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Surface Display Expression of *Bacillus licheniformis* Lipase in *Escherichia coli* Using Lpp'OmpA Chimera

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The lipase from *Bacillus licheniformis* ATCC14580 was displayed on the cell surface of *Escherichia coli* using Lpp'OmpA as the anchoring protein. The expressed Lpp'OmpA-lipase fusion protein has a molecular weight of approximately 35 kDa, which was confirmed by SDS-PAGE and western blot analysis. The Lpp'OmpA-lipase fusion protein was located on the cell surface, as determined by immunofluorescence confocal microscopy and flow cytometry. The enzyme activity of the surface-displayed lipase showed clear halo around the colony. The cell surface-displayed lipase showed the highest activity of 248.12 ± 9.42 U/g (lyophilized cell) at the optimal temperature of 37°C and pH 8.0. The enzyme exhibited the highest activity toward the substrate *p*-nitrophenyl caprylate (C8). These results suggest that *E. coli*, which displayed the lipase on its surface, could be used as a whole cell biocatalyst.

Keywords: surface display, *Bacillus lichenformis*, lipase, Lpp'OmpA, *Escherichia coli*

Introduction

Cell surface display systems have been developed to express peptides and proteins on the surface of bacteriophages, bacteria, yeasts, fungi, and mammalian cells (Schreuder *et al*., 1991; Georgiou *et al*., 1997; Li, 2000; Wittrup, 2001; Jo *et al*., 2011). Cell surface expression was first attempted by fusing phage coat proteins with peptides and small proteins as the anchoring motif (Smith, 1985). Next, various display systems were developed to express foreign proteins on the surface of microbial cells (Hofnung, 1991; Little *et al*., 1993; Fischetti *et al*., 1996; Georgiou *et al*., 1997). In these systems, a heterologous peptide or protein of interest, target protein is fused to various anchoring motifs that are outer membrane proteins, such as the maltoporin LamB, the phosphate-inducible porin PhoE, the outer membrane protein OmpA, the lipoproteins Lpp, and TraT, and the peptidoglycan-associated lipoprotein (PAL) (Agterberg *et al*., 1990a, 1990b; Janssen and Tommassen, 1994; Tommassen *et al*., 1994; Charbit *et al*., 1988). A particularly interesting motif is the Lpp'OmpA chimera, which can serve as an efficient targeting vehicle for the localization of any protein to the outer surface of *E. coli*. The Lpp'OmpA chimera is a fusion protein consisting of the signal sequence and first nine-terminal amino acids of the major *E. coli* Lpp and amino acids 46-159 of OmpA, and this chimera can expose targeting proteins on the *E. coli* outer membrane (Francisco *et al*., 1992). Thus far, target proteins, including enzymes, antibodies, receptors, toxin domains, and cellulose binding proteins have been successfully expressed on the surface of *E. coli* (Francisco *et al*., 1992; Stathopoulos *et al*., 1992; Francisco and Georgiou, 1994; Georgiou *et al*., 1997; Daugherty *et al*., 1998).

Lipases (EC 3.1.1.3) are lipolytic enzymes that hydrolyze long-chain triacylglycerides and represent the most important class of biocatalysts that are used for the many organic chemistry and biotechnology applications (Jaeger and Reetz, 1998). In addition, lipases catalyze not only the hydrolysis of oils but also the transesterification of esters (Jaeger and Reetz, 1998; Reetz and Jaeger, 2002). Because of these characteristics, lipases are essential for the sustainable development in various fields, including biopolymers, biodiesel and the synthesis of chemicals (Alcalde *et al*., 2006). Lipases are widely distributed in microorganisms, plants, and animals. Many bacterial lipases have attracted considerable attention because of their resistance to high temperatures, extreme alkaline pH, and other operational and/or storage conditions (Jaeger and Eggert, 2002). More than 70 lipases are now isolated from *Bacillus* and *Geobacillus* and almost all of them have been reported having basic biochemical properties (Guncheva and Zhiryakova, 2011). Although lipases from *Bacillus licheniformis* have been reported elsewhere (Khyami-Horani, 1996; Medan and Mishra, 2009), it is still worth to express and characterize another lipase from *B. licheniformis* as the potential candidate for industrial application.

In this study, we report the cloning of a lipase from *B. licheniformis* ATCC14580 and its expression on the surface of *E. coli* using the Lpp'OmpA chimera as an anchoring motif. In addition, we report the enzymatic characteristics of the surface-displayed lipase.

Materials and Methods

Strains, plasmids, and materials

B. licheniformis ATCC14580 was obtained from Korean Culture Center of Microorganisms (KCCM). The *E. coli* strain

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Table 1. Bacterial strains and plasmids used in this study

Strain and plasmid	Genotype or description	Reference
Strains		
B. licheniformis	ATCC14580	
E. coli TOP10F'	F'*[(lacI^{q}Tn10(Tet^r)]mcrAΔ(mrr-hsdRMS-mcrBC)(80lacZ ΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Strr) endA1 nupG*	Invitrogen Co.
Plasmids		
pGEM-T Easy	T/A cloning vector	Promega Co.
pGL	pGEM-T Easy containing *lip*	This study
pGS	pGEM-T Easy containing *lpp'ompA*	This study
pGSL	pGEM-T Easy containing *lpp'ompA-lip*	This study
pTSL	pTRC99A containing *lpp'ompA-lip, ΔlacI^q*	This study
pTRC99A	IPTG-inducible expression vector	Pharmacia Biotech Co.

TOP10F' (Invitrogen, The Netherlands) was used as the host strain for the surface display of the *B. licheniformis* ATCC 14580 lipase and conventional cloning. The plasmid pTRC99A was purchased from Pharmacia (Sweden), and the plasmid pGEM-T Easy, T4 DNA ligase, plasmid isolation kit and genomic DNA purification kit were purchased from Promega (USA). All strains and plasmids used in this study are shown in Table 1. The mouse anti-6× His monoclonal antibody was purchased from IG Therapy Co. (Korea). Goat anti-mouse IgG(Fc)-alkaline phosphatase conjugate and goat anti-mouse IgG(Fc)-FITC conjugate were purchased from Sigma Co. (USA). All DNA oligonucleotides were purchased from Bioneer (Korea). All enzymes including restriction enzymes, *Taq* polymerase and DNA modifying enzymes were purchased from New England Biolabs (USA).

Cloning of Lpp'OmpA-lip fusion gene and plasmid construction

For the construction of the Lpp-OmpA-lipase fusion protein, the first 9 amino acids of the Lpp signal peptide were fused to the OmpA amino acids 46-159 and were linked to the *B. licheniformis* ATCC 14580 lipase. This type of construct was previously reported by Franscisco *et al.* (1992). For construction of the hybrid gene, recombinant PCR was used as shown in Fig. 1A, and the primers used are shown in Table 2. Each hybrid construct containing the *lpp'ompA* and *ompA*-lipase (*lip*) gene was inserted into pGEM-T Easy. The resulting plasmids were designated pGS and pGL, respectively (Fig. 1A). The *lpp'ompA* fusion fragment of plasmid pGS was digested with *Nco*I, and introduced into the corresponding site of plasmid pTRC99A containing the strong *trc* promoter. The *lip* fragment of pGL was obtained by digestion with *Bam*HI and *Pst*I and was introduced into the same site of plasmid pTRC99A containing the *lpp'ompA* to obtain the *lpp'ompA-lip* fusion gene under control of the *trc* promoter. The resulting plasmid was designated pTSL. The *trc* promoter and *lpp'ompA-lip* fusion fragment from pTSL were amplified by PCR using the primers trcpF and LipB. The amplified fragment was inserted into pGEM-T Easy to obtain plasmid pGSL (Fig. 1B).

Expression and analysis of lipase by SDS-PAGE and western blotting

To express the fused lipase, the recombinant *E. coli* harboring pGSL was inoculated in 10 ml LB broth containing 50 μg/ml ampicillin and grown at 37°C for 12 h. Next, 1 ml culture was transferred to 100 ml LB medium and induced with 1 mM IPTG in a 500 ml flask and grown at 37°C for 12 h. The culture was then centrifuged at 3,500 × g for 10 min at 4°C. The cells were disrupted in 0.02 M sodium phosphate buffer (pH 7.2) with a Bead-Beater (Biospec products, USA). After centrifugation at 12,000 × *g* for 10 min at 4°C, the supernatant was collected and the cell pellet containing the total membrane fraction was further extracted with buffer containing 6 M urea in 0.01 M Tris-HCl (pH 6.8). The protein samples were analyzed by 12% SDS-PAGE. Western blot analysis was performed according to standard procedures (Sambrook *et al.*, 1989). The goat anti-6× His monoclonal antibody and goat anti-mouse IgG(Fc)-Alkaline phosphatase conjugate were used for immunodetection,. The Western Blue^R kit (Promega) was used for signal detection.

Halo assay

The transformants were transferred to LB agar plates con-

Table 2. Primer sequences used in this study

Primers	Sequences (5′→3′)
LipF	GGATCCGGCTTCCCACAATCCGGTC (*Bam*HI)
LipB	TTAATGATGATGATGATGATGTTTCCCGCTGGCGGTCAG (6× His tag)
LppF	CCATGGGTAAAGCTACTAAACTGGTA (*Nco*I)
LppB	CAAAGCCAACATACGGGTTAATACCCTGATCGATTTTAGCGTTG (*ompA* partial sequence)[a]
OmpAF	CAACGCTAAAATCGATCAGGGTATTAACCCGTATGTTGGCTTTG (*lpp* partial sequence)[b]
OmpAB	CCATGGGTTGTCCGGACGAGTGCC (*Nco*I)
trcpF	CCGACATCATAACGGTTC

[a, b] Partial sequences for recombinant PCR

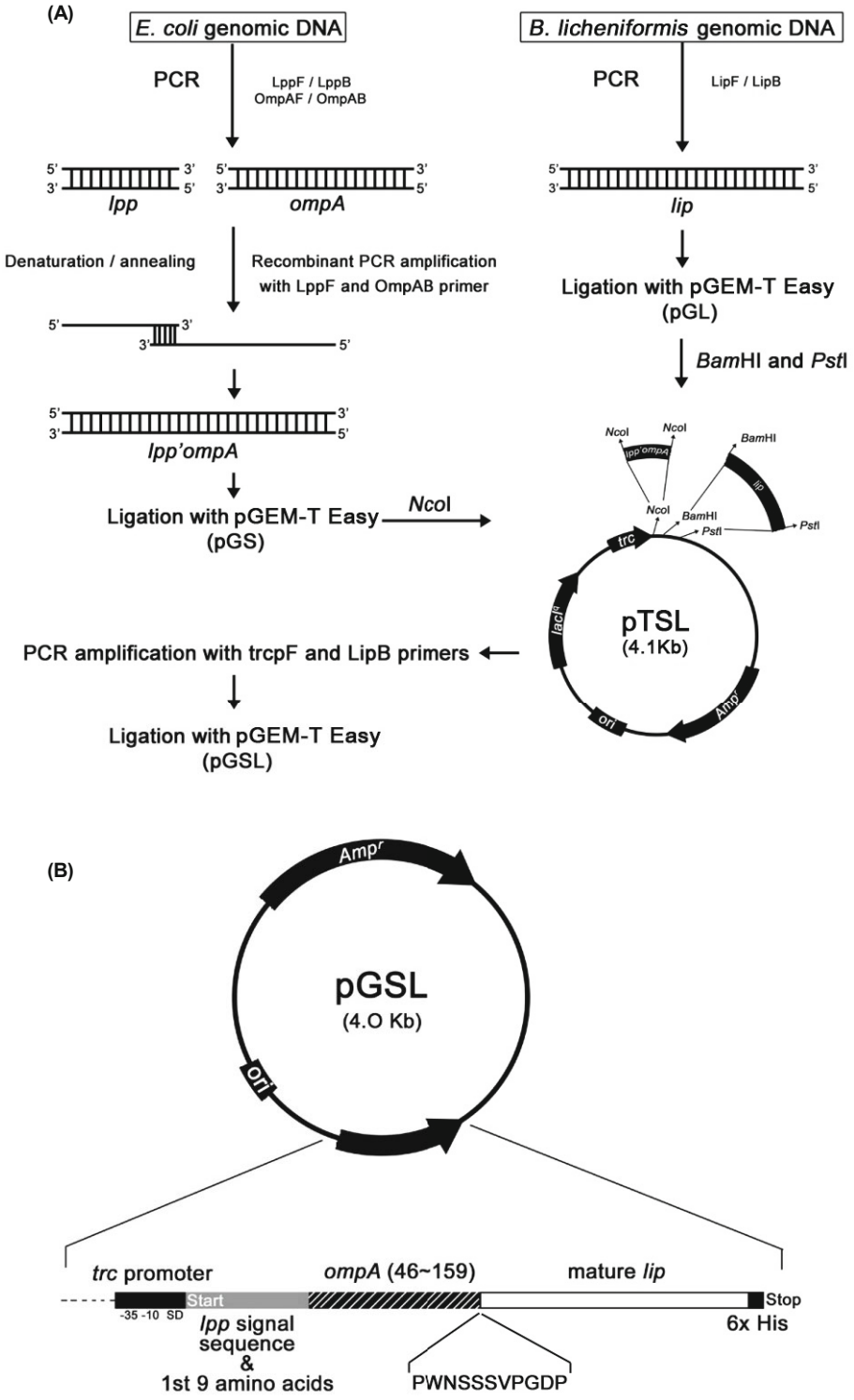

Fig. 1. Recombinant PCR (A) and schematic diagram of the recombinant plasmid pGSL containing the *trc* promoter and *lpp'ompA-lip* fusion fragments (B). The detailed construction is described in the 'Materials and Methods'. For PCR, all the primers used are shown, and the sequences are presented in Table 2. ori, bacterial replication origin; Amp^r, ampicillin resistance gene; *lpp* signal sequence and 1st 9 amino acids, the signal sequence and first nine N-terminal amino acids of *E. coli* lipoprotein, respectively; *ompA* (46–159) represents amino acids 46–159 of the *E. coli* outer membrane protein A. The 11-amino acids joining the *lpp'ompA* and *lip* are shown as a single letter. The 6× His represents the 6× His-tag.

taining 1% tributyrin (LBT), and the plates were incubated overnight at 37°C. The activity of the surface-displayed lipase was detected as clear halos formed around the colonies. The *E. coli* TOP10F' harboring the pGEM-T Easy vector was the negative control.

Immunofluorescence and flow cytometric analysis

After a fresh culture of recombinant *E. coli* was grown in LB medium at 37°C for 12 h, 1 ml cells was harvested by centrifugation at 6,000 × *g* for 5 min at 4°C, washed with phosphate-buffered saline (PBS) and resuspended in 300 μl PBS with goat anti-6× His monoclonal antibody (1:300 dilu-

tion) containing 1% BSA. After a 60 min incubation at 4°C, the cells were washed five times with PBS. The cells were then incubated at 4°C for 60 min with 300 µl PBS containing FITC (fluorescein isothiocyanate)-conjugated goat anti-mouse IgG (1:300 dilution) and 1% BSA. Finally, after washing with PBS, the cells were observed with a confocal laser scanning microscope system (LSM 700, Carl Zeiss GmbH, Germany). The localization of the surface-displayed lipase was also confirmed by flow cytometric analysis. The FITC-labeled cells were detected with a FACSCalibur flow cytometer (Becton Dickson). A total of 200,000 events were collected for each sample. *E. coli* TOP10F' cells transformed with pGEM-T Easy were used as the negative control.

Measurement of lipase activity

The lipolytic activity was measured with the lyophilized whole cells or cultured whole cells. The whole-cell lipase was assayed using a spectrometric method with measurement at 405 nm and *p*-nitrophenyl (*p*NPC) as the substrate according to previously described elsewhere (Lee *et al*., 2004). Briefly, *E. coli* cells expressing the surface-displayed lipase were cultured and expressed as described above followed by washing with distilled water. The cells were lyophilized with freeze-dryer (FDseries, Ilshin Lab., Korea) for 48 h. The lipase activity was measured by incubation of the lyophilized whole cells (0.1 mg) with the *p*-nitrophenyl (*p*NPC) as the substrate. For the assay with the cultured whole wet cells, 100 ml of the culture was harvested, washed, and weighed. The cells were re-suspended in 100 ml of 50 mM Tris-HCl (pH 8.0). Then, 0.1 ml of the cell suspension was used for the assay. The optimal temperature for the lipase was determined at pH 8.0 by performing the enzyme assay at different temperatures ranging from 20 to 80°C. The optimal pH for the lipase was determined at 37°C by performing the enzyme assay at various pH values ranging from 4.0 to 10.0. The substrate specificity of the lipase was determined by adding a 10 mM solution of *p*-nitrophenyl esters of various acyl chain lengths as substrates. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol *p*-nitrophenol via hydrolysis of pNPC substrate per min. The specific activity was defined as the lipase activity per gram of lyophilized or cultured whole wet cells. All measurements were performed in triplicate.

Table 3. Biochemical assay of surface-displayed lipase in *E. coli*

Strain	Total activity (U)	Wet cell weight (g)	Specific activity (U/g cell)
B. licheniformis	0.52 ± 0.05	0.42 ± 0.01	1.24 ± 0.14
E. coli Top10F'			
pGSL	18.68 ± 0.56	0.21 ± 0.01	88.95 ± 4.43
pGEM-T Easy	-[ND]	-	-[ND]

[a] 100 ml of *B. licheniformis* and *E. coli* were grown and whole-cells were used for the lipase assay.
[b] The values are the means ± SD of three independent experiments.
[c] ND, not detected.

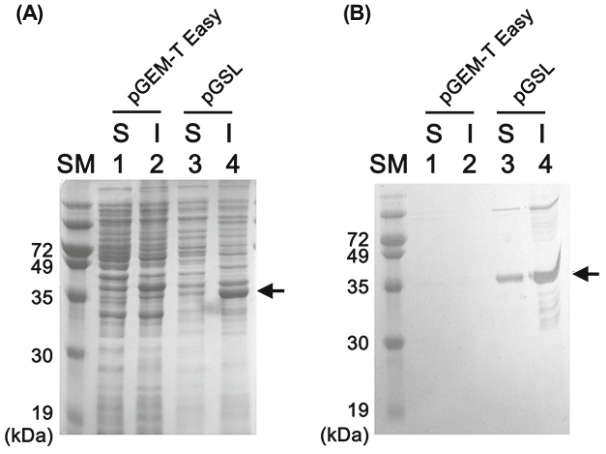

Fig. 2. SDS-PAGE (A) and western blotting (B) of the expressed lipase on the surface of *E. coli* TOP10F' cells harboring pGSL. Lanes: SM, molecular weight marker; 1 and 2, cells harboring pGEM-T Easy as the control; 3 and 4, cells harboring pGSL. The cells were lysed and extracted with 6 M urea as described in the text. Lanes: 1 and 3, soluble fraction (S) 2 and 4, insoluble fraction (I). The arrows indicate the expressed lipase.

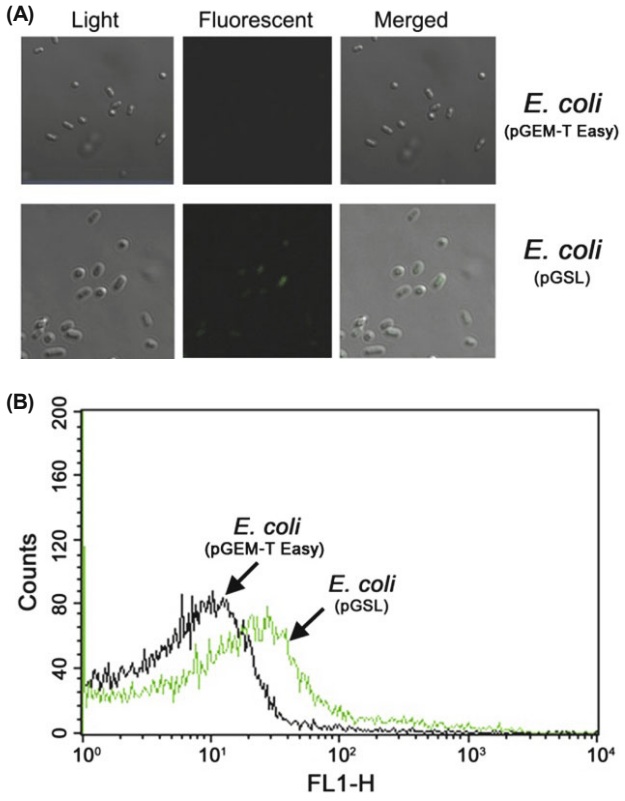

Fig. 3. Immunofluorescence and localization of the Lpp'OmpA-Lipase fusion protein in recombinant *E. coli*. (A) Fluorescence and confocal laser scanning microscopy analysis. The cells were labeled with FITC-conjugated goat anti-mouse IgG and analyzed as described in 'Materials and Methods'. The light microscope (left), immunofluorescence microscope (middle) and merged image (right) are shown. (B) FACS analysis. *E. coli* TOP10F' cells harboring pGEM-T Easy were used as the control.

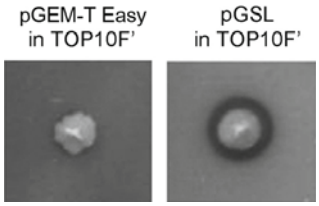

Fig. 4. Detection of enzyme activity on LB agar plates containing 1% tributyrin. The clear halo around the recombinant *E. coli* harboring pGSL (right) is shown. *E. coli* TOP10F' cells harboring pGEM-T Easy (left) were used as the control.

Results and Discussion

Cloning and expression of *B. licheniformis* lipase on the cell surface of *E. coli*

To display the *B. licheniformis* lipase on the surface of *E. coli*, we constructed the plasmid pGSL which encodes an Lpp'OmpA-lipase hybrid protein consisting of the signal peptide and N-terminal 9 amino acid residues of the *E. coli* lipoprotein Lpp, amino acids 46–159 of the OmpA, an 11-amino acid joining region derived from the MCS of the pTRC99A with the sequence of PWNSSSVPGDP, and the mature lipase sequence of *B. licheniformis* tagged with a 6× His at the C-terminal (Fig. 1). The hybrid gene was expressed by the 1 mM IPTG induction under the control of the *trc* promoter. The 6× His tag was used as an epitope for the goat anti-6× His antibody. Expression of the lipase in *E. coli* was analyzed by SDS-PAGE and western blotting. The fused protein had a molecular mass of approximately 35 kDa and was detected as a major band in the cell lysates precipitate (insoluble fraction, Figs. 2A and 2B, lane 4). The protein was not detected in the supernatant of the cell lysates (soluble fraction) by Coomassie blue staining (Fig. 2A, lane 3). However, western blot analysis showed that the fused protein was present in both the soluble and insoluble fractions (Fig. 2B, lane 3, 4). This result suggested that the majority of the expressed lipase was anchored in the cell wall and very small amount of the lipase can be extracted with urea. Therefore, almost all of the fused protein was detected in the insoluble precipitate. Several bands corresponding to degradation fragments with molecular weights less than the expected size of the fused protein were also detected with the anti-His antibody, suggesting that the fused protein underwent proteolysis in *E. coli*. According to Francisco *et al.* (1992), the relatively small amount of degraded fragments suggests that the majority of the fusion protein did not undergo proteolysis.

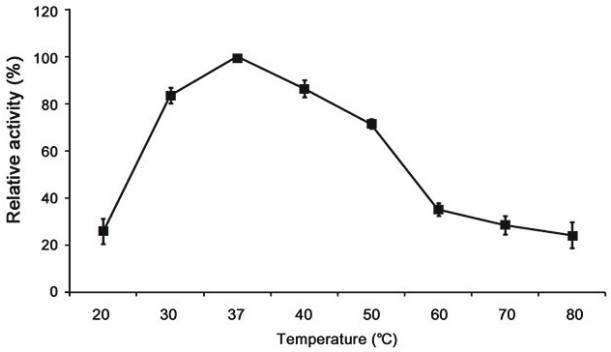

Fig. 6. Effect of temperature on the lipase activity of recombinant *E. coli* harboring pGSL. The lipase activity was determined at pH 8.0 at various temperatures using *p*NPC as the substrate. The relative activity was calculated by assuming that the activity observed at 37°C was 100%. The values are the means±SD of three independent experiments.

To verify the lipase activity from the surface-displayed *E. coli* cells, the cultured whole wet cells were used for the assay as described in 'Materials and Methods'. As shown in Table 3, recombinant *E. coli* whole cells showed much lipase activity. This result indicated again that the lipase was successfully displayed on the surface of *E. coli* cell. The less lipase activity of the *B. licheniformis* is caused by the assay with the whole cells instead of culture supernatant. This is because almost all the lipase of *B. licheniformis* is secreted in the supernatant.

Confirmation of lipase on the cell surface

The expression of the lipase on the *E. coli* cell surface was con-

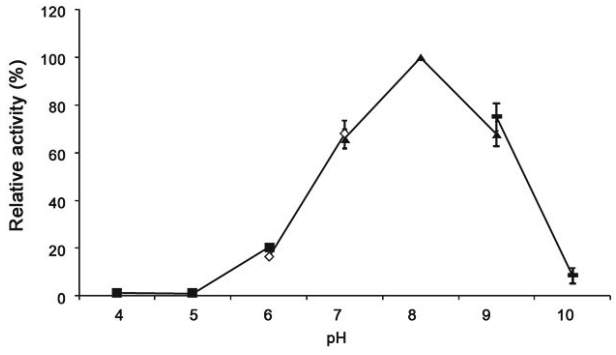

Fig. 5. Effect of pH on the lipase activity of recombinant *E. coli* harboring pGSL. The lipase activity was determined at 37°C at different pH values using *p*NPC as the substrate. For the assay, the enzyme solutions were prepared in the following buffers: citrate buffer (pH 4.0–6.0), phosphate buffer (pH 6.0–7.0), Tris-HCl buffer (pH 7.0–9.0) and carbonate buffer (pH 9.0–10.0). The relative activity was calculated by assuming that the activity observed at pH 8.0 was 100%. The values are the means±SD of three independent experiments.

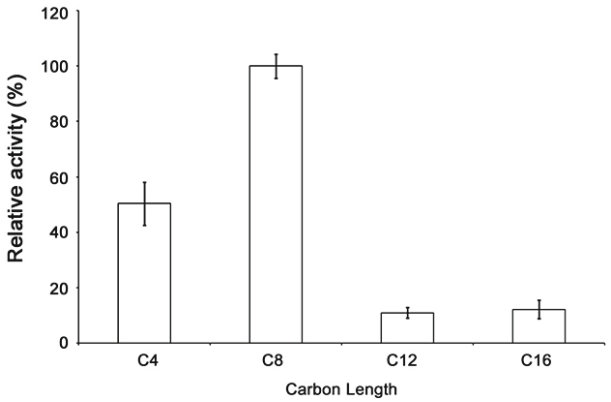

Fig. 7. Specificity of surface displayed-lipase on acyl-chain length. The substrate range of the lipase was determined using *p*-nitrophenyl fatty acyl esters of various carbon chain lengths from 4–16 at pH 8.0 and 37°C.: *p*NPB (C4), *p*NPC (C8), *p*NPL (C12), and *p*NPP (C16). The relative activity was calculated by assuming that the activity observed using *p*NPC was 100%. The values are the means±SD of three independent experiments.

firmed by immunofluorescence. Using the confocal laser scanning microscopy, fluorescence was observed on the cell surface of *E. coli* TOP10F' expressing the Lpp'OmpA-Lipase fusion protein but not on the surface of control cells (Fig. 3A). This result indicates that the lipase was successfully displayed on the cell surface and this was further verified by flow cytometric analysis. As shown in Fig. 3B, only the cells expressing the fused protein were detected by the surface-reactive anti-His antibody, indicating that the lipase was successfully expressed and immobilized on the surface of the *E. coli*. By contrast, negative cells harboring the pGEM-T Easy vector did not react with the anti-His antibody.

Biochemical characterization of cell surface-displayed lipase

The enzyme activity of the cell surface-displayed lipase was determined by spotting the recombinant *E. coli* TOP10F' cells onto LBT plates. The formation of a clear halo around the colony indicated that the cell surface-displayed lipase was enzymatically active (Fig. 4). To determine the optimal catalytic activity toward *p*NPC, whole-cell lipase activities of the lyophilized recombinant *E. coli* cells were measured in various buffers with pH values ranging from 4.0 to 10.0 and temperatures ranging from 20 to 80°C. The surface-displayed lipase showed activity over the pH range 6.0 to 10.0 with optimal catalytic activity (100%) at pH 8.0 (Fig. 5). At the pH 7.0 and 9.0, over 65% activity remained. However, the activity was reduced significantly to 1.1% and 8.3% at pH 4.0 to 5.0 and pH 10.0, respectively. The activity profiles at different temperatures are shown in Fig. 6. The activity was highest at 37°C and was significantly reduced at 20°C and 60°C (25% and 35% of the maximum activity, respectively). Finally, the substrate specificity of the lipase was investigated using *p*-nitrophenyl fatty acyl esters of various chain lengths. As shown in Fig. 7, the lipase showed the highest activity towards *p*-nitrophenyl caprylate (C8), and the activity was decreased towards substrates with longer chain lengths. *p*-Nitrophenyl laurate (C12) and *p*-nitrophenyl palmitate (C16) are long-chain fatty acid esters with relative activities of 10.9 and 12.1%, respectively. These results indicate that the cell surface-displayed lipase prefers short acyl chain length substrates. These biochemical characters suggested that the enzyme is slightly alkaline lipase. Although the surface display of lipase has many advantages to be used as whole-cell biocatalyst in the field of pharmaceutical, fine chemical, and production of chiral compound, the character of the enzyme is very important. To be used for the lipase in such fields, the enzyme must be stable at the high temperature, high pH and above all, show the stable activity for the prolonged storage. With this point of view, the lipase studied in this paper seems not adequate for the purpose. However, it is possible to be used as a whole-cell biocatalyst in the relatively mild conditions.

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