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Expression and Biochemical Characterization of Cold-Adapted Lipases from Antarctic *Bacillus pumilus* Strains S

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Two lipase genes (*bpl1* and *bpl3*) from Antarctic *Bacillus pumilus* strains were expressed in *Bacillus subtilis*. Both recombinant lipases BPL1 and BPL2 were secreted to the culture medium and their activities reached 3.5 U/ml and 5.0 U/ml, respectively. Their molecular masses apparent using SDS-PAGE were 23 kDa for BPL1 and 19 kDa for BPL3. Both lipases were purified to homogeneity using ammonium sulfate precipitation and HiTrap SP FF column and Superose 12 column chromatographies. The final specific activities were estimated to be 328 U/mg for BPL1 and 310 U/mg for BPL3. Both lipases displayed an optimum temperature of 35ºC, similar to other mesophilic enzymes. However, they maintained as much as 70% and 80% of the maximum activities at 10ºC. Accordingly, their calculated activation energy at a temperature range of 10−35ºC was 5.32 kcal/mol for BPL1 and 4.26 kcal/mol for BPL3, typical of cold-adapted enzymes. The optimum pH of BPL1 and BPL3 was 8.5 and 8.0, respectively, and they were quite stable at pH 7.0−11.0, showing their strong alkaline tolerance. Both lipases had a preference toward medium chain length (C_6-C_{10}) fatty acid substrates. These results indicate the potential for the two Antarctic *B. pumilus* lipases as catalysts in bioorganic synthesis, food, and detergent industries.

Keywords: Lipase, *Bacillus pumilus*, Antarctic, cold-adapted enzyme

Introduction

Lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) catalyze the hydrolysis of triacyglycerols to glycerols and fatty acids at oil-water interfaces. Lipases are ubiquitous in most living organisms, including animals, plants, and microbes [2, 8, 9, 24]. Aside from their natural importance, developments in enzyme technology have exploited lipases as important tools, with their potential applications including biodiesel production, biosensor construction, polymer synthesis, and functional lipid production [9, 21, 24]. Most of the lipases that are widely used industrially are microbial lipases, reflecting advantages that include diverse catalytic activities, high production yields, ease of genetic manipulation, and consistent supply due to the absence of seasonal fluctuations [6, 9, 21, 24].

Among the known bacterial lipases, *Bacillus* lipases have been classified as subfamily I.4. They are of interest from a research point of view and because of the industrial prowess. They are the smallest lipases known, with an approximate mass of 19 kDa, and possess minimum α/β hydrolase folds and a solvent-exposed substrate binding site (*i.e.*, absence of lid structure). In addition, *Bacillus pumilus* and *Bacillus subtilis* strains are "generally recognized as safe" (GRAS) microorganisms. Hence, there are no restrictions on the use of their lipases in diverse industries, including food and feedstock industries.

Psychrophilic lipases have attracted great attention for their increasing uses in the organic synthesis of fine chemicals owing to their high activity at low temperatures, which is a favorable property for the production of temperature-sensitive compounds [5, 7, 11, 19, 25]. In

addition, cold-adapted lipases are of great value in other practical applications such as bioremediation in fat-contaminated cold environments and laundry washing in cold water [5, 10, 22]. Cold regions, including the Arctic and the Antarctic, and intertidal mud-flats have proven to be good environments for isolating novel psychrophilic lipases [7, 20].

As the demand for the microbial lipases in many industries increases, the efficient and large-scale production of recombinant lipases is required. *Escherichia coli* can be a host for the production of recombinant lipases. However, the enzymes usually accumulate in the cytoplasm or periplasmic space of the gram-negative bacteria, necessitating destruction of the cells to obtain the enzymes and hindering their purification [3, 13, 23]. In contrast, in a *Bacillus* expression system, the recombinant lipases are secreted to the culture medium, which simplifies their recovery and purification.

In this study, two different recombinant lipase genes (*bpl1* and *bpl3*) from *B. pumilus* isolated from Antarctica [1] were expressed in a *B. subtilis* expression system using the pMA5 plasmid [26]. The enzymes were purified and characterized for their biochemical properties.

Materials and Methods

Materials

p-Nitrophenyl esters, tributyrin, tricaprylin, castor oil, coconut oil, olive oil, soybean oil, and sunflower oil were purchased from Sigma-Aldrich (St. Louis, MO, USA). Palm oil was acquired from NewDia (South Korea).

Cloning of Antarctic *Bacillus* Lipase Genes

The Antarctic *Bacillus* lipase genes (*bpl1* and *bpl3*) that were previously obtained [1] were used as template DNA for polymerase chain reaction (PCR). PCR primers were designed to carrying the *Nde*I restriction site at its 5′ end and the *Bam*HI restriction site at its 3′ end as follows: BPL1 forward primer 5′-GAACATATGAAAGTGATT CTTTTTAAG-3′, BPL3 forward primer 5′-GAACATATGAAA GTGATTCGTTTTAAG-3′, and the common reverse primer 5′-GCAGGATCCTTAATTCGTATTTTGTCCTCC-3′. PCR involved pre-denaturation at 94°C for 5 min, denaturation at 95°C for 30 sec, annealing at 47°C for 30 sec, elongation at 72°C for 1 min, and a post-elongation at 72°C for 7 min. The PCR products were ligated with the pGEM-T vector and the ligation mixtures were transformed into *E. coli* XL1-Blue. The resulting recombinant plasmids (pTBPL1 and pTBPL3) were purified from the *E. coli* cells.

E. coli-Bacillus shuttle vector pMA5 [26] was used to clone the lipase genes as follows. The vector was digested using *Nde*I and *Bam*HI, treated with alkaline phosphatase, and ligated with the *bpl1* gene (or *bpl3* gene) obtained from pTBPL1 (or pTBPL3).

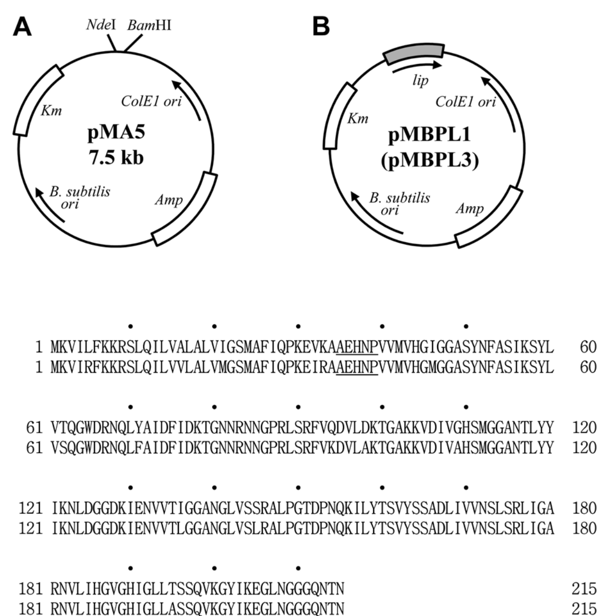

Fig. 1. Construction of the expression plasmids.

(**A**) An *E. coli-Bacillus* shuttle vector, pMA5, is shown. (**B**) The Antarctic *B. pumilus* lipase genes (*bpl1* and *bpl3*) were inserted into the *Nde*I and *Bam*HI sites of vector pMA5. The resulting pMBPL1 (or pMBPL3) is shown. (**C**) The amino acid sequences of BPL1 and BPL3 were aligned. The N-terminal sequences of their mature enzymes are underlined.

Ligation mixtures were transformed into *E. coli* XL1-Blue and the resulting recombinant plasmids (pMBPL1 and pMBPL3) were obtained from the *E. coli* cells (Fig. 1).

Expression of the Antarctic *Bacillus* lipases in *B. subtilis*

pMBPL1 and pMBPL3 were transformed into *B. subtilis* DB104 as follows. The recombinant plasmids (1 µg each) were mixed with 50 µl of competent *B. subtilis*, treated by a single pulse of 0.7 kV for electroporation as previously described [12, 17], added to 1 ml of LB-Broth, and incubated at 37°C for 1 h. The transformed *B. subtilis* cells were spread on a LB agar plate containing 10 µg/ml kanamycin and incubated overnight at 37°C. Colonies were streaked on another LB agar plate containing 1% tributyrin and 10 µg/ml kanamycin. The colonies that developed from the bacteria harboring pMBPL1 or pMBPL3 displayed a surrounding clear zone.

Modified LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1% K_2HPO_4, 0.04% $MgSO_4$, 4% glucose) was used to grow the recombinant *B. subtilis*. *B. subtilis* was cultured at 30°C for 24 h in a shaking (200 rpm) incubator, harvested by centrifugation (6,000 ×*g* for 10 min), and the culture supernatant was obtained. Ammonium sulfate was added to 30% saturation and the resulting precipitate was removed by centrifugation (10,000 ×*g* for 10 min). Ammonium sulfate was added a second time to the supernatant to 70% saturation and the resulting pellet was

collected by centrifugation (10,000 ×*g* for 10 min). The combined insoluble material was dissolved using distilled water. PD G10 desalting column chromatography was performed to remove residual ammonium sulfate. The Bradford protein assay was performed to check the protein concentration.

Lipase Assay

Tributyrin (TBN)-LB agar containing 1% TBN, 1× gum arabic solution, 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar was prepared. The gum arabic stock solution (10×) contained 10% (w/v) gum arabic, 200 mM NaCl, and 50 mM $CaCl_2$. Colonies with a surrounding halo were selected.

Lipase activity was measured using a *p*-nitrophenyl caprylate (pNPC) assay as follows. A pNPC reaction mixture (1 ml) consisted of 10 µl of 20 mM pNPC (in acetonitrile), 40 µl of ethanol, and 950 µl of Tris-HCl buffer (50 mM, pH 8). The reaction mixture was mixed with 10 µl of lipase solution and incubated at 37°C for 3 min and the absorbance at 405 nm was measured. One unit of enzyme activity was defined as the quantity of enzyme required to generate 1 µmol of *p*-nitrophenol via the hydrolysis of pNPC substrate in 1 min.

Lipase activity was also measured by titrating the free fatty acids released by hydrolysis of tributyrin (or tricaprylin), using the pH-stat method [12]. Substrate emulsion containing 1% substrate and 1% gum arabic was prepared by blending in a model 51BL31 Waring blender 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 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 1 unit.

Purification of BPL1 and BPL3

Ammonium sulfate-precipitated pellet (30−70% fraction) was dissolved using potassium phosphate buffer (20 mM, pH 7.0) with a ratio of 1 ml buffer to 100 ml culture supernatant. Dialysis was performed with a Spectra/Por 4 membrane (Spectrum Labs, USA) to remove the residual ammonium sulfate.

A HiTrap SP FF cation exchanger column (5 ml; GE Healthcare, Buckinghamshire, UK) in a fast protein liquid chromatography (FPLC) system was equilibrated with potassium phosphate buffer (20 mM, pH 7.0) and 10 ml of the aforementioned dialysate was loaded onto the column. Washing was done using the same buffer. Lipase bound to the resin was eluted using the buffer, with an increasing gradient of KCl from 0 to 0.5 M. Active fractions were collected and protein concentrations were measured.

A Superose 12 10/300 GL gel permeation column (GE Healthcare) in a FPLC system was equilibrated using a solution of 20 mM potassium phosphate and 150 mM NaCl (pH 7.0). Active fractions (1 ml) from the SP FF column were loaded and eluted using the same buffer. Active fractions were collected and the protein concentration was checked. The purity of lipase enzyme in the active fractions was checked using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Effect of Temperature on Lipase Activity and Stability

The optimal temperatures of the lipases were determined by assaying their hydrolytic activity toward pNPC at various temperatures (10−60°C), using an established spectrophotometric method. Lipase temperature stability was also examined by pre-incubation at various temperatures (10−40°C) for 30 min.

Effect of pH on Lipase Activity and Stability

The optimal pH for lipase activity was determined by assaying the hydrolytic activity toward pNPC at pHs ranging from 5−12, using an established spectrophotometric method. The stability of the lipases at various pHs was examined by pre-incubating 5 µl of enzyme in 45 µl of 50 mM sodium acetate (pH 5−6), 50 mM KH_2PO_4-K_2HPO_4 (pH 6−7.5), 50 mM Tris-HCl (pH 7.5−9), 50 mM KCl-glycine-KOH (pH 9−11), or 50 mM K_2HPO_4-K_3PO_4 (pH 11−12) buffers for 30 min and assaying spectrophotometrically.

Substrate Specificity

The hydrolysis rates of various substrates, including tributyrin, tricaprylin, olive oil, soybean oil, sunflower seed oil, coconut oil, palm oil, and castor oil, were measured using the pH-stat method [12] at 30°C. The hydrolysis rates of various synthetic substrates, including pNP-acetate (C_2), pNP-butyrate (C_4), pNP-caproate (C_6), pNP-caprylate (C_8), pNP-caprate (C_{10}), and pNP-laurate (C_{12}), were measured using an established spectrophotometric method. In addition, a different assay method was used for pNP-laurate (C_{12}), pNP-myristate (C_{14}), pNP-palmitate (C_{16}), and pNP-stearate (C_{18}). Lipase solutions were added to 0.88 ml of reaction buffer containing 50 mM Tris-HCl (pH 8.0), 0.1% gum arabic, and 0.2% deoxycholate. After a 3 min incubation at 30°C, the reaction was started by adding 0.1 ml of 8 mM substrate solubilized in isopropanol and incubating for 3 min at 30°C. The reaction was stopped by the addition of 0.5 ml of 3 M HCl. After centrifugation at 12,000 rpm for 2 min, 333 µl of the supernatant was mixed with 1 ml of 2 M NaOH and the optical density at a wavelength of 420 nm (OD_{420}) was measured.

Results and Discussion

Expression of Lipases BPL1 and BPL3 in *B. subtilis*

Antarctic *B. pumilus* strains producing lipases were isolated recently [1]. However, the lipase activities of their culture broth were low (approximately 0.1 U/ml), which hindered the purification of the lipase from the culture broth. These findings agreed with the previous report that *Bacillus* species possess stringent regulatory mechanisms of lipase synthesis and so do not exuberantly produce lipase [16]. For this reason, some research groups have tried to express recombinant *Bacillus* lipases in *E. coli* or *Bacillus* systems [12, 15, 16].

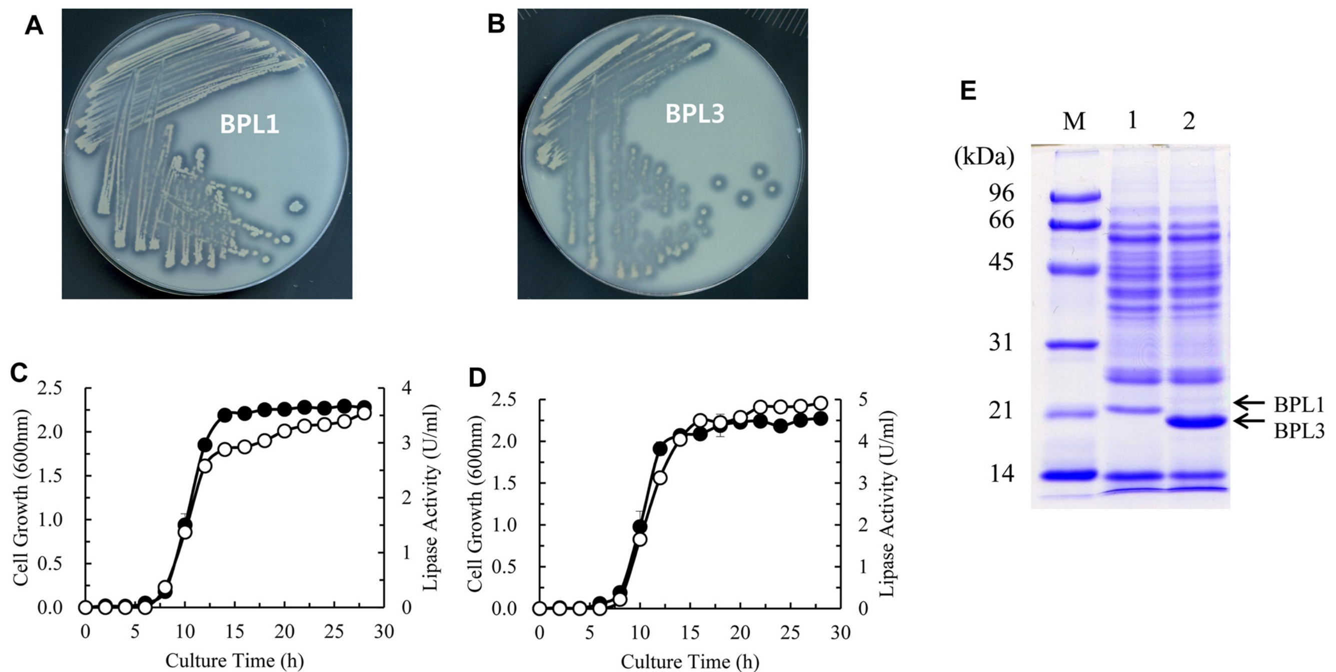

Fig. 2. Expression of *bpl1* and *bpl3* genes in *Bacillus subtilis*.

(**A**) *Bacillus* cells harboring *bpl1* were streaked on TBN agar plates. (**B**) *Bacillus* cells harboring *bpl3* were streaked on TBN agar plates. (**C**) The growth (black circles) and extracellular lipase activity (white circles) of *Bacillus* cells (*bpl1*) were measured with cultivation time course. (**D**) The growth (black circles) and extracellular lipase activity (white circles) of *Bacillus* cells (*bpl3*) were measured with cultivation time course. (**E**) The molecular masses of BPL1 and BPL3 were determined by SDS-PAGE. Lane M is protein size marker. Lanes 1 and 2 are the culture supernatants of *Bacillus* cells harboring *bpl1* and *bpl3*. BPL1 and BPL3 are indicated with arrows.

Presently, two recombinant plasmids (pMBPL1 and pMBPL3) containing the Antarctic *Bacillus* lipase genes (*bpl1* and *bpl3*) were constructed (Fig. 1) and used to transform *B. subtilis* DB104 cells. The resulting recombinant *Bacillus* cells showed clear zones on TBN-LB agar plates after 24 h incubation (Figs. 2A and 2B), which was evidence of extracellular lipase activity.

Cell growth and extracellular lipase activity were measured with cultivation time. The secretion of lipase was evident from both transformed *Bacillus* cells, beginning at 8 h of cultivation (Figs. 2C and 2D). The extracellular lipase activity increased along with the cell growth and attained 3.5 U/ml for BPL1 and 5 U/ml for BPL3 after 24 h of cultivation. Lipase activities in the culture broth of the wild-type *B. pumilus* strains (BPL1 and BPL3) were reported to be about 0.1 U/ml [1]. Thus, the present lipase activities of the recombinant *Bacillus* strains represent increases of 35-fold and 50-fold, respectively, in comparison with their wild-type strains. Culture supernatants were obtained from *Bacillus* cells harboring *bpl1* and *bpl3* after 24 h incubation and the lipases were partially purified by ammonium sulfate precipitation and desalting column chromatography. The specific activities of the crude lipases were 84.7 U/mg and 83.1 U/mg, respectively.

Many research groups have tried to express *Bacillus* lipases in *E. coli*. The various lipases were produced as intracellular enzymes and the N-terminal signal sequence was not removed [15]. Moreover, the specific activity of the crude cell extract was low (approximately 1.6 U/mg) [16].

SDS-PAGE of the crude enzyme preparation estimated the molecular masses of BPL1 and BPL3 to be 23 kDa and 19 kDa, respectively (Fig. 2E). Thereafter, N-terminal amino acid sequencing of the two protein bands was performed. Both N-terminal sequences were identified to be NH_2-Ala-Glu-His-Asn-Pro- (Supplementary Fig. S1), which implied that the two proteins were the mature part of the lipases and also that both 34-amino-acid signal sequences were removed correctly during their secretion to the culture medium. Both mature enzymes comprised 181 amino acid residues and their calculated molecular masses were 19,254 Da (BPL1) and 19,208 Da (BPL3) (Fig. 1). Thus, the calculated molecular mass and the observed mass on

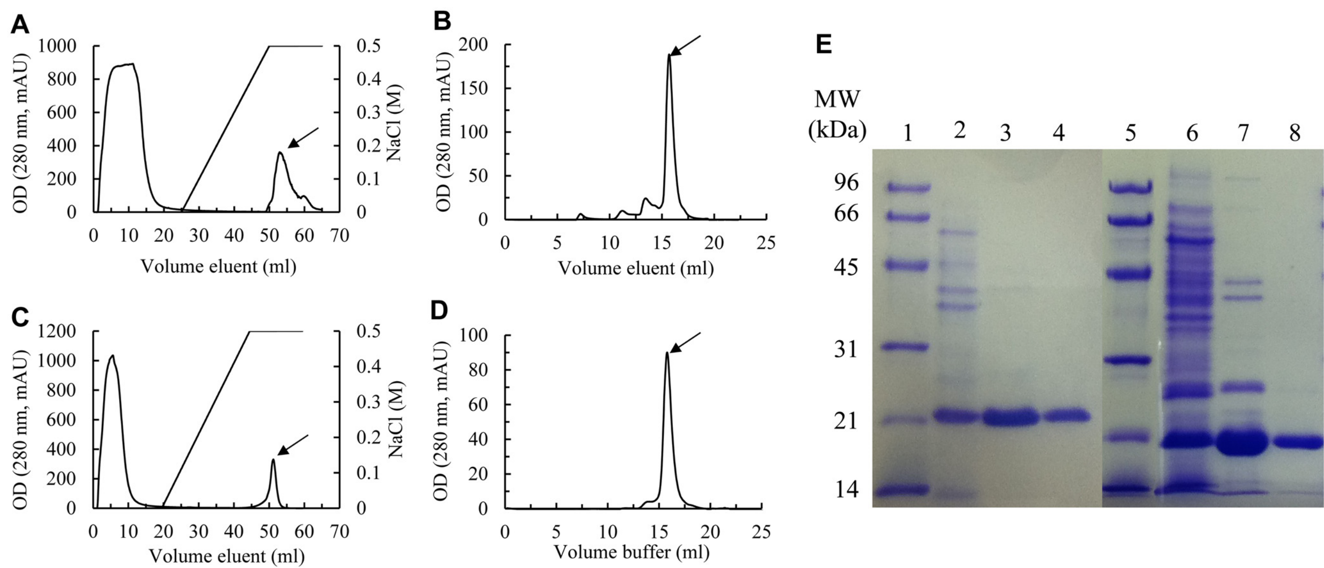

Fig. 3. Purification of BPL1 and BPL3.

BPL1 was purified by ion-exchange chromatography (**A**) and gel filtration chromatography (**B**). BPL3 was purified by ion-exchange chromatography (**C**) and gel filtration chromatography (**D**). (**E**) SDS-PAGE of BPL1 and BPL3 was performed. Lanes: 1 and 5, protein size marker; 2, BPL1 after ammonium sulfate precipitation; 3, BPL1 after ion-exchange chromatography; 4, BPL1 after gel filtration chromatography; 6, BPL3 after ammonium sulfate precipitation; 7, BPL3 after ion-exchange chromatography; 8, BPL3 after gel filtration chromatography.

SDS-PAGE were similar for lipase BPL3 but a little different for lipase BPL1. This discrepancy was unusual and difficult to explain at present.

Purification of Lipases BPL1 and BPL3

Ammonium-sulfate-precipitated protein samples were dialyzed against potassium phosphate buffer (20 mM, pH 7.0) and loaded onto a cation-exchange column (HiTrap SP FF). The calculated isoelectric (pI) value for BPL1 and BPL3 was 9.35 and 9.62, respectively, and a positive charge of 4.7 and 6.7, respectively, was evident at pH 7.0. Hence, both bound avidly to the negatively charged resin. Lipases BPL1 and BPL3 were eluted with a linear increasing gradient of KCl (Figs. 3A and 3C). The active fractions from the cation columns were collected and loaded onto a Superose 12 gel permeation column. The active fractions were collected again (Figs. 3B and 3D) and used for the following enzyme characterization experiments.

SDS-PAGE was performed using active fractions for each purification steps. The electrophoretic results indicated that both BPL1 and BPL3 were purified to homogeneity after gel permeation chromatography (Fig. 3E).

Summing up, the purification yield was 18.2% and the final specific activity was estimated to be 328 U/mg for BPL1 lipase (Table 1) and the purification yield of BPL3 was 23.3% and the final specific activity was 310 U/mg

Table 1. Purification results of BPL1 and BPL3.

	Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
BPL1	$(NH_4)_2SO_4$	7.14	605	84.7	100	1 .00
	HiTrap SP FF	1.43	340	238	56 .2	2.81
	Superose 12	0.335	110	328	18 .2	3.87
BPL3	$(NH_4)_2SO_4$	12.4	1030	83.1	100	1 .00
	HiTrap SP FF	2.57	797	310	77 .3	3.73
	Superose 12	0.775	240	310	23 .3	3.73

(Table 1) when pNPC was used as a substrate. The turnover numbers (k_{cat}) of BPL1 and BPL3 were calculated to be 105 s^{-1} and 99.3 s^{-1}, respectively.

The specific activity of *B. licheniformis* lipase [16] and *B. licheniformis* RSP-09 [15] is reported to be 130 U/mg and 288 U/mg, respectively. The specific activity of *B. pumilus* B26 was reported to be 138 U/mg [12]. The k_{cat} value of *B. pumilus* GMA1 was 1 s^{-1} [4]. In comparison, lipases BPL1 and BPL3 have higher activities than the most known *Bacillus* lipases.

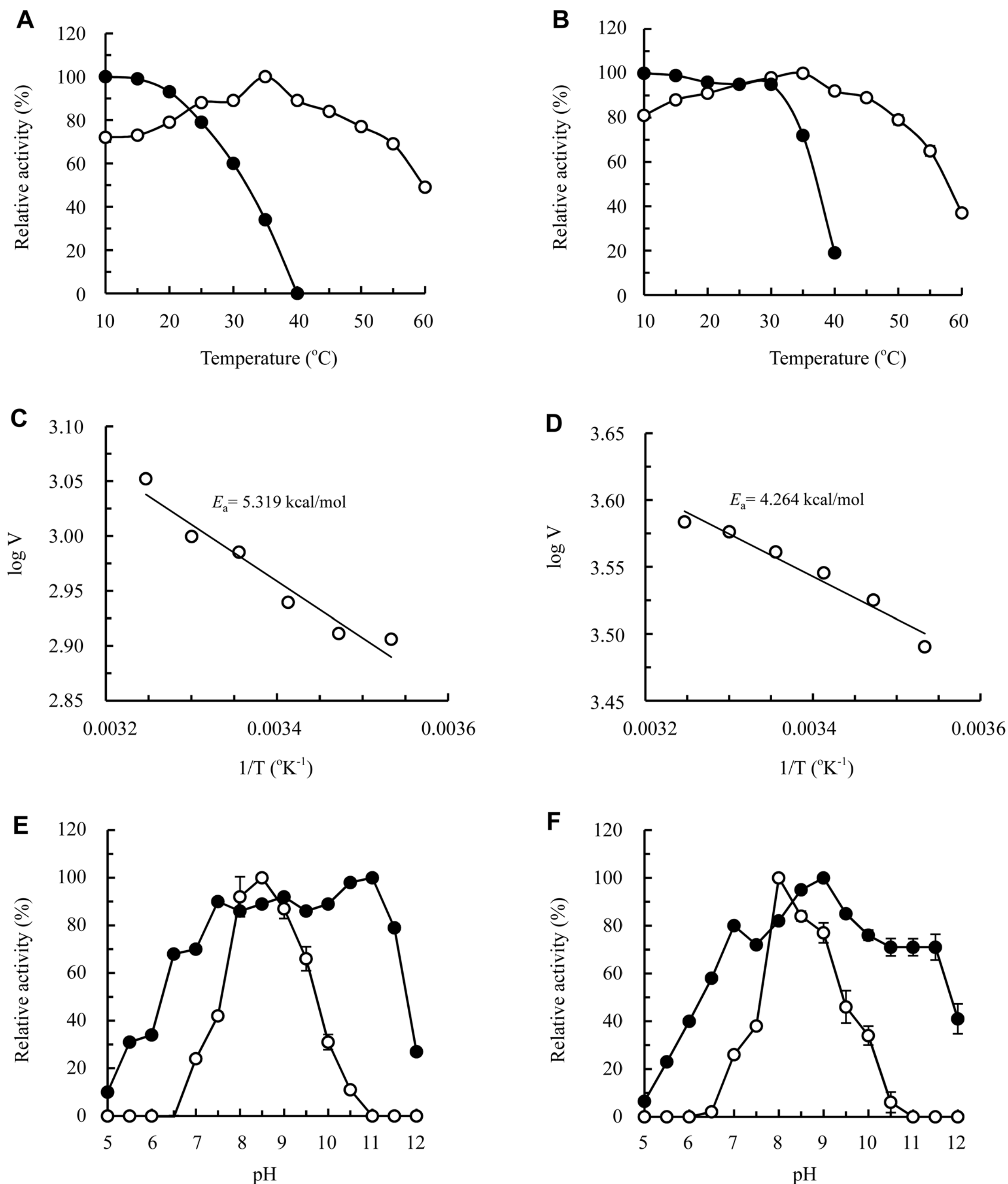

Fig. 4. Effects of temperatures and pHs on BPL1 and BPL3.

Lipases BPL1 (**A**) and BPL3 (**B**) activities at different temperatures were assayed by pNPC assay. The activation energies of BPL1 (**C**) and BPL3 (**D**) were calculated from their activities at different temperatures, using the Arrhenius equation. Lipases BPL1 (**E**) and BPL3 (**F**) activities at different pHs were assayed by pNPC assay. The error bar indicates the SD from three independent experiments. Open circles mean optimum activities and closed circles mean stabilities.

Effects of Temperature and pH on Lipase Activity and Stability

As lipases BPL1 and BPL3 were Antarctic *Bacillus* enzymes, we tried to examine the temperature effects on the enzymes. Both lipases showed maximum activities at 35°C (Figs. 4A and 4B). They displayed rather high activity at low temperatures. That is, as much as 70% and 80% of the maximum activity were detected at 10°C. Activation energies were calculated using the Arrhenius equation. Figs. 4C and 4D show linear graphs between the logarithms of velocity toward the reciprocals of absolute temperatures. Based on each slope, the activation energy was calculated to be 5.32 kcal/mol and 4.26 kcal/mol for BPL1 and BPL3, respectively. These low activation energies are the typical characteristic of most cold-adapted enzymes [11, 14, 18, 19].

The thermostabilities of lipases BPL1 and BPL3 were evaluated by determining the residual activity after incubation for 30 min. Lipase BPL1 was stable up to 25°C but lost its activity gradually above 30°C (Fig. 4A). Lipase BPL3 was stable until 30°C and lost its activity above 30°C (Fig. 4B). Both enzymes were unstable over 35°C. In comparison, the optimum temperature of *B. licheniformis* lipase is 55°C and is stable up to 45°C [16]. *B. licheniformis* RSP-09 lipase and *B. pumilus* B26 lipase have an optimum temperature of 40°C and are stable up to 40°C [15]. *B. pumilus* GMA1 lipase has an activation energy of 16.7 kcal/mol [4]. In comparison, when comparing with the other *Bacillus* lipases, the Antarctic *Bacillus* lipases BPL1 and BPL3 had comparatively lower optimum temperatures and very low activation energies as cold-adapted enzymes.

The optimum pHs were measured to be pH 8.5 for BPL1 and pH 8.0 for BPL3 (Figs. 4E and 4F). Lipase BPL1 had activities higher than 60% at pHs ranging from 6.5–11 (Fig. 4E), and lipase BPL3 had activities higher than 50% at pHs ranging from 6.5–11.5 (Fig. 4F). These results indicated the stability of both lipases over a wide pH range, in particular, alkaline pHs. In comparison, *B. licheniformis* lipases have an optimum pH of 10–11.5 and are stable up to pH 12, suggesting that they are alkaline lipases [15, 16]. *B. pumilus* B26 lipase was reported to have an optimum pH of 8.5 [12].

Substrate Specificity

To examine the substrate specificity of the BPL1 and BPL3 lipases, hydrolyzing activities toward *p*-nitrophenyl esters with various acyl chain lengths and oil types were measured. As shown in Fig. 5A, both lipases had the highest activity towards pNPC (C_8) substrate. In comparison, it was reported that most of the esterases and lipases from *Bacillus* species show specificity toward short and medium chain fatty acids [15].

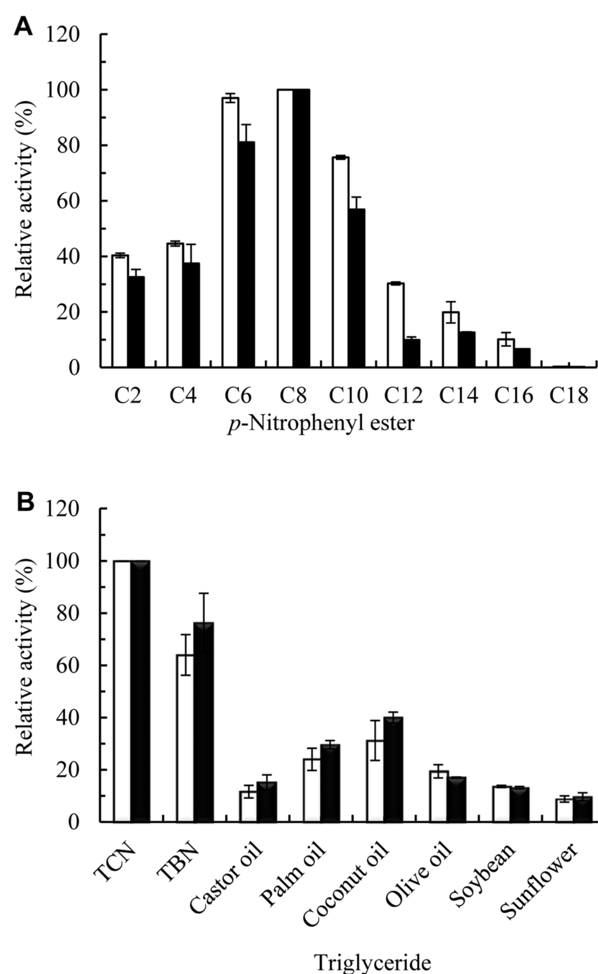

Fig. 5. Substrate specificities of lipases BPL1 and BPL3.
The hydrolytic activities of these lipases were measured toward various *p*-nitrophenyl esters (**A**) and triglycerides (**B**). An error bar indicates the SD derived from three independent experiments. White bars denote BPL1 and black bars denote BPL3.

Both lipases displayed the greatest activity toward tricaprylin (Fig. 5B). They could hydrolyze tributyrin, palm oil, castor oil, coconut oil, soybean oil, sunflower seed oil, and olive oil. These results confirmed that BPL1 and BPL3 are true lipases capable of hydrolyzing triglycerides.

In conclusion, two different Antarctic *B. pumilus* lipases were successfully produced in *B. subtilis*. They were purified to homogeneity using ion-exchange and gel permeation chromatographies. Both lipases were cold-adapted and alkaline-tolerant enzymes. These results suggest that they can be used in bioorganic synthesis and detergent industries.

Acknowledgments

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