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A novel proteolysis-resistant lipase from keratinolytic *Streptomyces fradiae* var. k11

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Abstract

A novel lipase gene, *lipS221*, was cloned from *Streptomyces fradiae* var. k11, which secretes multiple proteases. The 930-bp nucleotide sequence encodes 309 amino acid residues with a calculated molecular weight of 28.5 kDa. The protein sequence shows highest identity (82%) with a putative lipase of *Streptomyces coelicolor* A3(2). LipS221 was functionally expressed in *Pichia pastoris* and purified to homogeneity with a final specific activity of 569 U mg^{-1}. The purified enzyme showed the maximum activity at 55 °C and pH 9.8 for hydrolysis of *p*-nitrophenyl palmitate. Importantly, lipS221 was stable towards degradation by alkaline and neutral proteases, but activity decreased when hydrolyzed by savinase or proteinase K together with surfactant (SDS or CTAB). These results indicate that the proteolytic resistance of lipS221 may be related to its surface charge distribution.

Keywords: Lipase; *Streptomyces fradiae*; Proteolytic resistance; Characterization

1. Introduction

Lipases (triacylglycerol hydrolases; EC 3.1.1.3) have the ability to hydrolyze triacylglycerols at the interface of lipid–water and catalyze the reactions of ester synthesis and transesterification [1]. Because of their versatile functionality, lipases have recently emerged as key enzymes in the swiftly growing biotechnology. They play important roles in a wide array of industrial applications, such as biodiesel, organic synthesis, detergents, the food industry, leather manufacture and the paper industry [2–4].

Microbial lipases constitute an important group of biotechnologically valuable enzymes, mainly because of the versatility and stability of their applied properties compared to animal and plant lipases, but also for their ease of mass production [4]. The frequency of studies reporting microbial lipase purification, characterization and gene sequencing increased sharply in recent years [1,5,6].

The gram-positive bacteria, *Streptomyces*, can produce commercially important bioactive molecules including enzyme inhibitors, antibiotics and many valuable enzymes with attractive properties, such as proteases [7]. Several proteases have been isolated, purified and characterized from *Streptomyces fradiae* [8–10], and multiple secreted proteases have been reported by our group from *S. fradiae* var. k11, a strain that efficiently hydrolyzes feather and human hair [11,12]. However, only a few lipase genes have been cloned from *Streptomyces*, and even fewer have been expressed and characterized [13–15].

In this paper, one lipase gene was cloned from *S. fradiae* var. k11, in which lipase activity was detected, and expressed in *Pichia pastoris*. The purified recombinant lipase was characterized, the proteolytic stability was assessed in detail, and a preliminary mechanism was proposed.

2. Materials and methods

2.1. Strains, plasmids and reagents

S. fradiae var. k11 was conserved in our lab [11]. Plasmids pUC19 (Promega, USA) and pPIC9 (Invitrogen, USA) were used as cloning and expression vectors, respectively. *Escherichia coli* strain JM109 (TaKaRa, Japan) was used

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for gene cloning. *P. pastoris* GS115 (Invitrogen, USA) was used as host for gene expression. *p*-nitrophenyl palmitate (*p*NPP), *p*-nitrophenyl decanoate and *p*-nitrophenyl butyrate were purchased from Sigma (USA). Trypsin, α-chymotrypsin, subtilisin A, and collagenase were purchased from Sigma. Proteinase K was purchased from Amresco Inc. (USA). Proleather was obtained from Amano Enzyme Inc. (Japan). Savinase was from Novozymes (Denmark). Alkaline protease was produced from *Bacillus pumilus* SMJ-P [16].

2.2. Medium and culture conditions

The *S. fradiae* var. k11 was grown at 30 °C in Gause's synthetic medium (0.1% KNO_3, 2% soluble starch, 0.05% K_2HPO_4, 0.05% $MgSO_4$, 0.05% NaCl, 0.001% $FeSO_4$, w/v). Luria-Bertani (LB) medium (0.5% yeast extract, 1% tryptone and 1% NaCl, w/v) containing 100 μg ml^{-1} ampicillin was used to cultivate recombinant *E. coli*. The *P. pastoris* strain GS115 was cultivated at 30 °C in yeast extract peptone dextrose (YPD) medium. Buffered glycerol complex medium (BMGY), buffered methanol complex medium (BMMY), regeneration dextrose medium (RDB), minimal dextrose medium (MD), and minimal methanol medium (MM) were prepared according to the manual of the *Pichia* expression kit (Invitrogen).

2.3. Cloning of a lipase gene from S. fradiae var. k11

All molecular manipulations were performed according to standard protocols [17]. A ~500 bp fragment of a lipase gene had been previously sequenced from *S. fradiae* var. k11 by our group (data not shown). Thus a gene library of *S. fradiae* var. k11 was constructed as described [11,18]. Two specific primers (LIP-F, 5′-TCGCCCCCTACCTGGTGC-3′ and LIP-R, 5′-CCGATGGCCAGGTGCTCG-3′) were designed and used to amplify the sequence encoding the partial fragment of the lipase gene using a colony PCR method [18]. The PCR program was 32 cycles of 94 °C (30 s), 58 °C (30 s), and 72 °C (50 s) performed on a Mastercycler (Eppendorf, Germany). The positive clone was further sequenced by an ABI 3730 sequencer (Applied Biosystems Inc., USA) to obtain the full-length lipase gene (*lipS221*).

2.4. Sequence analysis of lipS221

The signal peptide was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP). Sequence assembles and the molecular mass of protein was determined using Vector NTI suite 7.0. DNA and protein sequence alignment and conserved domain analysis were carried out using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST).

2.5. Expression and high cell density fermentation of lipS221 in P. pastoris

The DNA fragment containing *lipS221* without signal peptide coding sequence was amplified with primers PIClipf (*Eco*RI site underlined), 5′-GTTGAATTCGCGACCCCGCCCGC-3′, and PIClipr (*Not*I site underlined), 5′-ATTGCGGCCGCTCAGCCGAGGACC-3′, using *S. fradiae* var. k11 genomic DNA as template. PCR reactions were carried out by 35 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 60 s. The PCR fragments were digested with *Eco*RI and *Not*I and inserted into the corresponding sites of pPIC9 to generate recombinant plasmid *pPIC9-lipS221*. pPIC9-lipS221 was linearized using *Bgl*II and then transformed into *P. pastoris* GS115 competent cells by electroporation. The transformed cells were plated on RDB plates and incubated at 30 °C for 2–3 days until colonies appeared. Selected HIS^+ transformants were transferred to MM and MD plates, and they were grown for 1–2 days at 30 °C until single colonies formed. The HIS^+ transformants were transferred from MD plates into 5 ml BMGY medium and cultivated at 30 °C for 48 h. The cells were pelleted by centrifugation, suspended in 1 ml BMMY containing 0.5% methanol, and then cultivated at 30 °C for 48 h. The culture was centrifuged and the supernatant was collected to detect lipase activity.

The recombinant *P. pastoris* strain with the highest lipase activity was selected for fermentation. Fermentation was carried out in a 3.7-l fermenter (Bioengineering KLF 2000, Switzerland) induced by methanol [19]. Cell density and lipase activity in the supernatant were assessed at 12-h intervals. Protein accumulation was evaluated by SDS–PAGE [20].

2.6. Enzyme activity assay

Lipase activity was measured by hydrolysis of *p*-nitrophenyl palmitate (*p*NPP) in sodium phosphate as described [21,22]. Shortly, isopropanol (10 ml) containing 0.0795 mmol of *p*NPP was mixed with 90 ml of 50 mM sodium phosphate (containing 0.2% of sodium deoxycholate and 0.1% of gum Arabic, w/v), pH 8.0. The freshly prepared substrate solution (2.4 ml) was preincubated at 37 °C, and then the reaction was initiated by the addition of 0.1 ml enzyme solution. After 15 min of incubation at 37 °C, absorbance was measured at 410 nm in 1-cm path-length cell with an Ultrospec 2000 UV–vis spectrophotometer (Amersham Pharmacia). One enzyme unit (U) was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol per min.

2.7. Purification of lipS221

After fermentation, the cell-free supernatant was harvested by centrifugation at 12,000 × *g* for 10 min and then concentrated using a Hollow-Fiber membrane UEOS503 from Motian (Tianjin, China). Subsequently, the concentrated crude enzyme was purified by anion-exchange chromatography on a HiTrap Q Sepharose XL column (Amersham Pharmacia, Sweden) equilibrated with buffer A (25 mM Tris–HCl, pH 8.0). The sample was eluted from the column with a linear gradient of NaCl from 0 to 1.0 M in buffer A. The fractions with lipase activity were pooled and concentrated by 10-kDa centrifugal concentrators (Pall, USA). Finally, 500 μl of the concentrated sample was applied to a Sephacryl S-200 HR FPLC column (Amersham Pharmacia) equilibrated with buffer A. The sample was eluted with buffer A at a flow rate of 0.5 ml min^{-1}. Fractions with lipase activity were collected and concentrated as above. The purity of the protein was analyzed by SDS–PAGE. The concentration of protein at each step was detected by the Bradford method with bovine serum albumin as standard [23].

2.8. Characterization of lipS221

The optimum pH of purified lipS221 was measured by assaying the relative activity at various pHs in buffers (50 mM Na_2HPO_4–citric acid, pH 2.2–8.0; 50 mM sodium phosphate, pH 6.0–8.0; 50 mM Tris-HCl, pH 7.1–9.8; and 50 mM K_2HPO_4–KOH, pH 10.0–12.0). The apparent optimum temperature of purified lipS221 was measured by assaying its activity at various temperatures (20–80 °C) in 50 mM sodium phosphate (pH 8.0).

To determine the pH stability of the enzyme, lipS221 was incubated at various pHs (pH 2.2–12) at 37 °C for 60 min. To examine the thermostability, the enzyme was incubated at various temperatures (40, 50, 60, or 70 °C) for 10, 20, 30 and 60 min in 50 mM sodium phosphate (pH 8.0). Then the residual lipase activity was measured at 37 °C and pH 8.0.

The substrate specificity of the enzyme was studied using *p*-nitrophenyl alkanoate esters of varying alkyl chain lengths (*p*-nitrophenyl decanoate, *p*-nitrophenyl butyrate, and *p*-nitrophenyl palmitate) as the substrate. Kinetic parameters (V_{max} and K_m) were determined by Lineweaver-Burk plots for the substrate *p*NPP at 37 °C and pH 8.0.

The effects of metal ions and chemical reagents on the lipase activity were determined by incubating purified lipase with metal ions or chemical reagents (final concentration was 1 mM) at 37 °C, pH 8.0, for 30 min. The remaining activity was measured as described above. The effects of SDS (0–20 mM) and cetyl trimethyl ammonium bromide (CTAB, 0–10 mM) on lipase activity were also determined under similar assay conditions.

2.9. Proteolytic resistance of lipS221

Trypsin (from bovine), α-chymotrypsin (type II from bovine), proteinase K (from *Tritirachium album*), subtilisin A (type VIII from *Bacillus licheniformis*) and collagenase (type IV from *Clostridium histolyticum*) were dissolved in 50 mM Tris–HCl (pH 7.5). Proleather (from *Bacillus subtilis*), savinase (from *Bacillus lentus*) and alkaline protease (from *B. pumilus*) were dissolved in 50 mM glycine-NaOH (pH 10.0). Purified lipS221 (0.032 mg ml^{-1}) was mixed