



Development of yeast cell-surface display system and it' s application for bioconversion process

谷野, 孝徳

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DEVELOPMENT OF YEAST CELL-SURFACE DISPLAY SYSTEM AND IT'S APPLICATION FOR BIOCONVERSION PROCESS

酵母細胞表層提示技術の開発ならびにバイオコンバージョンプロセスへの応用

TAKANORI TANINO

2007

PREFACE

This is a thesis submitted by the author to Kobe University for the degree of Doctor of Engineering. The studies collected here were carried out between 2001 and 2007 under the direction of Professor Akihiko Kondo in the Laboratory of Biochemical Engineering, Department of molecular Science and Material Engineering, Graduate School of Science and Technology, Kobe University.

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Takanori Tanino

Biochemical Engineering Laboratory

Department of molecular Science and Material Engineering

Graduate School of Science and Technology

Kobe University

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Introduction

In recent years, as the advances of biotechnology including genetic engineering and cell engineering, the organic compound production processes using biological functions, i.e. bioconversion process, is attracting more interest than ever.

Today at the time of facing the environmental and energy problems, development of environmentally friendly process, which enables continuous growth of industry, has been widely investigated. Most of enzymatic reaction, which controls biological functions, could be carried out under the common reaction condition such as ordinary temperature and pressure and neutral pH, and is suitable for environmentally friendly process. In addition, bioconversion process, such as application of metabolic pathway and enzymatic stereoselective reaction, is also suitable for the production of chemicals or pharmaceuticals with complex frame and optical activity.

From these backgrounds, increase in future usage of bioconversion process is undeniable and development of novel distributing system of enzymatic catalysis and function of organism are important. To fulfill these requirements, yeast cell-surface display system is one of the most attractive systems that could add a new function to yeast cell efficiently.

The present study investigated the development of the yeast cell-surface systems, and the bioconversion process with novel enzyme displaying yeast cell by yeast cell-surface display system as the whole cell biocatalyst.

Yeast cell-surface display system

Yeast cells displaying functional proteins and peptides have a wide range of applications in biotechnology (Kondo and Ueda 2003; Ueda and Tanaka 2000a), for instance, yeast cell displaying antibody and histidine oligo-peptide for analytical application and bioseparation (Boder and Wittrup. 1997; Feldhaus et al. 2003; Kuroda et al. 2001). In particular, enzyme displaying yeast cell as the whole cell biocatalyst have many advantages of high enzymatic activity without permeabilization treatment,

easy and cost-effective reproduction, easy separation from product and sequential reaction by combining enzymes displayed on yeast cell-surface with metabolic pathway, on the application area of bioconversion (Ueda and Tanaka 2000b).

In order to display functional proteins on yeast cell-surface, secretion signal sequence and anchor protein have to be genetically fused to the target protein. As the anchor system, agglutinin system using α -agglutinin (Boder and Wittrup 1997; Boder et al. 2000; Keike et al. 1999) and α -agglutinin (Murai et al. 1997a, b; Shibasaki et al. 2001a), is one of the most well-known system with putative glycosylphosphatidylinositol (GPI) anchor. The other famous system is the Flo1p anchor system using flocculation functional domain (Matsumoto et al. 2002, Watari et al. 1994). In both systems, target protein is produced as the fusion protein with anchor protein and immobilized on the cell wall.

In this present study, we developed a novel cell-surface localization system based on the invertase (β -fructofuranoside fructohydrolase, EC.3.2.1.26) from *Saccharomyces cerevisiae*. In addition to the cell wall, the periplasm, which is the space between the cell wall and the plasma membrane, can also be use to localize target proteins. Since yeast external invertase localizes in the periplasm (Beteta and Gascon 1971; Tammi et al. 1987), it is possible to localize target protein in the periplasm using invertase as an anchor protein.

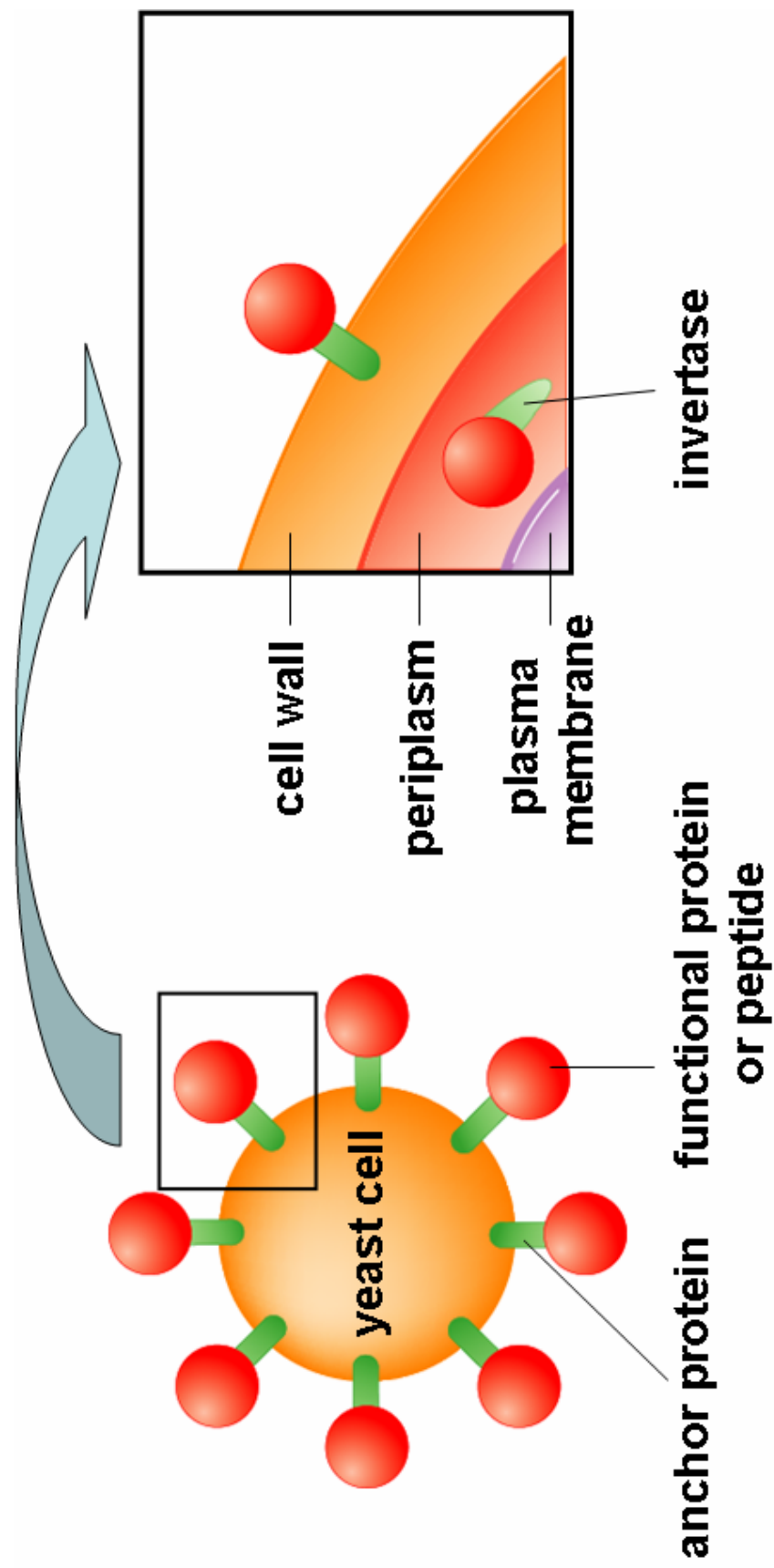


Figure 1 Yeast cell displaying functional protein on its cell-surface and structure of yeast cell-surface

Host strain of yeast cell-surface display system

Yeast cell has many advantages as a host strain of cell-surface display system. It has a rigid cell wall, about 200 nm thick and mainly consisted of β -linked glucans and mannoproteins, lying outside of the plasma membrane (Lipke and Ovalle 1998). Simplicity in genetic manipulation and ability to fold and glycosylate expressed heterologous eukaryotic proteins are also useful characteristics of yeast.

S. cerevisiae has “generally regarded as safe” (GRAS) status attributed to practical accomplishments in food and alcohol brewing industry, therefore it has been mainly used in development of yeast cell-surface display system (Schreuder et al. 1993). However, there are several other species that might be useful as the host strain of yeast cell-surface display system.

In the present study, we constructed a *Pichia pastoris* cell-surface display system based on Flo1p anchor system. In a large-scale manufacturing process, productivity of enzyme displaying yeast cells and stability of enzymes displayed on yeast cell-surface are required. *P. pastoris* can be cultivated to a very high density and produces the heterologous proteins under control of the alcohol oxidase 1 promoter (pAOX1) that is strongly inducible by methanol (Cereghino and Cregg 2000; Cregg et al. 2000). *P. pastoris* is thus a useful host for the host strain of yeast cell-surface display system.

Determination of cultivation condition for enzyme displaying yeast cell using Flo1p anchor system for effective utilization

An anchor system based on Flo1p is effective for the display of the proteins which have the functional domains such as catalytic domain and substrate binding domain near the C-terminus (Matsumoto et al. 2002; Shigechi et al. 2002). Fusion protein of Flo1p anchor and target protein is thought to localize on yeast cell-surface via noncovalent interaction between the flocculation functional domain of Flo1p and the mannan chain of the cell wall. Therefore, several cultivation conditions and medium components might inhibit the interaction between Flo1p anchor and cell-surface.

In order to investigate the effect of cultivation conditions, high stress tolerance and proliferation potential are required for the yeast cell used as the host strain of yeast cell-surface display system. Sake yeast, which is known to be a diploid cell with high stress tolerance and proliferation potential is one of the most attractive and suitable host strain to fulfill these requirements.

In this present study, by taking advantage of sake yeast, we investigated the effect of the cultivation conditions on the cell-surface display system using Flo1p anchor system and suitable condition to display the fusion protein on the cell-surface.

Applications of enzyme displaying yeast cell for bioconversion process

Bioconversion process with enzyme displaying yeast cells which achieved the most successful outcome is ethanol production from biomass materials. Several investigations for ethanol production from starchy materials with amylases displaying yeast cell have been reported (Kondo et al. 2002; Murai et al. 1997b; Shigechi et al. 2002), and recently, efficient and direct ethanol production from raw starch was achieved (Shigechi et al. 2004). Ethanol production processes from cellulosic biomass such as cellulose (Fujita et al. 2002a, 2004; Murai et al. 1997a, 2001) and hemicellulose (Fujita et al. 2002b; Katahira et al. 2004) have also been investigated.

In the present study, bioconversion with enzyme displaying yeast cell in organic solvent was investigated. There have been a few demonstrations of application of enzyme displaying yeast cell for bioconversion in organic solvent (Matsumoto et al. 2004; Nakamura et al. 2006), however, in addition to the above bioconversion process carried out under aqueous condition, utilization of enzyme displaying yeast cell in organic solvent is one of the most attractive field.

***Candida antarctica* lipase B displaying yeast cell**

Lipases (triacylglycerol lipase, EC 3.1.1.3) have emerged as key enzymes in swiftly growing biotechnology. As the source of lipase, microorganisms, mainly bacteria and fungi, are fruitful lipase source, and various lipases of microbial origin have been investigated and utilized in a wide range of industrial and biotechnological

applications, such as biodiesel, biopolymer, pharmaceuticals, cosmetics, agrochemicals and flavor compounds production (Gupta et al. 2004; Jaeger and Eggert 2002). Among the microbial lipases, *Candida antarctica* lipase B (CALB) is one of the most famous and practical lipase (Fig. 2). In many studies, CALB was shown to successfully catalyze many reactions, for instance, the oil alcoholysis reaction including biodiesel production (Modi et al. 2006; Torres et al. 2004), the kinetic selective reaction (Fransson et al. 2006; Lou and Zong, 2006) and the ester synthesis reaction (Larios et al. 2004; McCabe and Talor 2002). However, high cost of the enzyme often becomes problem in the industrial scene.

In this present study, we constructed a novel yeast whole-cell biocatalyst displaying CALB on the cell-surface. Furthermore, we applied the CALB displaying yeast whole-cell biocatalyst to the ester synthesis reaction using adipic acid and *n*-butanol, the model of the condensation reaction, at relatively high temperature.

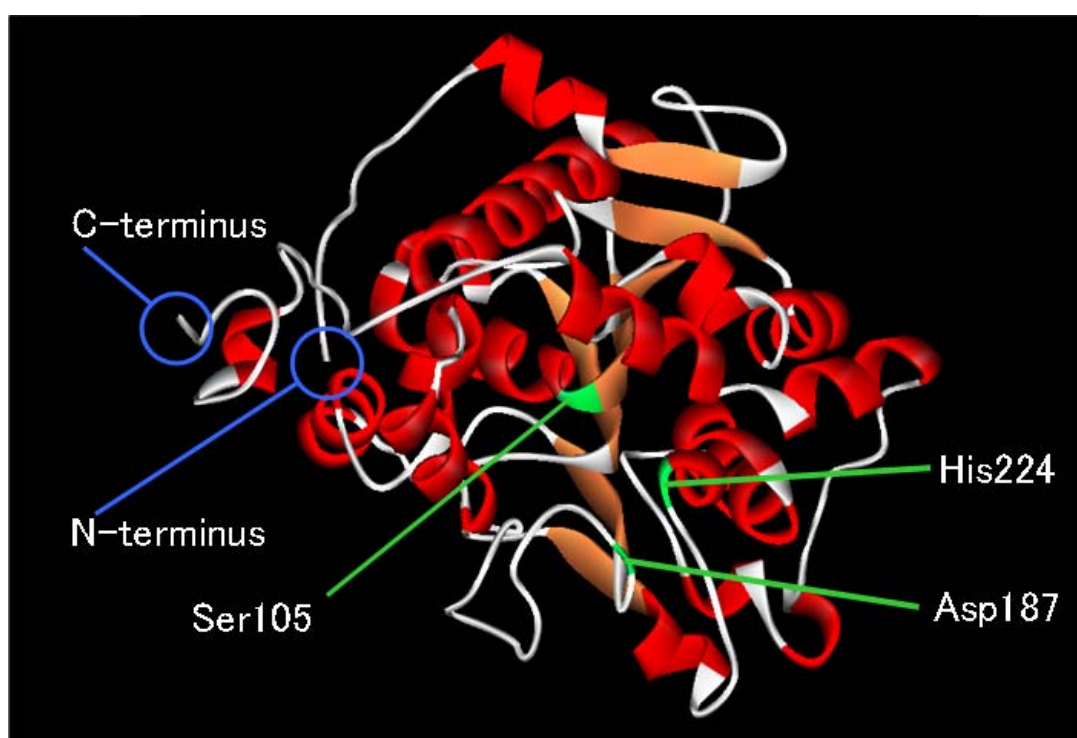


Figure 2 Schematic model of structure of *Candida antarctica* lipase B calculated by MODELLER8v1* with 1tca.pdb file (Uppenberg et al. 1994)

*University of California San Francisco, <http://salilab.org/modeller>

Modification of phospholipid by phospholipase

Phospholipids are polar lipid, which is one of the principal structural components of biomembrane and play an important role in signaling pathways (Exton 1994; Wang et al. 2006). Their applications in foods, pharmaceuticals including liposome preparation for drug delivery system and cosmetics industries keep increasing. Lecithin is industrially available phospholipid that could be prepared in large quantities at low cost from egg or soy bean, however, the bulk of lecithin is composed of phosphatidylcholine (PC) and effective modification is important to obtain desired phospholipids. Enzymatic modifications of phospholipids by phospholipases, transphosphatidylation by phospholipase D (PLD) (EC 3.1.4.4) (Dittrich and Ulbrich-Hofmann 2001; Sato et al. 2004) and transesterification by phospholipase A (Vijeeta et al. 2004) are suitable for selective exchange of base and fatty acids. .

In this present study, we constructed a PLD from *Streptoverticillium cinnamoneum* (Ogino et al. 1999) displaying yeast cell. By using the PLD displayed recombinant yeast, the phospholipid modification to PS from PC was investigated in a biphasic system. Furthermore, the transphosphatidylation reaction conditions of PS from PC were optimized by PLD displaying yeast cell.

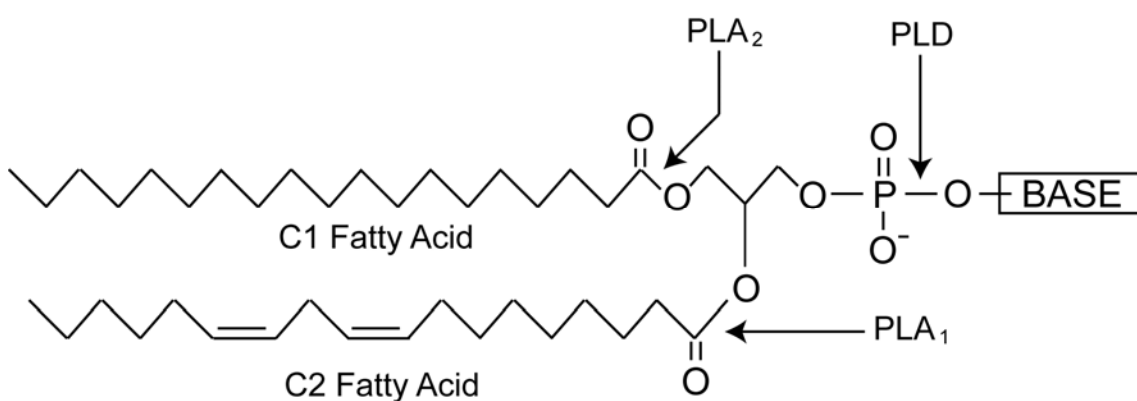


Figure 3 Structure of phospholipid and mode of action of phospholipases

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Synopsis

Part I. Construction of novel yeast cell-surface display system

Chapter 1. Construction of system for localization of target protein in yeast periplasm using invertase

We constructed a novel system for periplasmic localization of target proteins, using yeast external invertase (INV) as anchor protein, in which the C- or N-terminal of the target protein was fused to the invertase and the fusion proteins expressed under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (GAPDH). Unlike in conventional cell-surface display, the system enables the target fusion protein to localize in yeast periplasm in a free state. As a model, enhanced green fluorescence protein (EGFP) was localized in yeast periplasm using the new system. Yeast-periplasm localization of INV-EGFP and EGFP-INV fusion proteins was confirmed by fluorescence microscopy and immunoblotting: green fluorescence was observed at the cell outline and, in western blot analysis, most fusion proteins were detected in the cell-surface fraction, indicating that the fusion proteins had been transported to the cell-surface layer. In addition, in both C- and N-terminal fusion, invertase showed activity, indicating dimer formation. These results demonstrate that invertase is a useful anchor for localizing target protein in the yeast periplasm.

Chapter 2. Construction of *Pichia pastoris* cell-surface display system using Flo1p anchor system

Pichia pastoris cell-surface display system was constructed using a Flo1p anchor system, which was developed in *Saccharomyces cerevisiae*. The lipase from *Rhizopus oryzae* with a Pro sequence (ProROL) was used as the model protein and was genetically fused to the anchor consisting of amino acids 1 to 1099 of Flo1p (FS anchor). The resulting fusion protein FSPROROL was expressed under the control of the alcohol oxidase 1 promoter (pAOX1). The fluorescence microscopy of immunolabeled *P. pastoris* cells revealed that ProROL was displayed on the cell-surface, and western blot analysis revealed that the fusion protein FSPROROL was noncovalently attached to the cell wall and highly glycosylated. The lipase activity of *P. pastoris* cells was affected by the methanol concentration for the induction phase. Surprisingly, the activity of lipase displayed on the cells incubated at 60°C was not only stable, but also increased about 6.5 times of the initial value after 4 h incubation.

Part II. Optimization of enzyme display yeast cell preparation using Flo1p anchor system for effective utilization

Effect of cultivation conditions on cell-surface display of Flo1 fusion protein using sake yeast

The cell-surface display of the Flo1p anchor system with a flocculation functional domain was examined under various cultivation conditions. As a model system, lipase from *Rhizopus oryzae* with a Pro sequence (ProROL) was genetically fused to the Flo1 short (FS) anchor (FSProROL) and displayed on the sake yeast cell-surface under the control of the *SED800* promoter (p*SED800*). The nutrients and carbon source in the culture media affected the display of the fusion protein FSProROL on the sake yeast cell-surface. The lipase activity in whole cells cultivated in poor media, without peptone and/or yeast extracts, were higher than those cultivated in rich media. In addition, glucose and maltose were effective carbon sources for increasing the lipase activity in whole cells, and the addition of di- or tri-saccharide as the carbon source reduced the release of the lipase activity into the culture supernatants. The initial glucose concentration was found to influence the total lipase activity and it mainly affected the lipase activity in whole cells. Under the optimum condition, sake yeast was found to show high cell density and high lipase activity in short time cultivation.

Part III. Bioconversion with enzyme displaying yeast whole cell biocatalyst

Chapter 1. Development of yeast cells displaying *Candida antarctica* lipase B and their application to ester synthesis reaction

We isolated the lipase B from *Candida antarctica* CBS 6678 (CALB CBS6678) and successfully constructed CALB displaying yeast whole-cell biocatalysts using the Flo1p short (FS) anchor system. For the display of CALB on a yeast cell-surface, the newly isolated CALB CBS6678 exhibited higher hydrolytic and ester synthesis activities than the well-known CALB, which is registered in GENBANK. A protease accessibility assay using papain as a protease showed that a large part of CALB, approximately 75%, was localized on an easily accessible part of the yeast cell-surface. A comparison of the lipase hydrolytic activities of yeast whole-cells displaying only mature CALB (CALB) and those displaying mature CALB with a Pro region (ProCALB) revealed that mature CALB is preferable for yeast cell-surface display using the Flo1p anchor system. Lyophilized yeast whole-cells displaying CALB were applied to an ester synthesis reaction at 60°C using adipic acid and *n*-butanol as substrates. The amount of dibutyl adipate (DBA) produced increased with the reaction time until 144 h. This indicated that CALB displayed on the yeast cell-surface retained activity under the reaction conditions.

Chapter 2. Synthesis of functional phospholipids using phospholipase displaying yeast

Displaying of phospholipase D (PLD, EC 3.1.4.4) on the yeast cell-surface was performed by using *Saccharomyces cerevisiae* BY4741 Δ W as a host strain and Flo1 short anchor as an anchor protein, respectively. The PLD anchored yeast (BY4741 Δ W/PLD) exhibited 0.372 U/g-dry cell of hydrolysis activity for phosphatidylcholine (PC). In addition, the transphosphatidylation reaction to phosphatidylserine (PS) from PC using BY4741 Δ W/PLD in a biphasic system was archived and the optimized reaction conditions for transphosphatidylation reaction were investigated by changing the follow various parameters; organic solvents mixture ratio, pH, temperature, L-serine concentration and two-phase (aqueous; organic phase) volume ratio. As a result, PLD displayed yeast cells could effectively catalyze the transphosphatidylation reaction to PS from PC under the optimized condition, and the PS conversion yield of initial and final phases of the reaction improved from 19.7% to 46.0% and from 50.8% to 57.5%, respectively. Therefore, these results demonstrated that BY4741 Δ W/PLD was an effective catalyst for transphosphatidylation reaction and suggested that PLD displaying yeast could be employed in various phospholipids synthesis.

Part I. Construction of novel yeast cell-surface display system

Chapter 1. Construction of system for localization of target protein in yeast periplasm using invertase

Introduction

Cells that display enzymes on their surface can be used as whole-cell biocatalyst, and systems for display of heterologous proteins on the yeast-cell surface have therefore recently been widely studied (Fujita et al. 2002; Matsumoto et al. 2002; Murai et al. 1998; Nakamura et al. 2001; Sato et al. 2002). By combining the displayed enzymes with a metabolic pathway, it is possible to catalyze sequential reactions. For example, a yeast displaying endoglucanase II and β -glucosidase on the cell surface can produce ethanol directly from cellulosic material (Fujita et al. 2002). Yeast-based cell-surface display systems have the advantages of safety, simplicity of genetic manipulation, and rigidity of cell-surface structure.

In widely used cell-surface display systems, the target protein is immobilized on the cell-wall using the glycosylphosphatidylinositol (GPI)-anchor attachment signal sequence (Caro et al. 1997) or the flocculation functional domain of the Flo1 gene (Matsumoto et al. 2002). In addition to the cell wall, the periplasm, which is the space between the cell wall and the plasma membrane, is also advantageous for localizing target proteins. For instance, a whole-cell biocatalyst can achieve high activity through accumulation of enzyme in the periplasm layer, as such an arrangement circumvents the problem of substrate diffusion through the periplasm membrane. Moreover, by combining this novel periplasmic localization system with widely used cell-surface display systems, it will be possible to localize more enzymes in the cell-surface layer, namely outside of the plasma membrane.

In the present study, we developed a novel cell-surface localization system based on the invertase (β -D-fructofuranoside fructohydrolase, E.C.3.2.1.26; INV) from *Saccharomyces cerevisiae*. Since yeast external invertase localizes in the periplasm (Beteta and Gascon 1971; Tammi et al. 1987), it is possible to localize target protein in

the periplasm using invertase as an anchor protein. Enhanced green fluorescence protein (EGFP) was chosen as the target protein, as it allows localization to be visualized. EGFP was fused to the N- or C-terminal of invertase to investigate whether these latter could be used to achieve periplasmic localization of the protein.

Materials and methods

Strains and media

Escherichia coli NovaBlue (Novagen Inc., Madison, WI. USA) was used as the host strain for recombinant DNA manipulation. The *SUC2* gene was cloned from *S. cerevisiae* ATCC60715 (*MATa FLO8 his4 leu2-3 leu2-112 STA1*). *S. cerevisiae* MT8-1 (*MATa ade his3 leu2 trp1 ura3*) was used for production of INV/EGFP fusion proteins. *E. coli* was cultivated in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 0.5% sodium chloride) containing 100 µg/ml ampicillin. Yeasts were grown in complete medium (YPD: 1% yeast extract, 2% peptone, 2% glucose) or selective medium (SD: 0.67% yeast nitrogen base supplemented with appropriate amino acids and nucleotides, 0.5% glucose). For plate medium, 2% agar was added.

Construction of expression plasmids

Construction of plasmid pWGP3-SUC2 for expression of *SUC2*

SUC2 was amplified from *S. cerevisiae* ATCC60715 chromosomal DNA by polymerase chain reaction (PCR) with the following primers: SUC2-fw-SacI (5'-atcgagctcATGCTTTTGCAAGCTTTCCTTTTCCTTTTG-3') and SUC2-rev-SalI (5'-cgatgcgacCTATTTTACTTCCCTTACTTGGAAGTTGTC-3'). PCR was carried out using *pfu* hotstart turbo polymerase (Stratagene Cloning Systems, La Jolla, CA, USA). The amplified fragment was digested with *SacI* and *SalI* and inserted into the *SacI* and *SalI* sites of pWGP3 (Takahashi et al. 2001) (Fig. 1a). The resulting plasmid was named pWGP3-SUC2 (Fig. 1b)

Construction of plasmid pWGP3-ES for expression of EGFP-INV fusion protein

The secretion signal sequence and mature region of *SUC2* were amplified from *S. cerevisiae* ATCC60715 chromosomal DNA by PCR with the following primers: SUC2-fw-SacI and SUC2(signal)-rev-BglII-ctc-SalI (5'-cgatgtcgacgagagatctTGATGCAGATATTTTGGCTGC-3') for the secretion signal of SUC2; and SUC2(mature)-fw-SalI (5'-atcggtcgacATGACAAACGAACTAGCGATAGACCTTTG-3') and SUC2-rev-sphI-XhoI (5'-cgatctcgaggcctatTTTACTTCCCTTACTTGGAA-3') for the mature region of SUC2. The amplified fragments were digested with *SacI* and *SalI*, and with *SalI* and *XhoI*, respectively. First the fragment encoding the secretion signal sequence of *SUC2* was inserted into the *SacI* and *SalI* sites of pWGP3 (named pWGP3-S), and then the fragment encoding the mature region of *SUC2* into the *SalI* and *XhoI* sites of the plasmid. The resulting plasmid was named pWGP3-SS. The gene encoding EGFP (*EGFP*) was amplified from pEGFP (Clontech Laboratories Inc., Palo Alto, CA, USA) by PCR with the following primers: EGFP-fw-BglII (5'-atcgagatctATGGTGAGCAAGGGCGAGGAGCTGTTCACC-3') and EGFP(n)-rev-SalI (5'-cgatgtcgacCTTGTACAGCTCGTCCATGCCGAGAGTGAT-3'). The amplified fragment was digested with *BglII* and *SalI* and inserted into the *BglII* and *SalI* sites of pWGP3-SS and the resulting plasmid named pWGP3-ES (Fig. 1c).

Construction of plasmid pWGP3-SE for expression of INV-EGFP fusion protein

SUC2 without stop codon was amplified from *S. cerevisiae* ATCC60715 chromosomal DNA by PCR with the following primers: SUC2-fw-SacI and SUC2(n)-rev-SalI (5'-cgatgtcgacTTTTACTTCCCTTACTTGGAACTTGTCAT-3'). The amplified fragment was digested with *SacI* and *SalI* and inserted into the *SacI* and *SalI* sites of pWGP3 and the resulting plasmid named pWGP3-S(n). *EGFP* was also amplified from pEGFP by PCR with the following primers: EGFP-fw-SalI (5'-atcggtcgacATGGTGAGCAAGGGCGAGGAGCTGTTCATG-3') and EGFP-rev-SalI (5'-cgatgtcgacTTACTTGTACAGCTCGTCCATGCCGAGAGT-3'). The amplified fragment was digested with *SalI* and inserted into the *SalI* site of

pWGP3-S(n) and the resulting plasmid named pWGP3-SE (Fig. 1d).

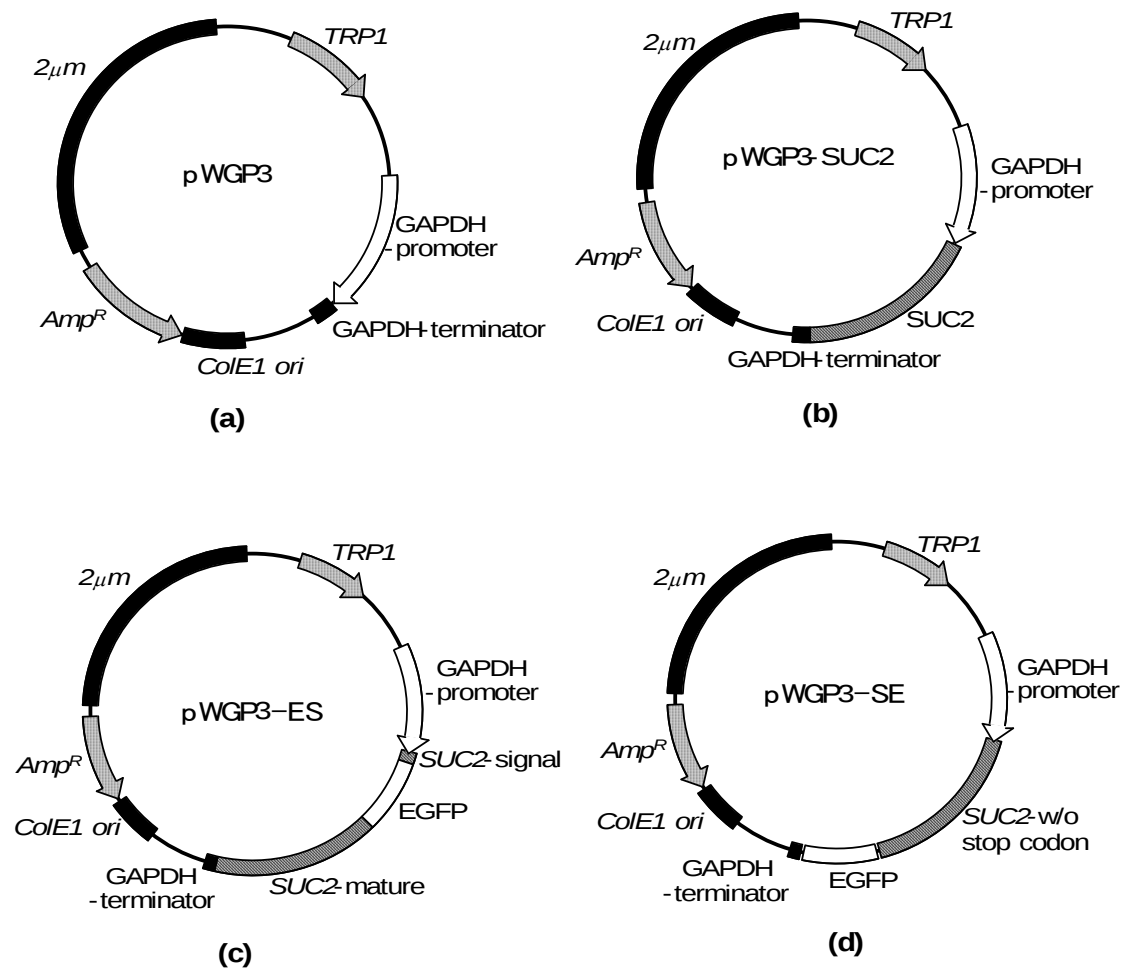


Figure 1 Control plasmid (a, pWGP3) and plasmids for expression of INV (b, pWGP3-SUC2), EGFP-INV fusion protein (c, pWGP3-ES), and INV-EGFP fusion protein (d, pWGP3-SE).

Yeast transformation

The expression plasmids prepared above were transformed into *S. cerevisiae* cells using YEAST MAKERTM (Clontech Laboratories Inc.) according to the protocol specified by the supplier.

Cultivation

Yeast transformants were precultivated in SD medium at 30°C for 30 h ($OD_{600} > 1.5$) and used as starters to inoculate 100 ml of SDCH medium (SD medium containing 2% casamino acids, 50 mM HEPES) in a 500-ml shaking flask to give initial OD_{600} of 0.03.

Measurement of EGFP fluorescence

FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA) was used to determine the fluorescence intensity of EGFP. Event rate was maintained at 500 cells/s and data for 10,000 events collected.

Fractionation of cell-surface proteins and intracellular proteins

Cells cultivated in SDCH medium for 72 h at 30°C were collected by centrifugation at 3,000 g for 10 min, washed twice with phosphate-buffered saline (PBS: 50 mM phosphate, and 150 mM sodium chloride, pH 7.4), and resuspended in 10 ml/g-cell Tris-SO₄ buffer (Tris 100 mM, 10 mM DTT, pH 9.4). The cell suspension was shaken (90 rpm) for 15 min at 30 °C, washed with 50 ml of Sorbitol/K⁺-Pi buffer (16 mM K₂HPO₄, 4 mM KH₂PO₄, 1.2 M Sorbitol, pH 7.4), and resuspended in 5 ml spheroplasting buffer (Sorbitol/K⁺-Pi buffer containing 1 mM PMSF and 0.5 µg/ml leupeptin). Zymolyase (Seikagaku Corp., Tokyo, Japan) was added to the suspension (5 g/g-cell), which was shaken at 90 rpm and 30°C for 60 min. After centrifugation at 3,000 g for 10min, a supernatant fraction (periplasmic fraction) containing cell-surface protein was obtained. The pellets were washed with spheroplasting buffer, resuspended in 5 ml of the same buffer, and the suspension agitated vigorously with acid-washed glass beads. After centrifugation at 3,000 g for 10 min, a supernatant fraction (intracellular fraction) containing intracellular proteins was obtained.

Endo Hf treatment

To remove *N*-glycosylated carbohydrates from the fusion proteins, endoglycosidase treatment was carried out by using Endo Hf (New England BioLabs, Beverly, MA, USA) according to the protocol specified by the supplier.

Western blot analysis of EGFP

Sodium dodecil sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using an 8% (wt/vol) gel was carried out. The proteins separated on the gel were electroblotted on a polyvinylidene difluoride (PVDF) membrane (Millipore Co., Boston, MA, USA) and allowed to react with primary rabbit anti-GFP IgG antibodies (Invitrogen Co., Carlsbad, CA, USA) and secondary goat anti-rabbit IgG alkaline phosphatase-conjugated antibodies (Vector Laboratories Inc., Burlingame, CA, USA). The membrane was then stained with nitro-blue tetrazolium chloride (NBT, Promega Co., Madison, WI, USA) and 5-bromo-4-chloro-3-indolylphosphate toluidine salt (BCIP, Promega Co.).

Invertase activity measurement

A modified version of the procedure of Goldstein and Lampen (Goldstein and Lampen 1975) was used. Substrate buffer was obtained by mixing 100 µl of 50 mM sodium acetate buffer (pH 5.0) containing 10% sucrose with 800 µl of 50 mM sodium acetate buffer to obtain substrate buffer, which was incubated at 30°C for 5 min before use. A cell suspension in 50 mM sodium acetate buffer (100 µl) was added to the substrate buffer and the mixture shaken at 30°C. After 20 min incubation, invertase activity was inhibited by addition of 400 µl of 1 M Tris-HCl, pH 8.8, and subsequent heating at 100°C for 2 min. The amount of released fructose was determined by high-performance liquid chromatography (HPLC).

Results

Detection of EGFP on cell surface

Yeast MT8-1 transformants harboring the plasmids pWGP3-SUC2 for overexpression of native type invertase, pWGP3-ES for EGFP-INV, pWGP3-SE for INV-EGFP, and pWGP3 for control were named MT8-1/pWGP3-SUC2, MT8-1/pWGP3-ES, MT8-1/pWGP3-SE and MT8-1/pWGP3, respectively.

Green fluorescence was clearly observed outlining both the MT8-1/pWGP3-ES (Fig. 2c) and MT8-1/pWGP3-SE (Fig. 2d) transformants, indicating that EGFP was successfully localized at their cell-surface layer. The fluorescence of MT8-1/pWGP3-ES was stronger than that of MT8-1/pWGP3-SE. In contrast, no fluorescence was detected in the control transformant MT8-1/pWGP3 or in MT8-1/pWGP3-SUC2 (Figs. 2a and 2b).

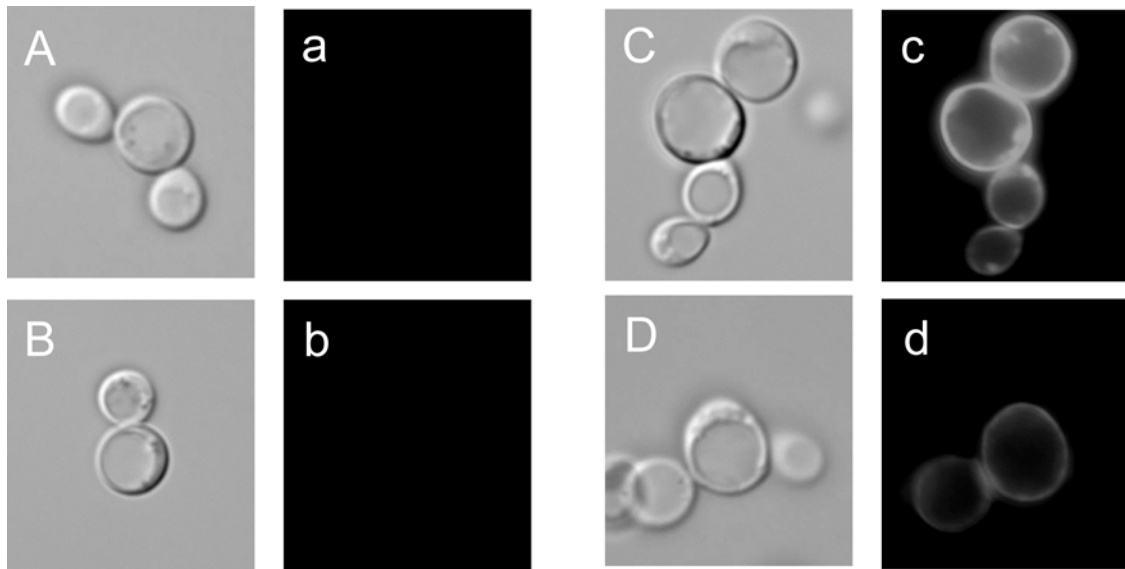


Figure 2 Differential interference contrast micrographs (left panels) and fluorescence micrographs (right panels) of yeast cells. (A, a) MT8-1/pWGP3; (B, b) MT8-1/pWGP3-SUC2; (C, c) MT8-1/pWGP3-ES; (D, d) MT8-1/pWGP3-SE.

Measurement of EGFP fluorescence intensity

To quantitatively analyze the amount of EGFP-INV and INV-EGFP, the fluorescence intensity of EGFP was measured using a flow cytometer. Figure 3 shows the time course of the average fluorescence intensity during cultivation. In both transformants the average fluorescence intensity increased with time. The average fluorescence intensity of MT8-1/pWGP3-ES exhibited maximal value at approximately 72 h and was approximately 3.5-fold greater than that of MT8-1/pWGP3-SE.

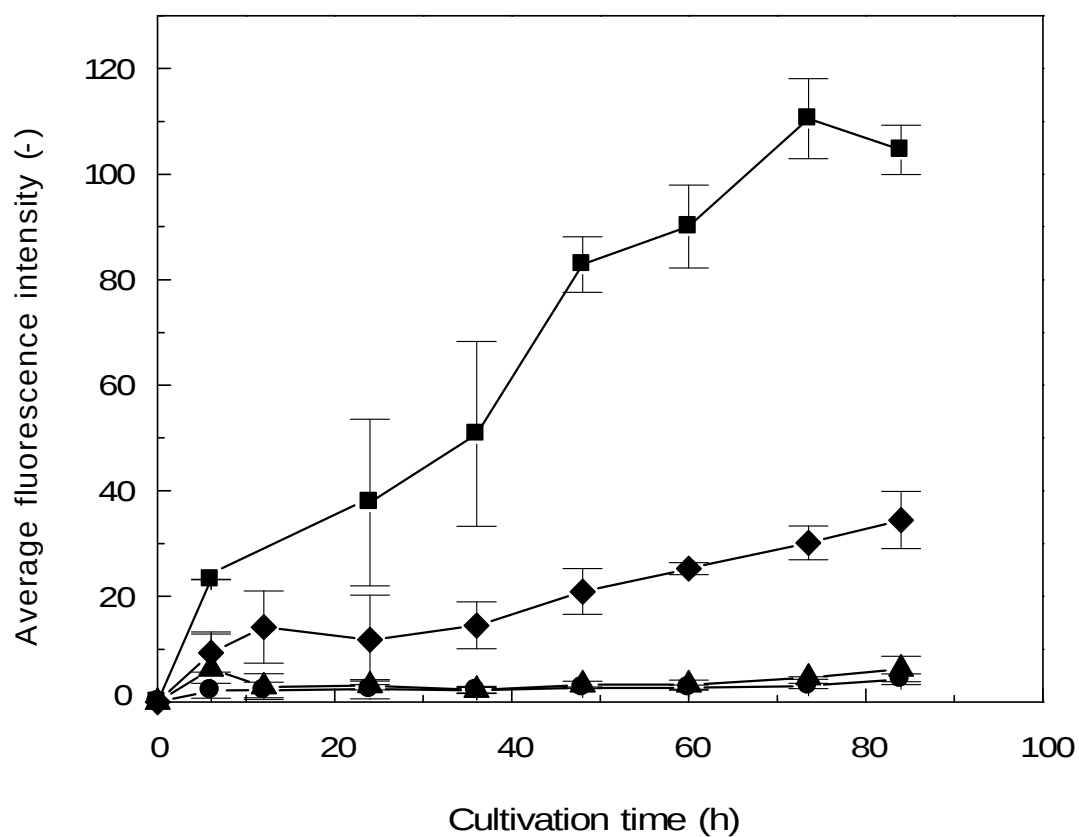


Figure 3 Time course of average fluorescence intensity of MT8-1/pWGP3-ES (squares), MT8-1/pWGP3-SE (diamonds), MT8-1/pWGP3 (triangles) and MT8-1/pWGP3-SUC2 (circles) during cultivation.

Western blot analysis of cell-surface and intracellular fraction

To determine the localization of the fusion protein, cell-surface proteins and intracellular proteins were fractionated. *S. cerevisiae* external invertase appeared to be a dimer of 270 kDa with approximately 50% D-mannose attached to the protein as 18 to 20 asparagine-linked polysaccharide units, and 9 to 10 units/protein subunit (Gascon et al. 1968; Tarentino et al. 1974; Trimble and Maley 1977). EndoHf treatment of the fractions was therefore carried out to remove N-glycosylated carbohydrates from the fusion proteins and analyze the molecular weight of protein. As shown in Fig. 4, most of the fusion proteins in the periplasmic fractions were detected at a position of approximately 87 kDa, which represents a similar molecular weight to the value calculated from the predicted amino acid sequence (85.9 kDa). On the other hand, only a very weak band was detected in the intracellular fraction.

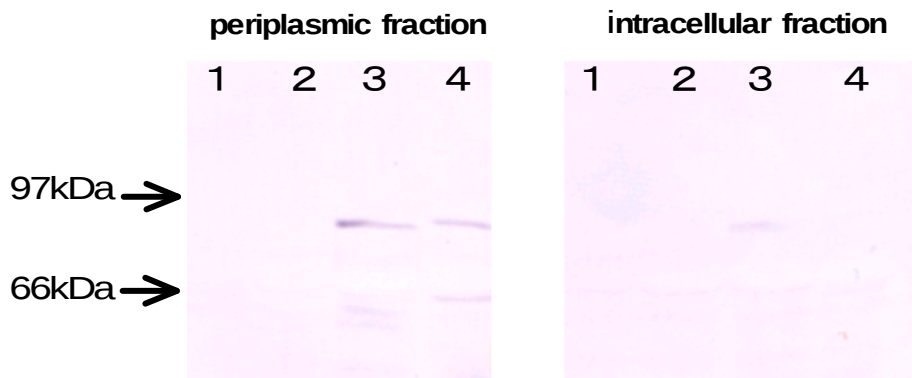


Figure 4 Immunoblotting of cell-surface fraction (left panel) and intracellular fraction (right panel). Lanes: **1** MT8-1/pWGP3, **2** MT8-1/pWGP3-SUC2, **3** MT8-1/pWGP3-ES, **4** MT8-1/pWGP3-SE. Arrows show molecular weight markers.

Activity measurement of fusion invertase

Invertase activity was measured to determine whether fusion protein was secreted to the cell surface in oligomeric form, as dimerization is required and sufficient to generate invertase enzymatic activity (Kern et al. 1992). Figure 5 shows the time course of the invertase activity of MT8-1/pWGP3-ES and MT8-1/pWGP3-SE. In both transformants, the invertase activities of yeast whole cells were clearly detected, although they were lower than that of MT8-1/pWGP3-SUC2. The lower activity of MT8-1/pWGP3-SE compared with MT8-1/pWGP3-ES is consistent with the lower fluorescence intensity of cells shown in Figs. 2 and 3.

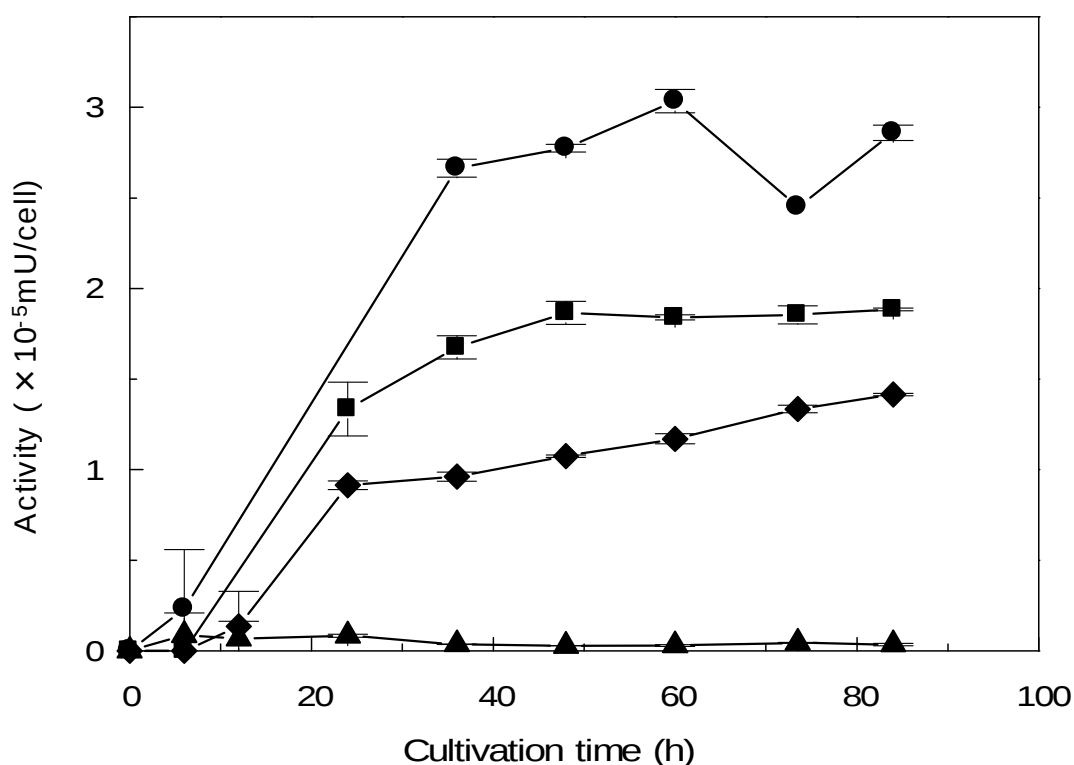


Figure 5 Time course of invertase activity of MT8-1/pWGP3-ES (squares), MT8-1/pWGP3-SE (diamonds), MT8-1/pWGP3 (triangles) and MT8-1/pWGP3-SUC2 (circles) during cultivation.

Discussion

In the present study, we successfully developed a novel periplasmic localization system for target proteins utilizing the yeast external invertase as anchor protein. Previously, Li et al. have reported that *SUC2* secretion signal sequence fusion to EGFP does not result in the localization at periplasm (Li et al. 2002). Kunze et al. have reported that invertase fused to the N-terminal of GFP is expressed in *S. cerevisiae*, and that green fluorescence is observed in the outline of the cells (Kunze et al. 1999). In the present study, as shown in Fig. 2, EGFP was localized in active form by either N- or C-terminal fusion with invertase. This system is expected to be effective for a wide variety of target proteins possessing functional domains near to the C- or N-terminal.

In both the MT8-1/pWGP3-ES and MT8-1/pWGP3-SE transformants, fluorescence was strongly localized at the cell outline (Fig. 2c, 2d), and both INV-EGFP and EGFP-INV fusion proteins were released from cells by Zymolyase treatment (Fig. 4). In addition, the intracellular fraction of MT8-1/pWGP3-ES and MT8-1/pWGP3-SE (Fig. 4) showed a very low signal. These results indicate that the major part of the fusion protein is secreted and localized in the periplasm in free state.

As shown in Fig. 4, after the removal of N-glycoside linkage, most fusion proteins in periplasmic fractions were detected at a similar molecular weight (87 kDa) to the value calculated from the predicted amino acid sequence (85.9 kDa). In a previous study, the carbohydrate-digested external invertase by endoglucosaminidase H was reported to be composed of two identical protein subunits of 60 kDa (Trimble et al. 1977). Since the molecular weight of EGFP is 27 kDa, the band of 87 kDa corresponds to the EGFP/INV fusion protein. This result suggests that both fusion proteins were successfully transported to the periplasm.

The time course of the average EGFP fluorescence intensity shows that MT8-1/pWGP3-ES has 3.5 times stronger fluorescence intensity than MT8-1/pWGP3-SE (Fig. 3). On the other hand, the difference between the transformants in the strength of the Western blot analysis band signal (Fig. 4) and in

invertase activity (Fig. 5) was smaller. This is probably because fusion of invertase to the C-terminal of EGFP is more efficient in maintaining the fluorescence of the latter. Which terminal to fuse the invertase anchor system to should therefore be chosen depending on the target protein. For example, to display recombinant lipase, which has its active site near the C-terminal, it is more effective to fuse the invertase to the N-terminal of lipase.

In the present study, we focused on the periplasm layer as the localization site of the target proteins. Sites outside of the plasma membrane are good localizations for target protein such as enzyme to make whole-cell biocatalyst more effective. By combining this novel periplasmic localization system with cell-surface display systems, it should be possible to localize more enzymes in the cell-surface layer to increase catalytic activity and/or catalyze sequential reaction. Further study to verify this hypothesis is important.

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Chapter 2. Construction of *Pichia pastoris* cell-surface display system using Flo1p anchor system

Introduction

Cell-surface display systems for proteins and peptides have been studied using many microorganisms as host strains (Stahl et al. 1997; Steidler et al. 1998; van der Vaart et al. 1997). Among these microorganisms, yeast *Saccharomyces cerevisiae* is the most widely studied and advanced host strain for cell-surface display systems. This is due to the advantages of yeast, such as safety, simplicity in genetic modification and cell wall structure rigidity are favorable for utilizing it as the host for the cell-surface display systems. Several anchor systems have been developed for yeast cell-surface display system, including a glycosylphosphatidylinositol (GPI)-anchor system (Boder and Wittrup 1997; Caro et al. 1977; Murai et al. 1998), a Flo1p anchor system using a flocculation functional domain (Matsumoto et al. 2002; Watari et al. 1994) and an invertase system (Tanino et al. 2004), and used to display various proteins (Nakamura et al. 2001; Shigechi et al. 2004; Ye et al. 2000) and peptides (Boder et al. 1997; Kuroda et al. 2001) on yeast cell surfaces.

As one of the promising application areas, novel reaction processes using enzyme-displaying cells have been developed (Fujita et al. 2004; Matsumoto et al. 2004; Shigechi et al. 2004). However, to use enzyme-displaying yeast cells for the production of chemicals or pharmaceuticals, further improvements in their performance are required. In particular, the productivity of enzyme-displaying yeast cells should be improved for their utilization in a large-scale manufacturing process. In addition, the stability of an enzyme displayed on a yeast cell surface should be improved.

As an approach to fulfill the requirements mentioned above, we focused on the methylotrophic yeast *Pichia pastoris* as the host strain for cell-surface display. *P. pastoris* is the yeast that is widely studied and can be cultivated to a very high density

(Cereghino and Cregg 2000; Cregg et al. 2000). In addition, the alcohol oxidase 1 promoter (pAOX1) that is strongly inducible by methanol is available in *P. pastoris*. Mergler *et al.* demonstrated that the GPI-anchor system is effective for cell-surface display in *P. pastoris* using *Kluyveromyces lactis* yellow enzyme (Mergler et al. 2004).

In this study, we constructed a *P. pastoris* cell-surface display system based on Flo1p. As the anchor for the cell surface-display system, amino acids 1 to 1099 of Flo1p, which is from *S. cerevisiae* and contains the flocculation functional domain, were used (FS anchor). As the model protein, the lipase from *Rhizopus oryzae* with a Pro sequence (ProROL) (Takahashi et al. 1998) was genetically fused to the FS anchor, and the fusion protein FSPROROL was expressed under the control of pAOX1. Degree of posttranslational modification of fusion protein FSPROROL, and the activity and stability of ROL in whole cells were investigated.

Materials and methods

Strains and media

Escherichia coli NovaBlue (Novagen Inc., Madison, WI) was used as the host strain for recombinant DNA manipulation. *P. pastoris* GS115 (Invitrogen Co., Carlsbad, CA) was used for the production of the fusion protein FSPROROL. *E. coli* was cultivated in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 0.5% sodium chloride) containing 100 µg/ml ampicillin. *P. pastoris* was cultivated in BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34% yeast nitrogen base, 4×10^{-5} % biotin, and 1% glycerol), and BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34% yeast nitrogen base, 4×10^{-5} % biotin, and 0.5-2.0% methanol).

Construction of expression plasmids

The FS anchor gene was amplified from the plasmid vector pWIFS (Matsumoto et al. 2002) by polymerase chain reaction (PCR) using the following

primers: *Bgl*III-Flo1 (5'-atcgagatctATGACAATGCCTCATCGCTATATGTTTTTG-3') and FS-*Bam*HI (5'-cgatggatccAGAGCTGGTGATTGTCCTGAAGATGATGATGACAAACTG-3'). PCR was carried out using a KOD-plus- polymerase (Toyobo Co., Ltd., Osaka, Japan). The amplified fragment was digested with *Bgl*III and *Bam*HI, and inserted into the *Bam*HI site of pPIC3.5K (Invitrogen, Co.). The resulting plasmid vector was named pPIC3.5KFS (Figure 1a). The gene encoding ProROL was amplified from the plasmid vector pWIFSPROROL (Matsumoto et al. 2002) by PCR using the following primers: *Bam*HI-ProROL (5'-atcgggatccGTTTCCTGTTTCTGGTAAATCTGGATCTTCC-3') and ProROL-*Eco*RI (5'-cgatgaattcTTACAAACAGCTTCCTTCGTTGATATCAAA-3'). The amplified fragment was digested with *Bam*HI and *Eco*RI, and inserted into the *Bam*HI and *Eco*RI sites of pPIC3.5KFS. The resulting plasmid vector was named pPIC3.5KFSPROROL (Figure 1b).

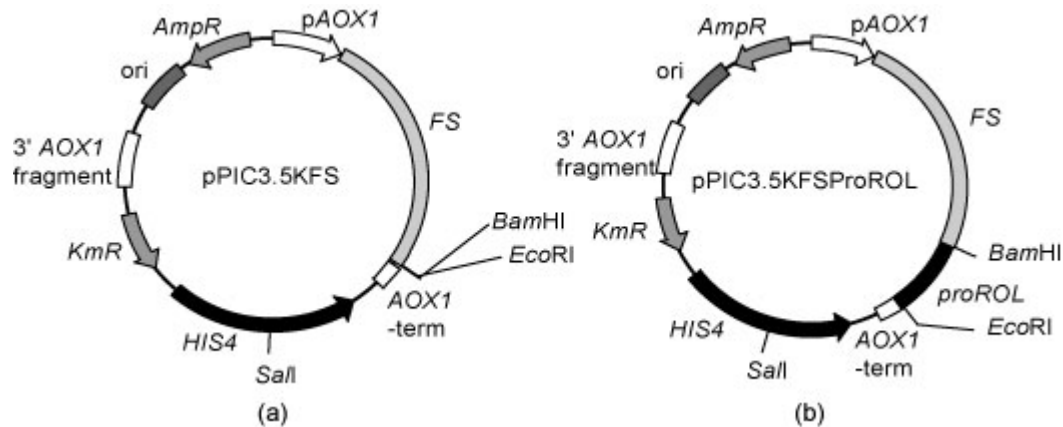


Figure 1 Plasmid vector containing FS anchor gene at downstream of pAOX1 ((a) pPIC3.5KFS), and plasmid vector for expression of fusion protein FSPROROL under pAOX1 control ((b) pPIC3.5KFSPROROL).

Yeast transformation

P. pastoris GS115 was transformed with *SalI* digested pPIC3.5KFSPProROL using a *Pichia* EasyComp kit (Invitrogen, Co.) according to the protocol specified by the supplier.

Cultivation conditions

The yeast transformant was precultivated in BMGY medium at 30°C for 16 h and used to inoculate 200 ml of BMGY medium in a 1 L baffles flask to give an initial OD₆₀₀ value of 0.1. After 24 h cultivation, the culture was centrifuged at 1,000 g for 10 min and resuspended in BMMY medium containing 0.5%, 1.0%, or 2.0% methanol. To maintain the induction of the fusion protein FSPProROL, 100% methanol was added every 24h to the culture to the final concentrations mentioned above.

Measurement of lipase activity in yeast cells

Lipase activity at 30°C was measured using Lipase kit S (Dainippon Sumitomo Pharma Co., Ltd. Osaka, Japan) according to the protocol specified by the supplier, and the resulting values were expressed in international units (IU). Lipase activity in yeast cells was measured as follows. Yeast cells harvested from culture broth were washed twice with distilled water and resuspended in distilled water. The cell concentration was determined by measuring the dried-cell weight, and an appropriate amount of the cell suspension was used for lipase activity measurement.

Immunofluorescence microscopy

Immunofluorescence microscopy was carried out as reported previously (Washida et al. 2001). Immunostaining was performed as follows. The rabbit polyclonal anti-ROL antiserum was raised against the recombinant ROL produced by *S. cerevisiae* (Takahashi et al. 1998) and used as the primary antibody. The antibody was preincubated with native *P. pastoris* GS115 to prevent nonspecific binding to the yeast cells. The cells were washed with phosphate-buffered saline (PBS; 50 mM phosphate, and 150 mM sodium chloride, pH 7.4). The cells and pretreated antibody were then

incubated with 2.0% bovine serum albumin at room temperature for 1.5 h. After the cells were washed with PBS, the secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibodies (Molecular Probes Inc., Eugene, OR), was diluted to 1:300 and allowed to react with the cells at room temperature for 1 h. The cells were then washed with PBS and observed with a fluorescence microscope.

Cell wall fraction preparation and protein extraction

The cell wall fraction was separated according to the previously reported method (Lipke and Ovalle 1998) with minor modifications. The cells were harvested by centrifugation at 1,000 g and washed with ice buffer (10 mM Tris-HCl (pH 7.8), and 1 mM phenylmethylsulfonyl fluoride). The cells, buffer, and glass beads (0.5 mm in diameter) were mixed at a ratio of 1:2:1 (wt/vol/wt) in a microcentrifugation tube and agitated vigorously with a vortex mixer at a maximum speed for 5 min at 0°C. The cell wall fraction was recovered by the centrifugation of the homogenate at 1,000 g for 5 min and washed with the buffer for three times. Sodium dodecyl sulfate (SDS) extraction was carried out according to the previously reported method (Bony et al. 1997).

Endo Hf and PNGase F treatments

To remove *N*-glycosylated carbohydrates from glycoproteins, endoglycosidase treatment was conducted using Endo Hf, which cleaves only a high-mannose structures and a hybrid structures of *N*-glycan chains from glycoproteins, or PNGase F, which cleaves nearly all types of *N*-glycan chains from glycoproteins, according to the protocol specified by the supplier (New England BioLabs Inc., Ipswich, MA).

Western blot analysis of FSpRoROL

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using an 8% gel was carried out. The proteins separated on the gel were electroblotted on a polyvinylidene difluoride (PVDF) membrane (Millipore Co., Boston, MA) and allowed to react with the primary rabbit anti-ROL IgG antiserum and the secondary

goat anti-rabbit IgG alkaline phosphatase-conjugated antibodies (Promega Co., Madison, WI). The membrane was then stained with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate toluidine salt (BCIP) (Promega Co.) according to the protocol specified by the supplier. The staining solution was prepared using 66 μ l of nitroblue tetrazolium and 33 μ l of BCIP and these solutions were sequentially added to 10 ml of alkaline phosphatase buffer (100 mM Tris-HCl (pH 9.0) containing 150 mM NaCl and 1 mM $MgCl_2$).

Measurement of thermal stability of lipase in yeast cells

The thermal stability of lipase in yeast cells was measured as follows. Yeast cells harvested from culture broth were washed with distilled water twice and resuspended in 50 mM potassium phosphate buffer (pH 6.0). The suspension was then used for heat treatment at 60°C with shaking at 150 rpm in a bioshaker. Periodically, aliquots of cell suspension were taken, washed with distilled water twice and used for residual lipase activity measurement at 30°C. The percentage of residual lipase activity was calculated by dividing the lipase activity after incubation by the initial lipase activity multiplied by 100. The thermal stability of the free lipase from *R. oryzae*, Lipase F-AP50 (Amano Enzyme Inc., Nagoya, Japan), was also measured.

Results

Immunofluorescence microscopy

The yeast expression plasmid vector containing the gene for FSPProROL was constructed, and the vector digested at the *SalI* site was transformed into *P. pastoris* GS115. The integration of the vector into the *HIS4* locus of the GS115 genome was confirmed by PCR analysis (data not shown), and the strain was named GS115/FSPProROL.

Immunofluorescence microscopy was carried out to investigate the presence of ProROL on the GS115/FSPProROL cell surface. Figure 2 shows the interference

contrast and fluorescence micrographs of immunolabeled GS115 (control) and GS115/FSPProROL cells with rabbit polyclonal anti-ROL antiserum and FITC-conjugated goat anti-rabbit IgG antibodies. The cells induced for 192 h in BMMY medium containing 2.0% methanol were used. The green fluorescence was observed on the GS115/FSPProROL outline (Figure 2A and 2a). In contrast, no green fluorescence was observed on the control cell (Figure 2B and 2b). This result indicates that the fusion protein FSPProROL is anchored and that ProROL is successfully displayed on the GS115/FSPProROL cell surface.

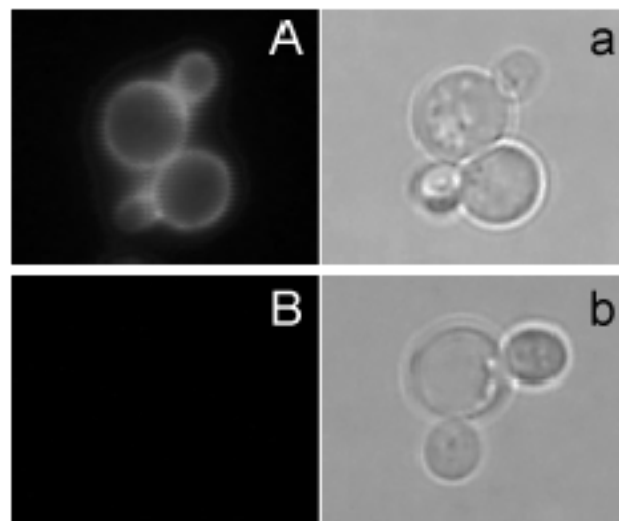


Figure 2 Differential interference contrast micrographs (right panels) and fluorescence micrographs (left panels) of yeast cells. (A and a) GS115/FSPProROL; (B and b) GS115.

Effect of methanol concentration on induction of lipase activity in yeast cells

The effect of methanol concentration on induction of lipase activity in GS115/FSPProROL cells was investigated. Figure 3 shows the time course of lipase activity in GS115/FSPProROL cells after induction. In all the cultures containing various concentrations of methanol for induction, the lipase activity of GS115/FSPProROL cells increased with induction time. The lipase activity of GS115/FSPProROL cells induced with 0.5% methanol was the highest until 120 h. Subsequently, the lipase activity of GS115/FSPProROL cells induced with 2.0% methanol was the highest and reached 15.3 IU/g-dry cell at 240 h.

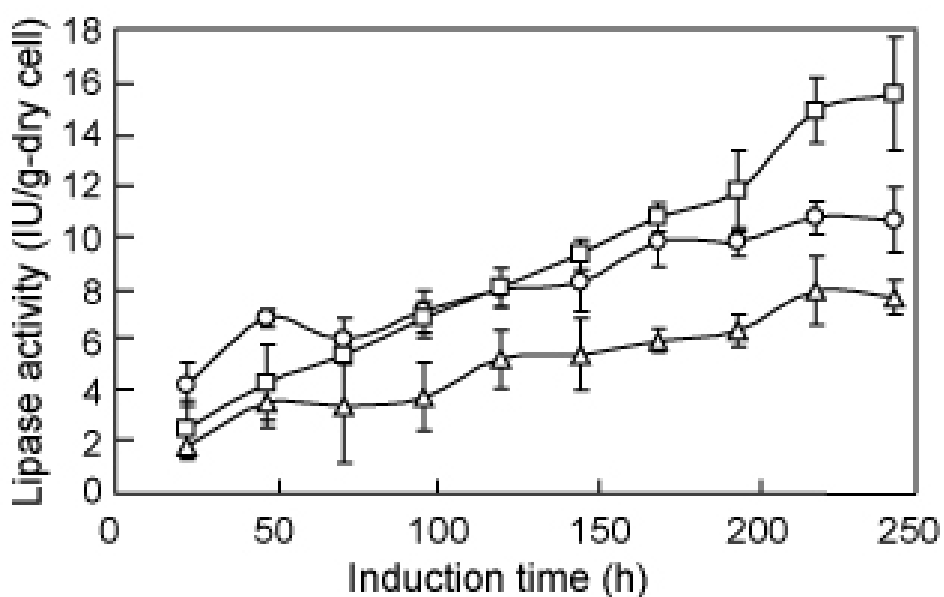


Figure 3 Time courses of lipase activity in whole cells of GS115/FSPProROL in several methanol concentrations, 0.5% (open circles), 1.0% (open triangles) and 2.0% (open squares). The vertical axis indicates lipase activity in whole cells per dry cell weight. The data points represent the average of three independent experiments.

Localization of fusion protein FSProROL

To determine the localization of the fusion protein FSProROL, the SDS-extracted fraction was used for western blot analysis. The cells induced for 240 h in BMMY medium containing 2.0% methanol were used. The SDS-extracted fraction was treated with Endo Hf or PNGase F. They were then applied to SDS-PAGE and blotted onto the PVDF membrane and immunostained. As shown in Figure 4, the fusion protein FSProROL was detected in SDS-extracted fraction (lane 1), indicating that the fusion protein FSProROL is noncovalently attached to the cell wall. The molecular mass of the fusion protein FSProROL was much larger than the calculated value (156 kDa) (lane 1). In addition, only a slight reduction in molecular mass by the Endo Hf and PNGase F treatments (lanes 2 and 3) was observed.

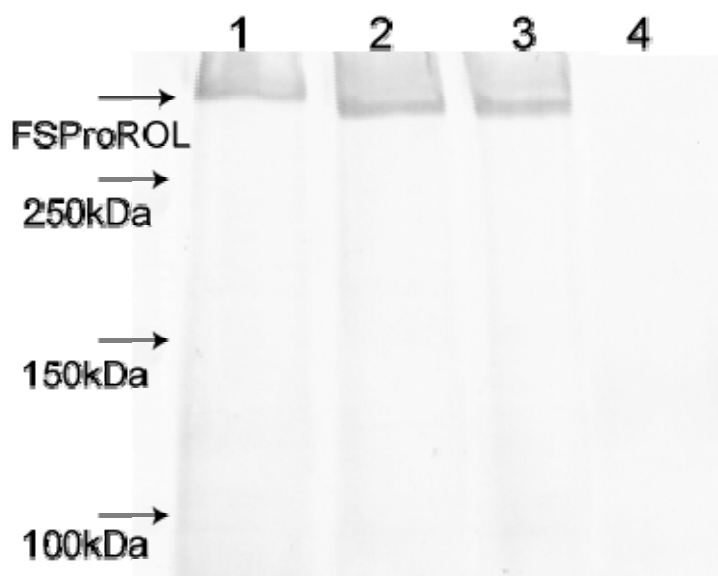


Figure 4 Immunoblotting of SDS extracts from GS115/FSProROL cell wall. Lane 1, fraction without endoglycosidase treatment; lane 2, fraction treated with Endo Hf; lane 3, fraction treated with PNGase F. Immunoblotting of SDS extracts from control cell (GS115) is also shown (lane 4).

Thermal stability of lipase in yeast cells

The thermal stability of lipase in GS115/FSPProROL cells, which was induced for 240 h in BMMY medium containing 2.0% methanol, was compared with that of the free enzyme at 60°C. Figure 5 shows the time course of residual lipase activity. The residual activity of the free lipase immediately decreased to ~6% after 2 h incubation, and was hardly detected after 16 h incubation. In contrast, the residual activity of lipase in GS115/FSPProROL cells surprisingly increased with incubation time, and reached the value that is about 6.5 times greater than the initial value measured at 4 h incubation. Subsequently, the residual activity of lipase in GS115/FSPProROL cells gradually decreased with incubation time, but it was still higher than the initial activity until 16 h incubation. This result suggests that the thermal stability of lipase activity in GS115/FSPProROL cells is superior to that of the free form.

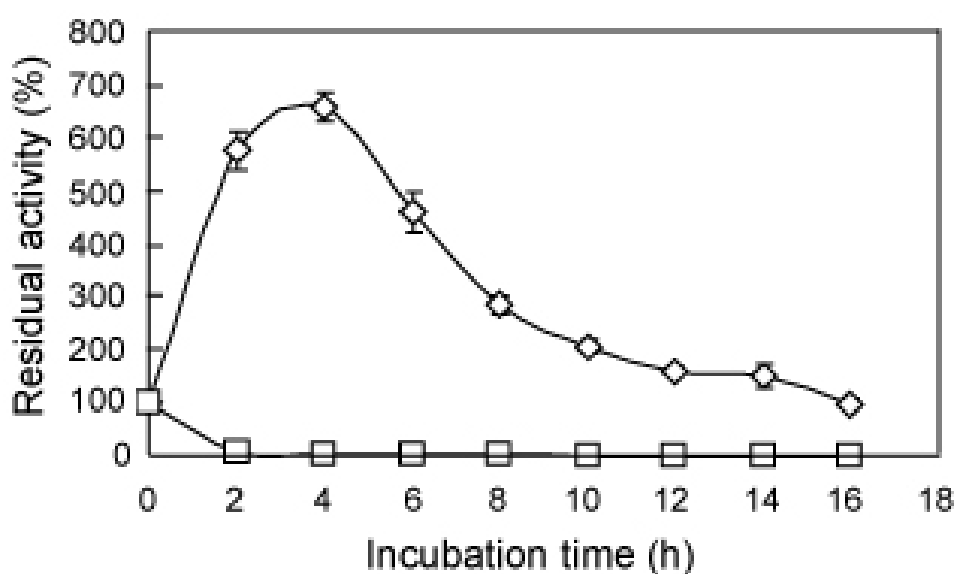


Figure 5 Time courses of residual lipase activity in whole cells of GS115/FSPProROL (open diamonds) and free ROL (open squares) at 60°C. The vertical axis indicates residual lipase activity which was calculated relative to the initial lipase activity, which was defined as 100%. The data points represent the average of six independent experiments.

Discussion

We have successfully demonstrated that the FS anchor system based on the flocculation functional domain of Flo1p is effective in *P. pastoris* as well as in *S. cerevisiae*. Previously, Mergler *et al.* demonstrated that the GPI-anchor system can be used in *P. pastoris* (Mergler *et al.* 2004). These results indicate that two major yeast cell-surface display anchor systems can be used in *P. pastoris*, and *P. pastoris* is a useful host strain for yeast cell-surface display.

In our study, ProROL was displayed on the cell surface (Figure 1). The fusion protein FSPProROL was noncovalently attached to the *P. pastoris* cell wall (Figure 4) as well as to the *S. cerevisiae* cell wall, although FSPProROL was also detected in the culture medium. The molecular mass of FSPProROL displayed on the cell surface was much larger than the calculated value (156 kDa) (Figure 4, lane 1) and those in the fraction treated with Endo Hf or PNGase F were still larger than the calculated value (Figure 4, lanes 2 and 3). In addition, no clear difference was observed between the two fractions. Therefore, FSPProROL appeared to be highly *O*-glycosylated, and oligosaccharide(s) *N*-linked to FSPProROL had high-mannose and/or hybrid structures. After PNGase F treatment, the molecular mass of FSPProROL displayed on *P. pastoris* cells was larger than that of FSPProROL displayed on *S. cerevisiae* cells (data not shown), indicating that the extent of *O*-glycosylation of FSPProROL in *P. pastoris* is greater than that in *S. cerevisiae*.

As shown in Figure 3, the methanol concentration of the induction phase affected lipase activity in GS115/FSPProROL cells. The effect of methanol (0.5-2%) on free ROL was found to be very small (data not shown). For short-time induction until 120 h, 0.5% methanol induction was found favorable. On the other hand, the highest activity, 15.3 IU/g-dry cell, was achieved on 2.0% methanol induction for 240 h. This index of lipase activity (IU/g-dry cell) was lower than that using *S. cerevisiae* as the host strain (Matsumoto *et al.* 2002). However, *P. pastoris* could be cultivated at a higher cell density than *S. cerevisiae*. Hence, another index of lipase activity (IU/litter of cultivation medium) was higher than that using *S. cerevisiae* as the host strain (data

not shown). It is very interesting that lipase activity in GS115/FSPROROL cells incubated at 60°C showed not only a high thermal stability but also a marked increase and reached the value that is about 6.5 times greater than the initial value after 4 h incubation. The free ROL was not stable at 60°C, and its activity immediately decreased after 2 h incubation. Although PROROL displayed on the *S. cerevisiae* cell surface showed a higher thermal stability than the free ROL (data not shown), a little increase of lipase activity in cell during incubation was observed. After 4 h incubation at 60°C, lipase activity in GS115/FSPROROL cells reached a value higher than that in *S. cerevisiae* cells. As discussed above, the difference in the extent of glycosylation of the cell-surface-displayed FSPROROL might affect the thermal stability of the displayed enzymes. And/or intracellular FSPROROL might also affect this phenomenon. The mechanism and detail of these phenomena are still to be investigated.

In summary, we demonstrated that our FS anchor system is effective in *P. pastoris* in the same manner as in *S. cerevisiae*. Lipase activity in GS115/FSPROROL cells was increased by incubation at 60°C, and the stability of lipase was highly improved by fusing the lipase to the FS anchor system and displaying the lipase on the *P. pastoris* cell surface. Therefore, enzymes displayed on *P. pastoris* cells are expected to be effective whole-cell biocatalysts.

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**Part II. Optimization of enzyme display yeast cell preparation using
Flo1p anchor system for effective utilization**

Effect of cultivation conditions on cell-surface display of Flo1 fusion protein using sake yeast

Introduction

Yeast cell-surface display systems have been widely studied and several anchor systems, including a glycosylphosphatidylinositol (GPI)-anchor system (Murai et al. 1998), a Flo1p anchor system using a flocculation functional domain (Matsumoto et al. 2002), and an invertase system (Tanino et al. 2004), have been developed. Many heterologous proteins were displayed on the yeast cell-surface using these anchor systems. Yeast cells displaying enzymes and functional peptides on their cell-surface could be used as whole-cell biocatalysts in several applications, such as enantioselective transesterification reaction (Matsumoto et al. 2004) and adsorption of heavy metal ions (Kuroda et al. 2001). In such applications, the reactions that combine the displayed enzymes with a metabolic pathway, for example, direct ethanol production from starch (Shigechi et al. 2004), are very exciting sequential reactions as they can be free of complex processes.

In the case of using yeast cells displaying enzymes for whole-cell biocatalyst high stress tolerance and proliferation potential are required for the yeast cells used as the host strain of the yeast cell-surface display system. To fulfill these requirements, sake yeast, which is known to be a diploid cell with high stress tolerance and proliferation potential, is one of the most attractive and suitable host strains for yeast cell-surface display systems. In addition, sake yeast has been used for traditional brewing and is generally recognized as being safe (GRAS).

In this study, we examined the expression of the Flo1p anchor system in sake yeast. As a model system, lipase from *Rhizopus oryzae* with a Pro sequence (ProROL) (Takahashi et al. 1998) was genetically fused to the Flo1 short (FS) anchor (Matsumoto et al. 2002). The *SED800* promoter (p*SED800*), in which 800bp of the 5' region of the *SED1* promoter is used to achieve high expression of heterologous proteins in sake

yeast, was employed to express the fusion protein FSproROL. By taking advantage of high stress tolerance and proliferation potential of sake yeast, we investigated the effect of the cultivation conditions on the cell-surface display system using Flo1p anchor system and suitable condition to display the fusion protein on the cell-surface.

Materials and methods

Strains and media

Escherichia coli NovaBlue (Novagen Inc., Madison, WI.) was used as a host strain for recombinant DNA manipulation. Sake yeast GRI-117-UK ([MATa/ α ura3/ura3 lys2/lys2] isolated by Gekkeikan Co.) was used as the host strain of the yeast cell-surface display system. *E. coli* was cultivated in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 0.5% sodium chloride) containing 100 μ g/ml ampicillin. Sake yeast was cultivated in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or SD medium (0.67% yeast nitrogen base supplemented with L-lysine and 2% glucose, unless otherwise stated). For the solid medium, 2% agar was added to the media described.

Construction of expression plasmids

The SED800 promoter (pSED800) sequence was amplified from pK113 by polymerase chain reaction (PCR) with the following primers: Sall-pSED800 (5'-atcggtcgcacTTGGATATAGAAAATTAACGTAAGGCAGTA-3') and pSED800-EcoRI (5'-cgatgaattcCTTAATAGAGCGAACGTATTTTATTTTGCT-3'). RCR was carried out using KOD-plus-polymerase (Toyobo Co., Osaka, Japan). The amplified fragment was digested with *Sall* and *EcoRI*, and inserted into the *XhoI* and *EcoRI* sites of pRS406 (ATCC87517). The resulting plasmid vector was named pUSED800. The DNA fragment containing the FS anchor gene, the ProROL gene and the terminator sequence of the isocitrate lyase gene of *Candida tropicalis* (term-ICL) (Kanai et al. 1996, Umemura et al. 1995) was amplified from the plasmid

pWIFSproROL (Matsumoto et al. 2002) by PCR using the following primers: EcoRI-Flo1 (5'-atcggaattcATGACAATGCCTCATCGCTATATGTTTTTG-3') and termICL-XbaI (5'-cgattctagagATTCTTTCTGGGTCTTTGTCTCCTTTAGT-3'). The amplified fragment was digested with *EcoRI* and *XbaI*, and inserted into the *EcoRI* and *XbaI* sites of pUSED800. The resulting plasmid vector was named pUSFS-ProROL (Fig. 1).

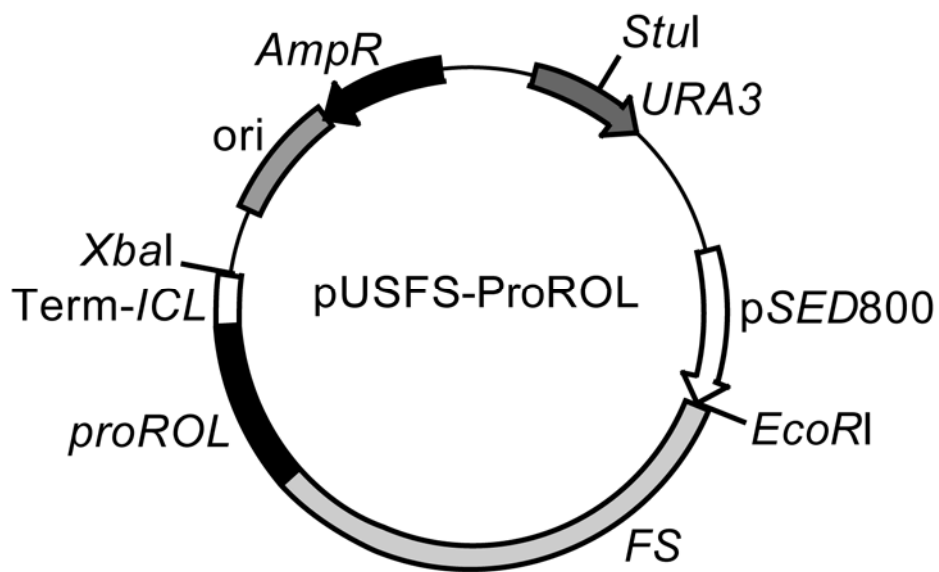


Figure 1 Plasmid vector for the expression of FSProROL fusion protein under the control of pSED800 (pUSFS-ProROL).

Yeast transformation

The expression plasmid pUSFS-ROL was digested with *StuI* and introduced into sake yeast cells using YEAST MAKER™ (TAKARA BIO INC., Otsu, Japan) according to the protocol specified by the supplier.

Effect of nutrients in culture medium on lipase activities in whole cells and culture supernatant

The transformant was precultivated in the SD medium at 30°C for 16 h and was used as starter to inoculate the SD, SDC (SD medium containing 2% Casamino acids), SDCP (SDC medium containing 2% peptone), SDCY (SDC medium containing 1% yeast extract), and SDCPY (SDC medium containing 2% peptone and 1% yeast extract) media. The culture was incubated at 30°C and the time courses of the lipase activities in whole cells and culture media were measured.

Measurement of lipase activity

The lipase activity was measured using a Lipase Kit S (Dainippon Sumitomo Pharmaceutical Co., Osaka, Japan) according to the protocol specified by the supplier and the activity was expressed in international units (IU). Lipase activity in whole cells was measured as follows. Yeast cells harvested from the culture were washed twice with distilled water and resuspended in distilled water. The cell density was determined by measuring the optical density at 600 nm and an appropriate amount of the cell suspension was used for lipase activity measurement.

Effect of carbon source on lipase activities in whole cells and culture supernatant

Transformants were precultivated in the SD medium at 30°C for 16 h and were used as starter to inoculate the SC (0.67% yeast nitrogen base supplemented with L-lysine and 2% Casamino acids) medium containing the carbon source at the final concentration of 2%. We employed mannose, glucose, sucrose, fructose, maltose and raffinose as the carbon source. The culture was incubated at 30°C, and after 12 h of cultivation, the lipase activities in whole cells and culture supernatant were measured.

Effect of initial glucose concentration on lipase activity in whole cells and total lipase activity

Transformants were precultivated in the SD medium at 30°C for 16 h and were used as starter to inoculate the SDC medium containing various concentrations of

glucose. The initial glucose concentration was varied between 1% and 8%. The culture was incubated at 30°C and time courses of the lipase activity in whole cells and the total lipase activities were measured.

Effect of cultivation temperature on lipase activities in whole cells and culture supernatant

Transformants were precultivated in the SD medium at 30°C for 16 h and were used to inoculate the SDC medium containing 4% glucose. Incubation temperature was varied between 16°C and 37°C. The time course of lipase activities in whole cells and culture supernatant were measured.

Results

Effect of nutrients in culture medium on lipase activities in whole cells and culture supernatant

The yeast expression plasmid vector pUSFS-ROL digested at the *StuI* site was transformed into sake yeast GRI-117-UK. Almost all transformants exhibited lipase activity, and approximately 5% of the transformants exhibited higher lipase activity than the other transformants. Those transformants exhibiting higher lipase activity contained a double-copy chromosomal integration of the FSPProROL expression cassette (data not shown). We selected one of these transformants for use in the subsequent experiments and named it GRI-117-UK/FSPProROL.

The effect of the nutrients in the culture medium on the lipase activities in whole cells and culture supernatant was investigated by incubating GRI-117-UK/FSPProROL in nutritionally poor media (SD and SDC) and rich media (SDCP, SDCY and SDCPY). Figure 2a shows the time course of the cell density, which was almost the same in all cultures until 12 h. After 12 h, the graph showing the change in the cell densities reached a plateau in the poor media, whereas the cell densities in the rich media continued to increase until 36 h and reached $OD_{600} = 20$.

Time courses of the lipase activities in whole cells and culture medium were also investigated (Figs. 2b, 2c). The lipase activities in whole cells cultivated in the poor media were higher than those cultivated in the rich media, and the highest lipase activity in whole cells was detected at 24 h of incubation in the SDC medium (6.61 IU/L/OD₆₀₀). In contrast, the lipase activities in whole cells cultivated in rich media were very low and those in the culture supernatant were high. Total lipase activities, the lipase activity in whole cells plus that in the culture supernatant, detected in the rich media were higher than those detected in the poor media at 8 h of incubation, whereas the total activity detected in the SDC medium was the highest after 24 h of incubation (Fig. 2d).

Effect of carbon source on lipase activities in whole cells and culture medium

The effect of the carbon source on the lipase activities in whole cells and culture medium was investigated. GRI-117-UK/FSPProROL was cultivated in the SC medium containing various carbon sources. Figures 3a and 3b show the lipase activities in whole cells and culture supernatant after 12 h of cultivation, respectively. Glucose and maltose were effective carbon sources for increasing the lipase activity in whole cells, whereas the lipase activity in whole cells cultivated in the medium containing raffinose as the carbon source exhibited the lowest value. Moreover, it was shown that mannose and fructose induced the release of lipase activity into the culture supernatant, particularly in the culture medium containing mannose as the carbon source, lipase activity was higher than that in whole cells. The lipase activities in the culture supernatant with glucose and sucrose were low and that in the culture with maltose or raffinose was not detected. No notable difference was detected in cell growth in all cultures except for that with maltose (Fig. 3c).

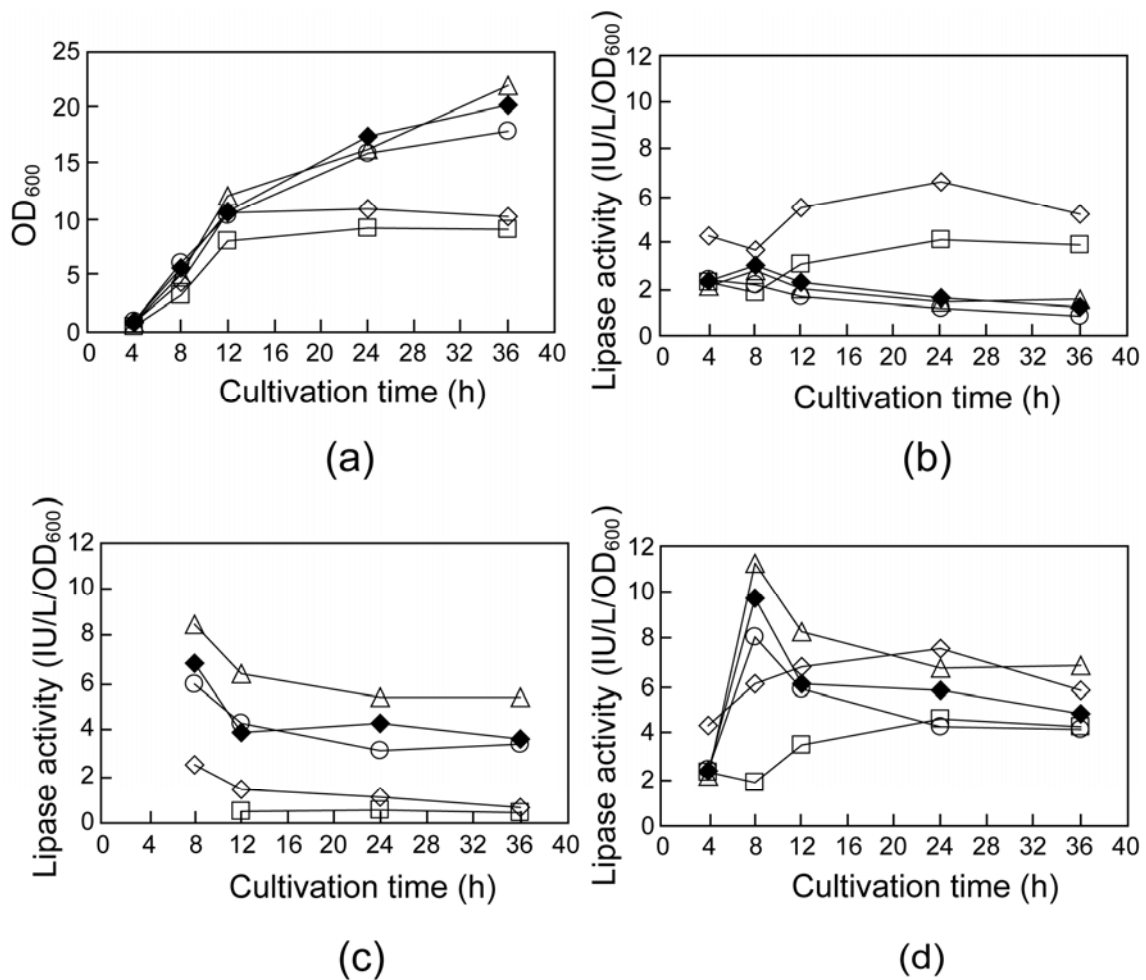


Figure 2 Effect of nutrients in culture medium on cell density (a), lipase activities in whole cells (b) and in culture supernatant (c), and total lipase activity (d) of GRI-117-UK/FSPProROL. Cultures were incubated at 30°C. Open squares, SD medium; open diamonds, SDC medium; open triangles, SDCP medium; open circles, SDCY medium; closed diamonds, SDCPY medium. The data points represent the mean value of three independent experiments.

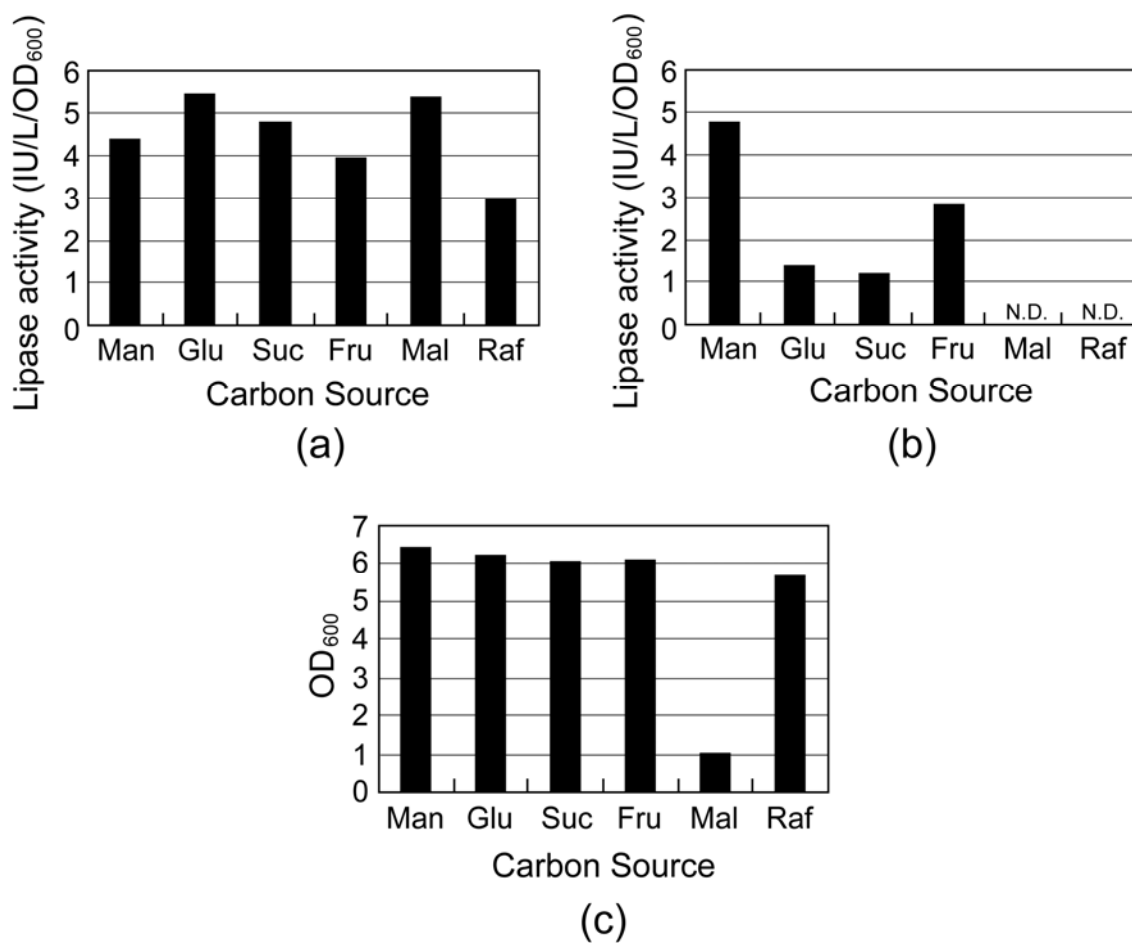


Figure 3 Lipase activities in whole cells (a) and culture medium (b), and cell density (c) of GRI-117-UK/FSPProROL after 12 h of cultivation in SC medium with various carbon sources. Man, mannose; Glu, glucose; Suc, sucrose; Fru, fructose; Mal, maltose; Raf, Raffinose. The data points represent the mean value of three independent experiments.

Effect of initial glucose concentration on lipase activity in whole cells and total lipase activity

The effect of the initial glucose concentration on the lipase activities in whole cells and on the total lipase activity was investigated. The lipase activity in whole cells increased with increasing initial glucose concentration up to 4% glucose, though the lipase activity in whole cells decreased above the initial concentration of 4% (Fig. 4a). At the initial glucose concentration of 4%, lipase activity in whole cells reached 10.5 IU/L/OD₆₀₀ at 16 h. The total lipase activity showed the same trend as the lipase activity in whole cells (Fig. 4b). These results suggest that the initial glucose concentration has an effect on the amount of fusion protein FSPProROL produced by GRI-117-UK/FSPProROL. On the contrary, cell density increased with increasing initial glucose concentration (Fig. 4c).

Effect of incubation temperature on lipase activities in whole cells and culture supernatant

GRI-117-UK/FSPProROL was cultivated at various temperatures and the time courses of the lipase activities in whole cells and in culture medium was investigated. The cultivation time required to reach the highest lipase activity in whole cells increased with decreasing cultivation temperature (Fig. 5a). A decrease in the cultivation temperature resulted in a slight increase in the highest lipase activity in whole cells and delayed the decrease in the lipase activity in whole cells. In contrast, at a higher cultivation temperature of 37°C, the lipase activity in whole cells was significantly reduced and did not increase. A decrease in the cultivation temperature slightly affected the cell growth until 23°C, and GRI-117-UK/FSPProROL was shown to be able to grow well even at 16°C (Fig. 5b). At the cultivation temperature of 30°C, the cell density quickly reached the highest value and the lipase activity in whole cells was sufficiently high.

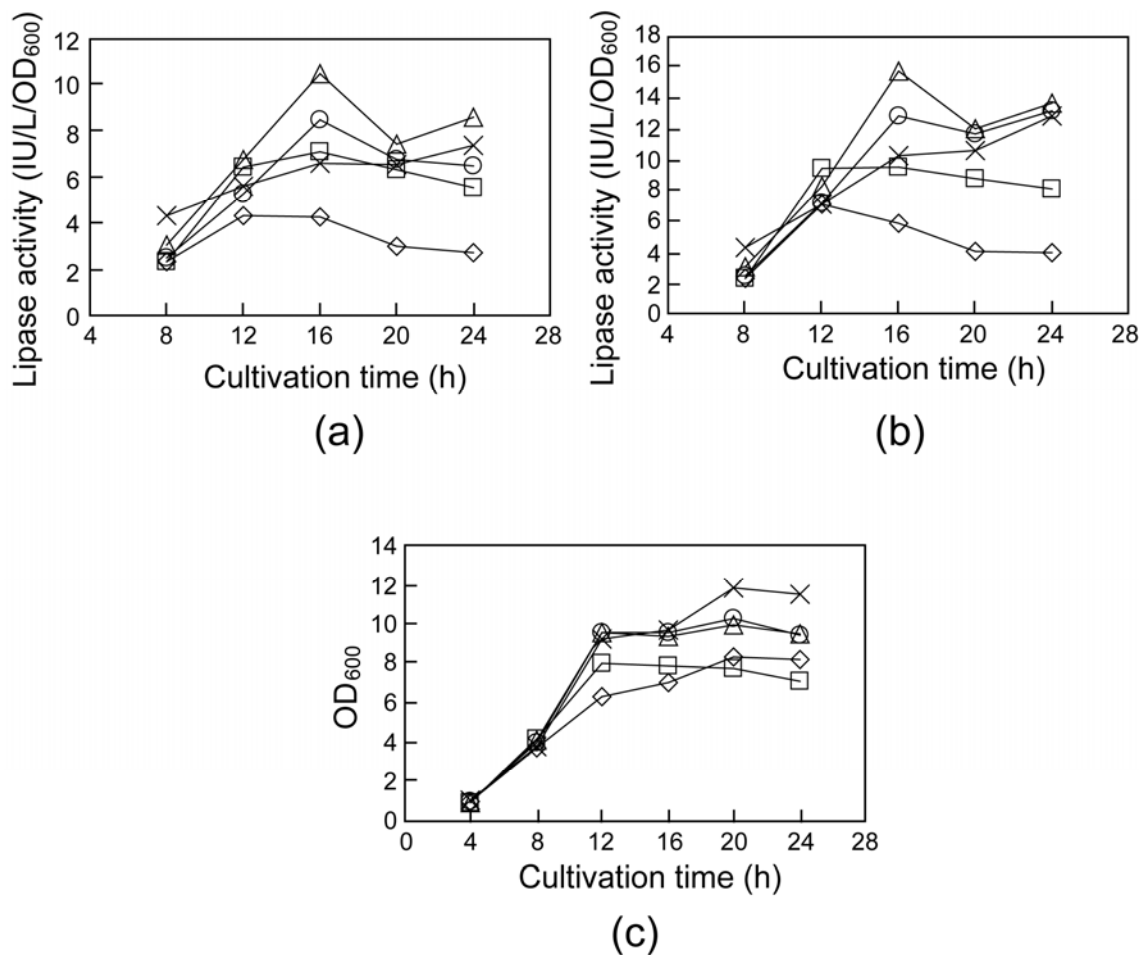


Figure 4 Effect of initial glucose concentration on lipase activity in whole cells (a), on total lipase activity (b), and on cell density (c) of GRI-117-UK/FSPProROL. Cultivation was carried out at 30°C in SDC medium. Open diamonds, 1% glucose; open squares, 2% glucose; open triangles, 4% glucose; open circles, 6% glucose; crosses, 8% glucose. The data points represent the mean value of three independent experiments.

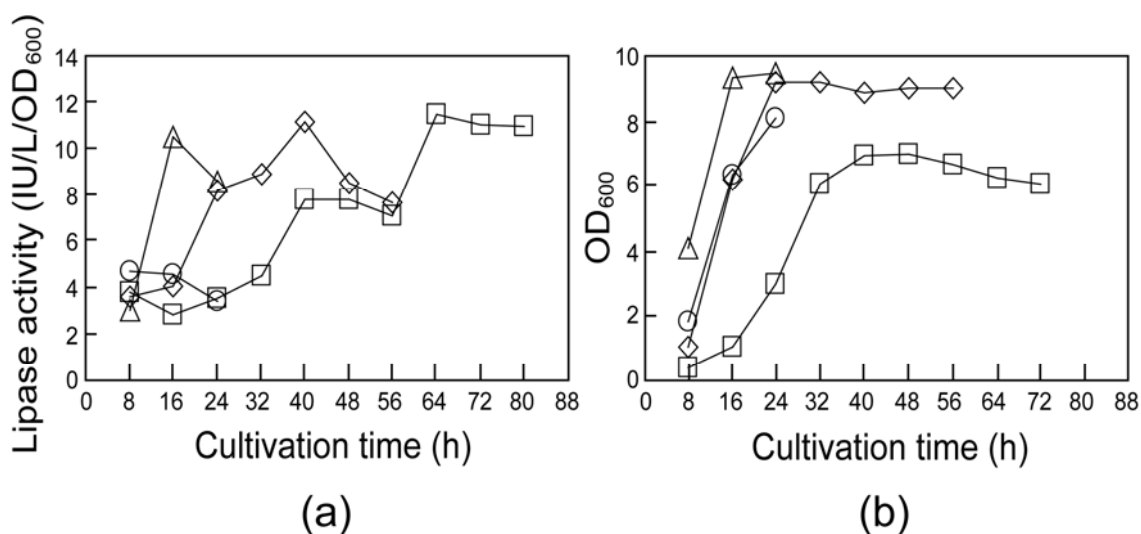


Figure 5 Effect of cultivation temperature on lipase activity in whole cells (a) and on cell density (b) of GRI-117-UK/FSPProROL. Cultivation was carried out in SC medium containing 4% glucose. Open squares, 16°C, open diamonds, 23°C; open triangles, 30°C; Circles, 37°C. The data points represent the mean value of three independent experiments.

Discussion

We evaluated the cell-surface expression of the Flo1p anchor system in sake yeast under various cultivation conditions. It was shown that the component of the culture medium affected the display of the fusion protein FSPProROL on the sake yeast cell-surface. SDC medium was found to be the most effective medium for displaying the target fusion protein on the sake yeast cell-surface among the media used in this study. There were several components of the culture medium and conditions that caused the release of the lipase activity into the culture supernatant. Cultivation in a rich medium, containing peptone and/or yeast extracts, induced the release of the lipase activity into the culture supernatant (Figs. 2b, 2c). By Western bolt analysis, we

compared the molecular masses of the fusion proteins displayed on the cell-surface and released to the culture supernatant, and it was revealed that the molecular masses were the same (data not shown). This indicates that the release of the lipase activity into the culture supernatant is not a result of the proteolysis of FSPROROL but is due to the inhibition of the interaction between the FS anchor and the yeast cell-surface. The release of the lipase activity into the culture supernatant might also result from the structural change of cell-surface attributed to the alteration of metabolic pathway by adding yeast extract and/or peptone. The key component(s) that induce the release of the lipase activity into the culture supernatant are under investigation.

The carbon source in the culture medium would also enable us to control the state of the target fusion protein. The use of glucose and maltose as the carbon source effectively increased the lipase activity in whole cells (Fig. 3a). In addition, the use of di- or tri-saccharide (sucrose and raffinose) as the carbon source reduced the amount of lipase activity released into the culture supernatant, in particular, the lipase activity in the culture supernatant was not detected in the culture containing raffinose as the carbon source. Although sucrose ($\text{Glu}\alpha 1\text{-}\beta 2\text{Fru}$) and raffinose ($\text{Gal}\alpha 1\text{-}6\text{Glu}\alpha 1\text{-}\beta 2\text{Fru}$) contain fructose, which induced the release of the lipase activity into the culture supernatant, the amount of enzymatic activity released was smaller than that in the culture containing fructose as the carbon source. This indicates that oligosaccharide reduces the amount of fusion protein released from the cell-surface. On the contrary, mannose and fructose, particularly mannose, induced the release of the lipase activity into the culture supernatant (Fig. 3b). The Flo1p anchor system uses a flocculation function domain (Matsumoto et al. 2002; Watari et al. 1994), and flocculation of the yeast cells induced by Flo1p is inhibited by mannose (Kobayashi et al. 1998). It is assumed that the release of the lipase activity into the culture supernatant occurs in the same manner as the inhibition of yeast cell flocculation by Flo1p. The mechanism of the release by the mannose might be different from that by peptone and/or yeast extract. This is because peptone, that is the enzyme hydrolysate of protein, does not contain mannose and yeast extract contains only a very low content of mannose.

As shown in Figure 4, the initial glucose concentration affected the total lipase activity and it mainly had an effect on the lipase activity in whole cells. The lipase activity in whole cells increased as the initial glucose concentration was raised and reached the highest value of 10.5 IU/L/OD₆₀₀ at the initial glucose concentration of 4%. In cultures with glucose concentrations above 4%, the total lipase activity decreased, possibly because of the effect of high osmotic pressure on the yeast cells and/or the arosteric effect on pSED800.

Decreasing cultivation temperature lead to slight increase in the highest lipase activity in whole cells and delayed the decrease in the lipase activity in whole cells (Fig. 5a). This temperature-sensitive phenomenon might be due to the typical protease degradation. In this short-time cultivation at 30°C, GRI-117-UK exhibited high cell density (Fig. 5b) which was 2.5 fold higher than that of the laboratory yeast MT8-1 with the same cell-surface expression system (data not shown). On the other hand, the lipase activity in sake yeast was almost same as that in laboratory yeast. These features of sake yeast are suitable for use as the whole cell biocatalyst in the production of chemicals and pharmaceuticals.

To conclude, we have found several characteristics of sake yeast with a Flo1-based cell-surface system. This information is valuable for developing a process using sake yeast with the cell-surface display system, and for further development of the cell-surface display system using sake yeast as the host strain and the Flo1 anchor system.

Acknowledgements

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Part III. Bioconversion with enzyme displaying yeast whole cell biocatalyst in organic solvent

Chapter 1. Development of yeast cells displaying *Candida antarctica* lipase B and their application to ester synthesis reaction

Introduction

Yeast cell-surface display systems have been widely studied and many heterologous proteins have been displayed on yeast cell-surfaces using several anchor systems. Yeast cells displaying functional proteins and peptides on their cell-surface can be used in several applications, such as the adsorption of heavy metal ions (Kuroda et al. 2001), protein isolation and purification (Kato et al. 2005), the high-through-put screening of combinatorial protein libraries (Shiraga et al. 2002) and in yeast whole-cell biocatalyst systems (Murai et al. 1998; Matsumoto et al. 2002). Yeast whole-cell biocatalysts displaying enzymes on their cell-surface can be produced at a low cost and show a high enzymatic activity without permeabilization treatment. Using enzyme-displaying yeast whole-cell biocatalysts for novel reaction processes to produce chemicals or pharmaceuticals is one of the promising application areas.

Previously, our laboratory successfully demonstrated that a yeast whole-cell biocatalyst displaying the lipase from *Rhizopus oryzae* with a Pro sequence (ProROL) using the Flo1p anchor system could effectively catalyze the methanolysis reaction in the solvent-free system (Matsumoto et al. 2002) and the enantioselective transesterification reaction (Matsumoto et al. 2004). ROL is one species of lipase, and the yeast whole-cell biocatalyst displaying the lipase on its cell-surface has been shown to be useful for bioconversion reactions.

Among the lipases, the lipase B from *Candida antarctica* (CALB) is one of the most famous and versatile lipase. In many studies, CALB has successfully catalyzed many reactions, for example, the oil alcoholysis reaction including biodiesel production (Torres et al. 2004; Modi et al. 2006), the kinetic selective reaction (Fransson et al. 2006; Lou et al. 2006) and the ester synthesis reaction (McCabe et al. 2002; Larios et al. 2004). However, the high cost of the enzyme often becomes

problem in the industrial scene.

In this study, we constructed a novel yeast whole-cell biocatalyst displaying CALB on its cell-surface using the Flo1p short (FS) anchor system (Matsumoto et al. 2002). Furthermore, we applied the CALB displaying yeast whole-cell biocatalyst to the ester synthesis reaction using adipic acid and *n*-butanol, the model of the condensation reaction, at the relatively high temperature of 60°C.

Materials and methods

Strains and media

Escherichia coli NovaBlue (Novagen Inc., Madison, WI) was used as the host strain for recombinant DNA manipulation. *Saccharomyces cerevisiae* MT8-1 (*MATa ade his3 leu2 trp1 ura3*) was used as the host strain for the yeast cell-surface display system. *E. coli* was cultivated in Luria-Bertani (LB) medium (1% (w/v) tryptone, 0.5% yeast extract and 0.5% sodium chloride) containing 100 µg/ml ampicillin. Yeast was cultivated in YPD medium (1% (w/v) yeast extract, 2% peptone and 2% glucose) or SD medium (0.67% (w/v) yeast nitrogen base supplemented with appropriate amino acids and nucleotides and 2% glucose). For solid media, 2% (w/v) agar was added to the media described.

Cloning of CALB

The *C. antarctica* lipase B gene with a pro region (ProCALB) was isolated from *C. antarctica* CBS 6678 chromosomal DNA by polymerase chain reaction (PCR) with the following primers containing a part of the sequence of the lipase B from *C. antarctica* LF058 (Uppenberg et al. 1994): BglII-pmCALB (5'-atcgagatctGCCACTCCTTTGGTGAAGCGTCTACCTTCC-3') and CALB-XhoI (-5'cgatctcgagTCAGGGGGTGACGATGCCGGAGCAGGTCCT-3'). PCR was carried out using Pyrobest DNA polymerase (TAKARA BIO Inc., Otsu, Japan). The amplified fragment was digested with *Bgl*II and *Xho*I, and inserted between the *Bam*HI

and *SalI* sites of pUC19 (TAKARA BIO Inc.). The resulting plasmid vector was named pUC19-ProCALB CBS6678.

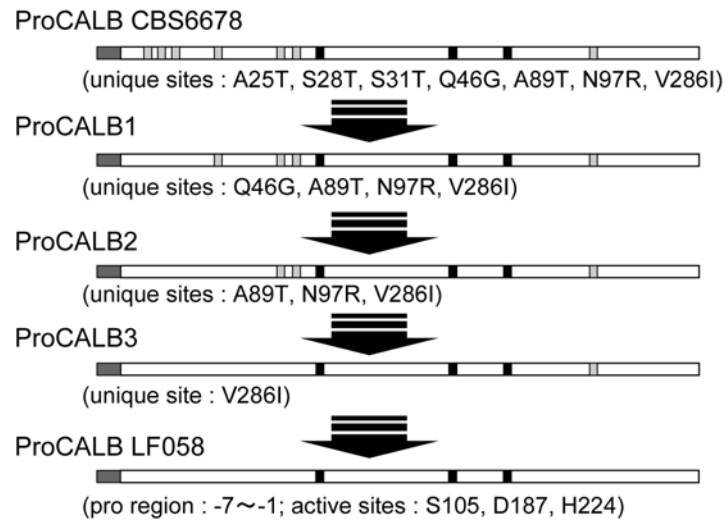
Substitution of amino acid residues by the site-directed mutagenesis

Figure 1a shows the scheme of the gradual substitution of amino acid residues in CALB. Four steps of substitution were carried out. The substitutions of T25A, T28S and T31S in CALB CBS6678 (A25T, S28T, S31T, Q46G, A89T, N97R and V286I) were achieved by PCR with two complementary mutagenic oligonucleotide primers incorporating the desired mutation using pUC19-ProCALB CBS6678 as a template. The thermal-cycle reaction mixture containing the mutated plasmid vector was treated with *DpnI* to digest the template plasmid pUC19-ProCALB CBS6678. The resulting mutated CALB and plasmid vector were named CALB1 (Q46G, A89T, N97R and V286I) and pUC19-ProCALB1, respectively.

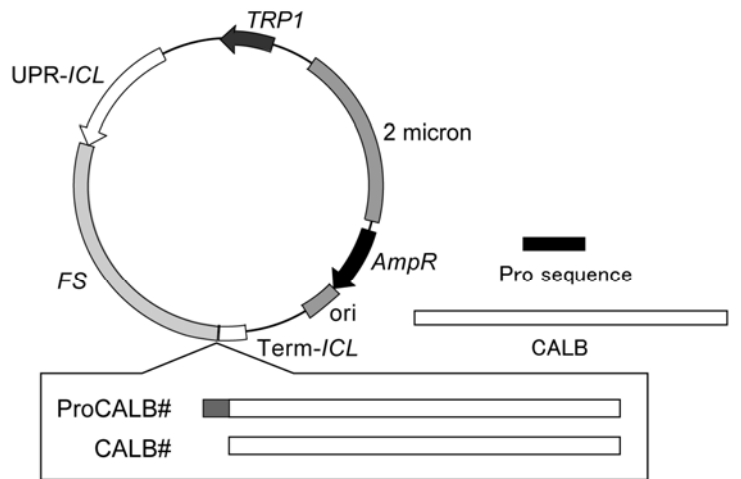
The gradual substitution of the amino acids at positions 46, 89, 97 and 281 in CALB was achieved in the same manner as that mentioned above and the resulting CALBs were named CALB2 (A89T, N97R and V286I), CALB3 (V286I) and CALB LF058. In addition, the plasmid vectors containing these CALBs were named pUC19-ProCALB2, pUC19-ProCALB3 and pUC19-ProCALB LF058, respectively.

Construction of expression plasmids

Figure 1b shows two types of CLAB# (# = CBS 6678, 1, 2, 3 and LF058) that were used for the display on the yeast cell-surface. ProCALB# and CALB# were amplified from pUC19-ProCALB# by PCR with the following primers: BglII-pmCALB and CALB-XhoI BglII-mCALB (5'-atcgagatctCTACCTTCCGGTTCGGACCCTGCCTTCTCC-3') and CALB-XhoI respectively. The amplified fragments were digested with *BglII* and *XhoI*, and inserted between the *BglII* and *XhoI* sites of pWIFS (Matsumoto et al. 2002). The resulting plasmid vectors were named pWIFS-ProCALB# and pWIFS-CALB#, respectively.



(a)



(# = CBS6678, 1, 2, 3, LF058)

(b)

Figure 1 Scheme of gradual substitution of amino acids in CALB (a) and plasmid vector for yeast cell-surface display of two types of CALB# (# = CBS6678, 1, 2, 3 and LF058) (b). The substitutions of amino acids were accomplished by PCR with two complementary mutagenic oligonucleotide primers incorporating the desired mutations. The ProCALB# and CALB# amplified by PCR were inserted downstream of the FS anchor in pWIFS. The resulting plasmid vectors were named pWIFS-ProCALB# and pWIFS-CALB#, respectively.

Yeast transformation

The expression plasmids prepared as described above were transformed into *S. cerevisiae* MT8-1 cells using YEAST MAKERTM (Clontech Laboratories Inc.) according to the protocol specified by the supplier.

Growth conditions

Transformants were preincubated in SD medium at 30°C for 16 h with shaking at 150 strokes/min, were used as starter to inoculate SDC medium (0.67% (w/v) yeast nitrogen base supplemented with appropriate amino acids and nucleotides, 0.5% glucose and 2% Casamino acids) to give an initial OD₆₀₀ of 0.03 and incubated at 30°C with shaking at 150 strokes/min.

Measurement of lipase hydrolytic activity

The lipase hydrolytic activity in the yeast whole-cells was measured by spectrometric method using *p*-nitrophenyl butyrate (PNPB) as a substrate. The final concentration of PNPB in a substrate solution containing 0.5% (v/v) ethanol was 0.5 mM. The assay mixture, with a total volume of 2.1 ml, contained 1.5 ml of the substrate solution, 400 µl of 20 mM potassium phosphate buffer (pH 7.0) and 100 µl of the yeast cell suspension. Yeast cells collected from the culture were washed twice with distilled water and resuspended in distilled water. The cell density was determined by measuring the optical density at 600 nm (OD₆₀₀) and an appropriate amount of the yeast cell suspension was used for lipase activity measurement. The assay mixture was incubated at 30°C for 10 min with shaking at 170 strokes/min, and the enzymatic reaction was stopped by adding 100 µl of 5% (w/v) trichloroacetic acid. The activity was assayed by measuring the absorbance of liberated *p*-nitrophenol (PNP) at 400 nm. One unit (U) of activity was defined as the amount of enzyme required to release 1 µmol PNP/min from PNPB at 30°C.

Protease accessibility assay

For whole-cell papain treatment, yeast cells collected from the culture were washed twice with distilled water and resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM L-cystein to give an OD₆₀₀ of 4. The cell-surface displayed CALBs were cleaved by incubating the suspension at 37°C for 3 h with papain at a final concentration of 37.4 U/ml. After the treatment, the cells were washed twice with distilled water by gentle centrifugation to remove the papain, and enzyme activity was measured as described above.

Preparation of lipase displaying yeast cells

In addition to the CALB displaying yeasts, a yeast displaying the lipase from *R. oryzae* with a pro sequence (ProROL), *S. cerevisiae* MT8-1 transformed with the expression plasmid pWIFSPROROL (named ProROL) (Matsumoto et al. 2002), was used. To prepare the lipase displaying yeasts as a whole-cell biocatalyst, the transformants were cultivated in SDC medium at 30°C for 120 h. After cultivation, the cells were collected, washed with distilled water twice, lyophilized with FreeZone FZ-1 (Labconco Corporation, Kansas, MO) for 36 h, and sieved to homogenize cell-particle size.

Ester synthesis reaction using yeast cells displaying CALB

The reaction mixture, consisting of 450 mg of adipic acid, 9 ml of *n*-butanol (dehydrated), 0.36 ml of distilled water and 150 mg of lyophilized cells, was incubated at 60°C with stirring at 400 rpm in ChemiStation PPW (Tokyourikagakukiki Co., Tokyo, Japan). An aliquot of the reaction mixture was centrifuged at 16,000 *g* and filtered with the 0.45-μm-filter unit Millex-LH (Millipore Co., Bedford, MA) to remove yeast cells. Subsequently, 20 μl of the reaction mixture was mixed with 980 μl of *n*-butanol (dehydrated). A 1.0-μl aliquot of the treated sample was injected into Shimadzu GC-2014 gas chromatograph (Shimadzu Co., Kyoto, Japan) connected to a InterCap 5 capillary column (0.32 mm x 30 mm; GL Sciences Inc., Tokyo, Japan) for the determination of monobutyl adipate (MBA) and dibutyl adipate (DBA) contents in

the reaction mixture. The column temperature was maintained at 120°C for 4 min, increased to 300°C at a rate of 20°C/min, and maintained at 300 °C for 10 min. The temperatures of the injector and detector were set at 300 and 350°C, respectively.

Results

Sequence analysis and substitution of amino acid residues in CALB

We isolated CALB from *C. antarctica* CBS 6678 (CALB CBS6678) and obtained additional three types of the CALB, namely, CALB1 (Q46G, A89T, N97R and V286I), CALB2 (A89T, N97R and V286I) and CALB3 (V286I), by the four steps of substitution of amino acids in CALB CBS6678 (A25T, S28T, S31T, Q46G, A89T, N97R and V286I) (see Materials and methods).

Enzyme activity

pWIFS-ProCALB# and pWIFS-CALB# were transformed into *S. cerevisiae* MT8-1. Expression experiments were performed by flask cultivation, and the time courses of the lipase hydrolytic activity of the whole cells of transformants displaying ProCALB CBS6678 and CALB CBS6678 are shown in Figure 2. In both transformants, the lipase hydrolytic activities of the yeast whole-cells increased with cultivation time, and no detectable lipase hydrolytic activity was determined in the culture medium. The yeast whole-cells displaying only mature CALB CBS6678 on the yeast cell-surface exhibited a higher activity than those displaying ProCALB CBS6678. The lipase hydrolytic activity of the yeast whole-cells reached a plateau in almost all the transformants displaying each type of CALB after 120 h of incubation (data not shown). Figure 3 shows a comparison of the lipase hydrolytic activities of the yeast whole-cells at this time. The yeast whole-cells displaying only mature CALB on the yeast cell-surface exhibited a higher activity than those displaying ProCALB in all the CALBs. Furthermore, the lipase hydrolytic activity of the yeast whole-cells displaying CALB CBS6678 (20.4 U/g-dry cell) was the highest of the activities of all the

transformants and was the higher than that of yeast whole-cells displaying CALB LF058.

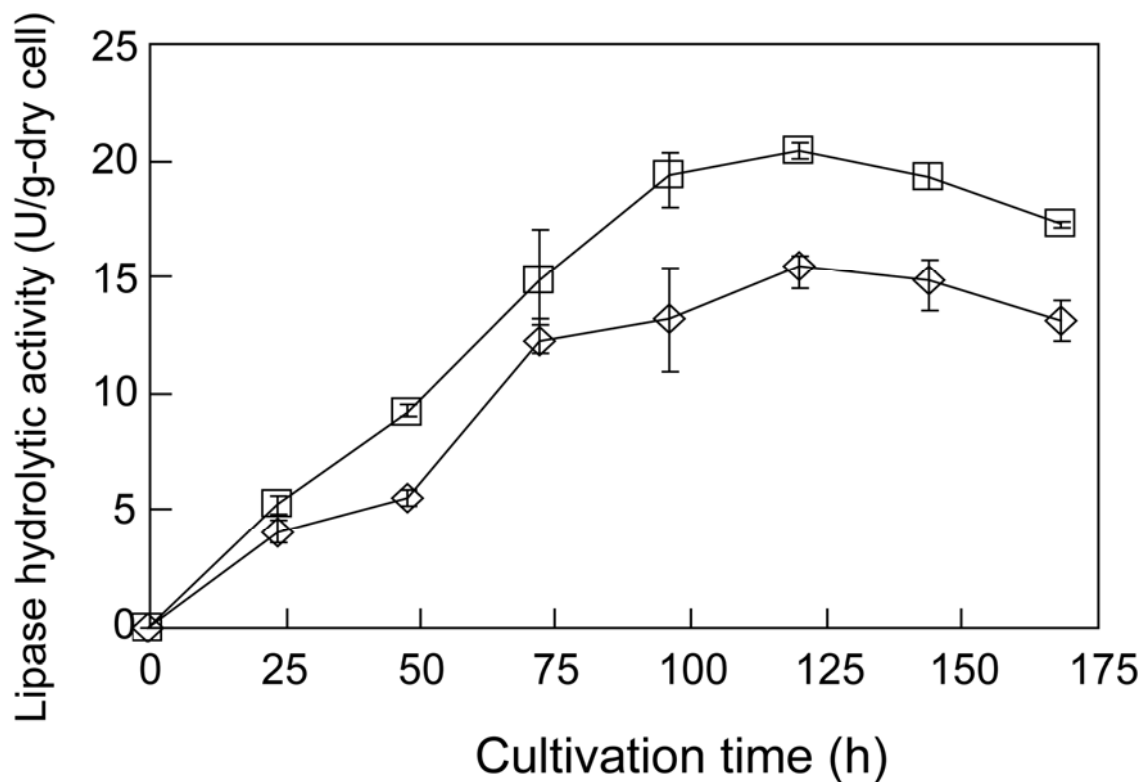


Figure 2 Time course of lipase hydrolytic activities of yeast whole-cells displaying both types of CALB CBS6678. Yeast cells displaying ProCALB (diamonds) and CALB (squares) were grown at 30°C in SDC medium. The data points represent the average of three independent experiments.

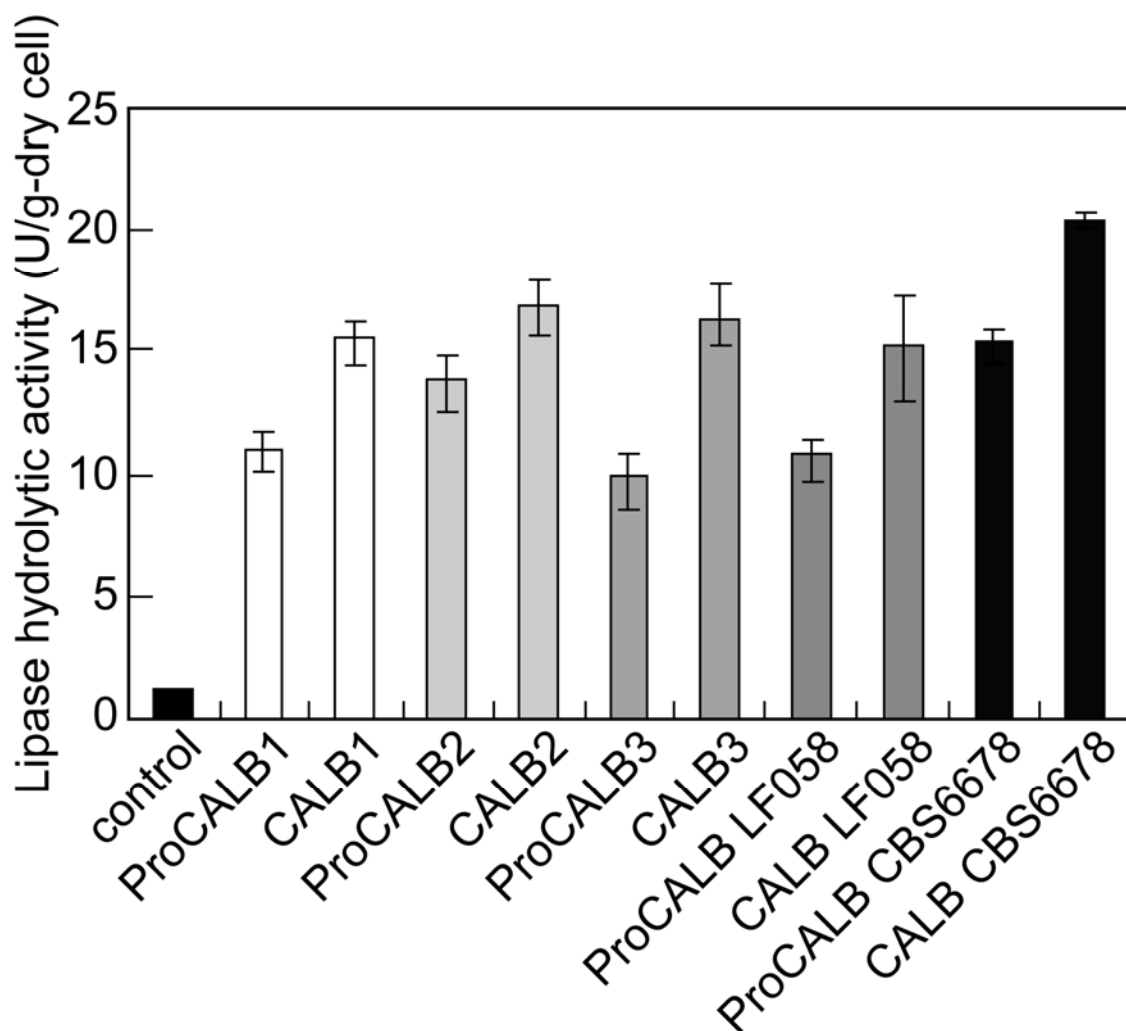


Figure 3 Comparison of the lipase hydrolytic activities of the yeast whole-cells displaying ProCALB# (# = CBS66678, 1, 2, 3 and LF058) and CALB# that were grown at 30°C in SDC medium after a 120 h incubation. Yeast cells harboring pWIFS were used as a control. The data bars represent the average of three independent experiments.

Localization of CALB fusion proteins

To determine the localization of the CALB fusion proteins, a protease accessibility assay using papain as a protease was carried out. The results of the protease accessibility assay using yeast cells displaying both types of CALB CBS6678 are shown in Figure 4. After papain treatment, the lipase activities of the yeast whole-cells decreased to approximately 25% of those of the untreated yeast whole-cells. These results indicate that a large part of the CALB was localized on an easily accessible part of the yeast cell-surface.

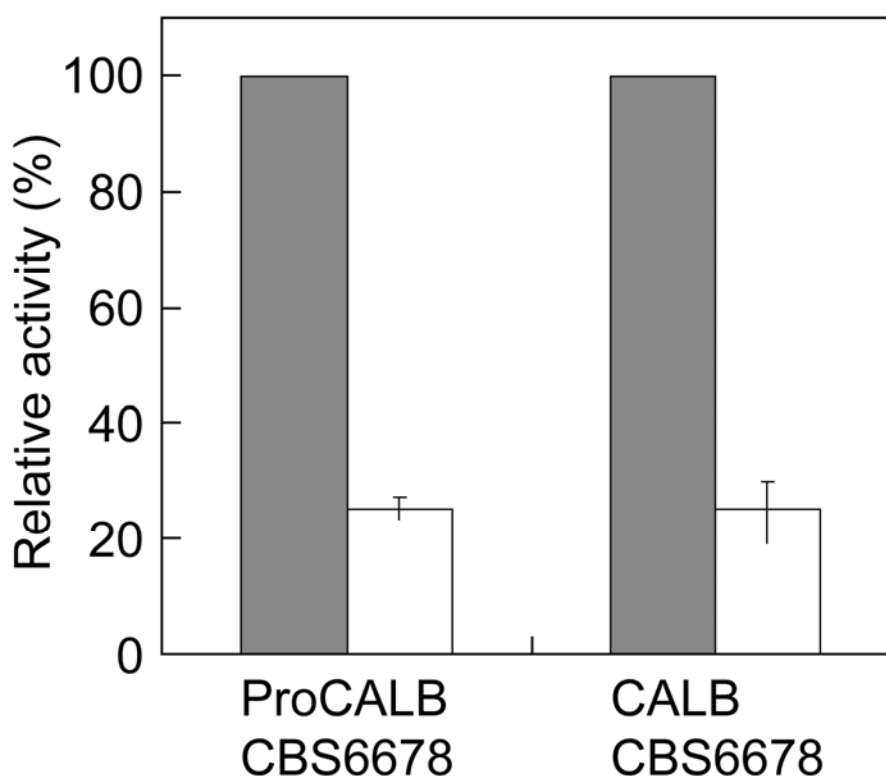


Figure 4 Protease accessibility assay using yeast whole-cells displaying both types of CALB CBS6678 at 37°C for 3 h with papain as protease. The vertical axis indicates the remaining lipase hydrolytic activity of the treated yeast whole-cells (open bars), calculated relative to the lipase hydrolytic activity of untreated yeast whole-cells (closed bars) which was defined as 100%. The data bars represent the average of three independent experiments.

Ester synthesis reaction using yeast cells displaying CALB

The ester synthesis reaction of adipic acid and *n*-butanol using the lyophilized lipase displaying yeast whole-cells was carried out at 60°C. The value of the GC areas of MBA and DBA obtained from the GC analysis of the reaction mixture after 3 h of reaction are shown in Figure 5. Significant differences were shown between the results of the reaction using the lyophilized lipase displaying yeast whole-cells and those of the reaction using the control yeast whole-cells (MT8-1/pWIFS cells). In the reaction mixture using the control cells, a small amount of MBA was detected, but DBA was not detected. In contrast, both MBA and DBA were detected in the reaction mixtures using the lyophilized lipase displaying yeast whole-cells. Moreover, in the reaction using the yeast whole-cells displaying ProROL, the value of GC area of MBA was the highest among all the reactions, but the proportion of DBA in the total product was not high in relation to that in the reactions using the yeast whole-cells displaying CALBs.

The reactions using the yeast whole-cells displaying only mature CALBs produced better results than those achieved using the yeast whole-cells displaying ProCALBs for each CALB (Fig. 5). In the reactions using the yeast whole-cells displaying CALB1 and CALB CBS6678, the value of the GC areas of DBA obtained from the GC analysis of the reaction mixtures were higher than those of MBA.

Figure 6 shows the time course of the ester synthesis reaction at 60°C using the lyophilized yeast whole-cells displaying only mature CALB CBS6678. Adipic acid content decreased with reaction time, and MBA content remained constant after 24 h of reaction. On the other hand, DBA content increased with reaction time and a large percentage of the adipic acid was converted to DBA until reaching 144 reaction hours. The lyophilized yeast whole-cells displaying only mature CALB CBS6678 successfully catalyzed the ester synthesis reaction at 60°C and retained ester synthesis activity for more than 144 h.

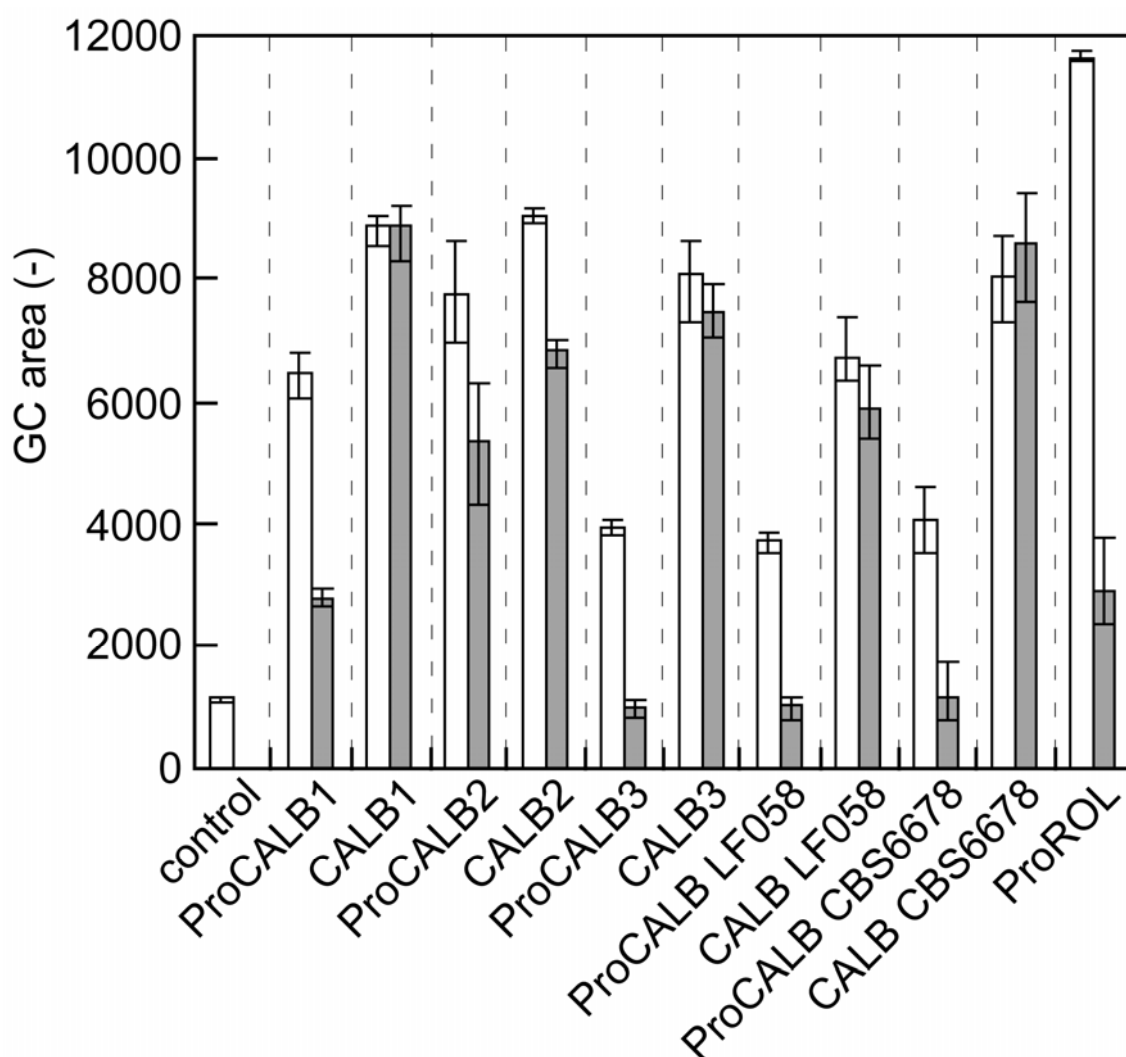


Figure 5 Comparison of lipase ester synthesis activities of yeast whole-cells displaying ProCALB# (# = CBS6678, 1, 2, 3 and LF058) and CALB#. Yeast cells harboring pWIFS and PWIFSProROL (Matsumoto et al. 2002) were used as a control and ProROL displaying yeast, respectively. The ester synthesis reaction of adipic acid and *n*-butanol using the lyophilized lipase displaying yeast whole-cells was carried out at 60°C. MBA (open bars) and DBA (closed bars) contents in the reaction mixture were determined by gas chromatography. The data bars represent the average of three independent experiments.

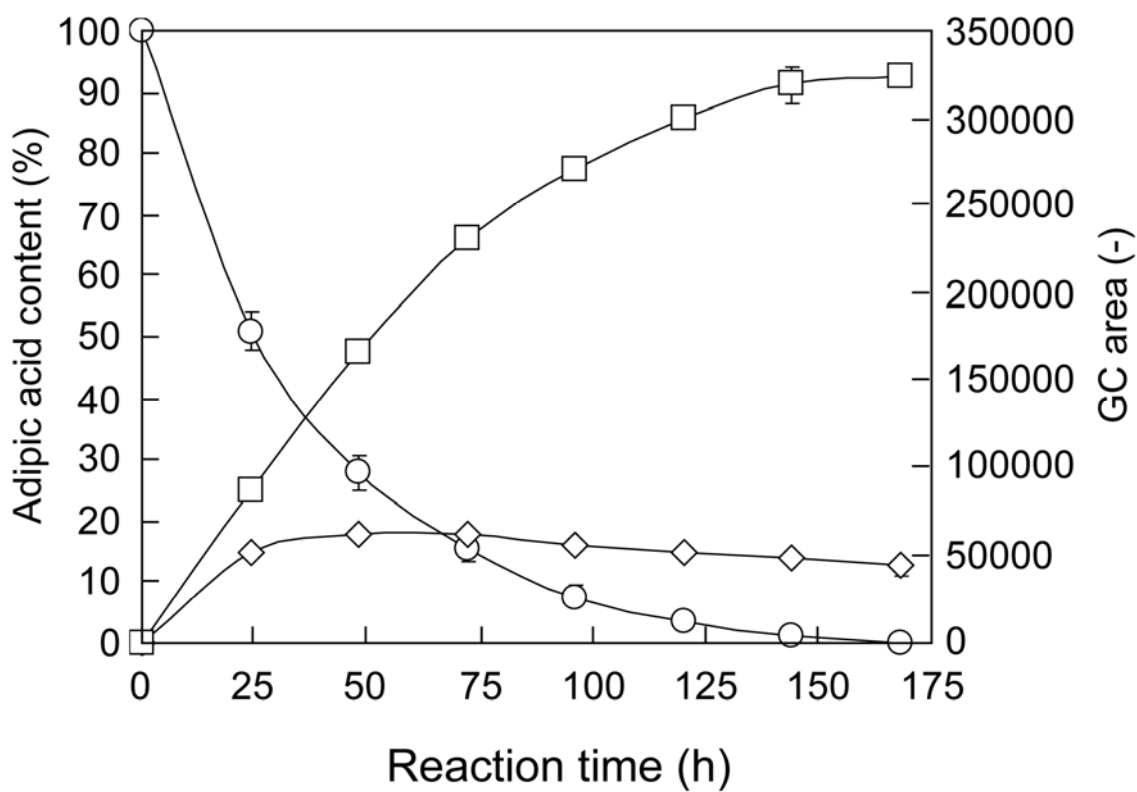


Figure 6 Time course of ester synthesis reaction at 60°C using lyophilized yeast whole-cells displaying CALB CBS6678, and adipic acid content (circles) calculated relative to initial value, which was defined as 100%. MBA (diamonds) and DBA (squares) contents in the reaction mixture were determined by gas chromatography. The data points represent the average of three independent experiments.

Discussion

In the present study, we cloned CALB CBS6678 and successfully constructed CALB displaying yeast whole-cell biocatalysts. The amino acid sequence of CALB CBS6678 was 97.8% identical to CALB LF058 (Uppenberg et al. 1994) with seven unique amino acids, A25T, S28T, S31T, Q46G, A89T, N97R and V286I. To compare

the activity of CALB CBS6678 with that of CALB LF058, we carried out four steps of substitution of the amino acids in CALB CBS6678 to obtain the amino acid sequence of CALB LF058. In addition to CALB LF058, we obtained three additional types of CALB (CALB1, CALB2 and CALB3) by substitution (Fig. 1a). We also compared the activities of these CALBs with that of CALB CBS6678 to investigate the possibility that CALB mutants that show a higher activity than the two above mentioned types of CALB. These five CALBs were displayed on the yeast cell-surface by two displaying types (with/without Pro region), and a protease accessibility assay revealed that a large part of CALB, approximately 75%, was localized on an easily accessible part of the yeast cell-surface (Fig. 4). This accessibility is almost the same as that detected for CALB displayed on the cell-surface of *E. coli* (Narita et al. 2006). A Western blot analysis using SDS-extracted fractions of cells displaying a CALB derivative (fused with the FLAG peptide tag (DYKDDDDK) at the N-terminus) revealed that the fusion proteins are noncovalently attached to the cell wall and highly glycosylated (data not shown).

The lipase hydrolytic activity of the yeast whole-cells increased with cultivation time and varied with the displaying type (Fig. 2). A comparison of the lipase hydrolytic activities of the yeast whole-cells displaying only mature CALB (CALB) and those displaying mature CALB with a Pro region (ProCALB) in all CALBs after 120 h cultivation revealed that only mature CALB is preferable for yeast cell-surface display using the Flo1p anchor system (Fig. 3). The Pro region contains the KEX2 protease site (-2K-1R) and this might have an effect on the post-translational modification of the CALB molecule during the transport to the cell-surface and the lipase hydrolytic activity of yeast whole-cells.

The lipase hydrolytic activity of the yeast whole-cells displaying CALB CBS6678 (20.4 U/g-dry cell) was the highest of the activities of all the transformants and was higher than that of yeast whole-cells displaying CALB LF058. This result suggests that CALB CBS6678 is superior to CALB LF058 for the display of CALB on the yeast cell-surface using the Flo1p anchor system. CALB CBS6678 contains three unique sites (A25T, S28T and S31T) compared with other CALBs; however, these do

not exist in important secondary structures (α -helix and β -strand) in CALB (Uppenberg et al. 1994). These sites are located near the N-terminus of CALB, which was fused to the Flo1p anchor, a structural change of the N-terminus of CALB attributed to these three unique amino acids might have affected the activity of CALB displayed on the yeast cell-surface. The mechanism of this phenomenon is still to be investigated.

The yeast whole-cells displaying CALBs and ProROL were applied to an ester synthesis reaction at 60°C using adipic acid and *n*-butanol as substrates. In analogy with the measurement of lipase hydrolytic activity, the yeast whole-cells displaying only mature CALB on the yeast cell-surface also exhibited a higher ester synthesis activity than those displaying ProCALB for all the CALBs, and even though the ratio of hydrolytic activity to ester synthesis activity is a little different, the tendencies in hydrolytic and ester synthesis activities between each CALB are almost the same (Fig. 5). Moreover, the yeast whole-cells displaying CALB CBS6678 exhibited a higher ester synthesis activity than those displaying CALB LF058, and this result also indicates that CALB CBS6678 is superior to CALB LF058 for the display of CALB on yeast cell-surface using the Flo1p anchor system. The mono ester synthesis activity of the yeast whole-cells displaying only mature CALB CBS6678 was lower than that of the yeast whole-cells displaying ProROL; however, the di ester synthesis activity of the former was almost 3-fold that of the latter. These results indicate that a function of mature CALB CBS6678 molecule, efficiently catalyzing the ester synthesis reaction, was shown to occur even the enzyme was displayed on the yeast cell-surface. These differences between CALB CBS6678 and ProROL could enable lipase displaying yeast whole-cells to become more efficient in the various application areas.

As shown in Figure 6, the DBA content increased with reaction time until 144 h; this indicated that mature CALB CBS6678 displayed on the yeast cell-surface retained activity under the reaction condition, which was a relatively high temperature (60°C), and reaction mixture that contained alcohol, for at least 144 h. The enzyme stability of the yeast whole-cell displaying CALB is favorable for use in bioconversion processes. These results indicated that the yeast whole-cell displaying CALB can be

used for condensation reactions. However, the MBA content remained constant after adipic acid was almost completely consumed. This indicates that the hydrolysis and synthesis of DBA reached an equilibrium state and that the control of the water activity of the reaction might be necessary.

To conclude, we constructed CALB displaying yeast whole-cells that exhibited favorable enzymatic activity and stability in relatively severe reaction conditions. Further studies to improve the activity and productivity of yeast whole-cells displaying CALB are under investigation.

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Chapter 2. Synthesis of functional phospholipids using phospholipase displaying yeast

Introduction

Phospholipids have various biological functions in living organisms (Nishizuka 1995, 2001). Recently, their applications in pharmaceuticals, food and cosmetics industries have been increased (D'Arrigo and Servi 1997; Takami and Suzuki 1994, 1995), since the polar head group of phospholipid affects the physiological and biological functions. However, major component of industrially available phospholipids is only phosphatidylcholine (PC). Therefore, the interconversion of polar groups is an important technique to obtain desired functional phospholipids. Phospholipase D (PLD, EC 3.1.4.4) is known to be a phospholipid metabolizing enzyme (Liscovitch et al. 2000; Ulbrich-Hofmann et al. 2005), and has hydrolysis and transphosphatidylation activities on the phosphatidic moiety of phospholipids. In order to obtain various phospholipids containing different polar head groups, which show versatile physiological functions (Amaducci et al. 1991; Crook et al. 1991, 1992; Hosokawa et al. 2001), PLD is the most suitable enzyme. The construction of inexpensive and convenient PLD enzyme production system would be highly attractive from an industrial standpoint.

Some yeast cell-surface display of proteins or peptides have been widely studied (Kondo et al. 2002; Kuroda et al. 2001; Nakamura et al. 2001), because of their safety, simplicity of genetic manipulation, and rigidity of cell-wall structure. Yeast cell-surface display allows display of target functional proteins on surface of yeast by fusing them to anchor proteins such as the glycosylphosphatidylinositol (GPI) anchor attachment signal sequence (Boder and Wittrup 1997; Murai et al. 1997) or the flocculation functional domain of the Flo1 protein (Matsumoto et al. 2002). Enzyme displaying yeast cell could be used directly as a whole-cell biocatalyst, which exhibits high enzymatic activity without permeabilization treatment.

In the present study, we constructed a PLD from *Streptoverticillium cinnamomeum* (Ogino et al. 1999) displaying yeast cell. In order to display this enzyme on yeast cell-surface, Flo1 short anchor (FS-anchor), amino acids 1 to 1099 of Flo1p, which contains the flocculation functional domain, was used. By using the PLD displayed recombinant yeast, the phospholipid modification to PS from PC was investigated in a biphasic system. Furthermore, the transphosphatidylation reaction conditions of PS from PC were optimized by PLD displaying yeast cell.

Materials and methods

Strains and media

Escherichia coli NovaBlue (Novagen Inc., Madison, WI) was used as cloning host for recombinant DNA manipulation. We constructed *TRP1*-gene-disruption yeast strain of *Saccharomyces cerevisiae* BY4741 (*MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0*) with PCR-mediated seamless gene deletion and marker recycling method (Akada et al. 2006). The mutant strain of BY4741 was designated BY4741ΔW (*MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 trp1Δ-7-722*) and used for the production of fusion protein. *E. coli* was grown in the Luria-Bertani medium (1% (w/v) tryptone, 0.5% yeast extract, 0.5% NaCl), supplemented with 100 μg/ml ampicillin. Yeast was grown in the complete medium (YPD medium: 2% (w/v) peptone, 1% yeast extract and 2% D-glucose) or the selective medium (SD medium: 0.67% (w/v) yeast nitrogen base supplemented with appropriate amino acids and nucleotides and 2% D-glucose).

Construction of recombinant PLD displayed Yeast

The DNA fragment of the mature region of the PLD gene fused with the gene encoding FLAG peptide tag at the N-terminus was amplified by PCR using the following primers BglII-FLAG-mPLD (5'-aat tagatctgactacaaggatgacgatgacaagAGCCCCTCGCCCGCCCCGCACCTGGACGCCGT GGAG-3') and mPLD-XhoI (5'-aattctcagTCAGAGCGAGCAGACGCCCC-3') with

the plasmid pUC702-PLD (Ogino et al. 2004) as a template. This fragment was digested with *Bgl*III and *Xho*I and inserted into *Bgl*III and *Xho*I sites of the plasmid pWIFS (Matsumoto et al. 2002). The resulting plasmid vector was named pWIFS-PLD (Fig. 1). The plasmid pWIFS-PLD was transformed into *S. cerevisiae* BY4741ΔW using YEAST MAKER (Clontech Laboratories Inc., Palo Alto, CA) according to the protocol specified by the supplier. The plasmid pWI3 (Kanai et al. 1996), which is original plasmid of pWIFS, was used as a negative control.

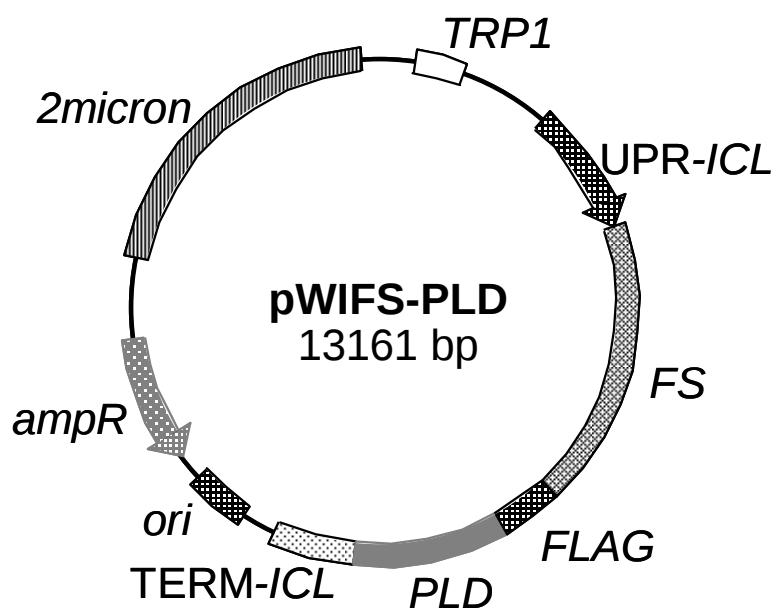


Figure 1 Plasmid vector for the expression of fusion protein FS-PLD (pWIFS-PLD).

Each yeast transformant was pre-incubated in the SD medium at 30°C for 24 h ($OD_{600} > 1.5$) and, after then inoculated to 400 ml of the SDC medium (SD medium containing 2% (w/v) casamino acids) in a 1 L baffle flask to give the initial OD_{600} of 0.03. The cultivation was performed at 30°C on reciprocating shaker with 150 rpm. After 7 days of incubation, the cell were collected, washed twice with distilled water, lyophilized with Free Zone FZ-1 (Labconco Corporation, Kansas, HO) for 1 h, and sieved to homogenize cell-particle size.

Measurement of PLD activity

PLD activity was typically measured by a spectrophotometric analysis using PC (Nacalai Tesque, Kyoto, Japan) as a substrate (Nakamura et al. 1995). The reaction mixture (total volume of 100 μ l) consisted of 0.5% (w/v) egg-yolk PC, 0.1% (v/v) Triton X-100, 40 mM Tris-HCl (pH 7.4), 20 μ l of ethanol and 40 μ l of 10 mg/ml lyophilized yeast cell solution. After incubation at 37°C for 20 min, the reaction was terminated by the addition of 50 μ l of solution containing 50 mM EDTA and 100 mM Tris-HCl (pH 7.4), and the PLD displaying yeast cell was immediately denatured by heating at 95°C for 5 min. After cooling the reaction mixture at room temperature for 5 min, 500 μ l of 20 mM potassium phosphate buffer (pH 7.4) containing 21 mM phenol, 0.6 mM 4-aminoantipyrine, 3000 U/L of *Anthrobacter* choline oxidase (Toyobo, Osaka, Japan), and 720 U/L of horseradish peroxidase (Toyobo, Osaka, Japan) were added to the reaction mixture. After incubation at 37°C for 5 min, the absorbance of the reaction mixture was measured at 505 nm. A calibration curve was obtained using a standard solution of choline chloride instead of the enzyme solution. One unit (U) of hydrolytic activity of PLD was defined as the amount of enzyme that produced 1 μ mol of choline per min from PC at 37°C.

Transphosphatidylation reaction using the yeast cell displaying PLD

Transphosphatidylation reaction was carried out in a biphasic system by ChemiStation PPW equipment (Tokyo rikagakukiki Co., Tokyo, Japan). The reaction mixture were consisted from 13 ml of organic phase of hexane and acetone (70:30) mixture containing 3.5 mM of PC, 13 ml of 2.5 M L-serine solution as a donor, 4 ml of 0.2 M phosphate buffer (pH 5.8), and 600 mg of lyophilized PLD displaying yeast cells equivalent to 0.223 units of PLD hydrolytic activity. The reaction mixture was incubated at 30°C with 600 rpm of stirring.

Optimization of transphosphatidylation reaction condition in biphasic system

The optimization of phospholipid modification condition in biphasic reaction was carried out by variation of following each parameter: (i) the effect of the organic

phase content on phospholipid conversion was investigated to determine the optimal organic solvent in the reaction. The volume ratio between hexane and acetone was varied to 100:0, 90:10, 80:20, 70:30, 60:40 and 50:50 (v/v). (ii) The effect of pH on reaction was investigated to determine the optimal pH of the reaction. The pH value of the reaction mixture was adjusted to 5.8, 6.4, 7.0, 7.6 and 8.2 by using phosphate buffer. (iii) The effect of temperature on reaction was investigated to determine the optimal temperature of the reaction. The incubated temperature was varied to 20, 30, 40 and 50°C. (iv) The effect of L-serine concentration on reaction was investigated to determine the optimal L-serine concentration of the reaction. L-serine concentration was varied to 0.625, 1.25, 1.875, 2.5 and 3.75 M. (v) The effect of volume ratio between the aqueous and organic phases on reaction was investigated to determine the optimal biphasic reaction. Water:organic solvent was changed to 1:2, 1:1 and 2:1 (v/v).

In each experiment, other reaction conditions were same as mentioned in section “Transphosphatidylolation reaction using the yeast cell displaying PLD”. Aliquots of the reaction mixture were collected at appropriate time for analysis.

Measurement of PC and PS in the reaction mixture

The phospholipids, PC and PS, in the reaction mixture were analyzed by high-pressure liquid chromatography (HPLC) system (Shimazu Co., Japan) (Ogino et al. 2002). A TSKgel Silica-60 column (4.6 mmI.D.×25 cm: TOSOH, Japan) was used together with a SPD-10A UV detector (wavelength 210 nm). The HPLC system was operated at 37°C with the mobile phase (acetonitrile: methanol: phosphoric acid (900: 95: 5, v/v), flow rate; 1.1 ml/min).

Reaction mixture was centrifuged at 6,000 g and separated into two phases, then, aliquots of the organic phase containing phospholipids were injected into the HPLC column. Relative concentrations of phospholipids were estimated from their peak area of integrator. Each phospholipid peak was identified by retention time using a standard phospholipid sample.

Results

Confirmation of PLD activity on the recombinant yeast cell

The PLD cell-surface expression plasmid vector pWIFS-PLD (Fig. 1) and pWI3 was introduced into *S. cerevisiae* BY4741 Δ W, and each transformants was named BY4741 Δ W/PLD and BY4741 Δ W/I3, respectively. In order to confirm the PLD activity of BY4741 Δ W/PLD, the spectrophotometric assay was carried out on BY4741 Δ W/PLD with BY4741 Δ W/I3 as a control. No PLD activity was observed in BY4741 Δ W/I3, whereas, the significant PLD activity was detected in BY4741 Δ W/PLD, and the activity was 0.372 U/g-dry cell.

Synthesis of PS in a biphasic system using BY4741 Δ W/PLD

For the phospholipid modification by transphosphatidylase activity of PLD, the biphasic reaction system had been usually performed by diethylether (Nakajima et al. 1994) or *n*-hexane-acetone mixture (Iwasaki et al. 2003; Juneja et al. 1989) used as an organic phase. Since the utilization of *n*-hexane was only permitted as a food additive component in Japan regulation, the *n*-hexane-acetone mixture was choose as an organic solvent in this study.

At first, the effect of organic solvent ratio on the transphosphatidylase reaction from PC to PS was investigated by variation of volume ratio between *n*-hexane, nonpolar solvent, and acetone, polar solvent (Fig. 2). No PS production was observed in the solvent containing only *n*-hexane. The yield of PS production increased with the increasing ratio of acetone up to 30% (vol of acetone/total vol), though that of PS decreased 30% over the ratio of acetone. The mixture ratio of *n*-hexane and acetone (70:30) achieved the highest yield of PS production (50.8%) after 48 h reaction among all organic solvent ratio tested. In addition, the PS conversion from PC using BY4741 Δ W/PL reached plateau after 24 h, and there was no significant difference between after 24 h and 48 h. Thus, it was assumed that the incubation for 24 h was sufficient for the reaction termination.

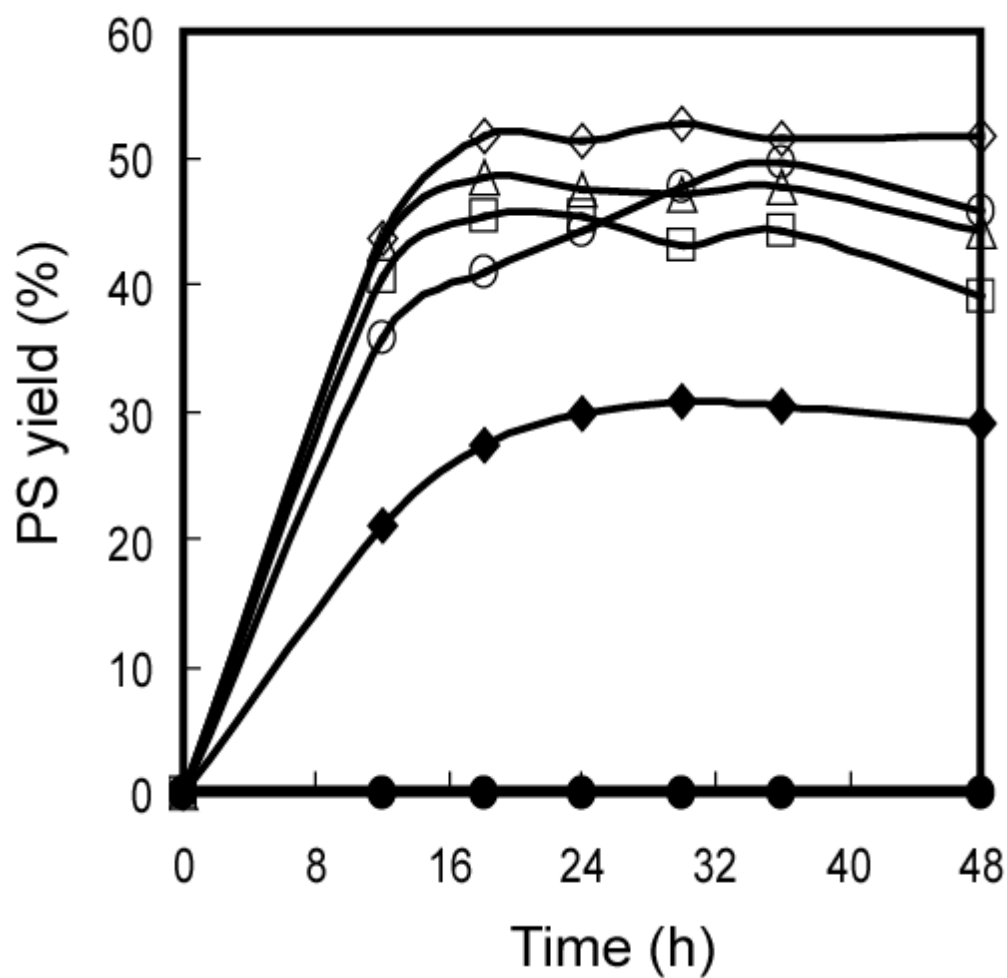


Figure 2 Effect of organic component ratio on transphosphatidyl reaction by BY4741ΔW/PLD. The reaction were carried out following various volume ratio of *n*-hexane:acetone: (open squares) 50:50, (open triangles) 60:40, (open diamonds) 70:30, (open circles) 80:20, (close diamonds) 90:10, (close circles) 100:0, respectively.

Effect of pH and temperature condition on the PS production at initial phase

The pH dependency on the transphosphatidylation by PLD was investigated by several researchers, and the optimal pH was considered at around 5.8 (Hatanaka et al. 2002; Ogino et al. 1999; Simbo et al. 1990). Therefore, the pH dependency of PLD displayed on yeast cells (BY4741 Δ W/PLD) was also investigated in biphasic system by varying pH from 5.8 to 8.2 (Fig. 3). Although the yields of PS after 24 h of reaction (final yield) were roughly the same at all pH values tested (data not shown), the initial PS yield within 4 h of reaction was affected on pH values. PLD displayed on yeast cells exhibited the high transphosphatidylation activity around the neutral pH and the highest yield at 4 h was achieved at pH 7.0.

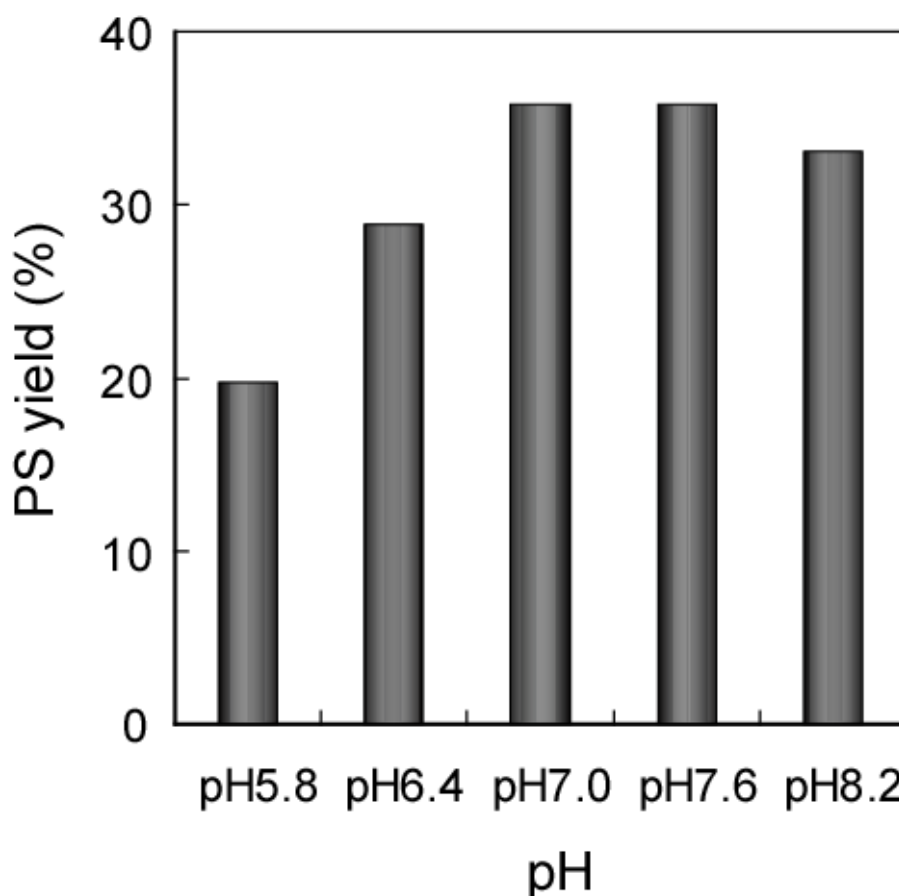


Figure 3 Effect of pH on transphosphatidylation reaction. The reaction was carried out at following various pH: 5.8, 6.4, 7.0, 7.6 and 8.2, respectively.

Next, effect of the temperature on the transphosphatidylation reaction using BY4741 Δ W/PLD was investigated at various temperatures. The final yield was almost the same at all reaction temperatures tested (data not shown). However, the reaction temperature affected the initial PS yield within 4 h (Fig. 4), and the phospholipid modification at 40°C was achieved the highly yield. Together these results, it was assumed that the optimized pH and temperature condition with the reduction of incubation time were 7.0 and 40°C, respectively.

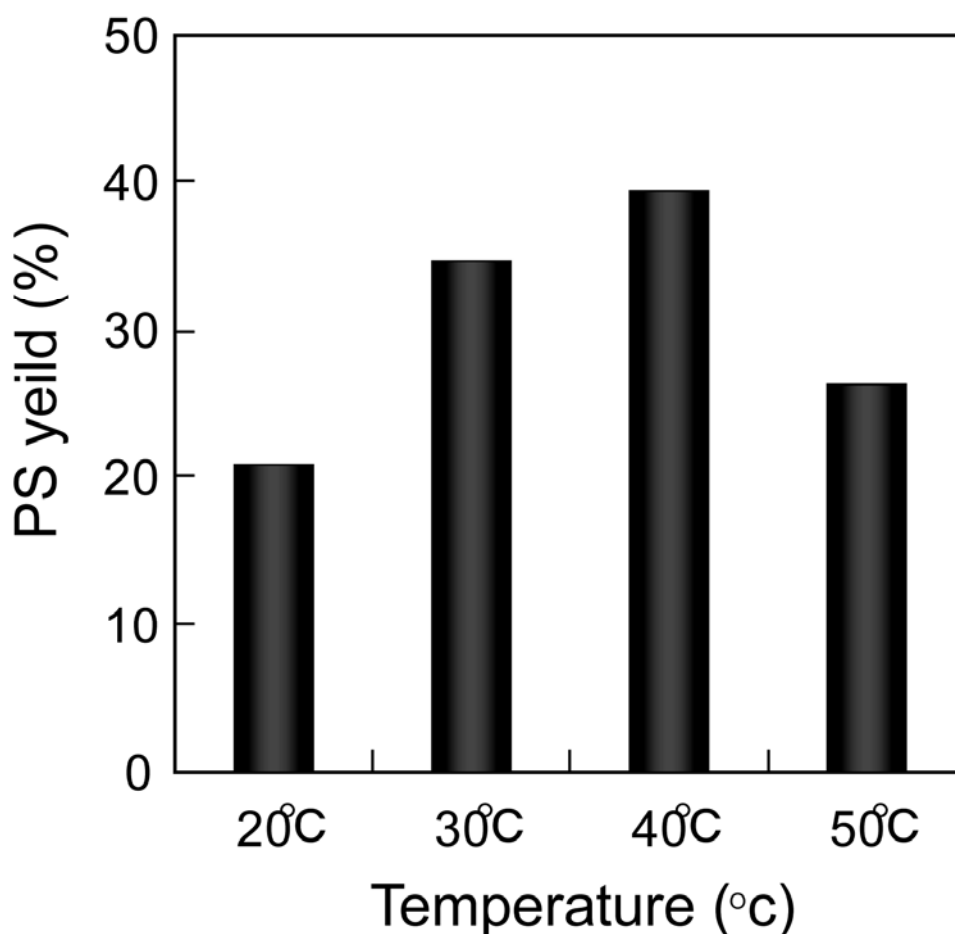


Figure 4 Effect of temperature on transphosphatidylation reaction. The reaction was carried out at following various temperatures: 20, 30, 40 and 50°C, respectively.

Effect of L-serine concentration on final PS conversion

The reduction of PA formation is key point for the following separation step of phospholipids products. For suppression of PC hydrolysis, the high concentration of L-serine solution had been used in previous papers (Iwasaki et al. 2003; Juneja et al. 1989). Therefore, the optimal L-serine concentration for the reaction using BY4741 Δ W/PLD was investigated by varying the L-serine concentrations from 0.625 to 3.75 M (Fig. 5). The L-serine concentration in the reaction mixture affected initial PS production rate (within 4 h) and final yields of PS, respectively. The PS production was increased with increasing of L-serine concentration. The highest PS yield (57.5%) was achieved at between 1.25 to 1.875 M of L-serine concentrations, and the high concentration of L-serine was suppressed of PS production.

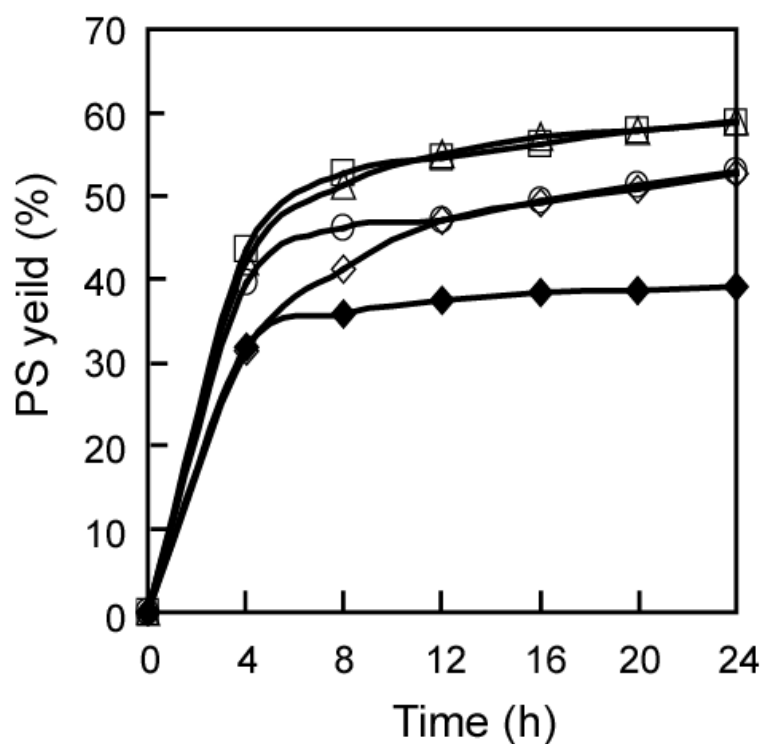


Figure 5 Effect of L-serine concentration on transphosphatidylase reaction. The reaction was carried out with following various L-serine concentrations: (open diamonds) 0.625, (open squares) 1.25, (open triangles) 1.875, (open circles) 2.5, (close diamonds) 3.75 M, respectively.

Effect of two-phase volume ratio on final PS conversion

Finally, the effect of two-phase volume ratio on the transphosphatidylation reaction was investigated by changing the volume ratio of aqueous phase and organic phase (Fig. 6). The highest early yield was recorded in the water:organic solvent ratio of 1 to 1. Therefore, the optimal two-phase ration was determined 1:1 (water:organic solvent).

By investigating the effects of various parameters on the transphosphatidylation reaction using BY4741 Δ W/PLD, the optimal condition was determined (Table 1). By comparing the initial condition, the final yield at optimized condition improved from 50.8% to 57.5%. In addition, the initial PS yield significantly increased from 19.7% to 46.0%

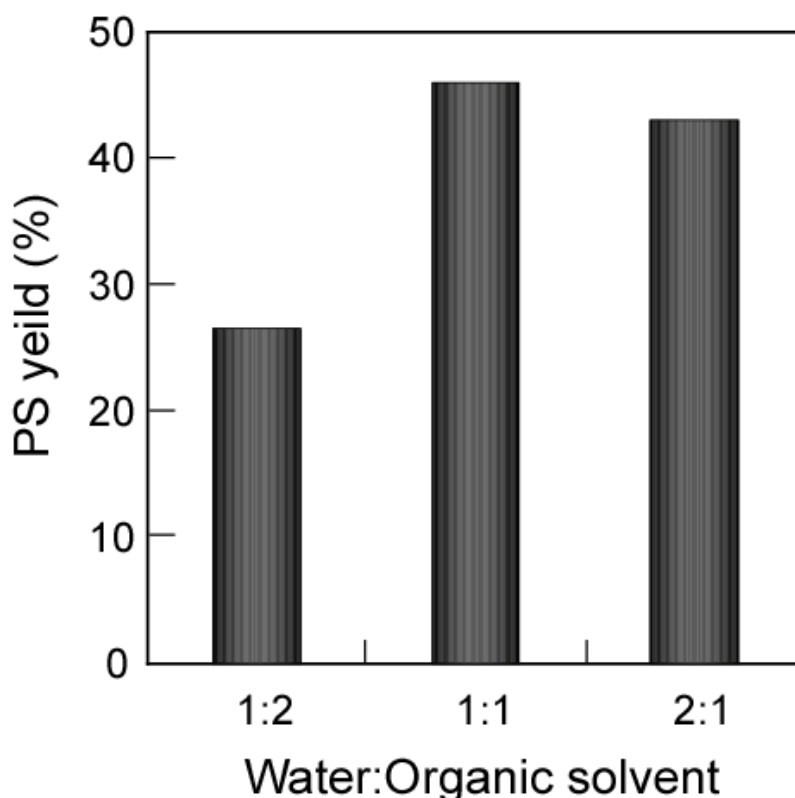


Figure 6 Effect of two-phase ratio on transphosphatidylation reaction. The reaction was carried out with following various ratio of water:organic solvent: 1:2, 1:1 and 2:1, respectively.

	Initial condition	Optimal condition
hexane:acetone	70:30	70:30
pH	5.8	7.0
temperature (°C)	30	40
serine concentration (M)	2.5	1.25
Water:organic solvent	1:1	1:1
early yield (after 4 h reaction)(%)	19.7	46.0
final yield (after 24 h reaction) (%)	50.8	57.5

Table 1 Initial condition and optimized condition for PS conversion in a biphasic system using BY4741 Δ W/PLD.

Discussion

The reaction model for transphosphatidylation of phospholipids by purified PLD powder or PLD immobilized on a carrier polymer have previously been reported (D'Arrigo et al. 2005; Dittrich and Ulbrich-Hofmann 2001). However, these strategies require the processes such as purification or immobilization to obtain PLD as a catalyst. Therefore, a strategy to obtain the PLD catalyst easily and inexpensively is long-awaited. In this study, we successfully developed a PLD displaying on to yeast cell surface of *S. cerevisiae* BY4741 Δ W by fusing to FS-anchor as an anchor protein. This method could be permitted us to obtain the PLD enzyme more efficiently than the conventional methods because the cell-surface display system do not need any complex processes for obtaining PLD as a catalyst.

We have developed a reaction system for the transphosphatidylation from PC to PS using BY4741 Δ W/PLD in this study. Although the activity of BY4741 Δ W/PLD was low (0.372 U/g-dry cell), the final yield of PS was greater than 50% under the initial condition. No detectable leakages of phospholipids and L-serine from BY4741 Δ W/PLD were also determined (data not shown). These results indicated that PLD displayed on yeast cell surface effectively catalyzed the transphosphatidylation reaction from PC to PS in a biphasic system. Therefore, it was confirmed that PLD displaying yeast was used as a whole-cell biocatalyst and has an equivalent potential to purified PLD powder or PLD immobilized on carrier polymer.

For improvement of the PS yield, the reaction conditions for transphosphatidylation reaction from PC to PS were optimized by changing following parameters; organic solvent component ratio, pH, temperature, L-serine concentration and two-phase volume ratio. Especially, the optimal L-serine concentration changed from 2.5 M to 1.25 M using BY4741 Δ W/PLD (Fig. 5). This result and tendency were not quite different with those of purified *Streptomyces* PLDs (Iwasaki et al. 2003; Juneja et al. 1989). The increasing of L-serine concentration in the reaction mixture will be induced the high liquid viscosity, and the diffusing of substrate to the cell surface was inhibited. Resulting, it was affected to the formation of the

PLD-phosphatidyl complex intermediates on the yeast cell-surface. Focused on the initial reaction rate, the optimal pH and temperature for transphosphatidylase activity of PLD was pH 7.0 (Fig. 3), and 40°C (Fig. 4), respectively. Since the pH stability of original PLD from *Streptomyces* species was between from 5.0 to 8.0, the optimized pH was also agreed with these previous results. However, the optimal pH for PS production had reported as around at 5.6~5.8 (Hatanaka et al. 2002; Simbo et al. 1990). Therefore, it is assumed that the catalytic function of PLD slightly changed by displaying on yeast cell-surface. The reaction temperature will be depended on the substrate solubility, *i.e.* the elevating of the temperature results in the increasing of the substrate solubility, however the enzymatic activity was not tolerance for long time at high temperature (Hatanaka et al. 2002; Ogino et al. 1999; Simbo et al. 1990). Therefore, it is assumed that the optimized temperature (40°C) is rational condition for both of the substrate stability and the enzymatic activity.

The optimal organic solvents component ratio, *i.e.* the mixture ratio of *n*-hexane and acetone, was determined as a 70% (v/v) of *n*-hexane (Fig. 2). The increasing of acetone ratio in the organic phase improved polarity of solvent, because PLD did not exhibit its activity in the high polarity solvent (Fig. 2). In addition, it was also improved PC solubility in the organic phase. However, the moreover of 30% of acetone contrary affected enzymatic activity because the enzyme structures are damaged. By changing the volume ratio of aqueous phase and organic phase, each ratio of 1:1 and 1:2 (aqueous: organic phase) showed almost same final conversion yield (Fig. 6). As shown the PS yield was represented as a concentration in Fig. 6, the absolute amount of PS product was greater using the ration of water to organic solvent of 1 to 2 than that of 1:1 case. Therefore, the mixture ratio of 1:2 (aqueous: organic phase) would be most effective condition on engineering aspect. Together with those results shown above, the initial and final PS conversion yield improved from 19.7% to 46.0% and from 50.8% to 57.5% in the optimal condition, respectively. Since it had been reported that the activation of PLD by various treatments and salt addition (Nakajima et al. 1994), it will possible to further improve the PS yield by combining the these activation methodology.

In this study, we successfully constructed a cell-surface display system of PLD using *S. cerevisiae* BY4741ΔW and demonstrated that BY4741ΔW/PLD has efficacy as a catalyst in transphosphatidylation reaction. Furthermore, this PLD displaying yeast could be employed in the synthesis of other phospholipids not involving PS. This technique also may be applicable to other phospholipases: *i.e.* phospholipase A₂ (Sugiyama et al. 2002), and lipase (Perez et al. 1993). If the cell-surface display systems of various phospholipases will be developed, it is expected that the development of facile functional phospholipids production system with the use of those whole-cell biocatalysts.

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General conclusion

This study was carried out to develop the novel distribution of enzymatic catalysis and function of organism by the yeast cell-surface display systems, and the novel bioconversion process by enzyme displaying yeast cell by yeast cell-surface display system as the whole cell biocatalyst.

Novel anchor system using invertase to localize target functional protein in periplasm layer was successfully constructed. EGFP was chosen as the target protein and fused to the N- or C- terminus of invertase (EGFP-INV, INV-EGFP). Green fluorescence of EGFP was clearly observed in the outline of the EGFP-INV or INV-EGFP fusion protein producing cells by fluorescence microscopy analysis. And both EGFP-INV and INV-EGFP fusion protein released from cells by Zymolyase treatment indicated that the fusion proteins are located in the periplasm in free state. By combining this novel system, it should be possible to localize more enzymes in the cell-surface layer to increase catalytic activity and/or catalyze sequential reaction.

As the additional approach to develop the yeast cell-surface display system, we constructed a *P. pastoris* cell-surface display system based on Flo1p anchor system using lipase from *R. oryzae* with a pro sequence (ProROL) as the model protein. The display of ProROL on the *P. pastoris* cell-surface was confirmed by immunomicroscopy analysis. Methanol concentration of 0.5% was favorable in short-time induction until 120 h, on the other hands, induction in 2% methanol achieved highest activity 15.3IU/g-dry cell for 240 h. This index of lipase activity (IU/g-dry cell) was lower than that using *S. cerevisiae* as the host strain. However, *P. pastoris* could be cultivated at a higher cell density than *S. cerevisiae*. Hence, another index of lipase activity (IU/litter of cultivation medium) was higher than that using *S.cerevisiae* as the host strain. Lipase activity in *P. pastoris* cell displaying ProROL showed high thermal stability, moreover that was increased about 6.5 times greater than initial value by incubation at 60°C. From these results, enzymes displayed on *P. pastoris* cells are expected to be effective whole-cell biocatalyst.

By taking advantage of high stress tolerance and proliferation potential of sake yeast, the effect of cultivation conditions on the cell-surface display expression of the Flo1p anchor system was investigated. Several cultivation conditions, especially component of the culture medium affected cell-surface display expression of the Flo1p anchor system. Cultivation in a rich medium containing peptone and/or yeast extract induced the release of the target fusion protein into the culture supernatant. The carbon source in the culture medium also affected the state of the target fusion protein. The oligosaccharide (sucrose and raffinose) reduced the amount of the fusion target protein, on the contrary, mannose and fructose, particularly mannose, induced the release of the target fusion protein. This information is valuable for further development of the cell-surface display system using Flo1p anchor system.

Novel bioconversions with enzyme displaying yeast cell were successfully demonstrated. First one is application of CALB displaying yeast cell, which was constructed in this study, for the di ester synthesis reaction using adipic acid and *n*-butanol as the model of the condensation reaction. We isolated CALB from CBS 6678 strain (CALB CBS6678), and yeast cell displaying newly isolated CALB CBS6678 exhibited higher hydrolytic and ester synthesis activity than the yeast cell displaying well-known CALB, which is registered in GENBANK. Di ester synthesis reaction using lyophilized yeast cell displaying CALB CBS6678 was successfully carried out at 60°C, and CALB CBS6678 displayed on the yeast cell-surface retained activity under the reaction condition, which was a relatively high temperature and contained alcohol, for at least 144 h. These results indicated that yeast cells displaying CALB can be used for condensation reactions. Moreover, this enzyme stability of yeast cells displaying CALB is favorable for use in bioconversion processes.

We also developed novel reaction system for transphosphatidylation from PC to PS as the model reaction of transphosphatidylation of phospholipids using *Streptoverticillium cinnamomeum* PLD displaying yeast cell. Although the activity of PLD displaying yeast cell was low (0.372 U/g-dry cell), PLD displaying yeast cell effectively catalyzed the transphosphatidylation reaction from PC to PS in a biphasic system and the final yield of PS was greater than 50% under the initial condition. We

optimized the reaction condition by changing several parameters and the initial and final PS conversion yield was improved from 19.7% to 46.0% and from 50.8% to 57.5% in optimal condition, respectively. Furthermore, this PLD displaying yeast could be employed in the synthesis of other phospholipids not involving PS. This technique also may be applicable to other phospholipases: *i.e.* phospholipase A₂, and lipase. If the cell-surface display systems of various phospholipases will be developed, it is expected that the development of facile functional phospholipids production system with the use of those whole-cell biocatalysts.

Publication list

Part I

Chapter 1

Tanino T, Matsumoto T, Fukuda H, Kondo A (2004) Construction of system for localization of target protein in yeast periplasm using invertase. *J Mol Catal B Enzym* 28:259-264

Chapter 2

Tanino T, Fukuda H, Kondo A (2006) Construction of a *Pichia pastoris* cell-surface display system using Flo1p anchor system. *Biotechnol Prog* 22:989-993

Part II

Tanino T, Noguchi E, Kimura S, Sahara H, Hata Y, Hukuda H, Kondo A (2006) Effect of cultivation conditions on cell-surface display of Flo1 fusion protein using sake yeast. *Biochem Eng J* *in press*

Part III

Chapter 1

Tanino T, Ohno T, Aoki T, Fukuda H, Kondo A (2007) Development of yeast cells displaying *Candida antarctica* lipase B and their application to ester synthesis reaction. *Appl Microbiol Biotechnol* *submitted*

Chapter 2

Shoji S, Tanino T, Ogino C, Kaneko S, Kondo A, Fukuda H (2007) Synthesis of functional phospholipids using phospholipase D displaying yeast. *Appl Microbiol Biotechnol* *submitted*

Other publications

Narita J, Okano K, Tatenno T, Tanino T, Sewaki T, Sung MH, Fukuda H, Kondo A (2006) Display of active enzyme on the cell surface of *Escherichia coli* using PgsA anchor protein and their application to bioconversion. *Appl Microbiol Biotechnol* 70:564-572

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Kato M, Fuchimoto J, Tanino T, Kondo A, Fukuda H, Ueda M (2006) Preparation of a whole-cell biocatalyst of mutated *Candida antarctica* lipase B (mCALB) by a yeast molecular display system and its practical properties. *Appl Microbiol Biotechnol* *in press*

Tamalampudi S, Talukder MMR, Hama S, Tanino T, Suzuki Y, Kondo A, Fukuda H (2006) Development of recombinant *Aspergillus oryzae* whole-cell biocatalyst expressing lipase encoding gene from *Candida antarctica*. *Apple Microbiol Biotechnol* *in press*