Process Biochemistry 48 (2013) 1679–1685

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Molecular cloning of a thermo-alkaliphilic lipase from *Bacillus subtilis* DR8806: Expression and biochemical characterization

Shirin Emtenani [a], Ahmad Asoodeh [a,b,*], Shamsi Emtenani [a]

[a] Department of Chemistry, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran
[b] Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran

ARTICLE INFO

Article history:
Received 18 May 2013
Received in revised form 17 August 2013
Accepted 26 August 2013
Available online 2 September 2013

Keywords:
Cloning
Lipase
Bacillus subtilis DR8806
Biochemical characterization
Ionic liquids

ABSTRACT

A thermo-alkaliphilic lipase from *Bacillus subtilis* DR8806 was functionally expressed as an N-terminal 6xHis-tagged recombinant enzyme in *Escherichia coli* BL21 using pET-28a(+) expression vector. Sequence analysis revealed an open reading frame of 639 bp encoding a 212-amino acid protein containing the well-conserved Ala-His-Ser-Met-Gly motif. One-step purification of the His-tagged recombinant lipase was achieved using Ni-NTA affinity chromatography with a specific activity of 1364 U/mg. The purified enzyme with an apparent molecular mass of 26.8 kDa demonstrated the maximum activity at 70 °C and pH 8.0 for hydrolysis of p-nitrophenylbutyrate as substrate. The enzyme activity was strongly inhibited by divalent ions of heavy metals such as Hg^{2+} and Cu^{2+}, while retained over 90% of the original activity in the presence of several reagents including DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)), SDS (sodium dodecyl sulfate), urea, DMF (dimethylformamide), DTT (dithiothreitol), glycerol and Triton X-100. While being considerably stable in organic solvents, imidazolium-based ionic liquids (ILs) had stimulatory effects on the activity of purified lipase. Remarkable stabilization of enzyme at alkaline pH and in ionic liquids as well as its thermostability/thermoactivity are among the most fundamental characteristics which offer great potential for various biotechnological applications including detergent formulation, bioremediation processes and biotransformation in non-aqueous media.

1. Introduction

Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) are a class of enzymes capable of hydrolyzing ester bonds in triglycerides at oil–water interface. Hydrolyzing the triglycerides, lipases also catalyze their synthesis from fatty acids and glycerol. Lipases, which are among the most widely used hydrolytic enzymes in industry, have become the object of particular attention for various biotechnological applications [1,2]. Thermostable lipases have garnered wide spread interest for potential application in the detergent, pharmaceutical, dairy, oil and fat industries due to their extreme stability at high temperatures and in organic solvents. In addition, operating bioprocesses at higher temperatures may result in an increase in diffusion rate and enhanced solubility of hydrophobic substrates as well as reduction in microbial contamination [3,4]. While harvesting a limited amount of enzymes from thermostable lipase producing bacteria, high expression level of protein has been achieved through cloning thermophilic genes into more appropriate mesophilic hosts [5]. Recently, ionic liquids (ILs) have gained much attention as an alternative to conventional organic solvents particularly in biocatalytic reactions. Owing to their unique properties including nonvolatility, thermal stability and ionic conductivity as well as nonflammability, ILs are considered as environmentally friendly green solvents [6]. The stimulatory effects of ILs on reactions involving lipases have been reported in previous studies [7,8]. A thermophilic lipolytic bacterium was previously isolated from Dig Rostam hot mineral spring in Iran. The strain was identified as *Bacillus subtilis* based on the 16S rDNA gene sequence (GenBank: JF309277) and has been deposited in Iranian Biological Resource Center under acquisition number of IBRC-M10742 [9]. The aim of the present study was to report the cloning, expression and purification of recombinant lipase from *Bacillus subtilis* DR8806, as well as biochemical properties of the purified enzyme.

2. Materials and methods

2.1. Bacterial strains and plasmids

A lipase producing strain *B. subtilis* DR8806 isolated from hot mineral spring was grown in nutrient broth at 37 °C. *E. coli* DH5α and BL21 (DE3) were used as cloning and expression hosts, respectively. The *E. coli* strains were cultivated in Luria-Bertani (LB; 1.0% tryptone, 0.5% yeast extract and 1.0% NaCl) medium at 37 °C. *B. subtilis* DR8806 was served as the source of genomic DNA. The plasmid pTZ57R/T (Fermentas, Maryland, USA) was used for cloning and sequencing of the lipase gene. The

* Corresponding author at: Department of Chemistry, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran. Tel.: +98 511 8795457; fax: +98 511 8795457.
E-mail address: asoodeh@um.ac.ir (A. Asoodeh).

1359-5113/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.procbio.2013.08.016

target gene was cloned into pET-28a(+) expression vector (Novagen, USA) under the control of strong T7 promoter to allow the insert to be maintained and expressed in *E. coli* host. To screen the transformants, medium was supplemented with ampicillin or kanamycin at a final concentration of 100 μg/mL and 50 μg/mL, respectively.

2.2. Cloning and sequencing of the lipase gene

A pair of oligonucleotide primers was synthesized based on coding sequences available for the *B. subtilis* lipase gene. The sequence of the forward primer LipF was 5′-CGCGGATCCGCGATTATGAAATTTGTAAAAAGAAGG-3′ and that of the reverse primer LipR was 5′-CCCAAGCTTGGGTCATTAATTCGTATTCTGGCC-3′. Genomic DNA from *B. subtilis* DR8806 was prepared by the method developed by Sambrook and Russell [10]. The lipase gene was amplified from genomic DNA using a set of primers (LipF and LipR) with incorporated restriction enzymes *Bam*HI/*Hind*III (Takara, Dalian, China), allowing the directional in-frame ligation of the amplified fragment into pET-28a(+) vector. The purified amplicon was cloned into the T/A cloning vector pTZ57R/T, in accordance with the manufacturer's instructions. Competent cells of *E. coli* were prepared by using a conventional $CaCl_2$ method [10]. Transforming into the *E. coli* DH5α competent cells using heat shock method [10], the cells were cultivated on LB-agar medium containing ampicillin (100 μg/mL). The plasmid DNA was isolated with Fermentas plasmid DNA isolation kit (Fermentas, Maryland, USA) following the manufacturer's instructions. Colony PCR using specific primers, restriction enzyme analysis and sequencing after plasmid extraction were performed to confirm the presence of the target gene. The nucleotide sequence of *B. subtilis* DR8806 lipase gene was determined and submitted to GenBank database. The insert was subcloned in pET-28a(+) vector by digesting the pTZ57R/T cloning vector containing the lipase gene with *Bam*HI/*Hind*III followed by ligation to the previously digested expression vector using T4 DNA ligase (Takara, Dalian, China). The resulting expression construct was confirmed by lipase gene amplification and double restriction digestion. The recombinant pET-28a(+) vector was employed for the expression of the lipase gene in *E. coli* BL21 (DE3) competent cells. The aforementioned construct placed the insert in frame with N-terminal region coding for six His residues facilitating the protein purification process.

2.3. Expression of the lipase gene

A transformant of *E. coli* BL21 harboring the recombinant plasmid (pET-28a(+)-lip) was cultured in LB medium containing 50 μg/mL kanamycin and incubated overnight at 37 °C with 150 rpm shaking. The pre-culture was inoculated (1% v/v) into 200 mL fresh LB medium supplemented with kanamycin at 37 °C until the OD_{600} reached 0.5. To induce the expression of recombinant lipase, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium at a final concentration of 1 mM and the growing culture was incubated at 25 °C for a further 8 h-period. Following induction, the aliquots were harvested by centrifugation (12,000 g, 20 min, 4 °C) and the pellets were resuspended in 10 mL lysis buffer (NaH_2PO_4 50 mM NaCl, 0.3 M imidazole 10 mM and 1 mM PMSF, pH 8.0). Induced cells were disrupted by sonication for six 30 s burst with a 30 s cooling period between each burst, the cell-free extracts were obtained by centrifugation.

2.4. Purification of the recombinant lipase enzyme

The N-terminally-attached His-tag lipase was purified under native conditions using the immobilized metal ion affinity chromatography (IMAC) column (Qiagen, CA, USA). The crude enzyme preparation was loaded onto the Ni-nitrilotriacetate (Ni-NTA) column previously equilibrated with native binding buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8.0). The column was washed sequentially with native wash buffer containing 20 mM imidazole. Finally, the bound target proteins were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 8.0) at a flow rate of 1 ml min^{-1}. The purification procedure was carried out at 4 °C.

2.5. Lipase assay and protein determination

Lipase activity was measured spectrophotometrically at 405 nm using 0.01 M p-nitrophenylbutyrate (pNPB) (Sigma–Aldrich, USA) as a substrate following incubation at 50 °C for 30 min and pH 8.0 (Tris-HCl buffer). One unit of lipase activity was defined as the amount of enzyme needed to liberate 1 μmol of p-nitrophenol per minute under standard assay conditions [2]. To determine the protein concentration, Bradford method was conducted using bovine serum albumin (Sigma–Aldrich, USA) as the standard [11]. The homogeneity of the purified enzyme and also the performance of affinity purification were analyzed on a 12% (w/v) SDS-PAGE polyacrylamide gel [12]. Protein bands were visualized by Coomassie brilliant blue R-250 staining. A protein standard (Vivantis, CA, USA) in the range of 10.5–175 kDa was used as molecular mass marker.

2.6. Enzyme characterization

2.6.1. Effect of pH on lipase activity and stability

To evaluate optimum pH of the recombinant enzyme, the lipolytic activity was assayed over a pH range from 2.0 to 10.0 using the following buffers (50 mM): glycine–HCl buffer pH 2.0–3.0, sodium acetate buffer pH 3.5–5.5, sodium phosphate buffer pH 6.0–7.5, Tris-HCl buffer pH 8.0–9.5, Na_2HPO_4–NaOH buffer pH 10. The pH stability of the lipase was determined after 60 min of pre-incubation at different pH values (2.0–10.0) at 50 °C. The residual lipolytic activity was determined under standard assay conditions. All enzyme assay experiments were conducted in triplicates.

2.6.2. Effect of temperature on lipase activity and stability

The effect of temperature on the activity of lipase was determined by monitoring the enzyme activity at various temperatures in the range of 30–80 °C for 30 min in Tris-HCl buffer, pH 8.0. The influence of temperature on lipase stability was analyzed by incubating the enzyme solution at above-mentioned temperatures for 60 min followed by the evaluation of the remaining activity according to standard assay method. To determine the enzyme half-life, the cloned lipase was kept at different temperatures ranging from 60 °C to 80 °C and the residual activity was assessed at 30 min intervals over a total period of 150 min.

2.6.3. Effect of metal ions on the purified lipase activity

The effects of various metal ions (Mg^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, K^+, Na^+, Fe^{2+}, Zn^{2+}, Co^{2+}, Pb^{2+}, Ba^{2+}, Cd^{2+} and Hg^{2+}) at 1 and 5 mM concentrations on the enzyme activity was assayed at 70 °C, pH 8.0 using p-nitrophenylbutyrate as substrate, upon pre-incubation of the purified lipase in each compound for 30 min. The enzyme activity of control sample (recombinant lipase without any metal ion) was taken as 100%. Lipase activity was measured as previously described.

2.6.4. Effect of organic solvents and ionic liquids on lipase performance

The effect of organic solvents on lipase activity was determined following pre-incubation of enzyme for 30 min at 25 °C under 150 rpm shaking in the presence of methanol, acetone, hexane, heptane, toluene, ethanol, chloroform, isopropanol, diethyl alcohol, butanol and isoamyl alcohol at concentrations of 10% v/v and 20% v/v. The incubation was conducted in closed screw cap tubes with silicone rubber gasket in order to prevent evaporation of the enzyme reaction.

The influence of imidazolium-based ionic liquids (ILs) on lipase activity was investigated after a 30 min-preincubation of the purified enzyme with different concentrations of ILs (ranging from 2 to 10% v/v) at 50 °C and pH 8.0. The remaining activity was analyzed by spectrophotometric method under the assay condition. The lipolytic activity was determined by triplicate experiments.

2.6.5. Influence of various effectors on enzyme activity

The effect of a variety of chemical reagents (1 and 5 mM) on the enzyme activity was investigated by pre-incubating the lipase for 30 min at 70 °C in 50 mM Tris-HCl buffer (pH 8.0) containing following chemical agents; oxidizing agents: ammonium persulfate, potassium iodide and H_2O_2, reducing agents: ascorbic acid and β-mercaptoethanol, chelating agents: sodium citrate and EDTA (ethylenediaminetetraacetic acid), detergents: SDS, CTAB (cetyltrimethylammonium bromide) and Triton X-100, additives: PEG 4000 (polyethylene glycol) and glycerol, inhibitors: PMSF (phenyl methyl sulfonyl fluoride), DTT, DMF, urea, DTNB, sodium fluoride, mercuric chloride and phenanthroline. The activity of the enzyme without additives was assumed as 100%.

2.6.6. Effect of commercial detergents on enzyme stability

The stability of the recombinant lipase in commercial enzyme-containing powder/liquid detergents was investigated. The solid detergents utilized in this study were as follows: Barf (Paxan, Iran), Vash (Henkel, Germany), Softlan (Pakshoo, Iran), Persil (Henkel, Germany) washing powder and handwash powder, Pril (Henkel, Germany), Shoma (TolyPers, Iran), Finish (Reckitt Benckiser, Canada) and Darya (TolyPers, Iran). The liquid detergents tested were Goli (Paxan, Iran), Persil (Henkel, Germany), Ave (Pakshoo, Iran) and Ganj (RaminGostar, Iran). Dissolving in tap water, the solid detergents were prepared at a final concentration of 5 mg/L, while a 100-fold dilution of liquid detergents was performed to simulate washing conditions. To inactivate the endogenous enzymes in aforementioned detergents, diluted detergent preparations were pre-incubated for 30 min at 80 °C before the addition of recombinant lipase. The enzyme was added to powder/liquid detergent solution following incubation at 70 °C for 60 min, followed by activity measurement. To allow further comparison, the effect of commercial detergents on the stability of a commercial lipase (porcine pancreatic lipase, PPL) was also studied under the same experimental conditions.

2.6.7. Determination of substrate specificity

Lipase substrate specificity was analyzed using the spectrophotometric assay, using 0.01 M p-nitrophenyl acetate (C2), butyrate (C4) and palmitate (C16) dissolved in ethanol as substrates.

3. Results and discussion

3.1. Cloning and sequence analysis of the lipase gene

The sequence of the lipase gene expressed in-frame with an N-terminal region coding for six His residues has been deposited in