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

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Abstract

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.

Keywords: *Saccharomyces cerevisiae*; Periplasm; Invertase; Enhanced green fluorescence protein; Fusion protein

1. 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 [1–5]. 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 materual [5]. 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 [6] or the flocculation functional domain of the Flo1 gene [3]. 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 [7,8], 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

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to investigate whether these latter could be used to achieve periplasmic localization of the protein.

2. Experimental

2.1. 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 (*MAT*a *FLO8 his4 leu2–3 leu2–112 STA1*). *S. cerevisiae* MT8-1 (*MAT*a *ade his3 leu2 trp1 ura3*) was used for production of INV/EGFP fusion proteins. *E. coli* was cultivated in 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.

2.2. Construction of expression plasmids

2.2.1. 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-*Sac*I (5′-atcggagctcATGCTTTTGCAAGCTTTCCTTTTCCTTTTG-3′) and SUC2-rev-*Sal*I (5′-cgatgtcgacCTATTTTACTTCCCTTACTTGGAACTTGTC-3′). PCR was carried out using *pfu* hotstart turbo polymerase (Stratagene Cloning Systems, La Jolla, CA, USA). The amplified fragment was digested with *Sac*I and *Sal*I and inserted into the *Sac*I and *Sal*I site of pWGP3 [9] (Fig. 1a). The resulting plasmid was named pWGP3-SUC2 (Fig. 1b).

2.2.2. 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-*Sac*I and SUC2(signal)-rev-*Bgl*II-ctc-*sal*I (5′-cgatgtcgacgagagatctTGATGCAGATATTTTGGCTGC-3′) for the secretion signal of *SUC2*; and SUC2(mature)-fw-*sal*I (5′-atcggtcgacATGACAAACGAAACTAGCGATAGACCTTTG-3′) and SUC2-rev-*sph*I–*Xho*I (5′-cgatctcgaggcatgcCTATTTTACTTCCCTTACTTGGAA-3′) for the mature region of *SUC2*. The amplified fragments were digested with *Sac*I and *Sal*I, and with *Sal*I and *Xho*I, respectively. First the fragment encoding the secretion signal sequence of *SUC2* was inserted into the *Sac*I and *Sal*I site of pWGP3 (named pWGP3-S), and then the fragment encoding the mature region of *SUC2* into the *Sal*I and *Xho*I site 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-*Bgl*II (5′-atcgagatctATGGTGAGCAAGGGCGAGGAGCTGTTCACC-3′) and EGFP(*n*)-rev-*Sal*I (5′-cgatgtcgacCTTGTACAGCTCGTCCATGCCGAGAGTGAT-3′). The amplified fragment was

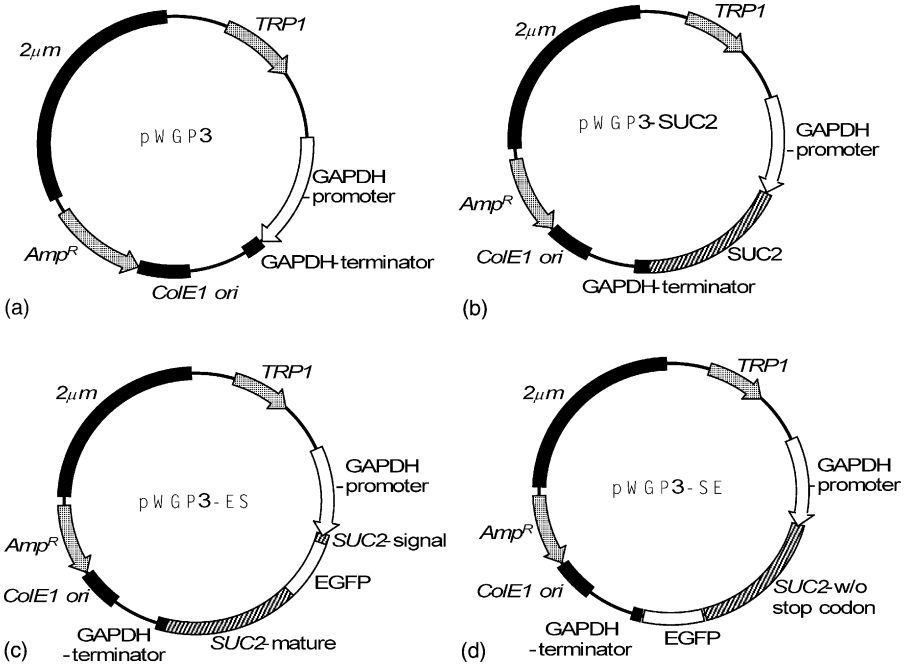

Fig. 1. Control plasmid (a, pWGP3) and plasmids for expression of INV (b, pWGP3-SUC2), EGFP-INV fusion protein (c, pWGP3-ES), and INV-EGFP fusion protein (d, pWGP3-SE).

digested with *Bgl*II and *Sal*I and inserted into the *Bgl*II and *Sal*I site of pWGP3-SS and the resulting plasmid named pWGP3-ES (Fig. 1c).

2.2.3. 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-*Sac*I and SUC2(*n*)-rev-*Sal*I (5′-cgatgtcgacTTTTACTTCCCTTACTTGGAACTTGTCA-AT-3′). The amplified fragment was digested with *Sac*I and *Sal*I and inserted into the *Sac*I and *Sal*I site of pWGP3 and the resulting plasmid named pWGP3-S(*n*). *EGFP* was also amplified from pEGFP by PCR with the following primers: EGFP-fw-*Sal*I (5′-atcggtcgacATGGTGAGCA AGGGCGAGGA GCTGTTCATG-3′) and EGFP-rev-*Sal*I (5′-cgatgtcgacTTACTTGTACAGCTCGTCCATGCCGAG-AGT-3′). The amplified fragment was digested with *Sal*I and inserted into the *Sal*I site of pWGP3-S(*n*) and the resulting plasmid named pWGP3-SE (Fig. 1d).

2.3. Yeast transformation

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

2.4. 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.

2.5. 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 per second and data for 10,000 events collected.

2.6. Fractionation of cell-surface proteins and intracellular proteins

Cells cultivated in SDCH medium for 72 h at 30 °C were collected by centrifugation at 3000 × *g* for 10 min, washed twice with PBS buffer (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl, pH 7.4), and resuspended in 10 ml/g-cell Tris–SO_4 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 K_2HPO_4, 4 mM KH_2PO_4, 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 3000 × *g* for 10 min, a supernatant fraction (periplasmic fraction) containing cell-surface proteins 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 3000 × *g* for 10 min, a supernatant fraction (intracellular fraction) containing intracellular proteins was obtained.

2.7. 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.

2.8. Western blot analysis of EGFP

Sodium dodecil sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using an 8% (w/v) 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.).

2.9. Invertase activity measurement

A modified version of the procedure of Goldstein and Lampen [10] 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).

3. Results

3.1. 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 b).

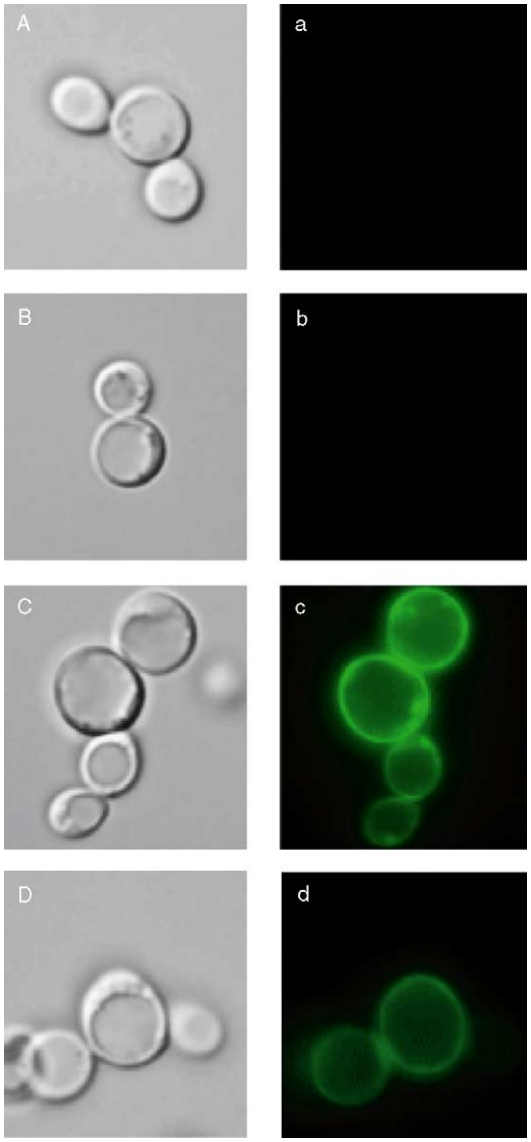

Fig. 2. Differential interference contrast micrographs (left panels) and fluorescence micrographs (right panels) of yeast cells. (A and a) MT8-1/pWGP3; (B and b) MT8-1/pWGP3-SUC2; (C and c) MT8-1/pWGP3-ES; (D and d) MT8-1/pWGP3-SE.

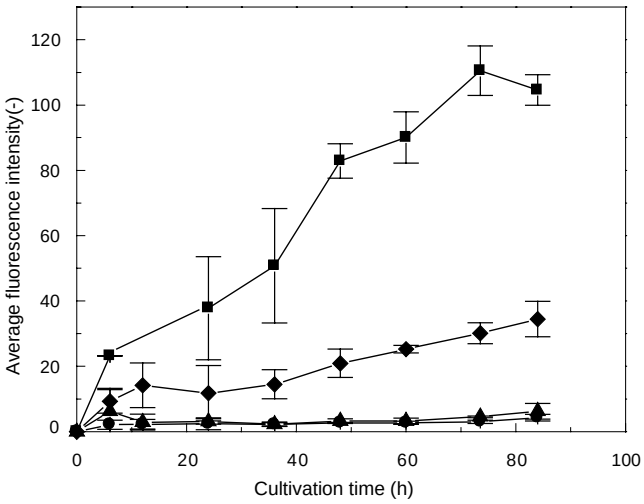

Fig. 3. Time course of average fluorescence intensity of MT8-1/pWGP3-ES (squares), MT8-1/pWGP3-SE (diamonds), MT8-1/pWGP3 (triangles) and MT8-1/pWGP3-SUC2 (circles) during cultivation.

3.2. 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. Fig. 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.

3.3. 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 18–20 aspargine-linked polysaccharide units, and 9–10 units per protein subunit [11–13]. Endo Hf 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.

3.4. Activity measurement of fusion invertase

Invertase activity was measured to determine whether fusion protein was secreted to the cell-surface in oligomeric

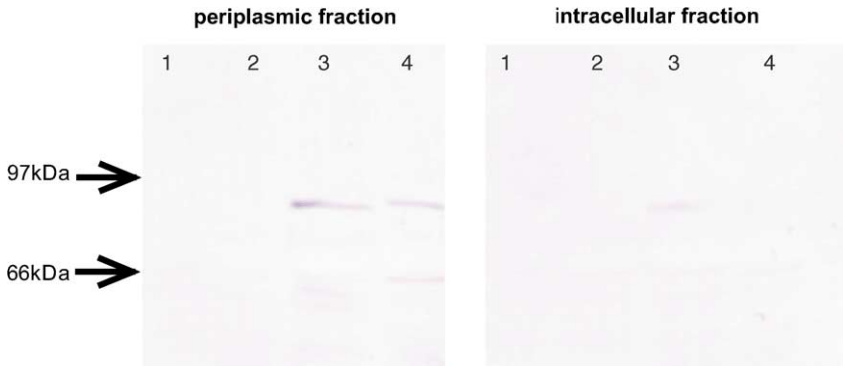

Fig. 4. Immunoblotting of cell-surface fraction (left panel) and intracellular fraction (right panel). Lane 1: MT8-1/pWGP3; lane 2: MT8-1/pWGP3-SUC2; lane 3: MT8-1/pWGP3-ES; lane 4: MT8-1/pWGP3-SE. Arrows show molecular weight markers.

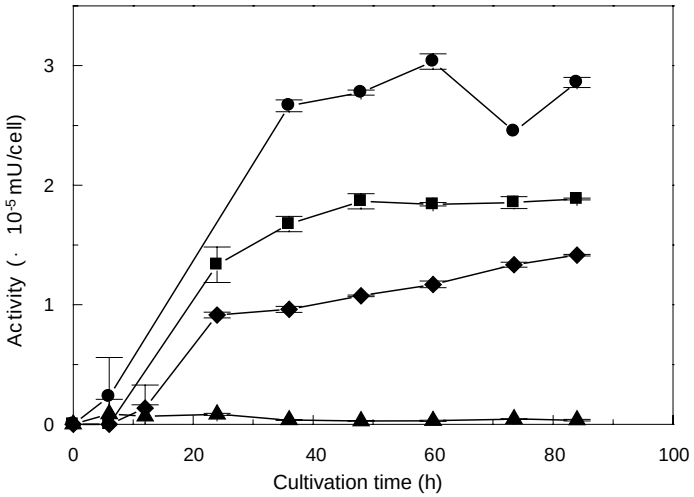

Fig. 5. Time course of invertase activity of MT8-1/pWGP3-ES (squares), MT8-1/pWGP3-SE (diamonds), MT8-1/pWGP3 (triangles) and MT8-1/pWGP3-SUC2 (circles) during cultivation.

form, as dimerization is required and sufficient to generate invertase enzymatic activity [14]. Fig. 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.

4. 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. [15] have reported that *SUC2* secretion signal sequence fusion to EGFP does not result in the localization at periplasm. Kunze et al. [16] 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. 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 and d), 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 [11]. 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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