Arch Microbiol (2009) 191:233–240
DOI 10.1007/s00203-008-0448-5

ORIGINAL PAPER

Characterization of two soil metagenome-derived lipases with high specificity for *p*-nitrophenyl palmitate

Ping Wei · Liping Bai · Wengang Song · Gangping Hao

Received: 25 May 2008 / Revised: 21 October 2008 / Accepted: 3 November 2008 / Published online: 29 November 2008
© Springer-Verlag 2008

Abstract Two novel genes (*pwtsB* and *pwtsC*) encoding lipases were isolated by screening the soil metagenomic library. Sequence analysis revealed that *pwtsB* encodes a protein of 301 amino acids with a predicted molecular weight of 33 kDa, and *pwtsC* encodes a protein of 323 amino acids with a predicted molecular weight of 35 kDa. Furthermore, both genes were cloned and expressed in *Escherichia coli* BL21 (DE3) using pET expression system. The expressed recombinant enzymes were purified by Ni-nitrilotriacetic acid affinity chromatography and characterized by spectrophotometric with different *p*-nitrophenyl esters. The results showed that PWTSB displayed a high degree of activity and stability at 20°C with an optimal pH of around 8.0, and PWTSC at 40°C with an optimal pH of around 7.0. *P*-nitrophenyl palmitate (*p*-NPP) was identified as the best substrate of PWTSB and PWTSC. The specific activities of PWTSB and PWTSC were 150 and 166 U/mg, respectively toward *p*-NPP at 30°C, about 20-fold higher than that toward *p*-nitrophenyl butyrate (C4) and caprylate (C8). In conclusion, our results suggest that PWTSB is a cold adapt lipase and PWTSC is a thermostable lipase to long-chain *p*-nitrophenyl esters.

P. Wei and L. Bai contributed equally to this work.

Communicated by Erko Stackebrandt.

P. Wei (✉) · W. Song · G. Hao
Department of Basic Medicine,
Taishan Medical University, Taian, Shandong, China
e-mail: wp360@yahoo.cn

L. Bai (✉)
Institute of Medicinal Biotechnology,
Chinese Academy of Medical Sciences and Peking
Union Medical College, Beijing, China
e-mail: lipingbai1973@yahoo.com.cn

Keywords Lipase · Metagenomic library · PWTSB · PWTSC

Introduction

Lipases (EC 3.1.1.3) are ubiquitous enzymes found in animals, plants, and microorganisms, including fungi and bacteria (Lee et al. 2006). A 'true' lipase is defined as a carboxylesterase, which catalyses the hydrolysis and synthesis of long-chain acylglycerols with trioleoylglycerol being the standard substrate (Jaeger and Eggert 2002). Recently, lipases have emerged as key enzymes in swiftly growing biotechnology, owing to their novel and multifold applications in food, oleochemistry, organic synthesis, detergent formulation and pharmaceutical industries (Rhee et al. 2005). Most of lipases are produced by microbes, and specifically bacterial lipases from *Bacillus*, *Psedomonas* and *Burkholderla* play vital roles in commercial ventures (Gupta et al. 2004). However, the properties of these enzymes do not always meet the requirements for a given application and the culturable microorganisms represent only a tiny fraction of the microbial diversity, which limits the spectrum of searching for new lipases for the bioprocess industry (Ferrer et al. 2005).

With metagenomic approach, some novel lipases and esterases have been isolated from soil (Lee et al. 2004; Elend et al. 2007), water (Kim et al. 2007; Hårdeman and Sjöling 2007) and thermal environments (Rhee et al. 2005). In Present study, we constructed a library of environmental DNA from soil sample collected from Taishan (China). Based on functional screening, two new genes encoding lipases were identified, overexpressed in *E. coli* and their characterization were studied.

Materials and methods

Sample sites, bacterial strains, and plasmids

Soil samples from different altitudes of Taishan (China) were collected to construct the environmental DNA libraries. *Escherichia coli* DH5α was used as host of the cloning experiments, and pUC18 was employed as vector. *E. coli* BL21(DE3) (Stratagene) and plasmid pET-30a (Novagen) were used for gene expression.

Construction of environmental DNA libraries

DNA extractions were performed using the method reported by Zhou et al. (1996) with minor modifications; furthermore, DNA was purified through direct extraction from the agarose gels. The purified DNA was partially digested with *Sau*3AI and size fractionated by sucrose density centrifugation [10 to 40% (wt/vol)]. Fractions containing DNA fragments over 2 kb were ligated into *Bam*HI-digested pUC18, and the products were transformed into *E. coli* DH5α. White colonies were collected into 96-well microtitre plates and cultured in Luria–Bertani (LB) media (Sambrook and Russell 2001) supplemented with 100 μg/ml ampicillin (Ap) at 37°C for 24 h before replication and long-term storage at −80°C.

Screening of lipase/esterase gene

To screen lipolytic activity, the soil libraries in 96-well plates were replicated in two Luria-Bertani (LB) agar plates containing 100 μg/ml ampicillin and 1% tributyrin. After incubation at 37°C until colonies were observed, the plates were further incubated at 50°C and 28°C, respectively. Active clones were identified by showing halo around individual colonies, and their plasmids were isolated and analyzed. The nucleotide sequence of soil DNA fragment cloned in pUC18 was checked using an ABI PRISM 377XL DNA Sequencer (Applied Biosystems). Lipase and esterase sequences for comparative study were retrieved from protein and nucleotide databases on the NCBI Entrez server at http://www.ncbi.nlm.nih.gov/Entrez/. Sequence similarity searches were conducted with the BLAST 2.0 program.

Enzyme overexpression and purification

The putative lipase gene *pwtsB* was amplified from the pTS1 plasmid using the primers: 5′-TTTACATATG ATGCTCTTAAAACGCCTG-3′ (*Nde*I adaptor restriction enzyme site underlined) and 5′-CAGACTCGAGCTATT CATCCAGCGCTTC-3′ (*Xho*I). The putative lipase gene *pwtsC* was amplified from the pTS2 plasmid using the primers: 5′-TTTACATATGGTGCCCCAGCACCTCCTC-3′ (*Nde*I) and 5′-CAGACTCGAGTTAATTCGTATTCTC CCCGG-3′ (*Xho*I). The PCR product was digested with *Nde*I and *Xho*I, ligated into the *Nde*I- and *Xho*I-linearized expression vector pET-30a (+) (Novagen), and then introduced into *E. coli* BL21(DE3) (Novagen). The nucleotide sequence of amplified DNA fragment cloned in pET30a was checked using an ABI PRISM 377XL DNA Sequencer (Applied Biosystems).

An overnight culture of transformants was diluted to 1:20 with LB broth and subjected to further incubation at 30°C until the absorbance at 600 nm reached ~0.6. Isopropyl-β-D-thiogalactoside (IPTG) was added to the culture at a final concentration of 0.1 mM. After incubation at 30°C for 8 h, the bacterial cells were harvested by centrifugation (3,000 rpm, 15 min) and suspended in the binding buffer (10 mM imidazole, 0.5 M NaCl, 20 mM Tris–Cl pH 8.0). The cells were sonicated and the supernatant was collected by centrifugation (12,000×*g*, 15 min) at 4°C. The sample was loaded onto a 5.0 ml Ni-NTA His-Bind resin column (Novagen) pre-equilibrated with binding buffer. The column was then eluted with 5 ml binding buffer (10 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl pH 8.0), 10 ml washing buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl pH 8.0) and 5 ml eluting buffer (250 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl pH 8.0) successively. The fractions containing the recombinant protein were collected and dialyzed with H_2O at 4°C. The purity of the protein was ascertained by SDS-polyacrylamide gel electrophoresis (PAGE).

Analysis of lipase characterization

The lipase activity of the purified proteins was quantitatively measured using a spectrophotometric method with *p*-nitrophenyl butyrate (C4), caprylate (C8) and palmitate (C16) (*p*-NPP) as substrates. The reaction mixture consisted of 0.1 M NaCl, 0.1 M NaH_2PO_4, 15% acetonitrile and 0.038 mM Triton X-100, pH 7.25 (Choo et al. 1998). The release of *p*-nitrophenol from *p*-nitrophenyl esters was continuously monitored spectrophotometrically at 400 nm at 30°C. One unit of enzyme activity was defined as the amount of activity required to release 1 μmol of *p*-nitrophenol/min from *p*-nitrophenyl ester.

Kinetic experiments were carried out in a total volume of 500 ml containing assay buffer and different p-nitrophenyl esters at 0.1–10 mM. Each reaction was incubated at 30°C for 30 min before termination by the addition of EDTA. The kinetic parameters K_m were determined by a linear least-squares fitting of a Lineweaver–Burke plot of the Michaelis–Menten equation. To identify the isoelectric point (PI) of PWTSB and PWTSC, isoelectric focusing was performed in a Pharmacia Phast gel system using calibrated pH gels according to the manufacturer's instructions.

To test the temperature ranges of the enzymes, the enzyme activities were measured at temperatures between 10 and 90°C under standard assay conditions. Blank reactions were performed with every measurement under different conditions to subtract the appropriate values for nonenzymatic hydrolysis of substrates from the results. Thermostabilities were analyzed by measuring the residual activity after incubating the enzymes at different temperatures for 30 min in Eppendorf tubes with mineral oil on top to prevent evaporation. Lipase activities were measured for a pH range from 4.0 to 11 using 50 mM of the appropriate buffers under standard assay conditions. Acetate buffer was used for pH 4.0–6.0, citrate/phosphate buffer (Macllvaine buffer) was used for pH 6.0–7.5, Tris–HCl was used for pH 7.5–9.0, and *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) was used for pH 9.0–11 (Voget et al. 2006). To test the enzyme stability for various metal ions, 5 mM $CaCl_2$, $CuCl_2$, $MgCl_2$, $FeSO_4$, $ZnCl_2$, $NiCl_2$, $MnCl_2$, $AgNO_3$, or $CoCl_2$ were added into the assay solution, assayed at pH 7.25 and 30°C (Lee et al. 2006).

Nucleotide sequence accession number

The sequences of the 906 bp *pwtsB* gene and 972 bp *pwtsC* gene fragments were deposited in the GenBank database with accession numbers EU305646 and EU305647, respectively.

Results

Construction and screening of the environmental libraries

Soil samples from Taishan (China) at three different altitudes (400, 800, 1,200 m) were used for the construction of environmental DNA libraries. Approximately 15 µg DNA was obtained from one gram soil and 800 clones were obtained from 1 µg of purified ground soil DNA. To determine the quality of three different environmental libraries, 50 clones per environmental library were randomly selected and the plasmids were digested. The results showed that the average insert size was 3 to 7 kb, and the percentage of plasmids containing inserts was 82% approximately.

Colonies of the library were replicated into Luria-Bertani (LB) agar plates containing tributyrin for screening lipase activity. As indicated by a transparent halo surrounding the colony, in the screening temperature of 28°C, a cold active lipase-positive clone pTS1 was selected from libraries of soil samples from Taishan in 1,200 m altitude (Fig. 1). In the screening temperature of 50°C, thermostable lipase-positive clone pTS2 was selected from libraries of soil samples from Taishan in 400 m altitude (Fig. 1). No lipase-positive clone was discovered from libraries of soil samples from Taishan at 800 m altitude.

Sequence analysis of the lipolytic active clones

The complete DNA sequences of the PTS1 and PTS2 were determined. The length of the insert DNA in PTS1 was 3,219 bp, and the length of the insert DNA in PTS2 was 3,918 bp. Based on a BLAST search, two complete lipase genes *pwtsB* (906 bp) and *pwtsC* (972 bp) were identified in PTS1 and PTS2, respectively. The deduced PWTSB encoded by *pwtsB* comprises 301 amino acid residues with a calculated molecular mass of 33 kDa. The PWTSB showed 64% identity to Triacylglycerol lipase (Q02104) from *Psychrobacter immobilis* (Arpigny et al. 1993), 60% identity to triacylglycerol lipase (CAA37863) from *Moraxella* sp. (Feller et al. 1991) and 57% identity to triacylglycerol lipase (CAJ90853) at uncultured soil bacterium (Zhang and Zeng. 2006). In addition, the signature sequences Gly–

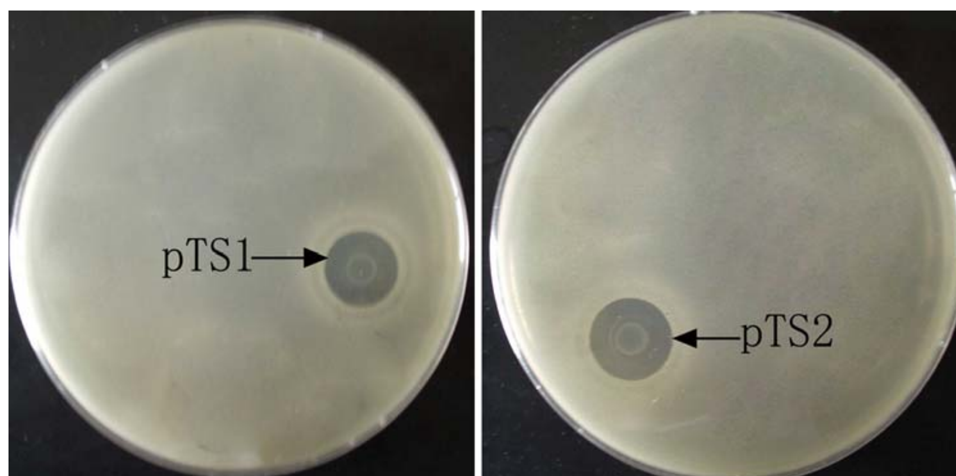

Fig. 1 Halo-formation around the colonies of pTS1 and pTS2

X–Ser–X–Gly conserved in lipase/esterase could also be found in the ORF (140–144, Fig. 2a).

The deduced PWTSC encoded by *pwtsC* comprises 323 amino acid residues with a calculated molecular mass of 35 kDa. Through amino acid sequence alignment, the PWTSC showed 81% identity (*N*-termination 121 amino acids) to triacylglycerol acylhydrolase of *Streptomyces* sp. (AAB51445; Pérez et al. 1993), 37% identity to lipase from *Streptomyces coelicolor* A3(2) (AAD09315; Valdez et al. 1999), and 37% identity to Triacylglycerol lipase from *Kineococcus radiotolerans* SRS30216 (YP_001363557). No typical motif of Gly–X–Ser–X–Gly was found in this lipase (Fig. 2b).

Expression and identification of the recombinant lipase PWTSB and PWTSC

To investigate the biochemical properties of the lipases encoded by *pwtsB* and *pwtsC*, these two genes were placed under the control of the T7 promoter by cloning in the expression vectors pET30a, respectively. The proteins of PWTSB and PWTSC were purified to homogeneity by the His-Bind resin affinity chromatography with a six-histidine tag at their C terminus. The sizes of PWTSB and PWTSC, as determined by SDS-PAGE (12%), were about 33 kDa (Fig. 3a) and 35 kDa (Fig. 3b), respectively, which are close to mass predicted from their deduced proteins.

Fig. 2 **a** Protein sequence alignment of lipase PWTSB and three similar enzymes. CAA37863: triacylglycerol lipase from *Moraxella* sp. (Feller et al. 1991); CAJ90853: triacylglycerol lipase at uncultured soil bacterium; Q02104: triacylglycerol lipase from *Psychrobacter immobilis* (Arpigny et al. 1993). **b** Protein sequence alignment of lipase PWTSC and three similar enzymes AAD09315: lipase from *Streptomyces coelicolor* A3(2) (Valdez et al. 1999); YP_001363557: triacylglycerol lipase from *K. radiotolerans* SRS30216; AAB51445: triacylglycerol acylhydrolase of *Streptomyces* sp. (Pérez et al. 1993)

```
A CAA37863     MLLKRLGLAALFSLSMVGCTTAPNTLAVNTTQKIIQYERSKSDLEVKSLTLASGDKMVYA 60
  CAJ90853     MLLIRIRLAAVYSPSMLGCTTAPNTLAVNTTQKIIQYERSKSDLEVKSLTLASGDKMVYA 60
  Q02104       MLLKRLCFAALFSLSMVGCTNAPNALAVNTTQKIIQYERNKSDLEIKSLTLASGDKMVYA 60
  PWTSB        MLLKRLCYAALISLSILGCSFAGNAIAVNTTHKIKQYDGN---VEIPGALHRNPAVLVSA 57
               *** *:  **: * *::**: * *::*****:** **: .    :*: .     .   :* *

  CAA37863     ENDNVTGEPL-LLIHGFG--GNKDNFTRIADKLEGYHLIIPDLLGFGNSSKPMTADYRAD 117
  CAJ90853     ENDNVTGEPI-LLIHGLG--GNKDNFTRIADKLEGYHLIIPDLLGFGNSSKPMTADYRAD 117
  Q02104       ENGNVAGEPL-LLIHGFG--GNKDNFTRIARQLEGYHLIIPDLLGFGESSKPMSADYRSE 117
  PWTSB        YHQGAHGQRLNAAFHRVGPQLNKDIFTRIADKLEANDLIIRDLLGFGNSSKRMTADYRAD 117
                : .. *: :   :* .*   *** ***** :**. .*** ******:*** *:****::

  CAA37863     AQATRLHELMQAKGLASNTHVGGNSMGGAISVAYAAKYPKEIKSLWLVDTAGFWSAGVPK 177
  CAJ90853     AQATRLHELMQAKGLASNTHVGGNSMGGAISVAYAAKYPKEIKSLWLVDTAGFWSAGVPK 177
  Q02104       AQRTRLHELLQAKGLASNIHVGGNSMGGAISVAYAAKYPKDVKSLWLVDSAGFWSAGIPK 177
  PWTSB        AQATRKHDLMQAKAIASITHVPGNSMGGAISVPYAAKLGKDVLSLCLVDSAGIWSAGIPK 177
               ** ** *:*:***.:**  ** **********.****   *:: ** ***:**:****:**

  CAA37863     SLEGATLENNPLLINSKEDFYKMYDFVMYKPPYIPKSVKAVFAQERINNKALDTKILEQI 237
  CAJ90853     SLEGATLENNPLLINSKEDFYKMYDFVMYKPPYIPKSVKAVFAQERINNKALDTKILEQI 237
  Q02104       SLEGATLENNPLLIKSNEDFYKMYDFVMYKPPYLPKSVKAVFAQERIKNKELDAKILEQI 237
  PWTSB        SLEGATLDYNGLQINSNEHFYIKVDAGMPVPLAEPKMIQANHAIEGVKILCQDREILEIG 237
               *******: * * *:*:*.**   *  *  *   ** ::* .* * ::    * :***

  CAA37863     VTDNVEERAKIIAKYNIPTLVVWGDKDQVIKPETTELIKEIIPQAQVIMMNDVGHVPMVE 297
  CAJ90853     VTDNVGERAKIIAKFNIPTLVVWGDKDQVIKPETTELITEIIPQALVMMMNDVAHVPMVE 297
  Q02104       VTDNVEERAKIIAQYKIPTLVVWGDKDQIIKPETVNLIKKIIPQAQVIMMEDVGHVPMVE 297
  PWTSB        VTPNVEEDAKTIAQLKIPTGVVWGDKDQIIKPETVNLIKKIIPQAQVIMMEDVGHVPMVE 297
               ** ** * ** **:  :*** ********:*****.:**.:***** *:**:**.******

  CAA37863     AVKDTANDYKAFRDGLKK-- 315
  CAJ90853     AVQDTASDYQAYGDAPKE-- 315
  Q02104       ALDETADNYKAFRSILEAQR 317
  PWTSB        ALDE---------------- 301
               *:.:
```

Fig. 2 continued

```
B
AAD09315        MQQNPHTHAAPG--AARPVLRGVRRRLAAVTAAVARVLVLGTLTGP-GAQAADNPYERGP 57
YP_001363557    ---MPNTLVRP---SRRRSARGFRASLAAS--AVALPLLVGVAP----AEAATNPYERGP 48
AAB51445        ---MPQHLLPARRQAARPSRPRTLTGLLAAAAATAGLLLSGLAPGAQAAAAAANPYERGP 57
PWTSC           ---VPQHLLPARRQRARPSRRRTLTGLVAAAARTAGLVLSALAPAAQPAAAAANGYERGP 57
                    *:     .      *          * *    .*  :: .  .     * ** * *****

AAD09315        APTESSIEALRGPYSVADTSVSSLAVTGFGGGTIYYPTSTSDGTFGAVVIAPGFTAYQSS 117
YP_001363557    APTNTSVEATRGSFAVSTTTVSNFAATGFGGGTIYYPTSTTSGTFGAVVIAPGYTASQSS 108
AAB51445        APTNASIEASRGPYATSQTSVSSLVASGFGGGTIYYPTSTADGTFGAVVISPGFTAYQSS 117
PWTSC           PATNRSIEPSRPAIATSQTSLSSLLASAFGGRRIYYPTSTADGTFGAVVISPGFTAYQSS 117
                ..*: *:*. * .  :.: *::*.: .:.***  *******:.********:**:** ***

AAD09315        IAWLGPRLASQGFVVFTIDTNTTLDQPDSRGRQLLAALDYLTGRSSVRGRIDSGRLGVMG 177
YP_001363557    MAWYGPRLASQGFVVFTIDTEGRYDQPASRGDQLQAALTYLTQRSTVRTRVDASRLAVMG 168
AAB51445        IAWLGPRLASQGFVVFTIDTNTTLDQPDSRGRQLLSALDYLTQRSSVRTRVDATRLGVMG 177
PWTSC           IAWLHG-----GYKVANVVTQGGANRLTTGKALPGTDPNQKILYTSIYSSRD--MYVMIY 170
                :**        *: * .: *:    ::   :        :         :::    *     ::

AAD09315        HSMGGGGTLEAAKSRPSLQAAIPLTPWNLDKSWPE---VSTPTLVVGADGDTIAPVASHA 234
YP_001363557    HSMGGGGTLEAVKDNPAIKAAIPLTPWNLDKTWPE---ISTPTLIVGAENDSTAPVASHS 225
AAB51445        HSMGGGGSLEAAKSRTSLKAAIPLTGWNTDKTWPE---LRTPTLVVGADGDTVAPVATHS 234
PWTSC           LSCIDRARYVHNHGVPHIALLISSEVYSLNPSIAQQILLSSTASLVYMAQDTCCSIALLI 230
                 *  .  .      :. .  :    *.    :.  : : .:    :  :.: :*     *: ..:*

AAD09315        EPFYSGLPSS----TDRAYLELNNATHFSPNTSNTTIAKYSISWLKRFIDDDTRYEQFLC 290
YP_001363557    EPFYGSIPTA----TDKAYLELRGASHFAPNSANTTIAKYSISWLKRYVDDDTRYTQFLC 281
AAB51445        KPFYESLPGS----LDKAYLELRGASHFTPNTSDTTIAKYSISWLKRFIDSDTRYEQFLC 290
PWTSC           SVYYRQAKCKGIYKKDCTAEDKYRIIKRKKITTDSTIXMNSISCLKRYNDSDRRIEQFLC 290
                . :*           * :   :       :     ::::**    *** ***: *.* *  ****

AAD09315        PLPRPSLTIEEYRG-NCPHGS------------ 310
YP_001363557    PAPGTSLAISEYRSTNC---------------- 298
AAB51445        PIPRPSLTIAEYRG-TCPHTS------------ 310
PWTSC           PIPPRSLTIAEYAGTWALTTKIEGENGAGENTN 323
                * *  **:* ** .   .
```

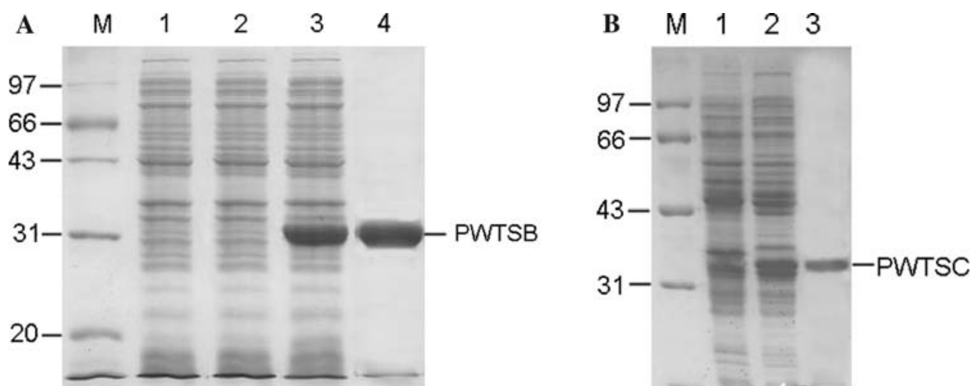

Fig. 3 **a** SDS-PAGE of recombinant PWTSB. *M* protein molecular weight markers, *1* total lysate of *E. coli* BL21(DE3) containing empty vector, *2* uninduced cell lysate, *3* induced cell lysate, *4* purified PWTSB protein. **b** SDS-PAGE of recombinant PWTSC. *M* protein molecular weight markers, *1* uninduced cell lysate, *2* induced cell lysate, *3* purified PWTSC protein

Subsequently, the activities of purified PWTSB and PWTSC were examined by using *p*-nitrophenyl esters with acyl chains of different lengths. Our results showed that the purified enzymes hydrolyzed various *p*-nitrophenyl esters, with the highest activity toward *p*-nitrophenyl palmitate (*p*-NPP). The purified PWTSB and PWTSC had specific activity of 150 and 166 U/mg toward *p*-NPP at 30°C, respectively. The catalytic efficiency toward *p*-NPP was approximately 20-fold higher than that toward *p*-nitrophenyl butyrate (C4) and caprylate (C8). Lipase activities and K_m of these proteins toward different *p*-nitrophenyl esters were displayed in Table 1. These values were verified by substitution into the Michaelis–Menten equation and fitting of the equation to a plot of *v* versus *s*.

Using DNASTAR software, the predicted PI of PWTSB was 6.87 and that of PWTSC was 9.75. We used calibrated pH 5.5–8.0 gels to determine the PI of PWTSB and calibrated pH 8.5–10.0 gels to determine the PI of PWTSC. As expected, lipases PWTSB and PWTSC have PI of 6.9 and 9.4, respectively.

Effects of temperature, pH and metal ions on enzyme activity and stability

The optimal temperatures of the recombinant PWTSB and PWTSC were 20°C (Fig. 4a) and 40°C (Fig. 4b), respectively. Notably, the PWTSB exhibited 40% of the maximal activity at 30°C and rapidly lost at 40°C then. The relative activity of PWTSC, respectively decreased to 70 and 50% at 50 and 60°C, and 10% of the maximal activity was detected at 20°C. The PWTSB showed the highest activity toward *p*-NPP at pH 7.0–9.0, and its optimal pH for the activity of lipase was 8.0 (Fig. 4c). The PWTSC showed the highest activity toward *p*-NPP at pH 6.0–8.0, with its optimal pH 7.0 (Fig. 4d).

Thermostability analysis showed that PWTSB was stable at 0–20°C, however, it lost approximately 40% of its activity at 30°C. PWTSB was unstable at 40°C and rapidly inactivated at 50°C with $t_{1/2}$ of less than 15 min (Fig. 5a). PWTSC was stable at 0–50°C, and retained more than 80% of the activity even after 1 h treatment at 60°C. However, its stability decreased at 80°C, with half-lives of approximately

Table 1 Activity and K_m for purified PWTSB and PWTSC using *p*-nitrophenyl esters

p-Nitrophenyl esters	Butyrate (C4)		Caprylate (C8)		Palmitate (C16)	
	Activity (U/mg)	K_m (mM)	Activity (U/mg)	K_m (mM)	Activity (U/mg)	K_m (mM)
PWTSB	5.2	1.3	6.6	0.8	150	0.15
PWTSC	6.9	1.6	8.1	0.4	166	0.09

One unit of enzyme activity was defined as the amount of activity required to release 1 μmol of *p*-nitrophenol/min from *p*-nitrophenyl ester

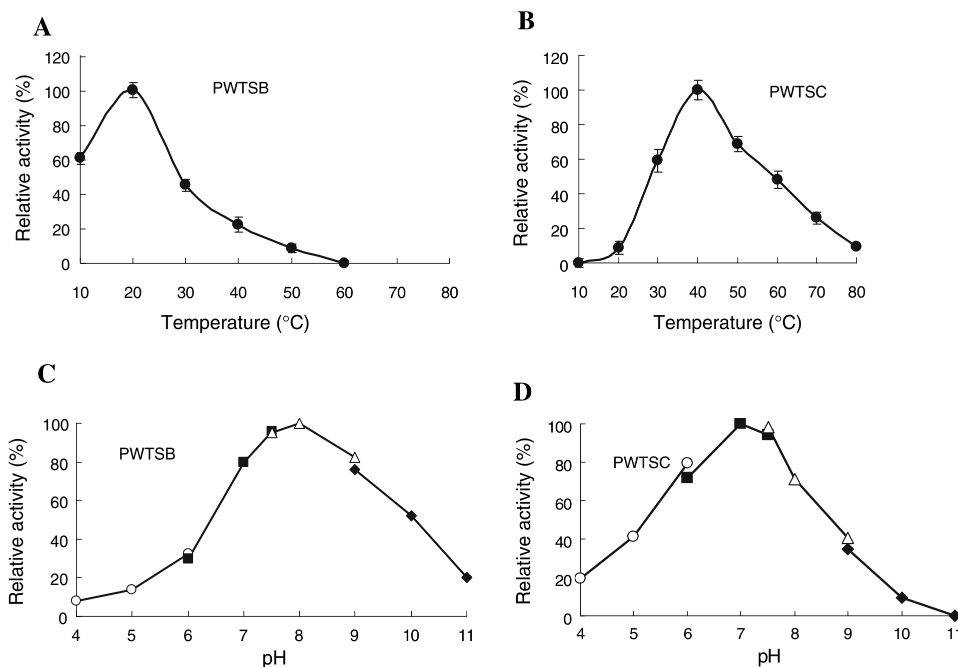

Fig. 4 Effects of temperature and pH on the activity of PWTSB and PWTSC. **a**, **b** Temperature-dependent activity of PWTSB and PWTSC toward *p*-NPP. Enzyme activity was measured at each temperature under standard assay conditions. **c**, **d** pH-dependent activity of PWTSB and PWTSC. Enzyme activity was measured at 30°C, Tests were done using four different buffer systems. *Open circle* Acetate buffer, pH 4.0–6.0; *filled square* citrate/phosphate buffer, pH 6.0–7.5; *open triangle* Tris–HCl, pH 7.5–9.0; *filled diamond*, *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS), pH 9.0–11

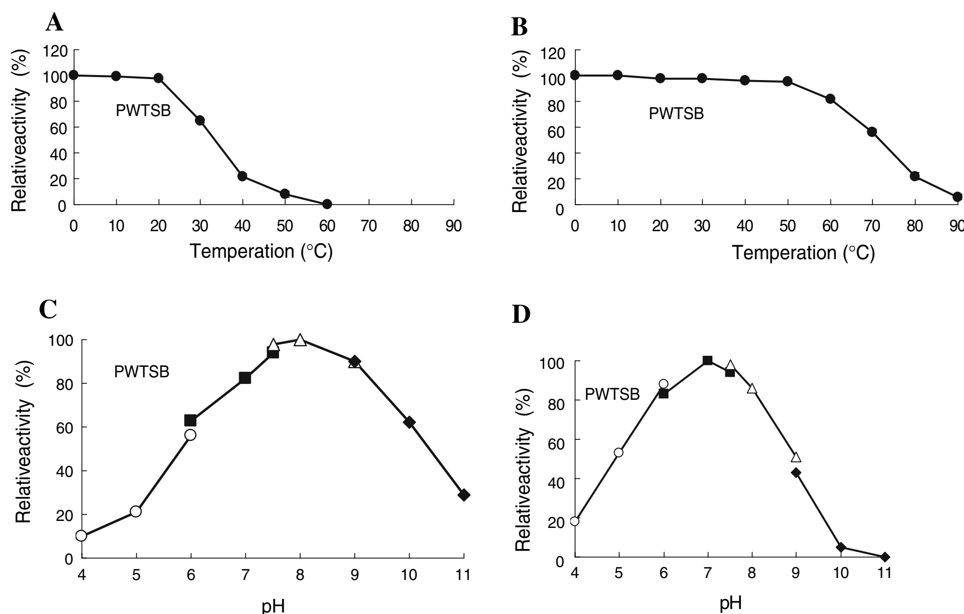

Fig. 5 Effects of temperature and pH on the stability of PWTSB and PWTSC. **a**, **b** Thermal stability of the recombinant PWTSB and PWTSC. Activity was measured under optimal condition. **c d** pH stability of PWTSB and PWTSC. Enzyme activity was measured at different pH values by a standard assay. *Open circle* acetate buffer, pH 4.0–6.0; *filled square* citrate/phosphate buffer, pH 6.0–7.5; *open triangle* Tris–HCl, pH 7.5–9.0; *filled diamond N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS), pH 9.0–11

Table 2 Effect of metal ions on enzyme stability. The lipase activity in the presence of a metal ion was compared with the control including no metal ion whose activity was taken as 100%

	PWTSB (Relative activity %)	PWTSC (Relative activity %)
None	100	100
$CaCl_2$	145	98
$CuCl_2$	110	112
$MgCl_2$	136	99
$FeSO_4$	88	125
$ZnCl_2$	92	56
$NiCl_2$	97	100
$MnCl_2$	96	96
$AgNO_3$	25	88
$CoCl_2$	66	82

15 min (Fig. 5b). PWTSB showed remarkable stability across a wide range of pH 6.0–10.0 (retaining >50% of the initial activity after a 24 h incubation at 20°C) (Fig. 5c). The PWTSC displayed remarkable stability under broad pH ranging from 5.0 to 9.0 (Fig. 5d).

The effect of divalent metal ions on the activities of PWTSB and PWTSC was illuminated in Table 2. Our data revealed that 5 mM $CaCl_2$ and 5 mM $MgCl_2$ increased the PWTSB activity markedly to 145 and 136%, respectively. Moreover, compared with the control, the activity of PWTSB was decreased to 25% in the presence of 5 mM $AgNO_3$. No apparent effects of other metal ions on PWTSB were observed. Similarly, PWTSC is not sensitive to most metal ions except Zn ion.

Discussion

Metagenomics has been used effectively to isolate novel biocatalysts from the environment as well as to acquire ecological data. Its scale and scope have been expanded since its concept was first introduced (Handelsman et al. 1998). In present study, metagenomic libraries were constructed by using the vector pUC18, and two lipase genes (*pwtsB* and *pwtsC*) were identified from soil environmental samples of Tanshan by functional screening. Analysis of homology revealed that *pwtsB* and *pwtsC* are totally novel genes. Like most genes screened from metagenome libraries, no information about a source microorganism could be obtained. *pwtsB* and *pwtsC* were expressed in *E. coli* to a high level in a soluble form.

Analysis of amino acid sequence showed similar size and approximately 64% identity between PWTSB and Triacylglycerol lipase from the *P. immobilis*. In addition, the sequence around Ser142 was Gly140–Asn141–Ser142–Met143–Gly144, which matches the characteristic Gly–X–Ser–X–Gly motif found in lipolytic enzymes (Fig. 2). The purified PWTSB showed maximal activity toward water insoluble long-chain triglycerides, optimal activity at 20°C and instability at 40°C. Under optimal assay conditions (20°C, pH 8.0), the catalytic activity of PWTSB can reach to 245 U/mg toward *p*-NPP. Above results indicated that PWTSB probably belongs to the family V (Arpigny and Jaeger 1999). The activity of PWTSB depends on the presence of calcium and magnesium, which is well known for lipases (Jaeger et al. 1999). It was a low-temperature-active lipase with low thermostability and probably derived from a low-temperature-active organism.

According to our knowledge, there is only one report describing a cold-active lipase of soil metagenomic origin to date (Elend et al. 2007). The potential applications for PWTSB with a relatively high activity at low temperatures could be detergent additives, or in the processing of volatile substances. Together, PWTSB makes it possible to reduce temperature and further bring energy costs down.

PWTSC was a thermostable lipase despite the low homology with some known lipases. It showed the highest activity toward *p*-NPP and the lower sensitivity to metal ions. Data in this study indicated that metagenome-derived lipase (PWTSC) might have the potential to be used as a biocatalyst.

Acknowledgments This work was supported by grant from Department of Health of Shandong Province (2007HZ036).

References

Arpigny JL, Jaeger KE (1999) Bacterial lipolytic enzymes: classification and properties. Biochem J 343:177–183

Arpigny JL, Feller G, Gerday C (1993) Cloning, sequence and structural features of a lipase from the antarctic facultative psychrophile *Psychrobacter immobilis* B10. Biochim Biophys Acta 1171:331–333

Choo DW, Kurihara T, Suzuki T, Soda K, Esaki N (1998) A cold adapted lipase of an Alaskan psychrotroph, *Pseudomonas* sp. strain B11–1: gene cloning and enzyme purification and characterization. Appl Environ Microbiol 64:486–491

Elend C, Schmeisser C, Hoebenreich H, Steele HL, Streit WR (2007) Isolation and characterization of a metagenome-derived and cold-active lipase with high stereospecificity for (R)-ibuprofen esters. J Biotechnol 130:370–377

Feller G, Thiry M, Gerday C (1991) Nucleotide sequence of the lipase gene lip3 from the antarctic *psychotroph Moraxella* TA144. Biochim Biophys Acta 1088:323–324

Ferrer M, Martínez-Abarca F, Golyshin PN (2005) Mining genomes and ‘metagenomes’ for novel catalysts. Curr Opin Biotechnol 16:588–593

Gupta R, Gupta N, Rathi P (2004) Bacterial lipases: an overview of production, purification and biochemical properties. Appl Microbiol Biotechnol 64:763–781

Handelsman J, Rondon MR, Brady SF, Clardy J, Goodman RM (1998) Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chem Biol 5:R245–R249

Hårdeman F, Sjöling S (2007) Metagenomic approach for the isolation of a novel low-temperature-active lipase from uncultured bacteria of marine sediment. FEMS Microbiol Ecol 59:524–534

Jaeger KE, Eggert T (2002) Lipases for biotechnology. Curr Opin Biotechnol 13:390–397

Jaeger KE, Dijkstra BW, Reetz MT (1999) Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. Annu Rev Microbiol 53:315–351

Kim S, Joo S, Yoon HC, Ryu Y, Kim KK, Kim TD (2007) Purification, crystallization and preliminary crystallographic analysis of Est25: a ketoprofen-specific hormone-sensitive lipase. Acta Crystallogr Sect F Struct Biol Cryst Commun 63:579–581

Lee SW, Won K, Lim HK, Kim JC, Choi GJ, Cho KY (2004) Screening for novel lipolytic enzymes from uncultured soil microorganisms. Appl Microbiol Biotechnol 65:720–726

Lee MH, Lee CH, Oh TK, Song JK, Yoon JH (2006) Isolation and characterization of a novel lipase from a metagenomic library of tidal flat sediments: evidence for a new family of bacterial lipases. Appl Environ Microbiol 72:7406–7409

Pérez C, Juárez K, García-Castells E, Soberón G, Servín-González L (1993) Cloning, characterization, and expression in *Streptomyces lividans* 66 of an extracellular lipase-encoding gene from *Streptomyces* sp. M11. Gene 123:109–114

Rhee JK, Ahn DG, Kim YG, Oh JW (2005) New thermophilic and thermostable esterase with sequence similarity to the hormone-sensitive lipase family, cloned from a metagenomic library. Appl Environ Microbiol 71:817–825

Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Valdez F, González-Cerón G, Kieser HM, Servín-González L (1999) The *Streptomyces coelicolor* A3(2) lipAR operon encodes an extracellular lipase and a new type of transcriptional regulator. Microbiology 145:2365–2374

Voget S, Steele HL, Streit WR (2006) Characterization of a metagenome-derived halotolerant cellulase. J Biotechnol 126:26–36

Zhang JW, Zeng RY (2006) Cloning, Expression and characterization of the cold lipase(lip3) from metagenomic DNA of an antarctic deep sea sediment. Prog Biochem Biophys 33:1207–1214

Zhou J, Bruns MA, Tiedje JM (1996) DNA recovery from soils of diverse composition. Appl Environ Microbiol 62:316–322