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Gene Cloning, Purification, and Characterization of a Cold-Adapted Lipase Produced by *Acinetobacter baumannii* BD5

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Acinetobacter baumannii* BD5 was isolated from waters of Baek-du mountain, and the lipase gene was cloned using a PCR technique. The deduced amino acid sequence of the lipase and lipase chaperone were found to encode proteins of 325 aa and 344 aa with a molecular mass of 35 kDa and 37 kDa, respectively. The lipase gene was cloned and expressed in *Escherichia coli* BL21 (*trxB*) as an inclusion body, which was subsequently solubilized by urea, and then purified using Ni-affinity chromatography. After being purified, the lipase was refolded by incubation at 4°C in the presence of a 1:10 molar ratio of lipase:chaperone. The maximal activity of the refolded lipase was observed at a temperature of 35°C and pH 8.3 when *p*-NP caprate (C10) was used as a substrate; however, 28% of the activity observed at 35°C was still remaining at 0°C. The stability of the purified enzyme at low temperatures indicates that it is a cold-adapted enzyme. The refolded lipase was activated by Ca^{2+}, Mg^{2+}, and Mn^{2+}, whereas Zn^{2+} and Cu^{2+} inhibited it. Additionally, 0.1% Tween 20 increased the lipase activity by 33%, but SDS and Triton X-100 inhibited the lipase activity by 40% and 70%, respectively.

Keywords: *Acinetobacter baumannii* BD5, genome walking PCR, Ni-affinity chromatography, refolding

Lipases, which are found in a wide range of organisms including bacteria and fungi, catalyze the hydrolysis of triglyceride at the interface between the insoluble substrate and water [18]. The structures of several triglyceride lipases have been elucidated, confirming that they are members of the α/β hydrolase superfamily, that is consisted of alternation of α-helices and β-sheets found also in other hydrolases [3, 25]. These enzymes share a semiconserved pentapeptide, GXSXG, where X represents any amino acid and contains the Ser, His, Asp catalytic triad characteristic of the serine proteases as well as a HG dipeptide, which is a portion of the N-terminal oxyanion hole [24]. In particular, microbial lipases are more stable than animal and plant lipases and readily available in large quantities because many of them can be produced in high yields by bacteria [12]. In addition, the crystal structures of many lipases are available, making it possible to design rational engineering strategies, and they do not usually require cofactors or catalyze side reactions [9].

Owing to their useful features, lipases secreted by microorganisms are used widely in various industries, including synthesis of pharmaceuticals and dairy hydrolysis of milk fat, detergents, the synthesis of pure compounds, and oil processing [17]. In fact, lipases produced by *Serratia marcescens* are used in the production of the drug Diltizem [3]. Lipases that are adapted to cold temperatures or that are generally thermostable are of particular interest for industrial processes.

In particular, cold-adapted lipases are attractive biocatalysts in biotechnology because they can be used as additives in laundry detergents to allow washing at cold temperatures, or for low-temperature treatment of fatty wastewaters produced by kitchens and the fast food industry [5, 26]. Additionally, cold-adapted lipases could potentially be used as catalysts for organic synthesis of chiral intermediates to produce relatively frail compounds at low temperatures [20]. Cold-adapted lipases and esterases produced by several microorganisms, including *Pseudomonas* sp. B11-1 [5], *Pseudomonas fragi* [1], *Moraxella* sp. TA144 [28], *Acinetobacter* sp. N16 [14], and *Psychrobacter* sp. Ant300 [14], have been studied. With the exception of lipases produced by *Acinetobacter* sp. O16 [4], however, cold-adapted lipases produced by the *Acinetobacter* sp. have rarely been reported.

Most of the lipases produced by *Acinetobacter* sp. have biochemical properties similar to those produced by *Pseudomonas* sp. and *Burkholderia* sp., and show stability

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and maximum activity under alkaline conditions at high temperatures. In addition, lipases produced by *Acinetobacter* sp. have been isolated from a variety of sources, including aquatic environments, soils, drugs, and human skin; however, they were not closely studied until much later than those produced by *Pseudomonas* sp. and *Burkholderia* sp. [18].

The cold-adapted lipase produced by *Acinetobacter* sp. may be a valuable biocatalyst in commercial industries. Therefore, we had earlier cloned the lipase gene that is present in *A. baumannii* BD5. In the present study, we cloned this lipase, expressed it in pET-32a(+) and subsequently purified. The purified lipase was characterized in regards with various substrates, temperatures, pH buffers, and metal ions.

MATERIALS AND METHODS

Bacterial Strains, Media, and Plasmids

A. baumannii BD5 (KACC13090) was isolated from water on agar plates of Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose) containing 1% tributyrin and deposited with the Korean Agricultural Culture Collection (KACC). One isolated strain forming the largest clear zone was selected on three kinds of agar plates as follows: The tributyrin LB medium (TBN), Tween 20 LB medium (TWN), and olive oil LB medium (OLB) were supplemented with LB medium containing 1% tributyrin, Tween 20, and olive oil emulsion, respectively. An emulsion was made from a solution containing 1% tributyrin, Tween 20, or olive oil, 10 mM $CaCl_2$, 200 mM NaCl, and 5% gum arabic for 2 min in a homogenizer [10]. *Escherichia coli* (*E. coli*) Top10F' was used as the cloning host, and the pGEM-T easy vector (Promega, Madison, WI, U.S.A.) and pET-32a(+) (Novagen) were used as the cloning vector and expression vector, respectively. *E. coli* Top10F' cells harboring the recombinant plasmids were grown in LB broth supplemented with 100 μg/ml ampicillin.

Table 1. Effect of various metal ions, detergents, and organic solvents on BDLipA activity.

Agent	Remaining activity (%) at a concentration of				
	0.1 mM	1 mM	10 mM	0.1%	10%
$CaCl_2$	272	331	409	-	-
$ZnCl_2$	113	159	54	-	-
$MgCl_2$	154	113	204	-	-
$MnCl_2$	227	263	72	-	-
$FeCl_2$	72	71	102	-	-
$CuCl_2$	84	69	27	-	-
Dithiothreitol	54	33	2.2	-	-
EDTA	62	66	42	-	-
2-Mercaptoethanol	18	18	17	-	-
SDS	-	-	-	40	-
Tween 20	-	-	-	133	-
TritonX-100	-	-	-	76	-
Methanol	-	-	-	-	93
Ethanol	-	-	-	-	92
Acetone	-	-	-	-	101
Isopropanol	-	-	-	-	96
DMSO	-	-	-	-	97

-, Not determined.

Chemicals

Tributyrin was purchased from Sigma (U.S.A.). A DNA walking SpeedUp premix kit was purchased from Seegene (South Korea), and a PCR purification kit was from Solgent (South Korea). *p*-Nitrophenyl (*p*-NP) acetate (C2), butyrate (C4), caprate (C10), laurate (C12), and palmitate (C16) were purchased from Sigma (USA), and *p*-NP myristate (C14) and stearate (C18) were from Fluka (U.S.A.).

DNA Manipulation and Analysis of 16S rDNA

Chromosomal DNA obtained from BD5 was prepared for analysis of 16S rDNA using phenol-chloroform. The genomic DNA was extracted with phenol, chloroform, and isoamylalcohol (25:24:1), and re-extracted with chloroform and isoamylalchol (24:1). The genomic DNA was precipitated with 100% ethanol and 3 M sodium acetate (pH 5.2), and washed with chilled 70% ethanol. Polymerase chain reaction was performed to amplify the 16S rDNA coding region using both primers 5'-GAGRRRGATCCTGGCTCAG-3' and 5'-AGAAAGGAGGTGATCCAGCC-3'. The reaction mixture was subjected to initial denaturation at 95°C for 10 min, followed by 10 cycles of denaturation at 95°C for 1 min, annealing at 37°C for 1 min, and extension at 72°C for 1.5 min. The cycle was followed by additional 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min, and final extension at 72°C for 10 min, using a thermal cycler (TaKaRa). The PCR product was subcloned into pGEM-T Easy vector. *E. coli* TOP10F' cells harboring the recombinant plasmid were then selected using LB agar plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galX-galactopyranoside (X-gal), isopropylthio-β-D-galactopyranoside (IPTG), and ampicillin. Nucleotide sequence was determined by Solgent, and phylogenetic trees were conducted using the ClustalX program.

Cloning of Lipase Gene Fragments

To amplify the gene encoding lipase from *A. baumannii* BD5, degenerate oligonucleotides were used, which originated from the highly conserved region of *Acinetobacter* sp. lipase including *Acinetobacter* sp. DYL129 [11], LipF (5'-AGATGGTTCCAGTTGT-AATTATCACGA-3'), and LipR (5'-GCAGAAAAAGTCAACTTG-ATTGGTCAT-3') and *Acinetobacter* sp. SY-01 [6], LipSF (5'-CCGCATACAAATCGCACGGACTATATAGCTG-3') and LipSR (5'-CAGGGAAATATAATGGAGAGCACTTACAGC-3'), and N-terminal and C-terminal conserved sequences, LP-N (5'-AACTGTA-ACGTAAGCAGTTG-3') and LP-C (5'-CTTTAGTTGGACCACC-AACA-3'), in previously reported lipase gene from *Acinetobacter*. They were then used in the PCR to amplify chromosomal DNA, which was used as the template. The reaction mix was subjected to initial denaturation at 95°C for 10 min, followed by 10 cycles of denaturation at 95°C for 1 min, annealing at 37°C for 1 min, and extension at 72°C for 1 min. This cycle was followed by an additional 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min using a thermal cycler. The PCR product was then electrophoresed on an 0.7% agarose gel. Next, the 0.6 kb product was isolated from the gel and ligated into

the pGEM-T Easy vector according to manufacturer's instructions. *E. coli* TOP10F' cells harboring the recombinant plasmid, pBD5lip containing 0.6 kb fragment, were then selected as previously described. Sequence analysis of the PCR fragment revealed that it encoded the partial lipase gene of *Acinetobacter* sp.

Cloning of Full-Length Lipase Gene Using Genome Walking PCR

To obtain the upstream and downstream sequences flanking the fragments, genome walking PCR was performed using a DNA walking SpeedUp Premix Kit according to the manufacturer's instructions. Three target-specific primers (TSP1, 2, 3) were designed, based on the upstream region of pBDlip5 encoding a partial lipase gene. The first PCRs with four individual DW-ACP primers and TSP1 were performed, and then the second PCR was performed as previously described with DW-ACPN primer and TSP2, but the purified product of the first PCR was used as a template in the second PCR. Finally, the third PCR was performed with Universal primer and TSP3 using the purified product of the second PCR. Third PCR products were subcloned into pGEM-T Easy vector and then the nucleotide sequence was determined. The analysis of sequence and database similarity searches were done using the server at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/Enterz). Homology alignment was performed with the ClustalW program and MacVector 6.5 software, and the signal peptide sequence was deduced by SignalP version 3.0.

Construction of Recombinant Plasmid pBDLipA and pBDLipB

The lipase gene produced by *A. baumannii* BD5 was expressed by subcloning the mature lipase gene and the lipase chaperone using the pET-32a(+) expression vector (Novagen). These genes were then amplified using forward and reverse primers that were specific for the lipase gene (5'-GAGGAATTCATGAAAAACTTAATATTT-3' that encodes a EcoRI site downstream of the start codon and 5'-AGCGTCGACTTATAATCCTTGAAGTTTTAA-3' that encodes a SalI site upstream of the stop codon) as well as primers specific for the chaperone gene (5'-GATGGATCCATGCAAAGGATGCAGAA-AAAG-3' that encodes a BamHI site downstream of the start codon and 5'-AGCGAATTCCTAGTAGTTAAAGGGTAGTTC-3' that encodes a EcoRI site upstream of the stop codon). The PCR reaction consisted of 30 cycles of denaturation at 95°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min. The constructed recombinant plasmids, pBDLipA and pBDLipB, were transformed into *E. coli* Top10F', and the isolated pBDLipA and pBDLipB plasmids were retransformed into *E. coli* BL21 (*trxB*), respectively.

Expression and Purification of pBDLipA and pBDLipB

Transformed *E. coli* BL21 (*trxB*) that contained the mature lipase gene and lipase chaperone was induced to express the lipases and chaperone encoded by these genes by incubating it at 37°C in 500 ml of LB broth containing 100 μg/ml ampicillin and 50 μg/ml kanamycin, respectively. After culture to an OD_{600} of 0.7, 0.03 mM IPTG was added to the culture, which was then incubated for an additional 8 h at 37°C. Next, the cultured cells were harvested by centrifugation at 8,000 rpm for 10 min, suspended in 20 ml of lysis buffer (20 mM Tris-HCl, pH 8.3, 0.1 M NaCl, 5% glycerol) containing 1% TritonX-100, and then frozen at −70°C for 30 min. To separate the proteins, the lysed cells were then centrifuged at 13,000 rpm for 20 min. To purify BDLipB, a His-Trap affinity column (Amersham Phamaciabiotech) was equilibrated with binding buffer (50 mM Tris buffer, pH 8.3, 100 mM NaCl, 20 mM imidazole), and the supernatant was then applied to the column. Next, unbound proteins were removed by washing the column with binding buffer, and the tagged protein was then eluted using elution buffer (50 mM Tris-HCl, pH 8.3, 100 mM NaCl, 500 mM imidazole). BDLipA was expressed as an inclusion body, and was therefore present in the pellet obtained from centrifugation. To purify BDLipA, the pellet was dissolved in solubilization buffer (8 M urea, 50 mM Tris buffer, pH 8.3, 1 mM DTT), and then subjected to centrifugation at 13,000 rpm for 20 min. The solubilized BDLipA was then purified following the same method that was used to purify BDLipB. BDLipB was concentrated by using Amicon Ultra-4 (Millipore). Urea in BDLipA was removed by dialysis against a 50 mM Tris-HCl buffer, pH 8.3, at 4°C and was concentrated by Amicon Ultra-4. The purified fusion proteins were digested with enterokinase (Novagen) for 16 h at 20°C to remove the thioredoxin fusion protein region, and unbound fractions were then collected using a His-Trap affinity column and used as purified enzymes.

Refolding of BDLipA

The BDLipA was refolded in distilled water in the presence of varying amounts (in molar ratios 1 to 0, 1, 10, 30, and 50) of the BDLipB. The inactive lipase (0.9 mg), BDLipA, was mixed with 9.6 mg of the lipase chaperone, BDLipB, in 1 ml of distilled water and then was incubated at 4°C for 36 h. The lipase activity was determined using *p*-NP caprate (C10) as a substrate.

Assay of Lipase Activity of the Refolded BDLipA

Lipase activity was determined using the colormetric method described by Kumar *et al.* [15]. The reaction mixture contained 0.5 mM *p*-NP ester in ethanol, 50 mM Tris buffer, pH 8.3, and 2 μl of enzyme in a final volume of 1 ml. The mixture was incubated at 37°C for 15 min and then frozen at −70°C for 10 min to inactivate the enzyme. The absorbance of *p*-NP liberated was then measured at 410 nm. One unit of enzyme activity was defined as the release of 1 μmol of *p*-nitrophenol per min. To determine substrate specificities, the lipase activity was assayed using various *p*-NP esters (*p*-NP acetate, *p*-NP butyrate, *p*-NP caprate, *p*-NP myristate, *p*-NP palmitate, and *p*-NP stearate), and the effect of temperature on lipase activity was assayed using *p*-NP caprate (C10) at various temperatures (0, 10, 20, 30, 35, 40, 50, 60, and 70°C). To study the effect of various pH buffers, 20 mM sodium acetate, Tris-HCl, and glycine-NaOH were used to obtain buffers of pH 4.8–6.0, 6.0–9.0, and 8.6–10.6, respectively. The effect of metal ions and inhibitors on the lipase activity was measured by incubating the refolded lipase with various metal ions and inhibitors (0.1 mM, 1 mM, and 10 mM), including Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, DTT, EDTA, and 2-mercaptoethanol, in 50 mM Tris-HCl buffer (pH 8.0) at 30°C for 1 h. After incubation, the remaining activity was determined by incubating the lipase with *p*-NP caprate at 50°C for 30 min. The effect of detergents on lipase was measured using 0.1% SDS, Tween 20, and Triton X-100. The effect of 10% organic solvent on the lipase activity was determined by the same method as that of metal ions.

GenBank Accession Number

The nucleotide sequences of lipase and 16S rDNA studied in this work have been assigned as the GenBank accession numbers EU78176 and EU78177, respectively.

RESULTS

Identification and Cloning of Strain BD5

Of hundreds of microbial strains isolated from water in Baek-du mountain, four strains produced a clear and powder-like zone around their colonies after being grown for 1–2 days on LB plates that contained 1% tributyrin and Tween 20, respectively. Of these strains, the colony that formed the largest clear zone was selected for further study. The optimal growth of this strain was observed at 37°C, although it also grew well at 50°C. Partial sequencing of its 16S rDNA indicated that it was most closely related to the genus *Acinetobacter*, with the highest similarity (99%) to *Acinetobacter baumannii*; therefore, this strain was identified as *A. baumannii* BD5. PCR amplification of the lipase gene from *A. baumannii* BD5 using degenerate primers (LipF and LipR) produced an approximately 600 bp fragment. The amplified products were then cloned and sequenced to identify the amino acids sequence, which was then subjected to BLASTP analysis. This search revealed a relatively high similarity between pBD5lip and other lipases, including those of *A. calcoaceticus* BD413 (53%), *P. mendocina* (53%), *P. aeruginosa* (49%), and *Acinetobacter* sp. SY-01 (48%). Based on the partial sequence of the lipase gene of BD5, we performed genome-walking PCR to determine the sequence of the lipase and its chaperone.

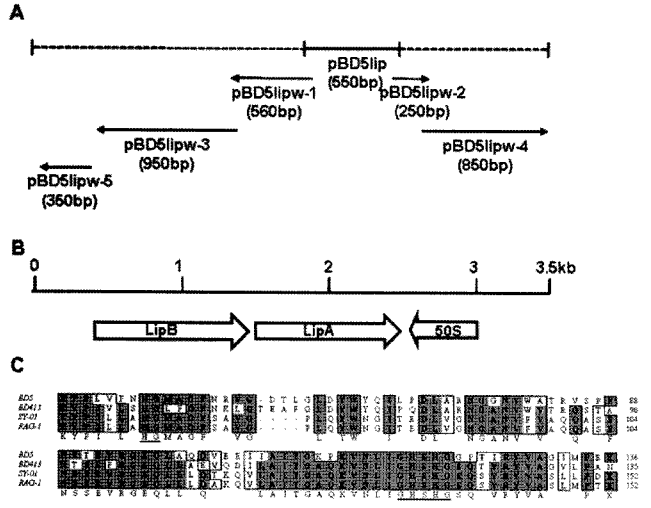

Fig. 1. Schematic diagram of genome walking PCR, the predicted three open reading frames, and the partial amino acid sequence alignment of lipase from *A. baumannii* BD5 with that of other lipases.

A. pBD5lip (550 bp), which was obtained by PCR using degenerate primers, was used for genome walking PCR. pBD5lipw-1 (560 bp), 2 (250 bp), 3 (950 bp), 4 (850 bp), and 5 (350 bp) were obtained by genome walking PCR. **B.** The 3,488 bp nucleotide sequences predicted three open reading frames including lipase chaperone, LipB (344 aa), lipase, LipA (325 aa), and 50S ribosomal (147 aa) protein. **C.** The amino acid sequence alignment of lipase from *A. baumannii* BD5 was compared with that of other *Acinetobacter* lipases including *A. calcoaceticus* RAG-1 (AF047691), BD413 (X80800), SY-01 (AF518410). The conserved amino acid motif, GXSXG, is shown in the amino acids sequence obtained from BD5 and other lipases. The HG sequence, which constitutes an oxyanion hole, is shown underlined and in bold.

Sequence Analysis of the Lipase and Lipase Chaperone

Analysis of the lipase (LipA) and lipase chaperone (LipB) gene present in *A. baumannii* BD5 revealed three complete open reading frames of 1,032 bp (positions 404 to 1,438), 975 bp (1,520 to 2,494), and 439 bp (2,987 to 2,547). The 1,032 bp ORF was found to be 26–49% homologous with the lipase chaperone gene from *Acinetobacter* sp. BD413, *Acinetobacter* sp. SY-01, *P. aeruginosa* PA7, and *Burkholderia*, and to encode a protein of 344 aa with a molecular mass of 38 kDa. The 975 bp ORF was found to be 48–56% homologous to the lipase gene from *Acinetobacter* sp. BD413, *P. mendocina*, *P. stutzeri* A1501, *Acinetobacter* sp. SY-01, and *Ralstonia* sp. M1, and to encode a protein of 325 aa with a molecular mass of 36 kDa. The 439 bp ORF was found to encode a 50S ribosomal protein. Unlike previously reported *Acinetobacter* sp. lipases, the gene encoding the lipase of *A. baumannii* BD5 is located 84 bp downstream of the gene encoding its chaperone. Putative ribosomal binding sites (RBSs) for the two genes were located 4 bp upstream of the *lipA* start codon and 10 bp upstream of the *lipB* start codon. Additionally, a putative transcription initiation site with a −10 region was located 46 bp upstream of the *lipB* start codon separated from the −35 region by 16 bp. In addition, a 23 amino acid residue signal peptide was found at the N-terminal of the lipase from *A. baumannii* BD5 with a cleavage position between Ala23 and Thr24, predicted by SignalP. Furthermore, an N-

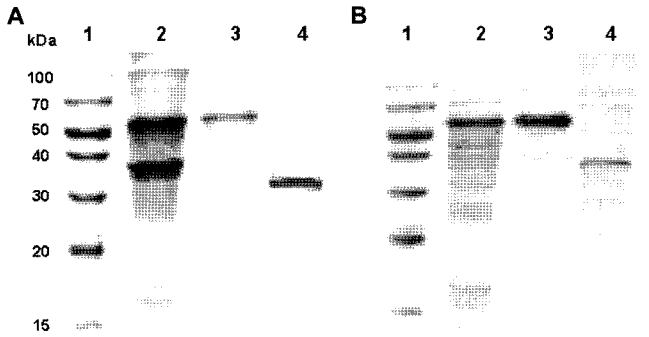

Fig. 2. SDS-PAGE analysis of BDLipA and BDLipB.

A. SDS-PAGE analysis of BDLipA. Lane 1, molecular mass markers (ELPIS, Korea); lane 2, insoluble proteins obtained from *E. coli* BL21 (*trxB*) cells harboring pBDLipA; lane 3, The purified fusion BDLipA obtained after Ni-affinity chromatography; lane 4, purified BDLipA digested by enterokinase. **B.** SDS-PAGE analysis of BDLipB. Lane 1, molecular mass markers (ELPIS, Korea); lane 2, total proteins obtained from *E. coli* BL21 (*trxB*) cells harboring pBDLipB; lane 3, purified fusion BDLipB obtained after Ni-affinity chromatography; lane 4, purified BDLipB digested by enterokinase.

terminal signal peptide was predicted in the lipase and transmembrane regions of its chaperone, suggesting that the lipase is a secreted enzyme or a membrane-bound protein. The typical lipase consensus catalytic triad, GXSXG, was found in the N-terminal region of the lipase, with Asp 269 and His 291 forming the catalytic triad. Finally, an HG sequence, which constitutes an oxyanion hole in a three-dimensional protein structure, was found in the lipase.

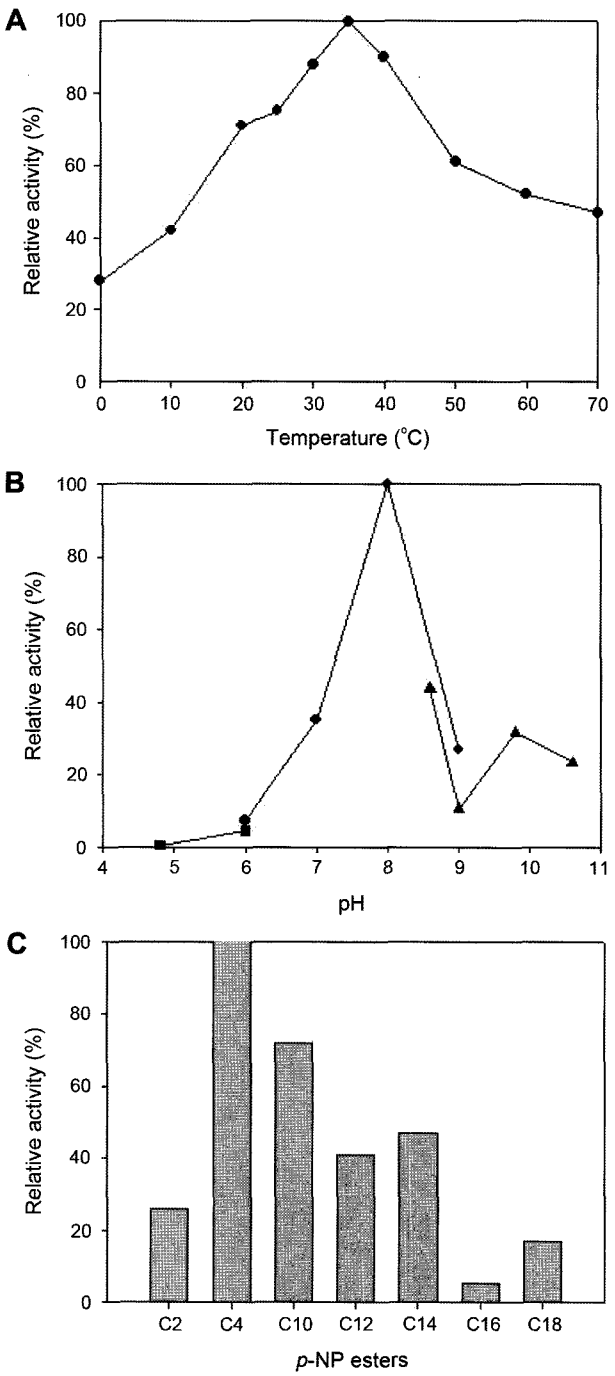

Fig. 3. Effects of temperature, pH, and substrate specificity on the activity of BDLipA.
A. Effect of temperature on activity was examined at temperatures ranging from 0–70°C in 50 mM Tris-HCl (pH 8.3) using *p*-NP caprate as the substrate. **B.** Effect of pH on activity was examined by incubating at 50°C for 30 min in various buffers containing *p*-NP caprate as the substrate. **C.** The substrate specificity was determined with various *p*-NP esters, using 50 mM Tris-HCl (pH 8.3) at 50°C for 30 min. The activities are reported relative to that of the maximum activity observed.

Expression, Purification, and Refolding of BDLipA and BDLipB

The recombinant plasmids, pBDLipA and pBDLipB, were expressed in *E. coli* BL21 (*trxB*) grown in the presence of 0.03 mM IPTG at 37°C. The results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that BDLipA was produced as an inclusion body, and that 36% of the total proteins present in the cells contained the expressed BDLipA. To prevent the aggregation and obtain an active form of BDLipA , urea in the enzyme preparation was removed by dialysis against 50 mM Tris-HCl buffer, pH 8.3, at 4°C for 6 days. The decrease in the aggregation of BDLipA was found to depend on the enzyme concentration during dialysis [21], with a very low concentration of the enzyme producing a stronger decrease of the aggregation of BDLipA. Therefore, the enzyme was concentrated to 3.6 mg using Amicon Ultra-4. Thereafter, it was purified and then analyzed by SDS-PAGE, which revealed the presence of one 35 kDa band. Unlike BDLipA, BDLipB was produced as a soluble protein, purified by Ni-affinity chromatography, and then concentrated. The purified BDLipB showed one band with a molecular mass of 37 kDa. Although inactive BDLipA in 8 M urea was refolded by dialysis against 50 mM Tris-HCl (pH 8.3), the refolded lipase protein was not active. Therefore, the denatured BDLipA was refolded after 36 h of incubation at 4°C in distilled water in the presence of various amounts of BDLipB (data not shown). The lipase refolded in the presence of various lipase:chaperone ratios (1 to 0, 1, 10, 30, and 50) showed specific activities of 0.16, 1.53, 1.1, 3.2, and 3.0 U/mg, respectively. In addition, a highly active lipase with a specific activity (3.2 U/mg) was obtained by refolding the protein *in vitro* at a molar ratio of 1:10 lipase:chaperone.

Effects of Temperature, pH, and Substrates on Lipase Activity

BDLipA showed its maximum activity toward *p*-NP caprate (C10) at 35°C and was most active between 0–70°C. At 0°C and 20°C, the lipase activity was 20% and 71% of the maximum activity, respectively, whereas the lipase maintained 47% of its optimal activity at 70°C. The optimal pH of BDLipA was determined to be 8.0, with the relative activity of 54% at pH 7.0 and activity of 100% at pH 8.0. Additionally, lipase activity was observed between pH 8.0 and 10.6; however, no activity was observed at pH 4.8. The substrate specificity of BDLipA was examined using *p*-NP esters of various chain lengths. Although the

maximum activity was obtained using *p*-NP butyrate (C4), the enzyme showed activity toward substrate esters with a wide range of chain lengths. In addition, although BDLipA optimally hydrolyzed short chain length esters, it shared about 71% and 54% of its maximum activity when tested against *p*-NP caprate (C10) and *p*-NP myristate (C14), respectively. Taken together, these results indicate that the ability of BDLipA to hydrolyze long-chain esters is similar to that of most bacterial lipases.

Effects of Metal Ions, Detergents, and Organic Solvents on Lipase Activity

It has been reported that metal ions and inhibitors affect the Group I and II lipases [23]. Therefore, the enzyme isolated in this study was mixed with various metal ions and then incubated at 30°C for 1 h. With the exception of Fe^{2+} and Cu^{2+}, the metal ions increased the lipase activity, and 10 mM Ca^{2+} was found to increase the lipase activity 4-fold of that of the control sample. Additionally, 0.1–1 mM Mn^{2+} and 10 mM Mg^{2+} were found to increase the lipase activity 2-fold, and 0.1–1 mM Zn^{2+} increased the lipase activity by 13–59%. However, the addition of 10 mM Zn^{2+} and Mn^{2+} decreased the activity by 46% and 28% of the control sample, respectively. Additionally, the presence of 0.1 mM and 1 mM Fe^{2+} inhibited the enzyme activity. All inhibitors, including DTT, EDTA, and 2-mercaptoethanol, decreased lipase activity. EDTA, a divalent metal-chelating agent, decreased the lipase activity by 40%, indicating that EDTA is accessible to the Ca^{2+} binding site of the enzyme and removes ions. Additionally, Tween 20 induced a 33% increase of the enzyme activity, most likely due to disaggregation of the lipase; however, 0.1% SDS and Triton X-100 decreased the lipase activity by 60% and 24%, respectively. Moreover, when BDLipA was incubated with various organic solvents for 1 h at 30°C, ≥90% activity was retained. Following incubation with acetone (10%), 100% of the initial activity was retained.

DISCUSSION

In this study, the partial sequence of the lipase gene of *A. baumannii* BD5, which was isolated from water in Baek-du mountain, was amplified by PCR and then cloned. The complete lipase and lipase chaperone genes were then obtained by genome walking PCR that uses primers designed based on the partial lipase gene sequence.

Based on the amino acid sequence homology, most lipases produced by bacteria have been identified and grouped into six families, with family I being further divided into 6 subfamilies with a total of 22 members [2, 8]. Sequence analysis of the lipase gene revealed that lipase from *A. baumannii* BD5 belongs to subfamily I.1.

Lipases belonging to subfamilies I.1 and I.2 are encoded in a single operon together with their cognate lipase chaperone, the so-called lipase-specific foldase [22]. In most cases, the lipase chaperone is located immediately downstream of the lipase gene, with the exception of *A. calcoaceticus* BD413 [13], *Acinetobacter* sp. DYL129 [11], and *Ralstonia* sp. M1 [18]. Compared with most *Acinetobacter* lipases, the position of the lipase chaperone present in *A. baumannii* BD5 was reversed, with the structural lipase gene being located immediately downstream of the lipase chaperone. Although the upstream gene in the lipase and lipase chaperone cluster has a promoter region, no promoter-like region was found upstream of the lipase gene, and there is only one ribosomal binding site. These results suggest that expression of the chaperone gene is dependant on that of the lipase gene.

In this study, fusion expression vectors such as pET-32a(+) and pGEX-6P-1 were used to overexpress lipase from *A. baumannii* BD5 in *E. coli*; however, both of the expressed lipases were insoluble proteins. According to Tang *et al.*, the solubility of the expressed proteins in *E. coli* may be increased by inducing the proteins at low temperature [27]. However, when inducing transformants at 15, 20, 25, and 30°C, no soluble target proteins were found. Therefore, the signal peptide in LipA was removed because the highly hydrophobic regions of the signal peptides contribute to the aggregation of target proteins; however, the lipase after removal of the peptide was also associated with insoluble proteins. Quyen *et al.* [18] found that lipases that were refolded in the presence of their chaperone were fully active. Therefore, these results suggest that folding of lipase depends on the chaperone that assists lipase proteins during folding to achieve an active conformation.

The most interesting property in this study was the effect of temperature on BDLipA. The optimal activity of BDLipA was 35°C, which is similar to that of *Pseudomonas* sp. KB700A [19] and lower than that of *Pseudomonas* sp. B11-1 [5]. Lipases produced by *Acinetobacter* sp. such as *Acinetobacter* sp. SY-01 [6], RAG-1 [23] and CMC-1 [7] have been shown to be optimally active at 40–55°C, whereas the optimal temperature of BDLipA was found to be lower than that of other *Acinetobacter* sp. lipases. BDLipA displayed a high activity at low temperature, indicating that it is a cold-adapted lipase. With the exception of *Acinetobacter* sp. strain No.6 [25], which produces esterase, and *Acinetobacter* sp. O16 [4], which produces lipase, no cold-adapted lipases have been obtained from *Acinetobacter* sp. Although BDLipA was inactive at high temperatures, it was more stable at higher temperatures than cold-adapted lipases produced by *P. fluorescens* [16], *Pseudomonas* sp. B11-1, *Pseudomonas* sp. KB700A, and *Psychrobacter* sp. 7195. Although inactivation at high temperatures is a typical property of cold-adapted lipases,

BDLipA showed activity at a broad range of temperatures. These results suggest that BDLipA is a unique lipase when compared with other cold-adapted lipases. The activity of BDLipA was increased by 4-fold in the presence of Ca^{2+}, which suggests that Ca^{2+} enhanced the activity of several bacterial lipases by accessing to the binding site for Ca^{2+} and stabilizing the protein. In contrast to BDLipA produced by *A. baumannii* BD5, lipase obtained from *A. calcoaceticus* RAG-1 [23] showed a high activity following incubation with 0.1 mM and 1 mM Fe^{2+}. High concentrations of Mn^{2+} and Cu^{2+} cause lipase aggregation, which may affect the structure of the enzyme. In addition, it has been reported that lipases from *A. calcoaceticus* RAG-1 [23] and *Ralstonia* sp. M1 [17] are inhibited by EDTA. Furthermore, DTT and 2-mercaptoethanol, which are both S-S reducing agents, also caused a 90% and 83% loss of activity, respectively. This finding indicates that an S-S bridge is required to maintain the structural conformation of the lipase, and that the cysteine residues forming the S-S bridge are included in the catalytic domain of the lipase.

In this study, lipase produced by *A. baumannii* BD5 exhibited activity at low temperatures and was not inactivated at relatively high temperatures. Unlike other known cold-adapted lipases, the enzyme in this study was able to hydrolyze substrates over a wide range of temperatures. These results suggest that the cold-adapted lipase produced by *A. baumannii* BD5 has the potential for applications that include the use of the enzyme as a biocatalyst and a detergent at low and high temperatures.

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