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Full Length Research Paper

Extracellular lipase of the antarctic bacterial isolate, *Pseudomonas* sp. INK1 as a potential tool for improving the flavor quality of dairy products

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An antarctic bacterial isolate displayed extracellular lipolytic activity. Based on the 16S rRNA sequence analysis, the strain was named *Pseudomonas* sp. INK1. The INK1 lipase was secreted into the production medium during transition to the stationary phase. The enzyme reached the apparent maximal activity at pH 8 to 9, with optimal activity at 50°C. The enzyme was active against a wide range of fatty acid esters of *p*-nitrophenyl, showing the highest activity towards *p*-nitrophenyl caprylate. It could also release caprylic acid from a natural substrate cream. The enzyme activity was strongly inhibited by Zn^{2+} and Mn^{2+}, and slightly enhanced by EDTA. INK1 lipase may be a potentially useful catalyst to enhance the buttery flavor of dairy products.

Key words: Antarctic, lipolytic, *Pseudomonas* sp., catalyst, dairy products.

INTRODUCTION

Lipases (EC 3.1.1.3) are hydrolases that act on carboxylic ester bonds and catalyze the hydrolysis of triacylglycerols to free fatty acids and glycerol (Houde et al., 2004; Snellman et al., 2002). Lipases are widely distributed in nature, occurring in the animal and plant kingdoms, as well as, in molds and bacteria (Gupta et al., 2004). So far, most lipases that have been extensively screened and characterized are derived from micro-organisms which are regarded as the best sources for biotechnological applications in the food, detergent, textile, cosmetic, paper, pharmaceutical and energy industries because of their favorable catalytic properties and easy bulk production (Hasan et al., 2006; Houde et al., 2004). Bacterial lipases from *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Chromobacterium* and *Pseudomonas* strains are presently commercially exploited (Gupta et al., 2004).

In particular, lipases have been extensively employed in animal husbandry-related industries such as dairy, meat processing, and leather manufacturing sectors (Hasan et al., 2006). Current applications include the hydrolysis of milk fat, flavor enhancement of cheeses, acceleration of cheese ripening, lipolysis of butterfat and cream, production of leaner meat like fish, fermentative improvement of sausage ripening, and eco-friendly degreasing of animal skins (Hasan et al., 2006; Lacumin et al., 2007; Muthukumaran and Dhar, 1982). Moreover, microbial lipases have been applied as a feed additive so as to increase fat digestion in the gut of young broiler chicks (Al-Marzooqi and Leeson, 1999) or to generate specific antimicrobial medium chain fatty acids in the foregut of piglets (Dierick et al., 2002a, b).

Some useful and unusual enzymes that exhibit quite different catalytic properties have been reported from so-called extremophiles that inhabit Antarctica (Demirjian et al., 2001). Considering that the number of microbes cultured to date remains only a tiny fraction of all microbial species on earth, the number of novel enzymes is expected to increase continuously (Park et al., 2007). Interestingly, a recent study reveals that glacier soil, one of extreme environments can be an attractive resource of microbial lipase diversity (Yuhong et al., 2009). Lipases from psychrophilic microbes might offer novel

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opportunities in many industrial processes in which high enzymatic activity or peculiar stereospecificity at low temperature and low thermal stability are required (de Pascale et al., 2008). For instance, heat labile enzymes can be easily inactivated by treatment for short periods of time at relatively low temperatures after being used for processing of food and other materials (Margesin and Schinner, 1994), which can prevent the materials from damage during heat inactivation of the enzymes (Choo et al., 1998). Furthermore, a lipase from the psychrophilic *Pseudomonas* sp. previously isolated in Alaska displayed higher activity towards short to mid chain fatty acids than long chain fatty acids (Choo et al., 1998), which might have the strongest impact on the flavor intensities of the cream (Saerens et al., 2008). In this report, data are presented concerning the partial characterization of extracellular lipolytic activity derived from an Antarctic bacterial isolate, *Pseudomonas* sp. INK1.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Bacterial isolate INK1 derived from Antarctic soil samples was supplied by the Korea Polar Research Institute operating the King Sejong Station (South Korea) in Antarctica. Screening for lipase activity was performed on selective agar plates (0.5% tributyrin (Sigma-Aldrich), 0.5% tryptone, 0.07% KH_2PO_4, 0.01% $CaCl_2.2H_2O$, 0.01% $MgSO_4.7H_2O$, 0.01% NaCl, 0.001% $FeSO_4.7H_2O$, 0.001% $MnSO_4.4H_2O$, 1.5% bacto agar (Difco); pH 7.4) at 30°C by observing a clear zone of hydrolyzed tributyrin around the colonies, as previously described by Wei et al. (2009). Growth and lipase production were investigated in 100 ml of a lipase production medium (1% soybean oil, 1% tryptone, 0.07% KH_2PO_4, 0.01% $CaCl_2.2H_2O$, 0.01% $MgSO_4.7H_2O$, 0.01% NaCl, 0.001% $FeSO_4.7H_2O$, 0.001% $MnSO_4.4H_2O$; pH 7.4) in a 500 ml Erlenmeyer flask, aerobically incubated with vigorous shaking (220 rpm), by monitoring the absorbance (O.D.600 nm) and lipase activity of the culture supernatant at 30°C at various times.

Taxonomic identification of strain INK1

Genomic DNA was extracted from strain INK1 using the FastDNA kit (Qbiogene) according to the manufacturer's protocol. The 16S rRNA gene was amplified from the genomic DNA by PCR using the universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (William et al., 1991). The amplified 1,400 bp sequences were determined by an automated ABI PRISM 3730 XL DNA analyzer (Applied Biosystems). The resulting sequences were compared with the GenBank database (NCBI) using BLAST (Altschul et al., 1990). Sequences showing a relevant degree of similarity were imported into the CLUSTAL W program (Thompson et al., 1994) and aligned. Evolutionary distances to other strains of *Pseudomonas* were computed using the Maximum Composite Likelihood method (Tamura et al., 2004), and the phylogenetic relationships were determined using MEGA version 4.0 software (Tamura et al., 2007).

Nucleotide sequence accession numbers

The nucleotide sequence of the 16S rRNA gene has been deposited in the GenBank database under Accession No. GU393026.

Preparation of partially purified enzyme

One liter of lipase production medium (1% soybean oil, 1% tryptone, 0.07% KH_2PO_4, 0.01% $CaCl_2.2H_2O$, 0.01% $MgSO_4.7H_2O$, 0.01% NaCl, 0.001% $FeSO_4.7H_2O$, 0.001% $MnSO_4.4H_2O$; pH 7.4) in two 2 L Erlenmeyer flasks was aseptically inoculated with a single colony of strain INK1 and aerobically cultivated with vigorous shaking (220 rpm) for 96 h at 30°C. The culture medium containing secreted lipase was centrifuged at 9,000 × g for 30 min at 4°C to remove the cells and proteins in the supernatant and was then precipitated with ammonium sulfate (75% saturation). The pellet was dissolved in 50 mM Tris-HCl (pH 8.0) and dialyzed overnight against 50 mM Tris-HCl (pH 7.4) at 4°C. The dialyzed solution was used as the lipase source throughout this work to examine its catalytic properties.

Zymography

Native polyacrylamide gel electrophoresis (PAGE) was carried out with a Modular Mini-Protein II Electrophoresis System (Bio-Rad) according to the manufacturer's instructions. After electrophoresis, the gel was washed sequentially with 50 mM Tris-HCl buffer (pH 8.0) containing 1% (v/v) Triton-X100 and twice with 50 mM Tris-HCl buffer (pH 8.0). The gel was overlaid on tributyrin agar prepared with bacto agar (1.5%) and tributyrin emulsion [1% tributyrin (Sigma-Aldrich), 20 mM NaCl, 1 mM $CaCl_22H_2O$, 0.5% gum arabic; pH 7.4]. The band of lipase activity against tributyrin was detected by appearance of a clear zone (Oh et al., 1999).

Measurement of enzyme activity and substrate specificity

Unless otherwise stated, the lipase activity was measured at 50°C by assaying the release of *p*-nitrophenol from *p*-nitrophenyl (*p*Np)-palmitate with a final substrate concentration of 114.8 µM in 2.3 ml of the reaction mixture containing 0.1 M NaCl, 0.5% Triton X-100, and 50 mM Tris-HCl (pH 7.4) (Choo et al., 1998). As for substrate specificity, activity on various *p*Np-esters such as *p*Np-acetate, *p*Np-butyrate, *p*Np-caprylate, and *p*Np-caprate was determined under standard assay conditions with a final substrate concentration of 114.8 µM. The release of *p*-nitrophenol from *p*Np-esters was monitored spectrophotometrically at 400 nm. One unit (U) of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of *p*-nitrophenol per minute under the given assay conditions.

Effect of pH and temperature on enzyme activity

The pH optimum for lipase activity with *p*Np-palmitate was determined at 30°C in 50 mM sodium acetate (pH 4.5-5), 50 mM Bis-Tris-HCl (pH 6 to 7), and 50 mM Tris-HCl (pH 7.4 to 9.0) buffers. To study the optimum temperature and enzyme activity, the enzyme reaction mixture was incubated at different temperatures from 5 to 80°C in 50 mM Tris-HCl (pH 7.4) buffer using *p*Np-palmitate as a substrate.

Effect of metal ions, inhibitors, and surfactants on enzyme activity

The effects of various metal ions (1 mM) including Mg^{2+}, Fe^{2+}, Ca^{2+}, Zn^{2+} and Mn^{2+} were investigated. The effects of enzyme inhibitors (1

Figure 1. Zymogram analysis of lipase activity in the enzyme preparation; Lane 1: Bovine serum albumin (negative control), Lane 2: INK1 lipase.

mM) were studied using phenylmethylsulfonyl fluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA). The effects of the surfactants were analyzed against 0.1% Triton X-100 and 1 mM SDS. Each additive was pre-incubated with the enzyme for 30 min at 25°C before the standard assay was performed and the residual activity was measured.

Measurement of caprylic acid liberated from cream by INK1 lipase

Raw cream with a fat content of 38% (w/w) was purchased from Seoul Milk Corporation (Seoul, South Korea). The cream emulsion was prepared by emulsifying 10 ml of raw cream in 40 ml of isooctane for 30 s at 13,500 rpm using the Ultra-Turrax T25 homogenizer (Ika-Labortechnik, Germany). The substrate emulsion (50 ml) was added to 40 ml of 50 mM Tris-HCl (pH 7.4) containing lyophilized INK1 lipase (0.5 U). The suspension was incubated for 6 h at 37°C in a shaking water bath. The suspension with no enzyme supplementation was also served as a reference. From the incubation mixtures, 0.2 ml of aliquots were removed at different time points and 0.8 ml of isooctane was added to the aliquots. Then, the liberated caprylic acids were quantified spectrophotometrically using cupric acetate-pyridine reagent as previously described by Kwon and Rhee (1986). A standard curve for caprylic acid was made within the range of 2 to 28 μmol.

Statistical analysis

The data were expressed as mean and standard errors from three experiments, and their significance was analyzed using Student's *t*-test.

RESULTS AND DISCUSSION

Identification of isolated strain INK1 and lipase production

To identify the strain INK1 exhibiting lipase activity (Figure 1), the 16S rRNA gene was cloned and its sequence was compared with those available in the GenBank database. A phylogenic tree based on the 16S rRNA gene sequences from 10 *Pseudomonas* strains revealed 99.2% sequence identity of INK1 with the type strain, *Pseudomonas frederiksbergensis* DSM 13022 (Figure 2). Therefore, it was named *Pseudomonas* sp. INK1. Time courses of cell growth and lipase activity are shown in Figure 3. The data indicated that extracellular lipase production in isolate INK1 was somewhat growth phase-dependent. Lipase production steeply increased during the transition to stationary phase, exhibiting maximal activity (0.0025 U/ml) at 149 h of incubation. Similar increases in extracellular lipase activity during transition to stationary phase have been observed in *Acinetobacter* strains (Fischer et al., 1987; Kok et al., 1993; Snellman et al., 2002).

Effect of pH and temperature on enzyme activity

The INK1 lipase exhibited apparent maximal activity at pH 8 to 9 (Figure 4A). This is very similar to previous studies of other bacterial lipase producers such as *Pseudoalteromonas haloplanktis* (de Pascale et al., 2008), *Serratia marcescens* (Abdou, 2003), *Pseudomonas* sp. strain S5 (Rahman et al., 2005), *Bacillus coagulans* (Kumar et al., 2005) and *Yarrowia lipolytica* (Yu et al., 2007). The optimal pH range of most microbial lipases occurs in the alkaline range (Chen et al., 2007). However, a recent study reported that the pH activity profile of the lipases from *Pseudomonas monteilii* (Wang et al., 2009) and *Staphylococcus epidermidis* (Esakkiraj et al., 2010) are maximal at pH 6 to 7. As shown in Figure 4B, the optimum temperature for lipase activity was 50°C and more than 60% of its highest activity was retained at 20 to 50°C. In addition, INK1 lipase exhibited 15% of its maximal activity at a low temperature of 5°C, which is in agreement with the character of many lipases from psychrotrophs (Abdou, 2003; Fox and Stepaniak, 1983).

Effect of various reagents on enzyme activity

The INK1 lipase activity in the presence of different metal

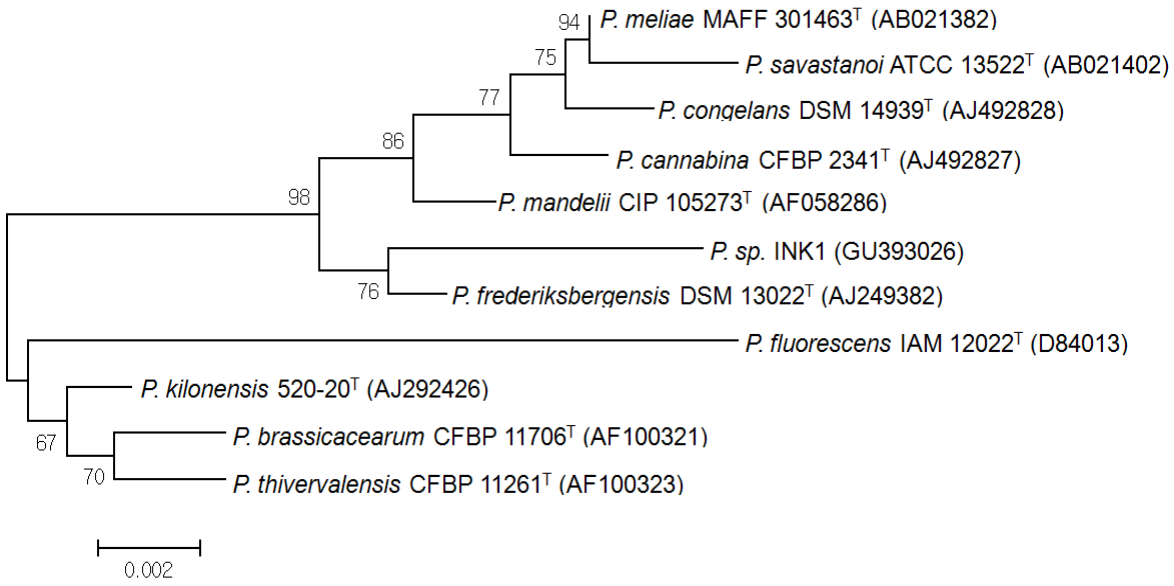

Figure 2. Phylogenic relationship of the 16S rRNA sequences of *Pseudomonas* sp. INK1 with other type strains of *Pseudomonas*. Bootstrap values (based on 1,000 trials and only values > 60%) are shown at the nodes. The GenBank accession numbers are indicated in parentheses. Bar, 0.002 substitutions per nucleotide position. *P.* represents the abbreviation of *Pseudomonas*.

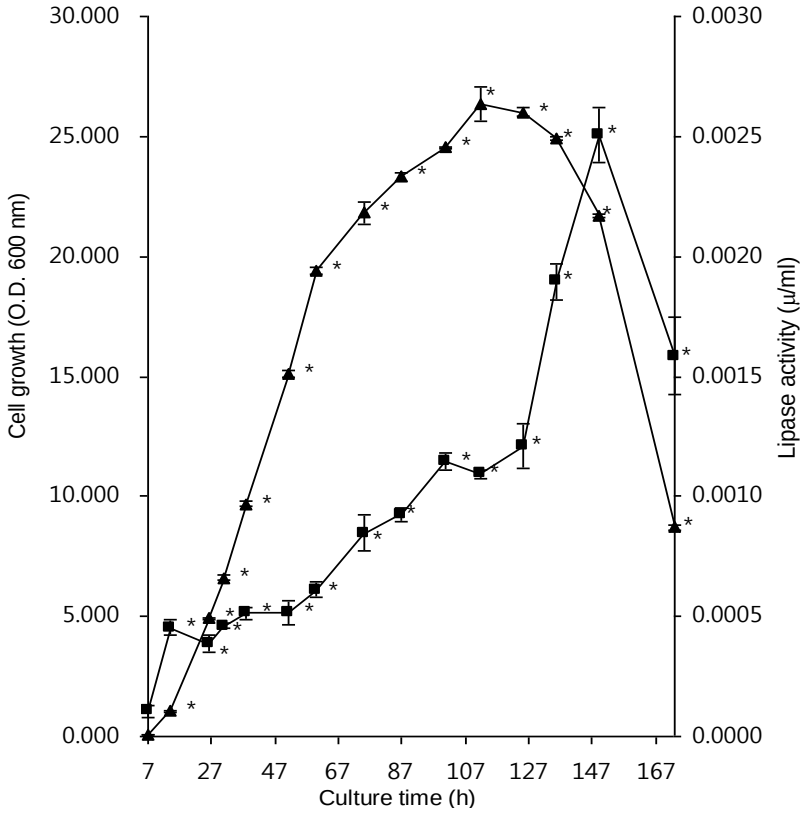

Figure 3. Growth and lipase production by *Pseudomonas* sp. INK1. Symbols represent lipase activity (filled square) and growth (filled triangle). Data represent means ± standard errors from three experiments. Asterisks indicate significant differences ($P<0.05$) versus the cell growth and lipase activity at 7 h, respectively.

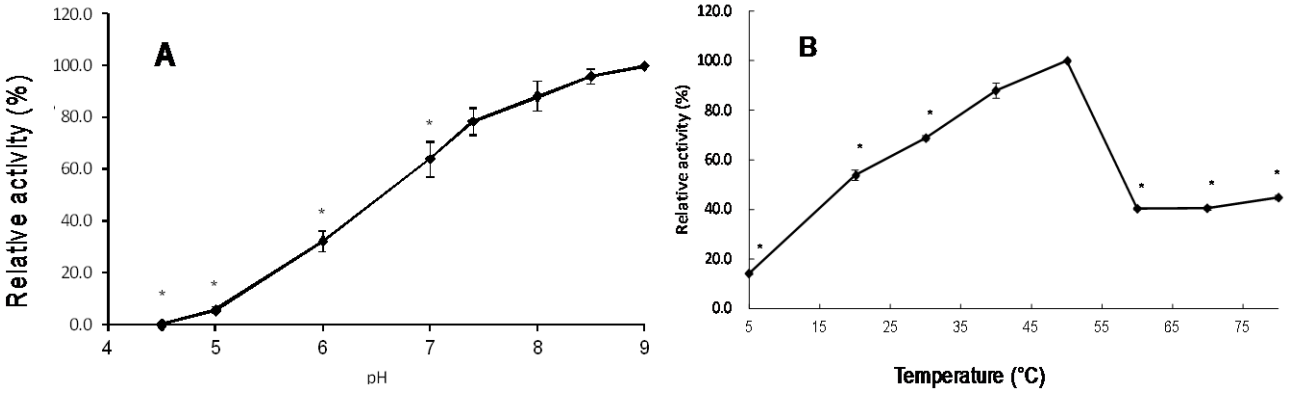

Figure 4. Optimal pH (A) and temperature (B) activity profiles of the INK1 lipase. (A) Relative activity at 30°C and various pHs, where 100% equates to 0.0013 U/ml. Asterisks indicate significant differences (P<0.05) versus the relative activity (100%) at pH 9. (B) Relative activity at pH 7.4 and various temperatures where 100% equates to 0.0014 U/ml. Asterisks indicate significant differences (P<0.05) versus the relative activity (100%) at 50°C. Both assays were performed at a final concentration of 114.8 μM *p*-nitrophenyl palmitate. Data represent means ± standard errors from three experiments.

Table 1. Effect of various reagents on the activity of INK1 lipase.

Reagents	Concentration	Relative activity (%)
None	0	100.0[a]
Mg^{2+}	1 mM	87.3 ± 1.5*
Fe^{2+}	1 mM	72.2 ± 1.9*
Ca^{2+}	1 mM	78.1 ± 4.4*
Zn^{2+}	1 mM	28.6 ± 2.4*
Mn^{2+}	1 mM	47.1 ± 1.7*
EDTA	1 mM	117.4 ± 1.7*
PMSF	1 mM	89.5 ± 0.6*
SDS	1 mM	91.1 ± 1.1*
Triton X-100	0.1%, w/v	112.3 ± 2.6*

[a]One hundred percent is assigned to the activity in the absence of the reagents, and equates to 0.0019 U/ml. Data represent mean ± standard errors from three experiments. Asterisks indicate significant differences (P<0.05) between the relative activity without reagent and with reagent.

ions, inhibitors, or surfactants is shown in Table 1. Among the tested metal ions, the enzyme was strongly inhibited by Zn^{2+} and Mn^{2+}. This result agrees well with *P. monteilii* F1 lipase, in which 77 and 72% of its initial activity was reportedly lost by the addition of 1 mM Zn^{2+} and Mn^{2+} respectively (Wang et al., 2009). Although, the Ca^{2+} binding site and the activation mechanism by Ca^{2+} of *Pseudomonas* sp. MIS38 lipase, which is the member of the I.3 family of lipases have been clarified (Amada et al., 2001), INK1 lipase was rather moderately inhibited by Ca^{2+}. Mg^{2+} and Fe^{2+} also moderately inhibited the enzyme activity. Generally, EDTA potently inhibits metallo-enzymes (Wang et al., 2009). However, EDTA slightly enhanced INK1 lipase activity. Similarly, the activity of a cold-adapted lipase (LipP) from an Alaskan psychrotroph, *Pseudomonas* sp. B11-1 was increased up to 9% in the presence of 5 mM EDTA, since the enzyme was not activated in the presence of 1 or 10 mM Ca^{2+} (Choo et al., 1998). On the other hand, 1 mM Ca^{2+} remarkably enhanced the stability of the *Acinetobacter* sp. RAG-1 lipase (LipA) that contains two putative Ca^{2+} binding amino acid residues, instead, EDTA strongly inhibited the enzyme leading to 90% loss of the activity (Snellman et al., 2002). Therefore, INK1 lipase does not appear to be a calcium-dependent metallo-enzyme. Enzyme activity was slightly inhibited by PMSF, a well-known serine protease inhibitor (Hutadilok-Towatana et al., 1999) (Table 1). A previous study reported that two surfactants, SDS and Triton X-100 strongly affected *P. monteilii* F1 lipase, with enzyme activity being decreased by 65% in the presence of 1 mM SDS and increased up to 254% in the presence of 0.1% Triton X-100 (Wang et al., 2009). However, in this study, these surfactants had no major effects on the activity of INK1 lipase (Table 1).

Substrate specificity and Release of caprylic acid from cream by INK1 lipase

The substrate specificity of INK1 lipase was examined using *p*-nitrophenyl (Np) esters with various chain lengths of the acid moiety (Table 2). The enzyme relatively showed broader specificity for substrates with a chain

Table 2. Substrate specificity of the INK1 lipase against *p*-nitrophenyl esters of different acyl chain lengths.

Substrates	Chain(C)-length	Relative activity (%)
*p*Np- acetate	C_2	44.5 ± 0.7*
*p*Np- butyrate	C_4	196.7 ± 3.2*
*p*Np- caprylate	C_8	231.9 ± 5.3*
*p*Np- caprate	C_{10}	171.4 ± 1.3*
*p*Np- palmitate	C_{16}	100.0[a]

[a] The maximum activity of the enzyme is taken as 100.0%, and equates to 0.0015 U/ml. Data represent mean ± standard errors from three experiments. Asterisks indicate significant differences (P<0.05) between the relative activity for *p*Np-palmitate and for other substrates.

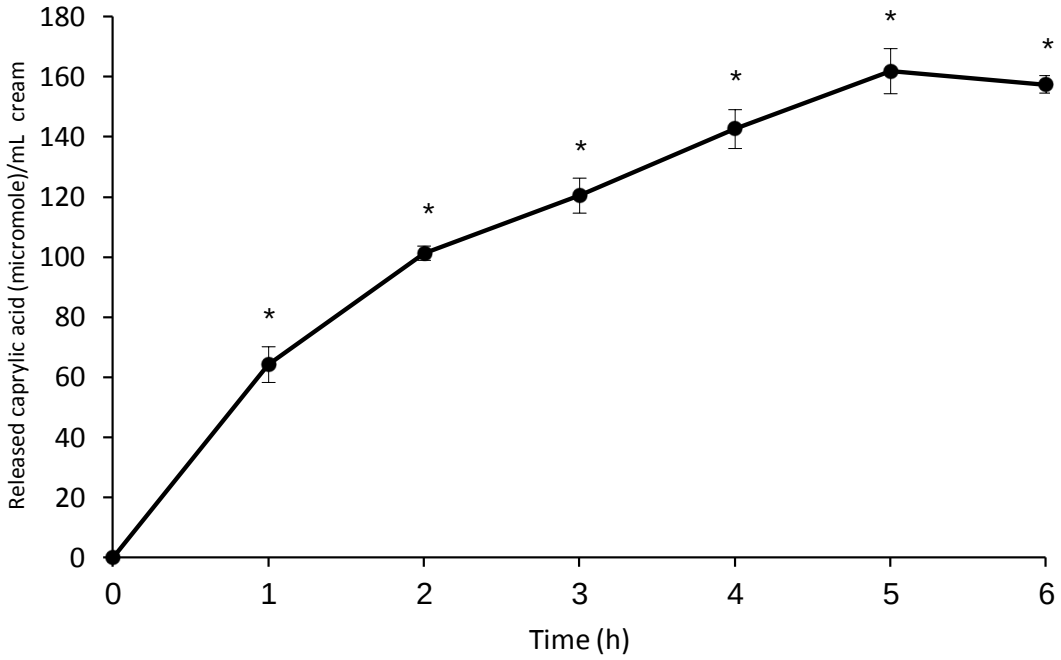

Figure 5. The release of caprylic acid from cream by the INK1 lipase. Lyophilized INK1 lipase (0.5 U) was incubated with cream at 37°C for 6 h in a shaking waterbath. The cream with no enzyme supplementation was also served as a reference. Data represent means ± standard errors from three experiments. Asterisks indicate significant differences (P<0.05) between 0 h and other time points.

length from C_2 to C_{16} showing the highest activity toward *p*Np-caprylate (C_8). On the other hand, the cold-active Lip1 and LipP lipase from another Antarctic bacterium, *P. haloplanktis* (de Pascale et al., 2008) and an Alaskan psychrotroph, *Pseudomonas* sp. B11-1 (Choo et al., 1998), displayed maximal activity toward *p*Np-caprate (C_{10}) and *p*Np-butyrate (C_4), respectively. As shown in Figure 5, INK1 lipase could also hydrolyze a natural substrate, cream, releasing 161.8 ± 7.5 caprylic acid (C_8)/ml cream for 5 h incubation. Flavor production by lipases in the dairy industry is due to a highly efficient and selective conversion process that relies on the enzymes' unique specificities (Saerens et al., 2008). In fact, milk fat is one of the most complex fats in nature with the relatively high number of short chain fatty acids (C_4 to C_8) being a typical characteristic of bovine milk fat (Jensen, 1992). Generally, long chain fatty acids (C_{16} to C_{18}) have negligible contributions to the flavor of a dairy product (Saerens et al., 2008). However, these short chain fatty acids play a pivotal role in the buttery flavor of a dairy product due to their low flavor threshold values in fat rich matrices (Kinsella, 1975; Schieberle et al., 1993). Therefore, one aims to release these fatty acids to enhance this highly appreciated flavor (Saerens et al., 2008). Additionally, butyric acid is regarded as a key component in the flavor of milk fat (Schieberle et al., 1993). In this regard, it is noteworthy that the INK1 lipase was more active on these short chain fatty acid esters (C_4

and C_8) than long fatty acid ester (C_{16}).

In conclusion, INK1 lipase may have potentials as a useful catalyst to enhance the buttery flavor in the dairy industry because of its relatively preferable specificity towards short chain fatty acid esters. Future research involving gene cloning, protein engineering, and fermentation technology will maximize the catalytic efficiency and productive yield of the enzyme.

REFERENCES

Abdou AM (2003). Purification and partial characterization of psychrotrophic *Serratia marcescens* lipase. J. Dairy Sci*.,* 86: 127-132.

Al-Marzooqi W, Leeson S (1999). Evaluation of dietary supplement of lipase, detergent, and crude porcine pancreas on fat utilization by young broiler chicks. Poultry Sci., 78: 1561-1566.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. J. Mol. Biol., 215: 403-410.

Amada K, Kwon HJ, Haruki M (2001). Ca^{2+}-induced folding of a family I.3 lipase with repetitive Ca^{2+} binding motifs at the C-terminus. FEBS Lett., 509: 17-21.

Chen S, Qian L, Shi B (2007). Purification and properties of enantioselective lipase from a newly isolated *Bacillus cereus* C71. Process Biochem., 42: 988-994.

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

Demirjian DC, Moris-Varas F, Cassidy CS (2001). Enzymes from extremophiles. Curr. Opin. Chem. Biol., 5: 144-151.

de Pascale D, Cusano AM, Autore F, Parrilli E, di Prisco G, Marino G, Tutino ML (2008). The cold-active Lip1 lipase from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 is a member of a new bacterial lipolytic enzyme family Extremophil., 12: 311-323.

Dierick N, Decuypere J, Molly K, Van Beek E, Vanderbeke E (2002a). The combined use of triacylglycerols (TAGs) containing medium chain fatty acids (MCFAs) and exogenous lipolytic enzymes as an alternative for nutritional antibiotics in piglet nutrition. I. *In vitro* screening of the release of MCFAs from selected fat sources by selected exogenous lipolytic enzymes in simulated pig gastric conditions and their effects on the gut flora of piglets. Livestock Prod. Sci., 75: 129-142.

Dierick N, Decuypere J, Molly K, Van Beek E, Vanderbeke E (2002b). The combined use of triacylglycerols (TAGs) containing medium chain fatty acids (MCFAs) and exogenous lipolytic enzymes as an alternative for nutritional antibiotics in piglet nutrition. II. *In vitro* release of MCFAs in gastric cannulated and slaughtered piglets by endogenous and exogenous lipases effects on luminal gut flora and zootechnical performances. Livestock Prod. Sci., 76: 1-16.

Esakkiraj P, Rajkumarbharathi M, Palavesam A, Immanuel G (2010). Lipase production by *Staphylococcus epidermidis* CMST-Pi 1 isolated from the gut of shrimp *Penaeus indicus*. Ann. Microbiol., 60: 37-42.

Fischer B, Koslowski R, Eichler W, Kleber H (1987). Biochemical and immunological characterization of lipase during its secretion through cytoplasmic and outer membrane of *Acinetobacter calcoaceticus* 69 V. J. Biotechnol., 6: 271-280.

Fox PF, Stepaniak L (1983). Isolation and some properties of extracellular heat-stable lipases from *Pseudomonas fluorescens* strain AFT 36. J. Dairy Res., 50: 77-89.

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

Hasan F, Shah AA, Hameed A (2006). Industrial applications of microbial lipases. Enz. Microb. Technol., 39: 235-251.

Houde A, Kademi A, Leblanc D (2004). Lipases and their industrial applications: an overview. Appl. Biochem. Biotechnol., 118: 155-170.

Hutadilok-Towatana N, Painupong A, Suntinanalert P (1999). Purification and characterization of an extracellular protease from alkaliphilic and thermophilic *Bacillus* sp. PS 719. J. Biosci. Bioeng., 87: 581-587.

Jensen RG (1992). Chapter 5: Fatty acids in milk and dairy products. In: Chow, CK (eds) Fatty acids in foods and their health implications, Marcel Dekker Inc, New York. pp. 95-135.

Kinsella JE (1975). Butter flavor. Food Technol., 5: 82-98.

Kok RG, Christoffels VM, Vosman B, Hellingwerf KJ (1993). Growth-phase-dependent expression of the lipolytic system of *Acinetobacter calcoaceticus* BD413: Cloning of a gene encoding one of the esterases. J. General Microbiol., 139: 2329-2342.

Kumar S, Kikon K, Upadhyay A, Kanwar SS, Gupta R (2005). Production, purification, and characterization of lipase from thermophilic and alkaliphilic *Bacillus coagulans* BTS-3. Protein Expr. Purif., 41: 38-44.

Kwon DY, Rhee JS (1986). A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. J. AOCS., 63: 89-92.

Lacumin L, Cocolin L, Cantoni C, Comi G (2007). Preliminary analysis of the lipase gene (gehM) expression of *Staphyloccus xylosus in vitro* and during fermentation of naturally fermented sausages (*in situ*). J. Food Prot., 70: 2665-2669.

Margesin R, Schinner F (1994). Properties of cold-adapted microorganisms and their potential role in biotechnology. J. Biotechnol., 33: 1-14.

Muthukumaran N, Dhar SC (1982). Comparative studies on the degreasing of skins using acid lipase and solvent with reference to the quality of finished leathers. Leather Sci., 29: 417-424.

Oh BC, Kim HK, Lee JK, Kang SC, Oh TK (1999). *Staphylococcus haemolyticus* lipase: biochemical properties, substrate specificity and gene cloning. FEMS Microbiol. Lett., 179: 385-392.

Park HJ, Jeon JH, Kang SG, Lee JH, Lee SA, Kim HK (2007). Functional expression and refolding of new alkaline esterase EM2L8 from deep sea sediment metagenome. Protein Expr. Purif., 52: 340-347.

Rahman RNZRA, Baharum SN, Basri M, Salleh AB (2005). High yield purification of an organic solvent-tolerant lipase from *Pseudomonas* sp. strain S5. Anal. Biochem., 341: 267-274.

Saerens K, Descamps D, Dewettinck K (2008). Release of short chain fatty acids from cream lipids by commercial lipases and esterases. Biotechnol Lett., 30: 311-315.

Schieberle P, Gassenmeier K, Guth H, Sen A, Grosch W (1993). Character impact odour compounds of different kinds of butter. Lebensm Wiss Technol., 26: 347-356.

Snellman EA, Sullivan ER, Colwell RR (2002). Purification and properties of the extracellular lipase, LipA, of *Acinetobacter* sp. RAG-1. Eur. J. Biochem., 269: 5771-5779.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolution Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol., 24: 1596-1599.

Tamura K, Nei M, Kumar S (2004). Prospect for inferring very large phylogenies by using the neighbor-joining methods. Proc. Natl. Aca. Sci. USA. 101: 11030-11035.

Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids. Res., 22: 4673-4680.

Wang SL, Lin YT, Liang TW, Chio SH, Ming LJ, Wu PC (2009). Purification and characterization of extracellular lipases from *Pseudomonas monteilii* TKU009 by the use of soybeans as the substrate. J. Ind. Microbiol. Biotechnol., 36: 65-73.

Wei P, Bai L, Song W, Hao G (2009). Characterization of two soil metagenome-derived lipases with high specificity for p-nitrophenyl palmitate. Arch. Microbiol., 191: 233-240.

William GW, Susan MB, Dale AP, David JL (1991). 16S Ribosomal DNA Amplification for Phylogenetic Study. J. Bacteriol., 173: 697-703.

Yu M, Qin S, Tan T (2007). Purification and characterization of the extracellular lipase Lip2 from *Yarrowia lipolytica*. Process Biochem., 42: 384-391.

Yuhong Z, Shi P, Liu W, Meng K, Bai Y, Wang G, Zhan Z, Yao B (2009). Lipase diversity in glacier soil based on analysis of metagenomic DNA fragments and cell culture. J. Microbiol. Biotechnol., 19: 888-897.