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ORIGINAL ARTICLE

Cloning and expression of a lipase gene from *Bacillus subtilis* FS1403 in *Escherichia coli*

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Abstract A moderate heat-resistant lipase gene from *Bacillus subtilis* FS1403 was cloned. Sequence analysis revealed that the lipase gene encodes a 212-amino-acid protein containing the conserved motif Ala-X-Ser-X-Gly that is present in other *Bacillus* lipases. Phylogenetic analysis suggested that the *B. subtilis* FS1403 lipase gene belongs to family I.4 of bacterial lipase genes. The lipase gene was inserted in pET-28a(+) and expressed in *Escherichia coli* BL21. SDS-PAGE analysis revealed that the recombinant lipase had a molecular weight of approximately 23 kDa, which corresponded to the predicted size (22.8 kDa) of the deduced protein. The lipase activity was the highest at 50°C and at pH 8.0. The enzyme retained over 70% of the maximal activity at temperatures of 40–60°C and pH 6.0–11.0.

Keywords *Bacillus subtilis* · Lipase · Gene cloning · Expression

Introduction

Lipases (EC 3.1.1.3) are a class of enzymes that hydrolyze ester bonds in triacylglycerides to form fatty acids and glycerol, or catalyze the reaction reversibly under certain conditions (Jaeger and Eggert 2002; Parawira 2009). These properties make lipases widely useful for the production of free fatty acids, interesterification of fats, synthesis of esters, etc. (Hasan et al. 2006).

The lipases from *Bacillus* species represent a particular group of enzymes with a consensus sequence of Ala-X-Ser-X-Gly surrounding the catalytic serine residue instead of the conserved pentapeptide of Gly-X-Ser-X-Gly present in canonical lipases (Dartois et al. 1992). Several lipase genes from the following *Bacillus* species have been cloned, expressed, and characterized: *B. subtilis* (Dartois et al. 1992; Eggert et al. 2000), *B. pumilus* (Rasool et al. 2005), *B. licheniformis* (Nthangeni et al. 2001), *B. megaterium* (Ruiz et al. 2002), *B. thermocatenulatus* (Schmidt-Dannert et al. 1996), *B. thermoleovorans* (Cho et al. 2000), *B. stearothermophilus* (Kim et al. 1998), and *B. sphaericus* (Rahman et al. 2003). However, lipases with special properties, such as thermostable lipases (Schmidt-Dannert et al. 1996; Cho et al. 2000), still receive much attention owing to their potential industrial applications such as in the food processing industry, etc. (Cho et al. 2000).

Microorganisms isolated from hot environments are an important source for the production of thermostable enzymes (Schmidt-Dannert et al. 1996; Kim et al. 1998; Li and Zhang, 2005). In a previous paper, we reported that *B. subtilis* FS1403, isolated from volcanic vent soil in Philippines and identified according to its 16S rDNA sequence, was found to produce a moderate heat-resistant lipase (Wu et al. 2008). In order to characterize and determine the function of this lipase, the lipase gene from *B. subtilis* FS1403 (*lip*) was cloned and expressed in *Escherichia coli* BL21 (DE3). A preliminary study of the properties of the recombinant lipase was conducted, and some implications for ecological environments are discussed.

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Materials and methods

Strains, plasmids, and culture medium

Bacillus subtilis FS1403 was isolated from a volcanic vent soil in Philippines and grown as described previously (Wu et al. 2008). The *E. coli* strains JM109 and BL21 were used for gene cloning and expression, respectively. All *E. coli* strains were cultivated in Luria-Bertani (LB; 1.0% tryptone, 0.5% yeast extract, and 1.0% NaCl) medium at 37°C. Kanamycin or ampicillin was added when necessary at a final concentration of 50 μg/mL or 100 μg/mL, respectively. The plasmid pMD19-T (Takara; http://www.takara-bio.com) was used for gene cloning, and pET-28a(+) (Novagen; http://www.merck-chemicals.com/life-science-research) was used as the expression vector.

Cloning of lipase gene

Genomic DNA from *B. subtilis* was prepared as described previously (Cheng and Jiang 2006). Lipase coding sequences (GenBank accession nos. M74010, DQ250714, EU482151, and FJ481899) from *B. subtilis* strains were aligned; stretches of conserved sequences at both ends of the coding regions were identified, and a set of primers was designed for the amplification of the lipase coding DNA from *B. subtilis* FS1403. The sequence of the forward primer F1 was 5′-CGGGCATGAAATTTGTAAAAAGAAG-3′ and that of the reverse primer R1 was 5′-CGGCATTAATTCGTATTC TGGC-3′. PCR was performed with an initial denaturation at 95°C for 5 min; this was followed by 35 cycles of 94°C for 45 s, 56.5°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. A 5 μL volume of PCR product was electrophoresed in 1% agarose gel for detection. The PCR product was purified using a DNA purification kit (TIANGEN, Beijing, PRC). The purified fragment was ligated with the pMD19-T vector, and the ligated product was transformed into *E. coli* JM109 competent cells using a standard protocol (Sambrook and Russell 2001). Positive transformants were selected and confirmed by colony PCR. The final validated positive clone of pMD19-T-lip was sent to Shanghai Sangon Co. Ltd (http://www.sangon.com) for sequence determination. The lipase gene sequence was submitted to GenBank.

Sequence analysis

Sequence similarity searches were performed in the GenBank database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were multiple aligned using the program ClustalX1.83. A phylogenetic dendrogram based on the aligned sequences was produced by the neighbor-joining method (Kimura 1980).

Construction of recombinant expression plasmid

Both the plasmids pMD19-T-lip and pET-28a(+) were isolated from *E. coli* JM109. The primers for the amplification of the lipase gene from pMD19-T-lip were as follows: F2 (forward primer), 5′-GCGCGGATCCATGAAATTTGTAAA AAGAAGG-3′ and R2, (reverse primer), 5′-ACCAAGCTT ATTCGTATTCTGGCCCCCG-3′. The underlined portions in the primer sequences represent the recognition sites for the restriction enzymes *Bam*HI and *Hin*dIII. These restriction sites are also present in pET-28a(+).

The lipase gene was amplified from the recombinant plasmid pMD19-T-lip. Both the amplified *lip* gene and the plasmid pET-28a(+) were digested with *Bam*HI and *Hin*dIII; the products were purified and ligated using T4 DNA ligase to form the expression vector pET-28a-lip. The recombinant expression vector was transformed into *E. coli* BL21 competent cells. Positive clones were identified by colony PCR and double restriction digestion. Detailed protocols were from Sambrook and Russell (2001).

Expression of lipase gene in *Escherichia coli* BL21

A transformant of *E. coli* BL21 harboring pET-28a-lip was cultured with vigorous shaking at 37°C overnight in 3 mL LB broth containing 50 μg/mL kanamycin. The culture was transferred using 1% inoculum to 100 mL fresh LB broth containing the same concentration of kanamycin; the culture was incubated with shaking at 37°C until the OD_{600} reached 0.6–0.8; IPTG was then added to a final concentration of 0.7 mmol/L. Cells were collected 2, 4, 6, and 8 h after IPTG addition for protein detection by SDS-PAGE. The negative control was prepared by the same method using cells not subjected to IPTG induction.

Identification of expression product

Samples obtained at different induction times were adjusted such that their OD_{600} value was 0.68; the samples were then mixed with 6X SDS gel loading buffer, boiled for 5 min, and centrifuged at 15,000 rpm for 10 min. Supernatant fractions of volume 20 μL were subjected to SDS-PAGE analysis. Cells from 30 mL culture were collected by centrifugation and resuspended in 20 mM Tris-HCl buffer, pH 8.0. The cell suspensions were sonicated and soluble lipase (crude enzyme) was obtained from the supernatant. The presence of lipase-expressing recombinants was confirmed on 2.5% (v/v) tributyrin agar plates (Samad et al. 1990). Clear zones would be formed by the addition of 20 μL sonicated supernatant onto a tributyrin agar plate.

Table 1 Sequence similarity between *Bacillus subtilis* FS1403 lipase (ABQ08591) and lipases from other *Bacillus*

Strains	Accession ID	Identity (%)
B. subtilis 168	AAA22575	99
B. subtilis	ABB54395	98
B. subtilis GHlip	ACK58682	98
B. subtilis B22	ACA60974	98
B. amyloliquefaciens FZB42	YP_001419931	76
B. pumilus ATCC7061	ZP_03056417	74
B. pumilus YZ02	ABC67449	75
B. pumilus MBB03	CAT00692	74
B. pumilus DBRL191	AAR84668	74
B. pumilus B106	ACA60975	73
B. licheniformis	CAB95850	72
B. pumilus F3	ABK80759	73
B. pumilus SAFR-032	YP_001487831	71
Bacillus sp. B26	AAL36938	72
B. pumilus XJU-13	ABO32303	72
B. pumilus SGMM8	ADD29798	71
Bacillus sp. NK13	ABY84673	68
B. subtilis 168/esterase	NP_388716	68
B. megaterium ATCC 9885/esterase	CAD23620	67
B. licheniformis ATCC 14580	YP_079680	63
B. clausii KSM-K16	YP_176491	42

Preliminary characterization of recombinant lipase

The effect of temperature on lipase activity was evaluated by applying 20 μL crude enzyme preparation to a tributyrin agar plate and incubating at temperatures of 35, 40, 50, 60, 70, and 75°C for 24 h. The sizes of the clear zones were determined and the relative activities of the lipase evaluated.

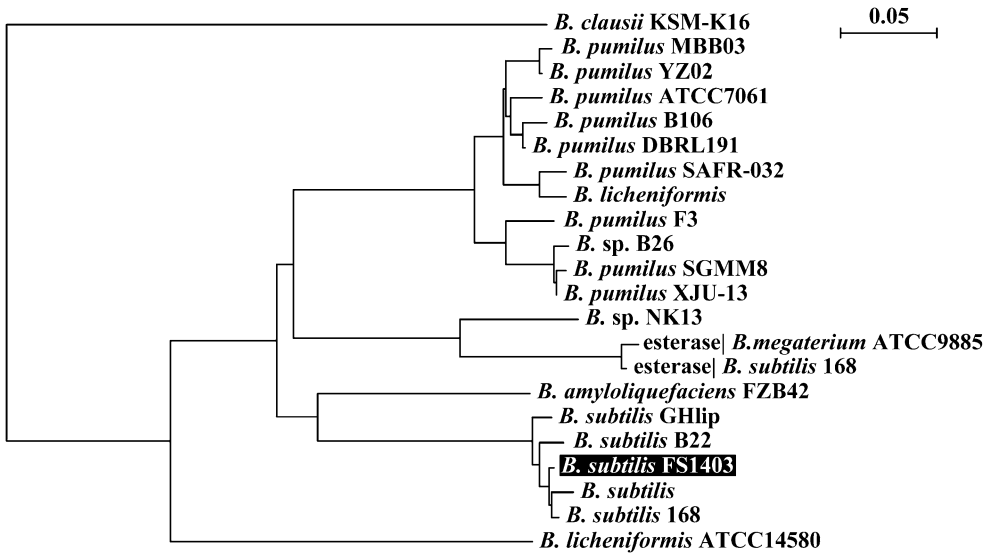

Fig. 1 Phylogenetic analysis of the lipase gene from *Bacillus subtilis* FS1403. Dendogram showing sequence relationships between *B. subtilis* FS1403 (ABQ08591) and *B. subtilis* 168 (AAA22574), *B. subtilis* (ABB54395), *B. subtilis* GHlip (ACK58682), *B. subtilis* B22 (ACA60974), *B. amyloliquefaciens* FZB42 (YP_001419931), *B. pumilus* ATCC7061 (ZP_03056417), *B. pumilus* YZ02 (ABC67449), *B. pumilus* MBB03 (CAT00692), *B. pumilus* DBRL-191(AAR84668), *B. pumilus* B106 (ACA60975), *B. licheniformis* (CAB95850), *B. pumilus* F3 (ABK80759), *B. pumilus* SAFR-032 (YP_001487831), *Bacillus* sp. B26 (AAL36938), *B. pumilus* XJU-13 (ABO32303), *B. pumilus* SGMM8 (ADD29798), *Bacillus* sp. NK13 (ABY84673), *B. subtilis* 168/esterase (NP_388716), *B. megaterium* ATCC 9885/esterase (CAD23620), *B. licheniformis* ATCC 14580 (YP_079680) and *B. clausii* KSM-K16 (YP_176491)

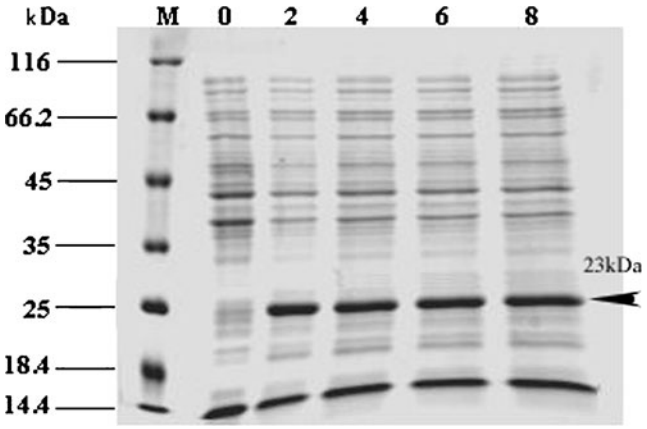

Fig. 2 SDS-PAGE analysis of the expressed protein. Lanes: *M* Protein molecular markers; *0* supernatant obtained upon sonication of *Escherichia coli* BL21 culture harboring pET-28a-lip before IPTG induction, i.e., negative control sample; *2*, *4*, *6*, *8* supernatants obtained on sonication of *E. coli* BL21 culture harboring pET-28a-lip after induction with IPTG for 2, 4, 6, and 8 h, respectively. The band of recombinant lipase is indicated by an *arrow*

The effect of temperature on thermostability of the lipase was determined by incubating the crude enzyme at temperatures of 40, 50, 60, 70, and 80°C for 60 min. The activity remaining was then determined. The crude enzyme without temperature treatment was used as the control.

The effect of pH on lipase activity was determined by the addition of 20 μL crude enzyme to tributyrin agar plates prepared at pH values of 4, 5, 6, 7, 8, 9, 10, and 11, and incubation at 55°C for 24 h; the relative activities of the crude enzyme were then estimated. Three parallel assays were performed under each experimental condition.

Results and discussion

Cloning and sequence analysis of *Bacillus subtilis* FS1403 lipase gene

Bacillus subtilis FS1403 was previously isolated from volcanic vent soil in Philippines, and exhibited lipolytic activity on tributyrin plates (Wu et al. 2008). A lipase-encoding gene was amplified from *B. subtilis* FS1403 by PCR; the sequence of the lipase gene was determined and submitted to GenBank (accession no. EF538417).

The sequence of the putative lipase gene contains a complete ORF of length 639 bp. The deduced protein consists of 212 amino acid residues with a putative molecular weight of approximately 22.819 kDa and a pI of 9.72. It possesses the conservative motif Ala-X-Ser-X-Gly, which is found in *B. subtilis* lipases, instead of the motif Gly-X-Ser-X-Gly found in common lipases (Gerritse et al. 1994).

The deduced amino acid sequence was multiply aligned with 21 *Bacillus* lipase sequences obtained from GenBank, and exhibited a high degree of sequence similarity at the predicted active site regions (Ala-His-Ser-Met-Gly). However, the overall similarities varied greatly between the different lipases analyzed (Table 1, identities from 42 to 99%). Phylogenetic analysis of the *Bacillus* lipases revealed that the lipase from *B. subtilis* FS1403 and LipA from

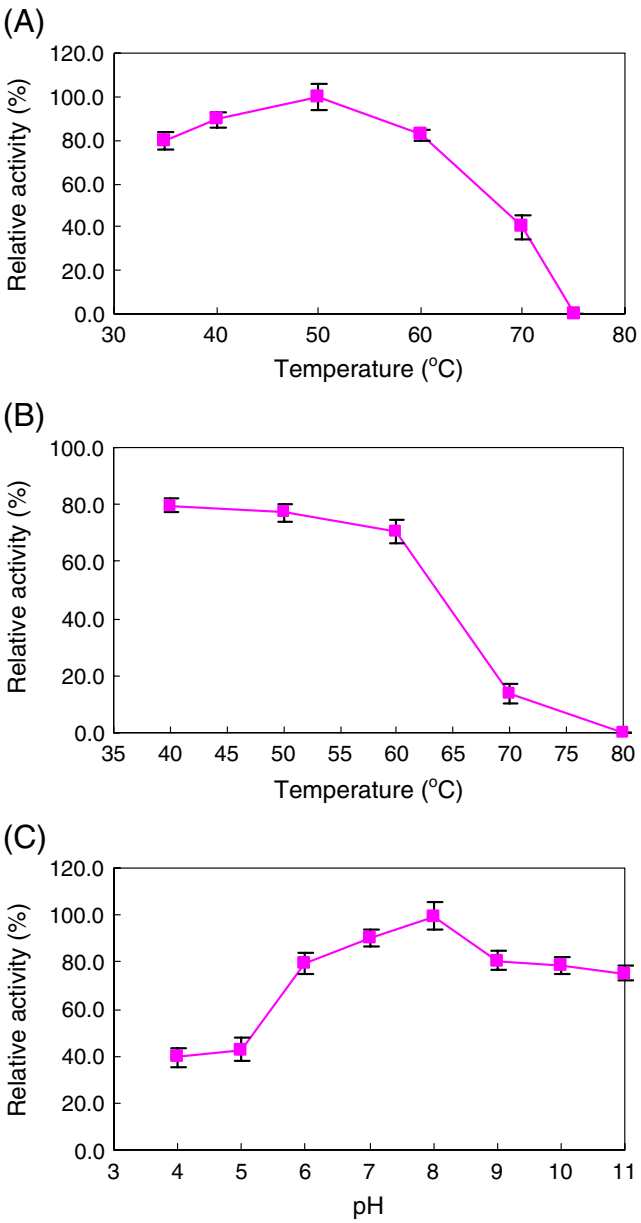

Fig. 3a–c Effect of temperature and pH on lipase activity and stability. **a** Temperature activity curve. The lipase activity was assayed at pH 9.4 using 2.5% tributyrin agar plates. **b** Temperature stability curve; activity was assayed after incubation at various temperatures for 60 min. **c** pH activity curve; activity was assayed after incubation at different pH values (4–12) for 24 h. The relative activity in **a** and **c** was calculated as a percentage of maximal activity; activity in **b** is expressed as a percentage of the initial activity without temperature treatment

B. subtilis 168 were clustered in the same sub-group owing to their high degree of sequence identity (Fig. 1). The results suggested that we had indeed cloned a LipA from *B. subtilis* FS1403 and that it belongs to subfamily I.4 of bacterial lipases (EC 3.1.1.3; Ruiz et al. 2002).

Expression of lipase in *E. coli* BL21

The recombinant plasmid (pET-28a-lip) was transformed into *E. coli* BL21. Expression of the lipase gene was driven by the T7 promoter and induced by the addition of IPTG. Protein expression was monitored and analyzed by SDS-PAGE. SDS-PAGE analysis revealed the presence of a new protein in the supernatant obtained after sonication treatment; this new protein had an approximate molecular weight of 23 kDa and was not observed in supernatants obtained without IPTG induction (Fig. 2). The size of the expressed protein agreed well with the predicted size of the lipase (22.819 kDa).

Very low activity was observed in the supernatants. The lipase activity of the supernatant obtained after sonication was highest after 6 h treatment with 0.7 mM IPTG, as determined by the size of the hydrolysis halos (diameter 1.7 cm) formed on the tributyrin plate. The results showed that most of the lipase expressed in *E. coli* existed intracellularly. Schmidt-Dannert et al. (1997) reported that the lipase from *Bacillus* species is not well processed by *E. coli* cells; Cho et al (2000) found that most of the lipase expressed in *E. coli* existed as inclusion bodies. Hence, a more efficient expression system with good protein secretion and correct processing should be developed.

Preliminary characterization of recombinant lipase

The effect of temperature and pH on activity of the recombinant lipase in *E. coli* was determined using tributyrin as the substrate. The recombinant lipase in *E. coli* was as thermostable as the wild-type lipase from *B. subtilis* FS1403 and had a similar pH range (Wu et al. 2008). The activity of the recombinant lipase was highest at a temperature of 50°C (Fig. 3a), and the lipase retained 80, 77, and 70% of its initial activity after incubation at 40, 50, and 60°C for 1 h, respectively (Fig. 3b). The enzyme exhibited maximal activity at pH 8.0, and retained over 80% of the maximal activity over a broad pH range of 6.0–11.0 (Fig. 3c). The recombinant lipase showed higher thermostability than the LipA from *B. subtilis* 168 (Lesuisse et al. 1993), which had an optimum temperature of 35°C and was stable at 40°C but lost its activity after incubation at 55°C for 30 min. The results indicated that the lipase we cloned might be valuable in the lipid processing industry, which requires the enzyme to be stable above 50°C (Cho et al. 2000).

Sequence data revealed that the amino acids at positions 29 and 133 in LipA differ between *B. subtilis* 168 (Dartois et al. 1992) and *B. subtilis* FS1403 (this study). Position 29 is occupied by Val in *B. subtilis* FS1403 lipase and Ala in and *B. subtilis* 168 lipase, while the corresponding amino acids at position 133 are Leu and Val, respectively. Whether the difference in thermostability of the lipase is due to these amino acid changes needs to be confirmed by experiments such as site-directed mutagenesis (Acharya et al. 2004; Ahmad et al. 2008). Our study suggests that enzymes from microbes (even the same species) from different ecological environments may possess different properties, and that the exploitation of microbes from extreme environments is an effective way of finding enzymes with new properties.

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