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Constitutive Expression of Lipase on the Cell Surface of *Escherichia coli* using OmpC Anchoring Motif

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Abstract – We have developed a constitutive display system of the *Pseudomonas fluorescens* SIK W1 TliA lipase on the cell surface of *Escherichia coli* using *E. coli* outer membrane protein C (OmpC) as an anchoring motif, which is an economical compared to induced system. For the constitutive expression of truncated OmpC-TliA fusion proteins, *gntT*104 promoter was employed. Cell growth was not affected by over expression of fusion protein during entire culture time, suggesting cell lysis was not a problem. The localization of truncated OmpC-TliA fusion protein on the cell surface was confirmed by immunofluorescence microscopy and measuring whole cell lipase activity. Constitutively displayed lipase was very stable, retaining activity enantioselectivity throughout the five repeated reactions. These results suggest that OmpC from *E. coli* be a useful anchoring motif for displaying enzymes on the cell surface without any inducers, and this stable surface display system can be employed for a broad range of biotechnological applications.

Key words: Cell surface display, Lipase, Constitutive expression, Outer membrane protein, *Escherichia coli*

1. Introduction

Lipases (triacylglycerol ester hydrolases, EC 3. 1. 1. 3) are ubiquitous enzymes found in various living organisms, including bacteria, plants and animals. Originally, this enzyme catalyzes the hydrolysis of oils, triglycerides and fatty acid esters under aqueous condition and also mediates the transesterification of esters under the non-aqueous solvents. It is one of the most commonly used enzymes in industry, because of its excellent enantioselectivity, commercial availability and broad substrate specificities to natural and unnatural esters of different structures [1-5]. However, there are some drawbacks for the practical usage of soluble enzyme, including difficulty of separation from solution and decrease or elimination of its activity under the non-conventional conditions such as organic solvents, extreme pH or temperature. These problems can be solved by immobilization of enzyme on the solid supports by physical methods such as adsorption on a water-insoluble matrix, gel entrapment, microencapsulation with a solid membrane and formation of enzymatic Langmuir-Blodgett film, and chemical methods including covalent attachment, crosslinking and co-crosslinking with other substances [6-10]. However, these methods also present disadvantages. For example, the interaction between enzymes and supports can be weak, which decreases total activity of immobilized enzyme. In the case of chemical methods, immobilization is rather expensive and covalent bonding often causes structural change of the enzyme [11].

Cell surface display, which is a technique to display peptides or proteins on the surface of gram-negative and gram-positive bacteria, fungi, or even mammalian cells by appropriately fusing them to surface anchoring motifs, can be used a non-conventional method for immobilization of enzyme [12-15]. Several researchers have been reported successful expression of lipase on the cell surface of bacteria such as *E. coli*, *Psedomonas* sp., and yeast strains by employing various anchoring techniques and cell surface displaying enzyme shows excellent enzymatic characteristics [16-19]. However, the expression of fusion proteins was induced by adding expensive isopropyl-β-D-thiogalactopyranoside (IPTG), which results in the increase of total cost. Finally, the industrial application is hampered for this reason. Therefore, a constitutive expression should be considered for further process development for economic consideration.

In this paper, we developed the constitutive display of *P. fluorescens* SIK W1 TliA lipase on the cell surface of *E. coli* using OmpC as an anchoring motif and further investigated stability of surface displayed lipase by repeated usage of genetically immobilized enzyme.

2. Materials and Methods

2-1. Bacterial Strains and plasmids

E. coli XL10-Gold Tet^r Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*^{q}ZΔ*M15* Tn*10* (Tet^r) Amy Cam^r] was used as a host strain for general cloning works and protein expression. Plasmids used in this study are listed in Table 1. All the DNA manipulations, including restriction digestion, ligation and agarose gel electrophoresis, were carried out by standard

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Table 1. Plasmids used in this study

Plasmid	Relevant Characteristics	Reference
p10499A	Ap^r; gntT104 promoter	Park et al., 2002
p104OC1	p10499A derivative; containing 339-bp fragment of ompC of *E. coli*	This study
p104OC2	p10499A derivative; containing 927-bp fragment of ompC of *E. coli*	This study
p104OC3	p10499A derivative; containing 1056-bp fragment of ompC of *E. coli*	This study
p104OC1PL	p104OC1 derivative; *P. fluorescens* SIK WI lipase gene	This study
p104OC2PL	p104OC2 derivative; *P. fluorescens* SIK WI lipase gene	This study
p104OC3PL	p104OC3 derivative; *P. fluorescens* SIK WI lipase gene	This study

procedures. Recombinant *E. coli* was cultivated at 37 °C and 250 rpm in a 250 mL flask containing 100 mL of Luria-Bertani (LB) medium (10 g/liter bacto-tryptone, 5 g/liter bacto-yeast extract and 5 g/liter NaCl) supplemented with 50 mg/mL of ampicillin.

2-2. DNA manipulation

Polymerase chain reaction (PCR) was performed with the PCR Thermal Cycler MP (Takara Shuzo Co., Shiga, Japan) using Expandä High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). DNA sequencing was carried out using the Bigdye terminator cycle sequencing kit (Perkin-Elmer Co., Boston, MA), Taq polymerase, and ABI Prism™ 377 DNA sequencer (Perkin-Elmer Co.). All DNA manipulations including restriction digestion, ligation, and agarose gel electrophoresis were carried out by standard procedures [20].

Primers used in this study are listed in 2. *E. coli* ompC were amplified from the genomic DNA of *E. coli* W3110 using the primers designed based on the reported genome sequences [21].

2-3. Immunofluorescence microscopy

Cells were harvested by centrifugation at 3,500 × g for 5 min at 4 °C, washed with phosphate buffered saline (PBS) solution and resuspended in PBS solution supplemented with 3% (wt/vol) of bovine serum albumin (BSA; Sigma Co., St. Louis, MO). Cells were incubated with mouse anti-6His antibodies diluted (1:1000) in PBS solution containing 3% (wt/vol) BSA at 4 °C for 4 h. After washing five times with PBS solution, the cell-antibody complex was incubated overnight at 4 °C with rabbit anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma) at a dilution of 1:3000. Prior to microscopic observation, cells were washed five times with PBS solution to remove unbound anti-mouse IgG conjugated with FITC. For the immunofluorescence microscopy, cells were mounted on poly-L-lysine coated microscopic slide glasses and examined by confocal microscopy (Carl Zeiss, Jena, Germany). Photographs were taken with a Carl Zeiss LSM 410.

2-4. Measurement of lipase activities

Cells were cultivated in a 250 mL flask containing 100 mL LB medium at 37°C and 250 rpm. Cells were harvested by centrifugation for 5 min at 5,590 g and 4 °C, washed with distilled water, and were lyophilized with freeze dryer (TFD5505, Ilshin Lab., Gyeonggi-do, Korea) for 48 h. Lipase activity was assayed by spectrophotometric method using p-nitrophenyl decanoate as a substrate. The p-nitrophenyl decanoate was dissolved in acetonitrile at a concentration of 10 mM. Ethanol and 50 mM Tris-HCl (pH 8.0) were subsequently added to make a substrate solution having a volume ratio of 1:4:95 (10 mM p-nitrophenyl decanoate in acetonitrile: ethanol: Tris-HCl). Lyophilized cells (0.15 mg), culture aliquot (200 uL) or culture supernatant (500 μL) was added to 3 mL substrate solution for the determination of lipase activity. After incubating the reaction mixture at 37 °C for 10 min, the activity was assayed by detecting the product, p-nitrophenol, spectrophotometrically at 405 nm. The reaction was terminated by adding 2 uL of 0.5 M ethylene diamine tetraacetic acid (EDTA).

For the examination of stability of cell surface displayed lipase, 10 mg of lyophilized cell was resuspended in 10 mL Tris-HCl (pH 8.0) and incubated at 37 °C for 2 weeks. The 0.1 mL aliquots were taken, and added to 1 mL substrate solution for the measurement of residual activity at 37 °C for 10 min.

2-5. Preparation of enantiomerically pure compound

For enantioselective hydrolysis, 300 mg of lyophilized cells was resuspended in 30 mL of 50 mM Tris-HCl (pH 8.0), into which 100 mg of racemic methyl mandelate (Aldrich, St. Louis, MO) was added. The reaction mixture was incubated at 37 °C and 250 rpm. Small aliquots of reaction mixture were removed at 6, 12, 24, 36 and 48h of reaction, and the products were analyzed by high performance liquid chromatography (HPLC; 1100 HPLC system, Agilent, Palo Alto, CA).

2-6. Reusability of the cell surface displayed lipase

300 mg of lyophilized cells was resuspended in 30 mL of 50 mM Tris-HCl (pH 8.0), into which 100 mg of racemic methyl mandelate was added. The reaction mixture was incubated at 37 °C and 250 rpm. The reaction was terminated by centrifugation for 5 min at 559 × g and 4 °C, and the supernatant was sequentially extracted with 30 mL each of ethyl acetate and ethyl ether twice at pH 3.0. Harvested cells were resuspended in 30 mL of 50 mM Tris-HCl (pH 8.0) and reused for the next reaction up to five times.

2-7. Analytical methods

Cell growth was monitored by measuring the optical density at 600 nm (OD600) using a spectrophotometer (BECKMAN DU650, Fullerton, CA). For the analysis of product ((S)-mandelic acid), Chiralcel

OD-H column (Daicel) was employed using a mixture of hexane, isopropanol and trifluoroacetic acid having a volume ratio of 80:20:1 as a mobile phase at a flow rate of 0.5 mL/min. Reaction products and substrates were detected by measuring the absorbance at 210 nm using a diode array detector (1100 HPLC DAD, Agilent).

3. Results

3-1. Development of constitutive display system

For the display of *P. fluorescens* SIK W1 TliA lipase on the surface of *E. coli*, we first searched for the possible anchoring motif. Previously, OmpC of *E. coli* had been used for the anchoring motif of surface display; we selected this as an anchoring motif [22]. For the constitutive expression of lipase on the cell surface, the truncated *ompC* (*ompC*$_t$) genes encoding the 92, 288 and 331 amino acids from the N-terminus were amplified by using primer sets shown in Table 2, and were cloned into the *Sac*I and *Xba*I sites of p10499A [23], which has the constitutive *gntT*104 promoter, to make p104OC1, p104OC2 and p104OC3, respectively. Serine and arginine were additionally inserted at the C-terminus by introducing the *Xba*I site at the 3' end of the *ompC*$_t$ genes. The *P. fluorescens* SIK W1 lipase gene from pTacOprF188PL [24] was inserted into the *Xba*I and *Hind*III sites of p104OC1, p104OC2 and p104OC3 to make p104OC1PL, p104OC2PL and p104OC3PL, respectively. Recombinant XL-10 Gold cells harboring all six plasmids were cultivated at 37°C. Growth defects were not severe for all of the recombinant cells throughout the culture time. Therefore, recombinant XL-10 Gold harboring p104OC1PL, p104OC2PL and p104OC3PL were used in further studies.

3-2. Confirmation of cell surface display

To examine the surface display of lipase, lipase on the *E. coli* cell surface was analyzed by immunofluorescence microscopy. As shown in Fig. 1B, *E. coli* XL-10 Gold (p104OC1PL) became fluorescent due to the binding of anti-His antibody followed by binding of FITC-conjugated secondary antibody. On the other hand, *E. coli* XL-10 Gold cells harboring p10499A was not fluorescent at all (Fig. 1A). These results suggest that lipase was successfully displayed in an active form using the OmpC$_t$ as an anchoring motif on the surface of *E. coli* without significant cell lysis.

After confirmation of successful display of lipase on the cell surface, whole cell lipase activity was measured during the cultivation. Fig. 2(A) shows the relative activity of cell surface displayed lipase after 24 h culture. The highest whole cell lipase activity was obtained in the case of p104OC2PL. Fig. 2(B) shows the time profiles of specific

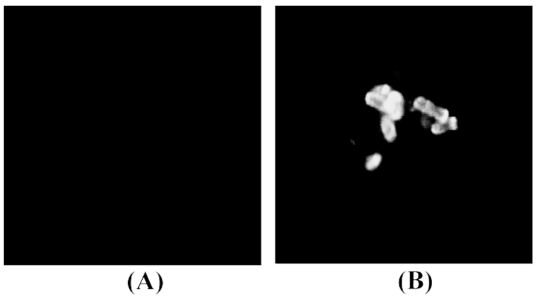

Fig. 1. Immunofluorescence micrographs of recombinant *E. coli* XL-10 Gold harboring p10499A (A) and p104OC1PL (B). Cells were incubated with rabbit anti-lipase probe antibody followed by probing with goat anti-rabbit IgG-FITC conjugate.

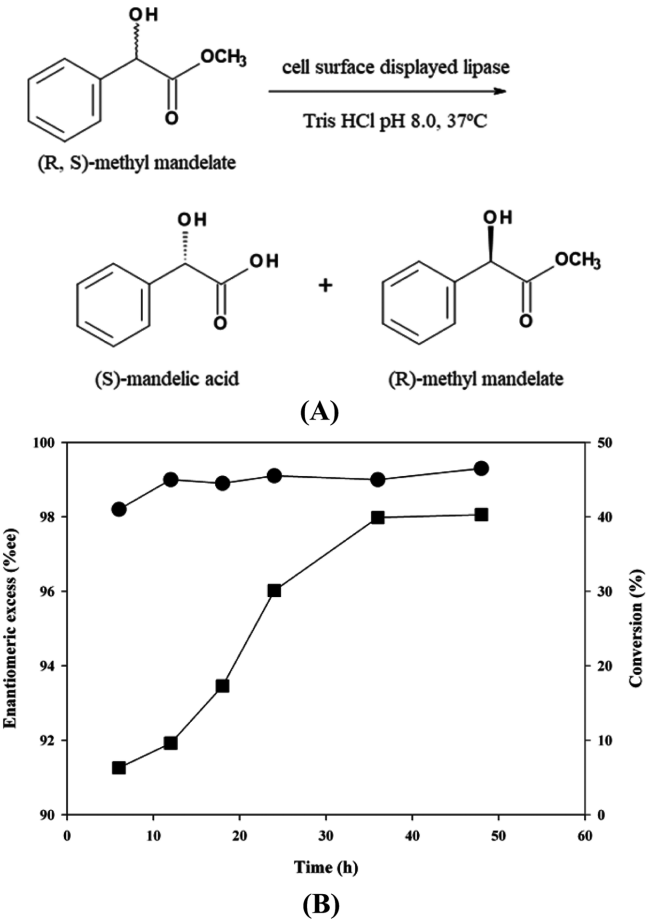

Fig. 2. Relative activity (A) and time profiles of the lipase activity (B) of recombinant *E. coli* XL-10 Gold harboring p104OC1PL (●), p104OC2PL (■) and p104OC3PL (○) continuously displaying lipase at 37 °C in LB medium.

lipase activity of XL10-Gold harboring p104OC1PL, p104OC2PL and p104OC3PL during cultivation at 37 °C. The whole cell lipase

Table 2. Primers used in this study

Primer	Primer sequence[a]	Gene to be amplified	Template DNA used
Primer 1	5'-c**gagctc**atgaaagttaaagtactgtcc	truncated *ompC*	*E. coli* W3110 chromosome
Primer 2	5'-ggc**tctaga**acgaccgttgttagttacgcc		
Primer 3	5'-ggc**tctaga**gccacgacccaggtttttacc		
Primer 4	5'-ggc**tctaga**gccagcgtcacgagtgaactg		

[a]Restriction enzyme sites are shown in bold.

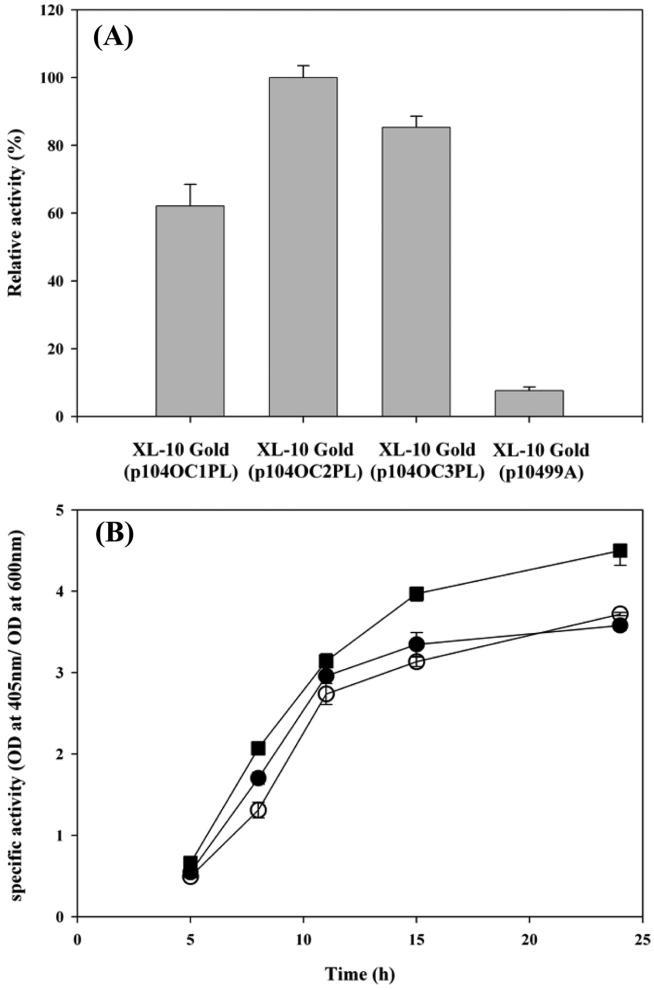

Fig. 3. Reaction scheme (A) and time profiles (B) of the enantioselective resolution of methyl mandelate using lipase displayed on the surface of *E. coli* XL-10 Gold (p104OC2PL). Time profiles of conversion (■) and enantiomeric excess (●) of reaction products are shown.

activity was detected from the early exponential phase to stationary phase in all recombinant *E. coli*. However, little lipase activity was detected in the supernatant. These results suggest that lipase was constitutively expressed on the cell surface with active form under the control of *gnt*T104 promoter without significant cell lysis during the entire culture period.

3-3. Biocatalytic applications of constitutive cell surface displayed lipase

For the possible application as a biocatalyst, we investigated the enantioselective resolution of racemic methyl mandelate. The scheme of this reaction is shown in Fig. 3(A). Time profiles of reaction during enantioselective resolution are shown in Fig. 3(B). The conversion of reaction and enantiomeric excess of the product, (*S*)-mandelic acid obtained in 48 h were over 40% and 99%, respectively.

Next, the stability of cell surface displayed lipase was investigated by carrying out repeated cell recycle reactions with methyl mandelate as a model substrate. The conversion and enantiomeric excesses of

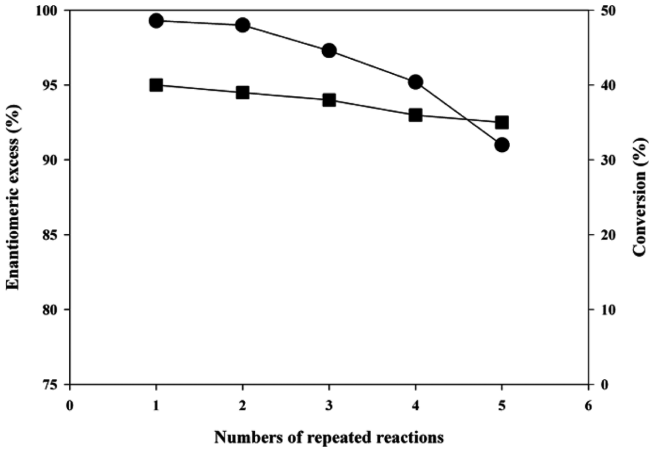

Fig. 4. Time profiles of conversion (■) and enantiomeric excess (●) during the repeated resolution of racemic methyl mandelate using XL-10 Gold (p104OC2PL).

product obtained after the repeated reactions are shown in Fig. 4. The catalytic activity of cell surface displayed lipase was maintained for five repeated reactions (120 h) with slight decrease of enantiomeric excess of mandelic acid (99% to 91.0%). These results suggest that the lipase displayed on the *E. coli* cell surface using the OmpC seemed to be maintained stably active during the repeated usage.

4. Discussion

Microbial cell surface display has been employed in a wide range of applications including live vaccine, adsorbents, biosensors, and whole cell biocatalyst along the enzyme displayed [12,13]. Among the various surface displayed enzymes, lipase expressed on the cell surface showed high stability and activity under harsh condition and could be used for the many biotechnological applications [11,16-19]. Expression of protein on the cell surface sometimes causes membrane instability or growth inhibition. This problem can be solved by optimization of fusion point, tightly regulation of expression [17,24].

In this paper, we developed a stable expression of lipase on the cell surface of *E. coli* by combination of constitutive expression and optimization of fusion point using outer membrane protein OmpC from *E. coli* as an anchoring motif. Previously, we reported that *E. coli* OmpC can be used as a successful fusion point for the display of poly-histidine peptides on the surface of *E. coli* by sandwich fusion strategy [22]. However, this strategy is not available for the display of larger protein. There have been several papers that show successful display of larger proteins by applying C-terminal deletion-fusion [18,24]. Therefore, we decided to employ the C-terminal deletion-fusion as a fusion strategy. After searching the possible anchoring motif and fusion strategy, we found the possible fusion point of anchoring motif for the C-terminal deletion. Based on the predicted secondary structure and information found in the literature, we chose Arg^{92}, Gly^{288} and Gly^{331} as potential fusion sites for displaying *P. fluorescens* SIK W1 lipase [22,25]. As shown in Fig. 2, all these sites

were suitable for the surface display of protein without induction, and membrane instability or growth inhibition was not shown in all cases.

For industrial applications, stability is one of the most important aspects. The constitutive display using OmpC anchor shows good stability during five repeated reactions. Whole cell lipase activity of surface displayed lipase and the enantiomeric excess of product maintained over 90% during the reactions (Fig. 4). The stability of cell surface displaying lipase has been reported, and the enzymatic characteristics were enough to apply to industry [18,24,26,27] However, all the reported systems were based on the inductive expression system. Therefore, addition of expensive inducer such as IPTG is needed before use, which results in the increase of total production cost. On the contrary, the constitutive display system, reported in this paper, does not need any inducer to express enough proteins on the cell surface suitable for the production of enantiomerically pure compounds without induction. These findings suggest that constitutive surface display system can be used as a new, cost-effective immobilized enzyme for the enantioselective biocatalysis.

In conclusion, we developed the constitutive and stable display of lipase on the cell surface using *E. coli* OmpC as an anchoring motif via C-terminal deletion-fusion strategy. The expression of enzyme can be maintained throughout the cultivation without expensive inducer such as IPTG. We also demonstrated the repeated usage of constitutive displayed lipase as an enantioselective biocatalyst of racemic methyl mandelate, suggesting that it can be used for a cost-effective biocatalyst for the production of enantiomerically pure compounds.

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