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Isolation and Biochemical Characterization of *Bacillus pumilus* Lipases from the Antarctic[S]

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Lipase-producing bacterial strains were isolated from Antarctic soil samples using the tricaprylin agar plate method. Seven strains with relatively strong lipase activities were selected. All of them turned out to be *Bacillus pumilus* strains by the 16S rRNA gene sequence analysis. Their corresponding lipase genes were cloned, sequenced, and compared. Finally, three different *Bacillus pumilus* lipases (BPL1, BPL2, and BPL3) were chosen. Their amino acid sequence identities were in the range of 92–98% with the previous *Bacillus pumilus* lipases. Their optimum temperatures and pHs were measured to be 40°C and pH 9. Lipase BPL1 and lipase BPL2 were stable up to 30°C, whereas lipase BPL3 was stable up to 20°C. Lipase BPL2 was stable within a pH range of 6–10, whereas lipase BPL1 and lipase BPL3 were stable within a pH range of 5–11, showing strong alkaline tolerance. All these lipases exhibited high hydrolytic activity toward *p*-nitrophenyl caprylate (C_8). In addition, lipase BPL1 showed high hydrolytic activity toward tributyrin, whereas lipase BPL2 and lipase BPL3 hydrolyzed tricaprylin and castor oil preferentially. These results demonstrated that the three Antarctic *Bacillus* lipases were alkaliphilic and had a substrate preference toward short- and medium-chain triglycerides. These Antarctic *Bacillus* lipases might be used in detergent and food industries.

Key words: *Bacillus pumilus*, lipase, Antarctic

The Antarctic is the Earth's southernmost continent, containing the geographic South Pole. The cold environments have been successfully colonized by numerous organisms, particularly bacteria, yeasts, unicellular algae, and fungi [8, 12, 19]. These organisms are potential sources of valuable new enzymes [11].

Lipase (triacylglycerol acylhydrolase; E.C. 3. 1. 1. 3) catalyzes the hydrolysis of the ester bonds of triacylglycerols in oil–water interfaces, and the synthesis of ester bonds *via* transesterification in anhydrous organic solvents [22]. Lipases are ubiquitous in nature and isolated from various plants, animals, and microorganisms. Lipases of microbial origin are diverse in their catalytic activities and substrate specificities, which make them attractive tools for industrial applications [9, 10, 24].

In particular, cold-adapted lipases that display high lipolytic activity at low temperature are very attractive biocatalysts for biotechnological applications. Therefore, they have great potential in production of pharmaceuticals and food products, bioremediation in fat-contaminated cold environments, and as additives in detergents for cold washing [11, 23]. Psychrophilic and psychrotrophic microorganisms are good candidates to produce these types of lipases.

The cold adapted or cold active lipolytic enzymes include those isolated from psychrophilic Antarctic bacteria *Moraxllea* Tal 44 [7] and *Psychrobacter immobilis* B10 [3]; cold active esterase produced by Antarctic bacteria *Psychrobacter* sp. Ant300 [14]; lipase (PFL) produced by *Pseudomonas fragi* (X14033) [2]; cold adapted lipase (KB-Lip) produced by a psychrotrophic *Pseudomonas* sp. strain KB700A [20]; lipase (LipP) produced by an Alaskan psychrotrophic *Pseudomonas* sp. strain B11-1 [6]; and cold adapted lipase from an Antarctic deep-sea psychrotrophic *Psychrobacter* sp. [25].

In this study, many microbial strains with lipase activities were isolated from the Antarctic. Among them, three *Bacillus pumilus* lipases were chosen and their biochemical properties characterized for potential use as biocatalysts in biotechnological applications.

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[S]Supplementary data for this paper are available on-line only at http://jmb.or.kr.

Materials and Methods

Screening of Lipase-Producing Bacteria

Fifty strains isolated from the Antarctic were obtained from Korea Polar Research Institute (KOPRI). These strains were grown at 25°C for 48 h on 1% tricaprylin (TCN) agar plates containing 1× gum arabic solution, 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar. Gum arabic stock solution (10×) contained 10% (w/v) gum arabic, 200 mM NaCl, and 50 mM $CaCl_2$. Strains producing big halo zones were inoculated into 5 ml of LB broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) and incubated at 20°C for 48 h with shaking at 200 rpm. Culture supernatants from these strains were separated by centrifugation (6,000 ×*g*, 10 min, and 4°C) and their lipase activities were determined by assaying their hydrolytic activities toward *p*-nitrophenyl caprylate (*p*NPC)

Production and Concentration of Lipases

Bacillus pumilus strains were cultivated in 800 ml of LB broth at 20°C for 54 h with shaking at 200 rpm. Culture supernatants were separated from bacterial cells by centrifugation (6,000 ×*g* and 10 min) at 4°C. Ammonium sulfate was added to 20% saturation and centrifugation (10,000 ×*g* and 10 min) was performed to remove non-protein polymers and protein aggregate from the supernatant. Ammonium sulfate addition was continued up to 70% saturation. Protein precipitates were collected by centrifugation (10,000 ×*g* and 10 min), dissolved in distilled water, and dialyzed with Spectra/Por 4 membrane (Spectrum Labs, USA) to remove residual ammonium sulfate.

Lipase Activity Assay

Lipase activity was measured by the pH titration method and spectrophotometric method. In the case of pH titration method, the lipase activity was assayed by titrating free fatty acids released by hydrolysis of olive oil using the pH-stat method. An olive oil emulsion containing 1% olive oil and 1% gum arabic was prepared by blending in a Waring blender (model 51BL31) at maximum speed for 2 min. After the pH of the substrate emulsion (20 ml) was adjusted to 8.0 by the addition of 10 mM NaOH solution, an appropriate amount (0.1–0.2 ml) of the enzyme solution was added. The release rate of free fatty acid was measured at 35°C for 5 min with a 718 Titrino pH titrator (Metrohm, Switzerland). The amount of enzyme catalyzing the release of 1 μmol of fatty acid per minute was defined as one unit.

For the spectrophotometric detection of lipase activity, *p*NPC was used as a substrate. The reaction mixture consisted of 10 μl of 10 mM substrate in acetonitrile, 40 μl of ethanol, and 950 μl of 50 mM Tris-HCl buffer (pH 8.0) containing an appropriate amount (4 μl) of the enzyme. One unit of enzyme activity was defined as the quantity of enzyme required to generate 1 μmol of *p*-nitrophenol *via* the hydrolysis of *p*NPC substrate in 1 min.

Molecular Mass Determination

SDS-PAGE and zymography were performed to determine the molecular masses of the lipases. SDS-PAGE was performed using polyacrylamide gel (10%) as describe by Laemmli [15]. Proteins were stained with Coomassie Brilliant Blue R-250. Lipolytic activity of the protein on the SDS-PAGE gel was detected by using a TCN agar plate. After the electrophoresis, the gel was washed twice with 1% (v/v) Triton X-100 for 10 min, twice again with 50 mM Tris-HCl buffer (pH 8.0) for 10 min, and then with distilled water for 10 min twice. The gel was overlaid on a TCN agar plate and incubated at 25°C until a transparent band appeared.

Effect of Temperature on Lipase Activity and Stability

The optimal temperatures of the lipases were determined by assaying their hydrolytic activity toward *p*NPC at various temperatures (10–70°C) using the spectrophotometric method. Their temperature stability was also examined by preincubation at various temperatures for 30 min before assay using the spectrophotometric method.

Effect of pH on Lipase Activity and Stability

The optimal pHs for the lipases were determined by assaying their hydrolytic activity toward *p*NPC at various pHs (pH 4–12) using spectrophotometry. The stabilities of the lipases at various pHs were examined by preincubating 20 μl of the enzyme in 180 μl of 0.1 M sodium acetate (pH 4–6), 0.1 M KH_2PO_4-K_2HPO_4 (pH 6–7.5), 0.1 M Tris-HCl (pH 7.5–9), 0.1 M KCl-glycine-KOH (pH 9–10), or 0.1 M K_2HPO_4-K_3PO_4 (pH 10–12) for 30 min and assayed using spectrophotometry.

Analysis of Substrate Specificity

Hydrolysis rates of these lipases toward various substrates including tributyrin, tricaprylin, olive oil, soybean oil, sunflower oil, fish oil, and castor oil were measured using the above pH STAT method. Hydrolysis rates toward various synthetic substrates (*p*NP-acetate, *p*NP-butyrate, *p*NP-caprylate, *p*NP-caprate, and *p*NP-laurate) were measured by the typical spectrophotometric method. A different assay method was used for *p*NP-laurate, *p*NP-myristate, *p*NP-palmitate, and *p*NP-stearate as follows. Twenty microliters of lipase solution was added to 880 μl of reaction buffer containing 50 mM Tris-HCl (pH 8.0), 0.1% gum arabic, and 0.2% deoxycholate. After 3 min incubation at 30°C, the reaction was started by adding 100 μl of 8 mM substrate (in isopropanol) and incubated again for 3 min at 30°C. The reaction was stopped by addition of 0.5 ml of 3 M HCl. After centrifugation at 12,000 ×*g* for 2 min, 333 μl of supernatant was mixed with 1 ml of 2 M NaOH and O.D. measured at 420 nm.

Cloning of *B. pumilus* Lipase Genes

Primers BPUM1F (5'-GAGTCGTATAAGATGAATAAGGGGGAATG-3') and BPUM1R (5'-TTAATTCGTATTTTGTCCTCCGCCGTTC-3') were designed based on previous *B. pumilus* lipases genes [4] and they were used to amplify the corresponding lipase genes by the following protocol; an initial denaturation at 95°C for 10 min, 35 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min, and a final extension for 10 min at 72°C. The PCR products were ligated into pGEM T Vectors (Promega, USA) and then the ligated DNAs were transformed by electroporation into *E. coli* XL1-Blue cells. Transformed *E. coli* cells were selected on LB plates containing 100 μg/ml of ampicillin, 1 mM of IPTG, and 50 μg/ml of X-gal. The resulting recombinant plasmids were purified and sequenced by T7 and SP6 promoter primers.

Lipases Gene Sequence Analyses

Percent identities of obtained DNA sequences were analyzed using the BLAST program of the NCBI. The DNA sequences were translated into amino acid sequences by using the EditSeq application from the DNASTAR program. Amino acid sequences were aligned with other known *B. pumilus* lipases using the ClustalW method in

the MegAlign application of the DNAStar program. Protein divergence (in millions of years since species divergence) was calculated based on amino acid substitutions using cytochrome S as a "molecular clock" [17]. Protein divergence (π) at each node in the phylogenetic tree (Fig. 2) was calculated according to the formula $\pi = \Sigma\ \pi_{ij}/2n$, where π_{ij} is the pairwise divergence between the i^{th} protein in one branch and the j^{th} protein in the other.

RESULTS AND DISCUSSION

Screening and Cloning of Lipase-Producing Bacteria

Fifty strains isolated from the Antarctic environment (KOPRI, Korea Polar Research Institute) were streaked on TCN agar plates to detect their lipolytic activities. As shown in Fig. 1A, 24 strains out of 50 showed clear halos around their colonies. The culture supernatants of these 24 bacteria were prepared and their extracellular lipase activities were measured by using the *p*NPC assay method. Seven bacteria (KOPRI No. 23867, 25005, 23047, 22952, 23908, 23745, and 22850) exhibiting lipase activities above 0.1 U/ml were chosen (Fig. 1B). Their 16S rRNA gene sequence analysis showed that all of them were *Bacillus pumilus* strains (data not shown). This is the first report that *Bacillus pumilus* strains producing lipases were isolated from the Antarctic. Until now, some *B. pumilus* lipases have been isolated from soil and characterized. However, their extracellular lipase activities in the culture media have not been reported, for they were too low to measure (less than 0.1 U/ml). Therefore, some research groups have tried to express recombinant *B. pumilus* lipases [13] and also tried to improve their catalytic activities [1, 5].

To clone the lipase genes of these bacteria, we performed a PCR with primers designed based on the *B. pumillus* lipase genes reported in a previous paper [3]. Six (KOPRI No. 22850, 22952, 23047, 23745, 23867, and 25005) lipase genes were cloned. All of them consisted of an open reading frame of 648 bp encoding a protein of 215 amino acids (Supplementary Fig. S1). One lipase gene (KOPRI No. 23908) was not cloned using the primer set described above.

Their protein sequences were compared both among them and with other known *B. pumilus* lipases using the BLAST program. Four lipase genes (KOPRI No. 22952, 23047, 23745, and 25005) were exactly the same and had the highest identity of 98.1% with *B. pumilus* DBRL-191 (Accession No. AAR84668). Lipase 23867 had the highest identity of 99.5% with *B. pumilus* F3 (Accession No. ABK80759). Lipase 22850 lipase had the highest identity of 93.5% with *B. pumilus* B26 (Accession No. AAL36938) (Supplementary Fig. S2).

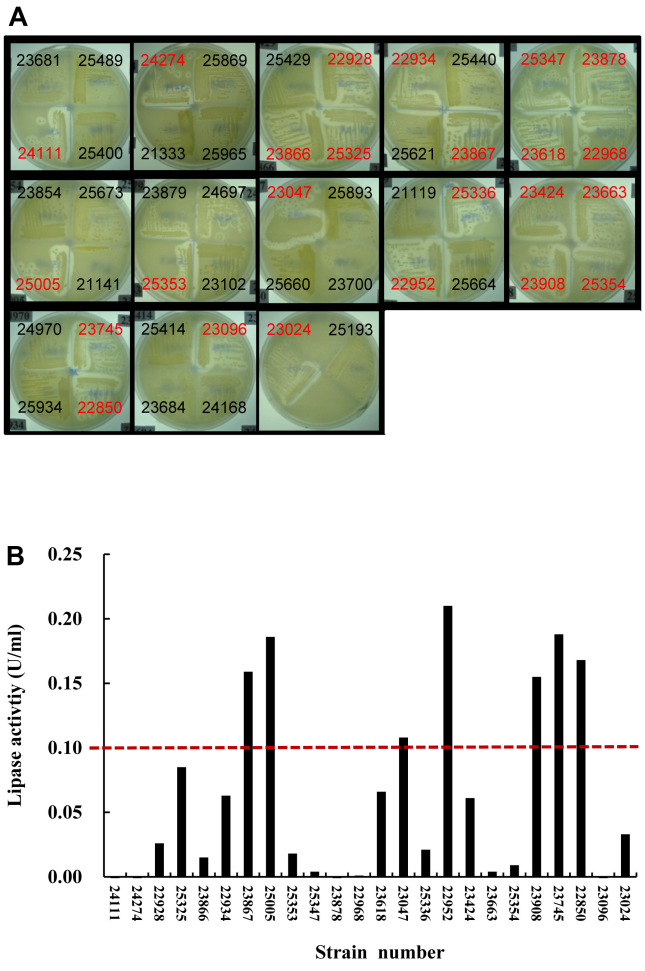

Fig. 1. Screening for lipase-producing bacteria.
(**A**) The fifty strains isolated from the Antarctic were streaked on TCN agar plates. Twenty-four strains (KOPRI No. 24111, 24274, 22928, 23866, 25325, 22934, 23867, 25347, 23878, 23618, 22968, 25005, 25353, 23047, 25336, 22952, 23424, 23663, 23908, 25354, 23745, 22850, 23096, and 23024 indicated by red color letters) out of 50 strains had a big clear zone around their colonies. (**B**) Lipase activities of the 24 bacteria were measured by *p*NPC assay.

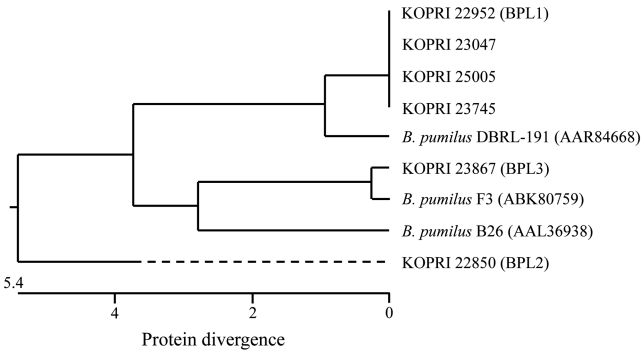

Fig. 2. Phylogenetic analysis of KOPRI No. 22952, 23047, 25005, 23745, 23867, and 22850 lipases.
The phylogenetic tree of these lipases was constructed based on complete amino acid sequence alignment with *Bacillus pumilus* DBRL-191, F3, and B26. The tree was analyzed using ClustalW in the MegAlign application.

Phylogenetic analyses clearly showed lipases 22952, 23047, 23745, and 25005 to be closely related to *B. pumilus* DBRL-191 lipase and lipase 23867 related with *B. pumilus* F3 lipase (Fig. 2). Lipase 22850 was relatively apart from most *Bacillus* lipases in the phylogenetic tree. We renamed the Antarctic *Bacillus* lipases 22952, 22850, and 23867 to lipases BPL1, BPL2, and BPL3, respectively.

Determination of Lipase Molecular Mass

The cell growth and lipase activity of *B. pumilus* 22952 were measured with culture time course. *B. pumilus* 22952 was chosen to check the cell growth, for its lipase activity was the highest among the seven strains. As shown in Fig. 3A, *B. pumilus* 22952 reached the stationary phase and started to secrete the lipase enzyme into the culture medium at 36 h of cultivation. This extracellular lipase activity dramatically increased until 54 h of cultivation time.

Next, to determine the molecular mass of the extracellular lipase, the culture supernatant at 54 h of incubation was obtained and partially purified by ammonium sulfate precipitation and dialysis. The crude enzyme was used in SDS-polyacrylamide gel electrophoresis and zymography analysis. The TCN zymogram showed formation of one band in lane 1 (Fig. 3B). The molecular mass of lipase BPL1 was estimated to be approximately 23 kDa. As mentioned earlier, the amino acids sequence of lipase BPL1 showed 98.1% identity with that of *B. pumilus* DBRL-191 lipase, consisting of a signal sequence of 34 amino acids and a mature part of 181 amino acids [21]. Lipase BPL1 was also suggested to have a mature part of 181 amino acids and the molecular mass was calculated to be 19,254 Da based on the amino acid sequence. It was somewhat different from the molecular mass observed on the zymogram. Thus, it is necessary to purify lipase BPL1 and to confirm its amino acid sequence in a further study.

Effects of Temperature and pH on Lipases Activity and Stability

After ammonium sulfate precipitation and dialysis of the culture supernatants, the lipase activities of BPL1, BPL2, and BPL3 at standard condition (pH 8.0, 35°C) were 1.19, 1.10, and 1.56 U/ml, respectively. To analyze the biochemical properties of lipases BPL2, BPL1, and BPL3, their optimal temperature and pH were tested at various temperatures ranging from 10°C to 70°C and at pHs from 6.0 to 10.0 using the standard *p*NPC assay. The highest activity of all lipase was observed at 40°C and pH 9.0 (Figs. 4A and 4C).

B. pumilus DBRL-191 lipase was reported to be optimally active at 37°C and pH 9.0 [21]. *B. pumilus* B26 had optimum temperature and pH at 35°C and 8.5, respectively [13]. Therefore, those Antarctic *Bacillus* lipases are similar to most mesophilic *Bacillus* lipases in the respect of optimal temperature and pH.

In the case of lipase BPL1, the activities at 15°C and 10°C were measured to be 60% and 40% of the maximum activity, respectively. Thus, lipase BPL1 seemed to be a typical cold adapted enzyme.

The thermostabilities and pH stabilities of the three lipases were evaluated by determining lipase activity after incubation for 30 min at temperatures from 10°C to 45°C and at pH from 4 to 12. Lipases BPL1 and BPL2 were stable up to 30°C, which had their enzymes activities higher than 70% and 80% of the maximum activity, respectively. Lipase BPL3 was stable up to 20°C, but it lost its activity gradually above 25°C (Fig. 4B). Therefore, the three Antarctic lipases were unstable over 35°C, which indicated that they were psychrotolerant enzymes.

Lipases BPL1 and BPL3 maintained their activities higher than 40% at pH 5~11, whereas lipase BPL2 maintained its activity higher than 70% at pH 6~10 (Fig. 4D). Thus, our

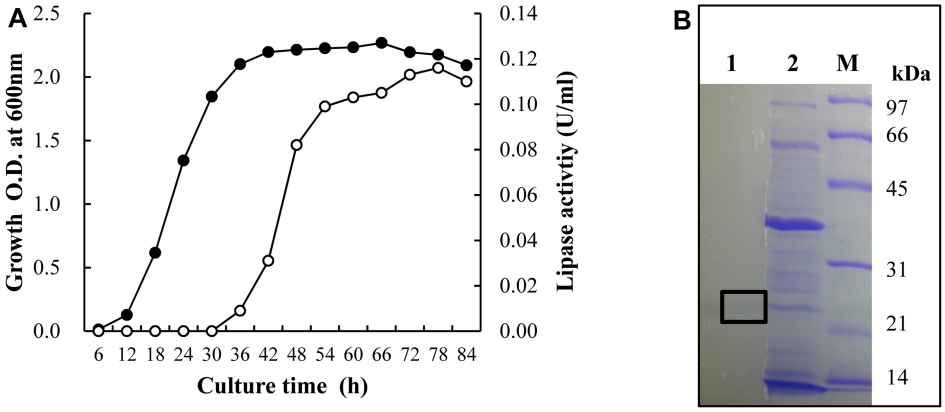

Fig. 3. Characterization of extracellular lipase BPL1.
(**A**) The growth (closed circles) and extracellular lipase activity (open circles) of *B. pumilus* 22952 were measured by cultivation time. (**B**) The molecular mass of lipase BPL1 was determined by TCN zymography (lane 1) and SDS-PAGE (lane 2). Lane M is protein size maker. The size of the band in the square was estimated to be approximately 23kDa.

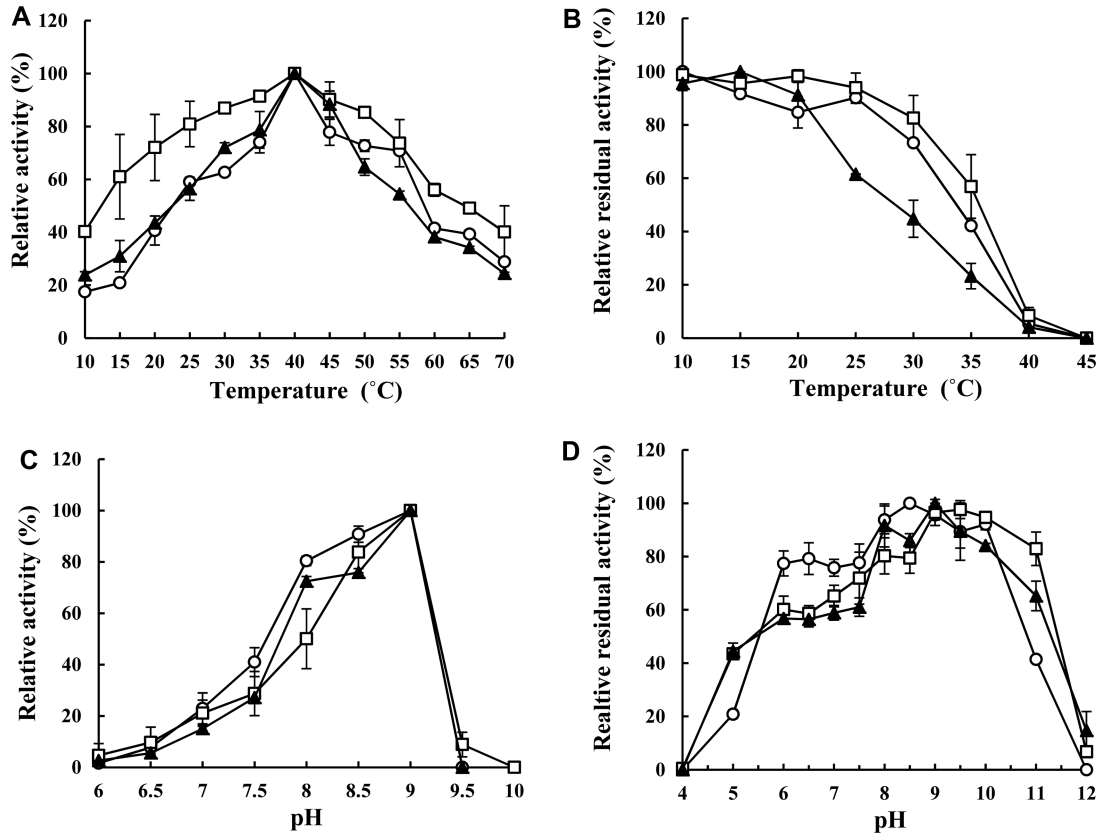

Fig. 4. Effects of temperature and pH on lipases BPL1, BPL2, and BPL3.
These lipases activities at different temperatures (**A**) and pH buffers (**C**) were assayed by *p*NPC assay. These lipases were incubated at various temperatures (**B**) and pH buffers (**D**) for 30 min and the residual activity was assayed. An error bar was used to show the SD derived from three independent experiments. Open squares mean BPL1, open circles mean BPL2, and closed triangles mean BPL3.

results indicate that the Antarctic *Bacillus* lipases are stable in a wide pH range, in particular, in alkaline pH. Thus, these enzymes can be applied in detergent industries.

Analysis of Substrate Specificity

To examine the substrate specificity of the three lipases, we tested the hydrolyzing activities toward *p*-nitrophenyl

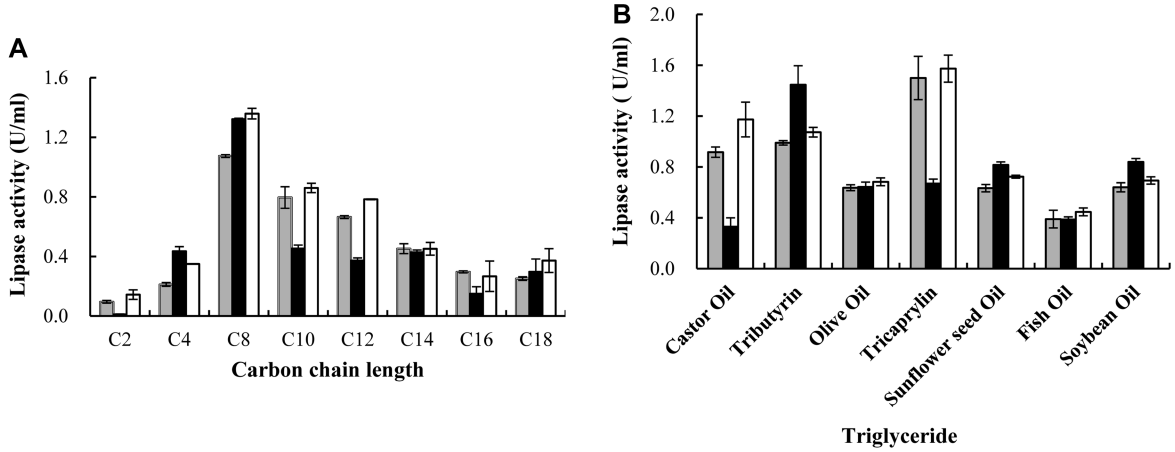

Fig. 5. Substrate specificities of lipases BPL1, BPL2, and BPL3.
Hydrolytic activities of these lipases were measured toward various *p*-nitrophenyl esters (**A**) and triglycerides (**B**). An error bar was used to show the SD derived from three independent experiments. Black bars mean BPL1, gray bars mean BPL2, and white bars mean BPL3.

esters with various acyl chain lengths (acetate, C_2; butyrate, C_4; caprylate, C_8; caprate, C_{10}; laurate, C_{12}; myristate, C_{14}; palmitate, C_{16}; stearate, C_{18}) and oil types. As shown in Fig. 5A, the three lipases had the highest hydrolysis activity toward *p*-nitrophenyl caprylate (C_8) substrate. Lipases BPL2 and BPL3 had broader specificity toward long-chain substrates (C_{10} to C_{18}) in comparison with lipase BPL1. This result was quite different from other reports showing that cold active lipase *Moraxella* TA144 lipase 2 [7] showed maximum relative activity on butyrate (C_4, 100%) and very low activities toward C_6 (4%), C_{16} (3%), and C_{18} (2%) esters.

Furthermore, lipase BPL1 showed high activity toward tributyrin. Lipases BPL2 and BPL3 showed high activity toward tricaprylin (Fig. 5B). The three lipases hydrolyzed many natural oil substrates such as castor oil, olive oil, sunflower seed oil, fish oil, and soybean oil (Fig. 5B). Most of the lipases from *Bacillus* sp. were reported to have substrate specificity toward short-chain fatty acid esters [13, 16, 18]. In contrast, the Antarctic *Bacillus* lipases showed strong hydrolytic activity toward short and/or medium-chain fatty acids (C_8~C_{12}), and many natural triglycerides.

In conclusion, three *B. pumilus* lipases were screened from the Antarctic. The three enzymes were cold active and alkaline-tolerant lipases and showed the highest hydrolytic activity with *p*-nitrophenyl caprylate among various *p*-nitrophenyl esters examined. In the future, functional expression of these enzymes in a heterogeneous host may develop their economical and practical applications in the detergent and food industries.

Acknowledgments

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