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Homologous expression, purification and characterization of a novel high-alkaline and thermal stable lipase from *Burkholderia cepacia* ATCC 25416

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ABSTRACT

The lipase secreted by *Burkholderia cepacia* ATCC 25416 was particularly attractive in detergent and leather industry due to its specific characteristics of high alkaline and thermal stability. The lipase gene (*lipA*), lipase chaperone gene (*lipB*), and native promoter upstream of *lipA* were cloned. The *lipA* was composed of 1095 bp, corresponding to 364 amino acid residues. The *lipB* located immediately downstream of *lipA* was composed of 1035 bp, corresponding to 344 amino acid residues. The lipase operon was inserted into broad host vector pBBRMCS1 and electroporated into original strain. The homologous expression of recombinant strain showed a significant increase in the lipase activity. LipA was purified by three-step procedure of ammonium sulfate precipitation, phenyl-sepharose FF and DEAE-sepharose FF. SDS-PAGE showed the molecular mass of the lipase was 33 kDa. The enzyme optimal temperature and pH were 60 °C and 11.0, respectively. The enzyme was stable at 30–70 °C. After incubated in 70 °C for 1 h, enzyme remained 72% of its maximal activity. The enzyme exhibited a good stability at pH 9.0–11.5. The lipase preferentially hydrolyzed medium-chain fatty acid esters. The enzyme was strongly activated by Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and apparently inhibited by PMSF, EDTA and also DTT with SDS. The enzyme was compatible with various ionic and non-ionic surfactants as well as oxidant H_2O_2. The enzyme had good stability in the low- and non-polar solvents.

1. Introduction

Lipases (E.C.3.1.1.3) are glycerol ester hydrolases that catalyze the hydrolysis and synthesis of a variety of acylglycerols at the interface of lipid and water [1]. Alkaline lipases have high catalytic efficiency for the hydrolysis of acylglycerols under the alkaline conditions. Due to the great advantages of enzyme catalysis, such as high efficiency, mild reaction conditions, safety and innocuity, alkaline lipases have been widely utilized in biotechnical applications including detergent industry, leather industry, food and textile industry. For example, in the detergent industry lipases improve the removal of fatty residues in laundry, dishwashers and for cleaning of clogged drains [2]. The importance of lipase in washing agents results not only from their high efficacy, but also from reason of saving energy. Using enzymes (lipase, protease and amylases) in detergent formulations allows laundering at lower temperatures and thus reduces energy expenditure [3]. In leather manufacturing, degreasing is one of the important unit operations which involve removal of natural fat present in the skin or hide [4]. Applying the lipase preparations into the degreasing process, as a clean technology, could remove the natural fat wit high efficiency, make the degreasing process easy and decrease the amount of environmentally sensitive organic solvents (such as white spirit or trichloroethylene) and emulsifiers. The ideal enzymes used in detergent industry and leather manufacturing should be stable at high pH and temperature.

One of alkaline and thermal stable lipase-producing bacterial genera is *Pseudomonas*. Some of alkaline lipases secreted by *P. menodocina*, *P. alcaligenes* and *P. glumae* have already been commercially exploited [5]. *Burkholderia cepacia* lipases which represent an important group of *Pseudomonas* lipases have high transesterification capacity and broad substrates adaptability and have often been used as biocatalysts for enantioselective synthesis [6]. However, few of *B. cepacia* lipases reported has high pH optimum as well as perfect stability to extremely alkaline environment. We first found that *B. cepacia* ATCC 25416 could secrete a high alkaline and thermostable lipase capable of withstanding high temperature and high alkalinity, which was unusual in *B. cepacia* lipases. The optimal pH of the lipase was even higher than some *Pseudomonas* lipases used in detergent industry, such as *P. mendocina* and *P. pesudoalcaligenes*. The properties of lipase secreted by *B. cepacia* ATCC 25416 showed this enzyme must be a good candidate for biotechnical applications, especially in detergent and leather industry, and the enhancement of the enzyme production would have great meaning for industrial

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application. The high-level production of lipase could be achieved through the efficient overexpression of the corresponding genes.

Until now, the cloning, sequencing and expression of a variety of *Pseudomonas* lipase genes have been reported. Based on the gene sequences, Jaeger et al. ascribed *Pseudomonas* lipases into three groups [7]. *Pseudomonas* lipases of group I and group II including the lipases of *P. aeruginosa* strains (group I), lipases of *B. cepacia* and *B. glumae* strains (group II), have N-terminal signal sequence and two cysteine residues to form inter chain disulfide bond as well as need a chaperone whose gene (*lipB*) is located downstream of the lipase gene (*lipA*) to help the LipA fold correctly and secrete efficiently. Lipases of group III include *P. fluorescens* lipases, which have no typical N-terminal signal sequence, cysteine residues in the primary structure and do not need the chaperone for folding and secretion. *Pseudomonas* lipases are secreted through two different secretion pathways. Group III lipases use the ABC pathway that secretes the protein by the one-step mechanism [8]. Group I and group II lipases are secreted by GSP pathway containing over 12 secretory proteins and dependent on signal sequence and chaperone for correct folding and translocation [9–11]. So far, the genes of group I and group II lipases have been tested for heterologous expression in a variety of hosts, such as *Bacillus licheniformis*, *Escherichia coli*, *Streptomyces lividans*, *Aspergillus niger*, and *Kluyveromyces lactis* [12,13]. The high-level expression of the active and soluble lipase in heterogonous strains has yet not been achieved due to the high GC content of *lipA* and *lipB*, complex process of folding and secretion of the lipase protein. The most lipases expressed are present in the bacterial cytoplasm as inactive inclusion bodies and the chaperone could not be expressed at high levels. Thus, in vitro refolding process of the lipase is necessary for achieving the high activity [6]. However, high-level expression of active and soluble lipases from *P. alcaligenes* [12] and *B. cepacia* G63 [14] in the original strains exhibited the great advantage of homologous expression of *Pseudomonas* lipases belonging to group I and group II in secretion and production of the active soluble enzymes. Thus, in this work we reported for the first time the homologous expression of *lipA* and *lipB* from *B. cepacia* ATCC 25416 as well as purification and characterization of the lipase.

2. Materials and methods

2.1. Materials

Peptone and yeast extract were obtained from OXOID (England). Phenyl-sepharose FF, DEAE-sepharose FF and low molecular mass standards (*Mr* of 14.4 kDa, 20.1 kDa, 31.0 kDa, 43.0 kDa, 66.2 kDa and 97.4 kDa) were purchased from Pharmacia (Shanghai, China). Ultrafiltration membrane with 30,000 molecular weight cutoff (MWCO) was purchased from Millipore (USA). *p*-Nitrophenyl esters was from Sigma (USA). All reagents were of analytical grade unless otherwise stated.

2.2. Strains and plasmids

For this study bacterium *B. cepacia* ATCC 25416 was used. *E. coli* DH5α [supE44ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1] was used for cloning. The broad host vector pBBRMCS1 donated by Chen in Tsinghua University was used to construct expression vector in *B. cepacia*. The construction of pBBRMCS1 was first reported by Kovach et al. [15]. This plasmid is a Cm^R (chloromycetin resistance) bhr plasmid that is only 4.7 kb and contains 16 unique cloning sites within the *lacZα* gene and is relatively stable retained in vitro (>10 days) and in vivo (>4 weeks) without antibiotic selection [15]. The complete nucleotide sequence for pBBRMCS1 has been assigned GenBank accession no. U02374.

2.3. Recombinant DNA techniques

Chromosomal DNA was extracted from *B. cepacia* ATCC 25416 using bacteria genomic DNA extraction kit from Omega (USA). Plasmid DNA was prepared using mini plasmid kit from Omega (USA). PCR product was purified by DNA gel extraction kit from Omega (USA). DNA was ligated using T4 DNA ligase obtained from Takara (Dalian, China). Taq DNA polymerase, restriction endonucleases and pMD19-T simple vector were purchased from Takara (Dalian, China) and used under the recommended conditions.

2.4. Construction of cloning vector and expression vector

Based on the conserved sequence upstream of *B. cepacia* lipase gene and sequence deposited in GenBank with the accession number AY682925, a pair of primers lipUp (5′-CCCAAGCTTCGTGTCTAGTCAGGGCGCAAAC-3′) and lipDn (5′-TGCACTAGTCTAGAGATCTCACGCGCGCGGCATAAC-3′) were used to amplify the lipase operon including *lipA* and *lipB*, the promoter upstream of *lipA* and transcription terminator downstream of *lipB*. PCR product was then ligated into pMD19-T simple vector to construct cloning vector pMD19-T*lipAB* and sequenced by ABI 3730 DNA analyzer. The pMD19-T*lipAB* was double digested with *Hind*III and *Xba*I, the fragment of the lipase operon was then subcloned from pMD19-T*lipAB* into the *Hind*III and *Xba*I restriction sites of broad host vector pBBRMCS1 to yield expression plasmid pBBRMCS1-*lipAB* (Fig. 1).

2.5. Electroporation and isolation of recombinant strains

Electroporation of plasmids into *E. coli* and *B. cepacia* was carried out as described by Calvin and Hanawalt [16]. Transformants of *E. coli* or *B. cepacia* with recombinant plasmid pBBRMCS1-*lipAB* were selected on LB agar plates with 34 μg/mL chloromycetin.

2.6. Media and cultivation

For small- and large-scale DNA isolations, recombinant *E. coli* and *B. cepacia* strains were propagated in LB medium (10 g/L of tryptone, 5 g/L of yeast extract, 5 g/L of NaCl, pH 7.0) supplemented with appropriate antibiotic (ampicillin, 100 μg/mL; chloromycetin, 34 μg/mL). Colonies were plated on LB agar plates with 34 μg/mL chloromycetin. For the expression of lipase gene, wild-type *B. cepacia* ATCC 25416 and recombinant strain with pBBRMCS1-*lipAB* were cultured in the shaking flask. The seed cultures were grown for 12 h with shaking at 200 rpm at 30 °C in the LB medium (for the culture of recombinant strain, 34 μg/mL chloromycetin was contained). For the main fermentation, the medium used contained maltose 0.5%, yeast extraction 0.5%, K_2HPO_4 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, and emulsified olive oil 2%, pH 7.0. The seed cultures were then inoculated into fermentation medium with the ratio 1:100 at 30 °C, 200 rpm for 48 h.

2.7. Lipase assay

Lipase activity was measured spectrophotometrically using an assay based on the hydrolysis of *p*-nitrophenyl palmitate (*p*NPP) at 0.79 M. The reaction mixture was composed of 2.4 mL of *p*NPP solution and 0.1 mL of lipase solution. The *p*NPP solution was prepared as follows: 10 mL isopropanol containing 30 mg *p*NPP was added to 90 mL 0.05 M Sorensen's phosphate buffer (pH 8.0) supplemented with 207 mg sodiumdeoxycholate and 100 mg gum arabic. The enzyme reaction mixture was incubated at 37 °C for 2 min. *p*-Nitrophenol produced in the reaction mixture was quantified spectrophotometrically at 410 nm. One unit was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol per 1 min under the condition mentioned above [17].

2.8. Lipase purification

Proteins in the supernatant (1 L) were extracted with 30–60% ammonium sulfate. The precipitate was dissolved in 50 mM Tris–HCl buffer (pH 7.5) and dialyzed against 1.2 M ammonium sulfate in 50 mM Tris–HCl buffer (pH 7.5). The crude preparation was loaded onto a phenyl-sepharose FF column equilibrated with 1.2 M ammonium sulfate buffer. The lipase was eluted from the column in a linear concentration gradient of 1.2–0 M ammonium sulfate in the same buffer, and the most of enzyme was eluted with 0% ammonium sulfate. Active fractions were pooled and dialyzed against 50 mM Tris–HCl buffer (pH 7.5). The solution was then applied to a DEAE-sepharose FF column equilibrated with 50 mM Tris–HCl buffer (pH 7.5). The elution fraction at 0.3 M NaCl showed the most of lipase activity was collected, and again dialyzed against 50 mM Tris–HCl buffer (pH 7.5). Dialyzed solution was then concentrated by using an ultrafiltration membrane with 10,000 MWCO. The molecular mass of the lipase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed with 12.5% (w/v) SDS polyacrylamide gels by the method of Laemmli [18].

2.9. Effects of pH on lipase activity and pH stability of the lipase

The effect of pH on the lipase activity was investigated by the method using *p*NPP as the substrate. The buffers used were 50 mM Tris–HCl buffer (pH 8.0–9.0), disodium hydrogen orthophosphate–NaOH buffer (pH 9.0–11.0), and glycine–NaOH buffer (pH 11.0–12.0). Then the enzyme reactions were carried out at different pH values at 37 °C. As for the pH stability of the lipase, the reaction mixtures were incubated at various pH values at 37 °C for 1 h and the residual enzyme activity was measured by the method in lipase assay 37 °C, pH 11.0.