACT

Lipase A from *Candida antarctica* (CAL A) was purified to apparent homogeneity in a single step using hydroxyapatite (HAP) chromatography. CAL A bound to HAP was eluted with 10 mM Na-phosphate buffer, pH 7.0 containing 0.5% Triton X-100. The protocol resulted in a 3.74-fold purification with 94.7% final recovery and 400.83 U/mg specific activity. Silver staining after SDS–PAGE revealed the presence a single band of 45 kDa. The enzyme exhibited a temperature optimum of 60 °C, was unaffected by monovalent metal ions, but was destabilized by divalent metal ions (Zn^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}) and stimulated by 50 mM Fe^{2+}. Detergents at 0.1% concentrations did not affect lipase activity. Except for Triton X-100, detergent concentrations of 1% had a destabilizing effect.

1. Introduction

Lipases (triacyl glycerol acylhydrolases EC 3.1.1.3) catalyze the hydrolysis of fatty acid ester bonds in aqueous environments and their synthesis in non-aqueous medium.

Lipases exhibit a very complex catalytic mechanism, including phenomenon of "interfacial activation" (Verger, 1997). Lipases may exist in two different forms, a closed and inactive form, where the active center is protected from the reaction medium by a polypeptide chain "lid", and open and active form, where the lid is displaced and the active center is exposed to the reaction medium (Fernández-Lorente et al., 2003; Ericsson et al., 2008). In the presence of hydrophobic interfaces, the lipases are adsorbed via interactions between the very hydrophobic internal side of the lid and the surroundings of the active center, and such hydrophobic surface, shifting the equilibrium towards the open form (Palomo et al., 2002a).

Lipases are important industrial enzymes that are used in detergents and in the synthesis of pharmaceuticals and food ingredients (Sharma et al., 2011). Since many industrial processes require temperatures higher than 45 °C, lipases should ideally have good activity and stability around 50 °C (Dheeman et al., 2011).

There has been an increasing interest in the study of enzymes from extremophiles (Cowan and Fernandez-Lafuente, 2011), because of their higher thermostability and resistance to chemical agents and extreme pH values compared to their mesophilic homologous (Sun et al., 2009). The yeast *Candida antarctica* produces two different lipases, lipases A and B. While lipase B (CAL B) has received most attention (Ericsson et al., 2008), lipase A could also be of interest because it maintains its hydrolytic activity in organic solvents at higher temperatures and over a broader pH range than CAL B (de Maria et al., 2005). CLA A accepts tertiary and sterically hindered alcohols (Palomo et al., 2002b), recognizes the Sn-2 recognition during hydrolysis of triglycerides, and is selective towards trans-fatty acids (Kirk and Christensen, 2002). Additionally, CAL A is an excellent biocatalyst for the asymmetric synthesis of amino acids/amino esters, because of its chemoselectivity towards amine groups (de Maria et al., 2005). In most commercial preparations, lipases are immobilized on different carriers or lyophilized as crude products that often contain other enzymes, some of which might be active against target compounds (Palomo et al., 2002b). Thus, pure lipase preparations are needed in order to understand and control the process (Fernandez-Lorente et al., 2005). Traditionally, the purification of lipases was achieved by a multi-step process (Taipa et al., 1992), and yields usually only reached 2–20% (Kambourova et al., 2003). Thus, today's industries look for purification strategies that are inexpensive, rapid, high-yielding and amenable to large-scale operations.

Purification of CAL A requires a combination of several precipitation and chromatographic steps (Patkar et al., 1993; Hoegh et al., 1995) to obtain nearly homogeneous (Pfeffer et al., 2006). Since

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hydroxyapatite (HAP) has been used for purification of lipases from different sources (Taipa et al., 1992), its use for purification of CLA A was studied and a simple and inexpensive method has been developed that provides the enzyme in high yield and homogeneity.

2. Methods

2.1. Chemicals

p-Nitrophenyl palmitate, sodium dodecyl sulfate (SDS), coomassie brilliant blue R-250, and bromophenol blue were purchased from Sigma Chemical Company, USA. All other chemicals were of analytical grade and of the highest purity available. Hydroxyapatite was prepared in the laboratory as described previously (Atkinson et al., 1973).

2.2. Enzyme production and enzyme assay

C. antarctica DSM 70725 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Enzyme production under submerged conditions and a lipase assay based on the hydrolysis of *p*-nitrophenyl palmitate (*p*NPP) were described earlier (Dimitrijevic et al., 2011). One unit of activity was expressed as the amount of enzyme that released 1 μmol of *p*-nitrophenolate ion/min under the assay conditions. The amount of protein was estimated by the Bradford dye-binding method, using bovine serum albumin (BSA) as a standard.

2.3. Binding of C. antarctica lipase A (CAL A) on hydroxyapatite

The kinetics of binding of CAL A on HAP was examined by incubating 100 μL of HAP (equilibrated in 10 mM Tris buffer pH 7.0) with 1 mL of *C. antarctica* fermentation broth. At various time points, aliquots were withdrawn from the supernatant after centrifugation (10,000*g*, 5 min), diluted (1–10 times) with buffer (10 mM Tris buffer pH 7.0) and lipase activity was determined by the *p*NPP assay.

In order to determine the optimal volume of fermentation broth to be applied to HAP, 1 mL of HAP (equilibrated in 10 mM Tris–HCl pH 7.0) was incubated with different volumes of fermentation broth, and the amount of unbound enzyme as a function of fermentation broth volume was determined.

2.4. Elution of C. antarctica lipase A (CAL A) from hydroxyapatite

Elution of lipase from HAP was tried with 0.2 M Na-phosphate buffer pH 7, 0.1 M NaCl, 10 mM $CaCl_2$, 1.5% Triton X-100, 1.5% Tween 80, 50% ethanol and 40% ethylene glycol. Gradient elution with Triton X-100 was performed using 0–1.5% of Triton X-100 in 12 column volumes (CV). The sample, column and fraction volumes were 10, 1 and 0.5 mL, respectively. Isocratic elution with Triton X-100 was performed in batch. One-hundred microliters of HAP (17 mg) with bound lipase was incubated with 1 mL of solutions containing different amounts of Triton X-100. After 30 min, lipase activity of the whole suspension was measured.

2.5. Activation of CAL A on HAP

In order to examine interaction between CAL A and HAP, 100 μL of HAP (equilibrated with 10 mM Tris buffer) was incubated with 1 mL of fermentation broth. After 30 min, lipase activity in the suspension was determined and compared with the initial activity in the fermentation broth.

2.6. Single step isolation and purification of CAL A

After 5 days of *C. antarctica* cultivation in optimal medium, when the highest lipase production was reached (Dimitrijevic et al., 2011), cells were removed by centrifuging (4500×*g*, 30 min) and the supernatant (V = 270 mL; pH 5.6; conductivity 5 mS) was directly applied on the HAP column (V = 45 mL), previously equilibrated with 10 mM Tris buffer pH 7.0. The column was washed with three CV of 0.5 M NaCl dissolved in 10 mM Tris–HCl pH 7.0, followed by one CV of 10 mM Tris–HCl pH 7.0 to remove salt. Elution was performed isocratically with 0.5% Triton X-100 dissolved in Tris–HCl pH 7.0. Fractions of 6 mL were collected and those with lipolytic activity were pooled.

2.7. Removal of Triton X-100 from purified CAL A

Triton X-100 was removed from purified CAL A using previously described method (Horikawa and Ogawara, 1979). Briefly, CAL A solution containing Triton X-100 was extracted with chloroform (1:1) by mixing for a few seconds. After centrifugation at 7000×*g* for 20 min, an aqueous phase which contained CAL A, but not Triton X-100 was recovered. Triton X-100 remained in chloroform phase. Residual Triton X-100 was determined by measuring absorbance at 275 nm.

2.8. Characterization of CAL A

2.8.1. Electrophoresis

The molecular mass of lipase was determined by SDS–PAGE with 12% acrylamide. Proteins were visualized by silver stain.

2.8.2. Effect of temperature on CAL A activity and stability

The optimal temperature of the enzyme was determined by measuring the enzyme activity at temperatures of 30–70 °C. Thermal stability was determined by incubating the purified enzyme at 60, 65, 70, 75, 80 °C. After 0, 5, 15, 30, 60, 150 and 210 min, aliquots were withdrawn and the residual activity was measured. The inactivation constant (k) at every temperature was calculated from the slope of the linear part of the enzyme activity curve (results not shown). Using equation $t_{\frac{1}{2}} = -\ln 0.5/k$, the enzyme half-life at every temperature was determined.

2.8.3. Effect of ions and detergents on CAL A stability

Fifty microliters of purified CAL A was incubated with 1 mL of 50 mM cations from NaCl, KCl, $(NH_4)_2SO_4$, $ZnSO_4$, $CaCl_2$, $MgCl_2$, $CuSO_4$, $MnSO_4$, $FeSO_4$ and residual activity was measured after 30 min. Purified CAL A was incubated in 0.1% and 1% solutions (w/v) of Triton X-100, Triton 1339, Tween 20, Tween 80, SDS, lauryl sarcosine, Brij 35 and deoxycholate at 30 °C and residual activity was determined after 1 h.

3. Results and discussion

3.1. Binding of CAL A on hydroxyapatite

Binding of CAL A on hydroxyapatite was monitored in time as described in Section 2.3 (Fig. 1A). Around 50% of the activity was bound on the HAP within 10 min and 80% of the activity was adsorbed in 30 min, incubation of up to 190 min only increased binding by 10% (Fig. 1A).

In order to determine optimal ratio of fermentation broth and HAP needed for complete binding of lipase, different volumes of fermentation broth were incubated with 1 mL of HAP and lipase activity in supernatant was measured (Fig. 1B).