

Review

Novel application of yeast molecular display system to analysis of protein functions

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This review fundamentally covers the functional analysis of proteins and the preparation of modified proteins for the creation of novel functions using a yeast molecular display system as a molecular tool. Yeast molecular display is a genetic technique of producing proteins or peptides on the yeast cell surface, such as the cell wall or cell membrane. In this review, recent studies on the yeast molecular display system in the field of not only applied studies but also fundamental studies are described with illustrations.

Keywords: molecular display, protein functions, preparation of modified proteins, high-throughput screening

Introduction

A molecular display system is used for protein production with which proteins or peptides can be displayed on the cell surface of microorganisms. In this system, proteins or peptides are displayed as fusion forms with some anchoring proteins. There are some display systems using microorganisms, such as phages (1, 2), bacteria (3-5), and yeasts (6). In the case of the yeast *Saccharomyces cerevisiae*, α -agglutinin (7), a-agglutinin (8), or flocculin (9) has been used as an anchor protein. These proteins exist on the yeast cell surface and have glycosylphosphatidylinositol (GPI) anchors that play important roles in the surface localization of proteins (10). The GPI-anchored proteins translocate to the cell surface through the secretory pathway of *S. cerevisiae* (11). The GPI attachment signal is assigned to the C-terminus of GPI-anchored protein.

The advantage of using yeast in a display system is that it is a eukaryote. Proteins produced by yeast cells with a

quality control system undergo post-translational modification and proteins having large molecular masses could be displayed on the cell surface, as compared with display systems using phages and bacterial cells. Furthermore, effective strategies using the yeast molecular display system for rapidly analyzing, improving or modifying the activities and functions of

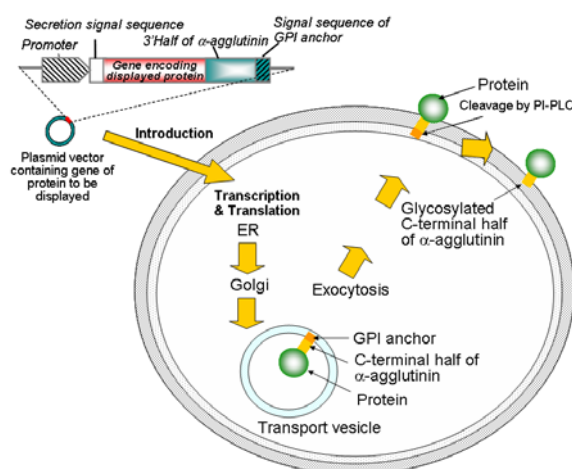


Fig. 1. Construction of plasmid to display the desired protein and transportation to cell wall of yeast cell.

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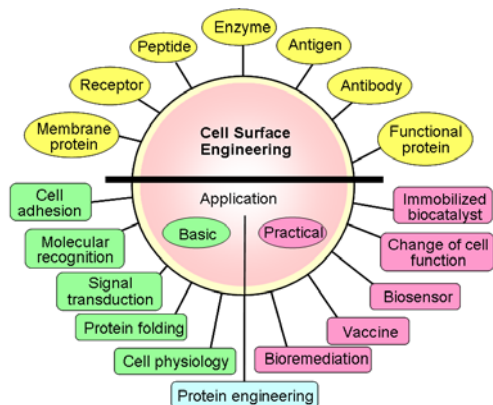


Fig. 2. Development of molecular display system using yeast to fundamental and practical studies.

proteins can be established, because protein-displaying cells are used as a single-protein cluster and multiple protein purification procedures can be avoided. In the case of using α -agglutinin as an anchor protein, active proteins and peptides are displayed on the yeast cell surface in the form of fusion proteins linked to the C-terminal half of α -agglutinin, which is a serine- and threonine-rich region and contains a GPI-anchor attachment signal at the C-terminus (7). The protein synthesized and attached to the GPI anchor in the endoplasmic reticulum (ER) is transported to the plasma membrane by the conventional vesicular transport system, *via* the Golgi apparatus. Part of the GPI anchor of the GPI-anchored protein transported to the plasma membrane is cleaved by phosphatidylinositol-specific phospholipase C (PI-PLC) and transferred to the outermost surface of the cell wall. The GPI-anchored protein transported to the cell surface is fixed by the addition of β 1,6-glucan to the residual GPI anchor ((12) and Fig. 1). Since 10^4 - 10^5 molecules can be easily displayed on one yeast cell in many cases (13, 14), it is possible to consider a protein- or enzyme-displaying yeast cell as a protein cluster and a whole-cell biocatalyst in the case of an enzyme in particular. The yeast molecular display system has been used to

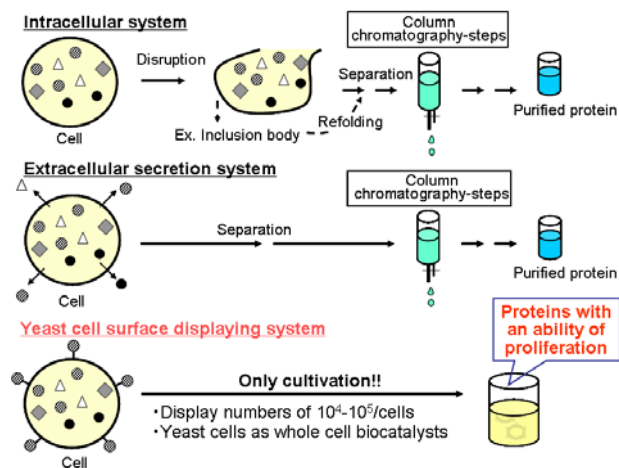


Fig. 3. Comparison of conventional systems and yeast molecular display system for protein production and purification.

date in various applications (15-17) (Fig. 2). The principle of this system and some applications were summarized in detail in a previous review (18).

In this article, we deal with the fundamental and application studies using the yeast molecular display system.

1. Application to fundamental studies

1-1. Construction of novel protein purification system

In conventional overproduction systems for protein preparation, there are mainly the intracellular production system and extracellular secretion production system. However, these conventional methods require a series of chromatography procedures, which are time-consuming and tedious. Furthermore, a refolding procedure is additionally required when a protein is produced as an inclusion body. Since it is necessary to prepare many mutant proteins, to clarify the relationship between structure and functions of them, these conventional procedures are also labor-intensive and time-consuming. On the other hand, the yeast molecular display system is an effective tool for rapidly analyzing, improving, or modifying functions of proteins, since protein displaying cells are used as protein clusters and many laborious protein-purification procedures described above can be avoided

(Fig. 3). If the protein displayed on the cell surface could be cleaved, it would be possible to isolate the protein easily without requiring conventional purification procedures.

To cleave proteins displayed on the yeast cell surface, the selective cleavage system of GFPuv (19) as a model protein was constructed. A cell-surface-displaying vector harboring genes of the GFPuv- α -agglutinin fusion protein containing the amino acid sequence -Ile-Glu-Gly-Arg-Lys- recognized by the blood coagulation factor, factor Xa, between GFPuv and α -agglutinin was constructed (Fig. 4a) (20). The factor Xa is a site-specific serine-type endoprotease that preferentially cleaves the C-terminal peptide bond of the recognition sequence. The display of the fusion protein (GFPuv-factor Xa recognition sequence- α -agglutinin) on the cell surface was confirmed by the GFPuv fluorescence around the yeast cells. When the fusion-protein-displaying yeasts were treated with the factor Xa, the fluorescence of GFPuv disappeared, indicating that the GFPuv was successfully cleaved from the cell surface (Fig. 4b). Furthermore, the cleavage of GFPuv from the cell surface was confirmed by quantifying GFPuv using a chemiluminescence system and by electrophoresis. This additional system to the molecular display system makes it easy to isolate and purify the desired protein without

many purification procedures. It is suggested that this system of introducing the protease recognition sequence between the desired protein and the anchor protein has the attractive potential for use as a novel protein purification method instead of the conventional purification method.

1-2. Analysis of processing system of vacuolar proteases

The vacuole of *S. cerevisiae* has several physiological functions, including proteolysis, pH regulation, osmo regulation, and the storage of amino acids, ions, and polyphosphates (21, 22). Proteolysis is one of the very important functions of the vacuole. In yeast cells, as in mammalian cells (23), the transport pathway is defined as leading from the site of synthesis, the endoplasmic reticulum (ER), through the Golgi apparatus to the lysosomal component, the vacuole. In general, vacuolar hydrolytic enzymes are synthesized in the form of inactive precursors, which upon arrival at the vacuole are proteolytically cleaved to yield mature active enzymes, so as not to digest themselves and other proteins before arrival at the destined organelle (Fig. 5a). Proteinase A (PrA, EC 3.4.23.25) encoded by *PEP4* is also synthesized as an inactive preproenzyme and it contains information for its targeting to the vacuole via the secretory route (24, 25). PrA plays an essential role in the processing of vacuolar hydrolases (26) including

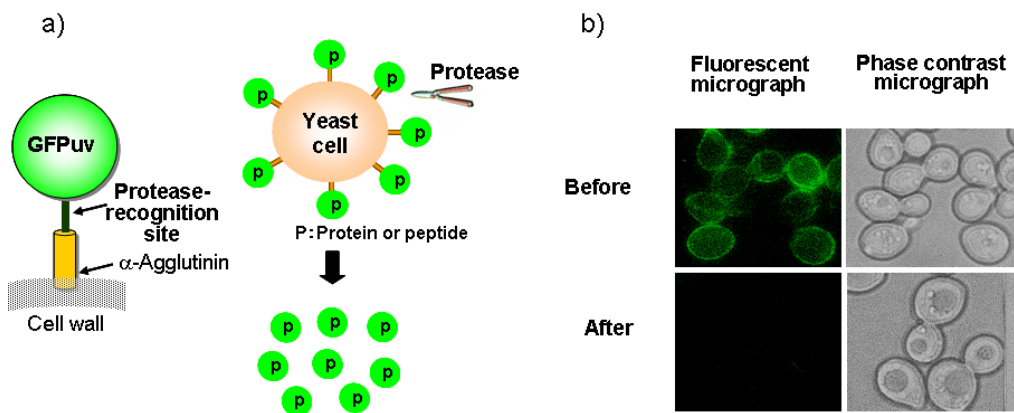


Fig. 4. A image of display of GFPuv on the cell surface and strategy for selective cleavage (a) and demonstration of cleavage of GFPuv from cell surface by protease (b).

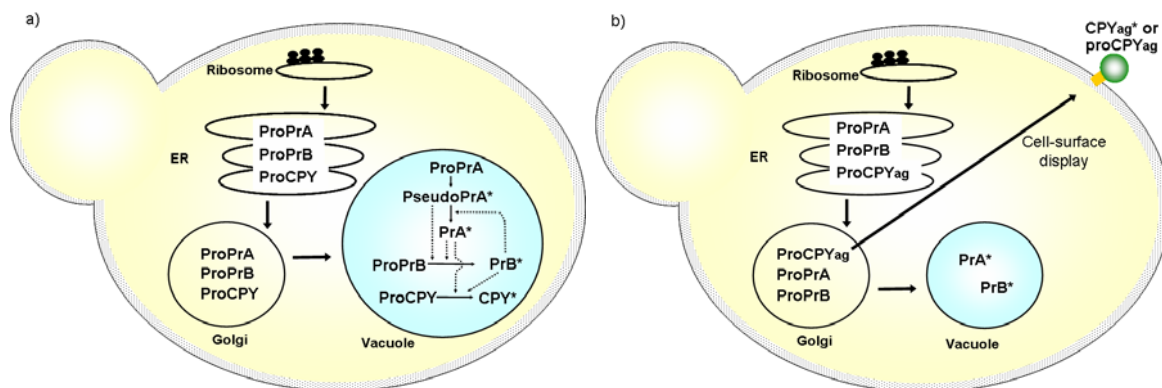


Fig. 5. Compartment of the main vacuolar proteases and their processing cascade (a) and strategy for processing analysis of PrA by altering the transport route of CPY (b). Active forms are indicated by an asterisk. Solid and dotted lines in the vacuole show the conversion to active enzymes and activation, respectively. The “ag” of CPYag and proCPYag indicates the fusion proteins with α -agglutinin.

proteinase B (PrB, EC 3.4.21.48), carboxypeptidase Y (CPY, EC 3.4.16.5), alkaline phosphatase, and aminopeptidases.

Although the mechanism of each processing of PrA, PrB, and CPY has been reported, where their proteases undergo processing to be converted into mature enzymes remains unclear. In particular, the location of maturation of proproteinase A (proPrA), which is a precursor of PrA still remains to be solved. PrA is a key enzyme as an initiator of a processing cascade for vacuolar proteases. Although it was predicted that the conversion of inactive proPrA to active PrA including pseudoPrA occurs autocatalytically owing to the acidic pH in the vacuole (26-28), it was reported that vacuolar acidification was not necessary for zymogen activation by the experiment using vacuolar H-ATPase mutant yeast strain in which the vacuolar pH is neutral (29).

To demonstrate the location of proPrA activation, a processing analytical system using yeast molecular display was constructed. The plasmid containing procarboxypeptidase Y (proCPY)-encoding gene for displaying the proCPY on the yeast cell surface as the proCPY- α -agglutinin fusion protein (proCPYag) was introduced in various protease-knockout yeast strain. ProCPYag produced in the cell changes the transport pathway to the cell surface, not to

the vacuole. The phenotype by the introduced gene was analyzed by measuring the activity of CPY displayed on the cell surface (Fig. 5b). The activity of CPY displayed on the cell surface was measured after the conversion of inactive proCPYag to active CPYag by treatment of whole cells with proteinase K, which converts proCPY to CPY *in vitro* (30) (Fig. 6a). When *proCPY* was expressed in the wild-type, CPY-knockout, and PrB-knockout strains, CPY was displayed in its active form (mature CPY), but in the PrA-knockout strain, CPY was present in its inactive form (proCPY). That is, CPY was displayed in its active form only when PrA existed in cells (Fig. 6b). PrA was not transported to the extracellular medium, as determined by measuring the activity of PrA in the extracellular fraction. Furthermore, PrA is an aspartic protease with a structural similarity to other aspartic proteases such as cathepsin D (31), a lysosomal enzyme in higher eukaryotes. Cathepsin D is present in its active form in the endosome (32), which is the transport compartment from the Golgi apparatus to the lysosome. These findings indicate that inactive proPrA had already been converted into active pseudoPrA or PrA before its transport to the vacuole and that active PrA might convert proCPY into CPY before the transport of proCPY to the vacuole. The

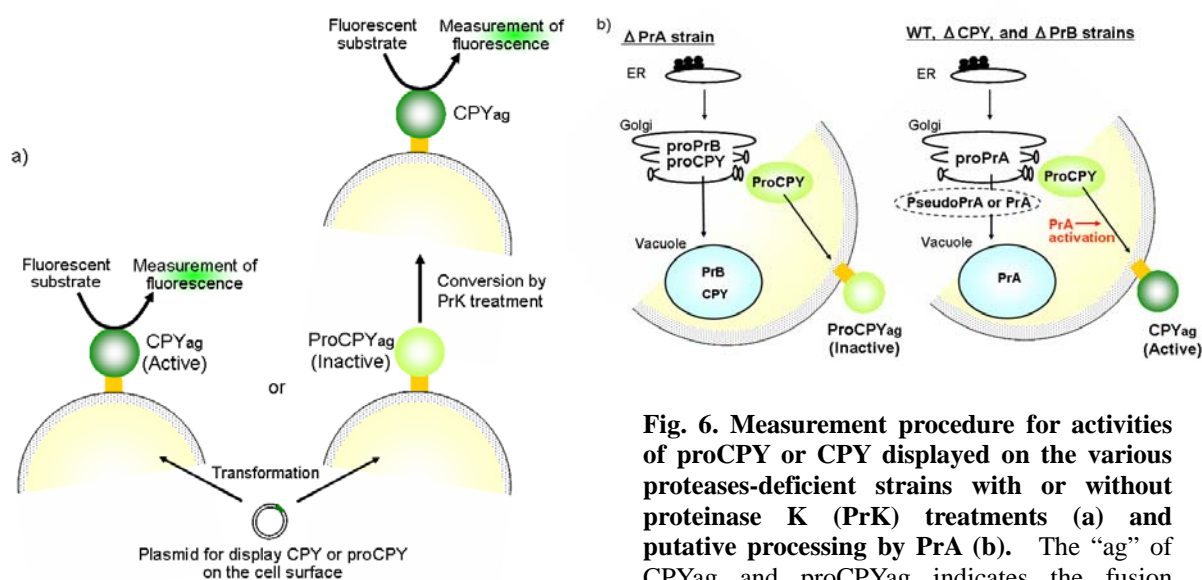


Fig. 6. Measurement procedure for activities of proCPY or CPY displayed on the various proteases-deficient strains with or without proteinase K (PrK) treatments (a) and putative processing by PrA (b). The “ag” of CPYag and proCPYag indicates the fusion protein with α -agglutinin.

results obtained using the yeast molecular display system, suggest that the autocatalytic activation to active PrA might occur during the early transport to the vacuole from the Golgi apparatus (Fig. 6b).

2. Application to practical studies

2-1. Preparation of whole-cell biocatalysts

Lipases are used in many applications in chemical processing of organic solvents and detergent formulations, the synthesis of biosurfactants, the dairy industry, paper manufacture, nutrition, cosmetics, and pharmaceutical processing (33). Lipases can be effective for asymmetric synthesis by utilizing their high stereo-specificity in chemical synthesis and for esterification in preparing esters from fatty acids by taking advantage of their substrate specificity (in particular, chain-length specificity). *Candida antarctica* lipase B (CALB) (34) is one of the most widely used biocatalysts (35) and the present industrial preparations of CALB are Novozym 435 (Novozymes A/S, Bagsvaerd, Denmark) and Chirazyme L-2 (Roche Molecular Biochemicals, Mannheim, Germany) as immobilized products, but they are expensive (36). To easily prepare CALB at a low cost, CALB was displayed on the cell surface using a yeast molecular

display system (13). When CALB was displayed, it showed a preference for advantageous for producing flavors, although *Rhizopus oryzae* lipase (ROL) preferred fatty acids with intermediate lengths (37). Furthermore, CALB displayed on the cell surface showed high thermal stability. These findings suggest that a CALB-displaying yeast strain is also practical for industrial use as a whole-cell biocatalyst.

When ROL-displaying yeasts were used in synthesis reactions, they could catalyze synthesis reactions in organic solvents. The activity of ROL-displaying yeasts used for esterification with palmitic acid and *n*-pentanol in heptane containing 0.2 % H₂O was 3.8×10^4 -fold higher than that of a commercial lipase (38). To increase lipase activity, mutation was introduced into ROL by the error-prone PCR method, which has been widely used to improve enzyme functions, in combination with the molecular display system. Some of the ROL mutants showed activities 3- to 6-fold higher than the original ROL-displaying yeast. The combination of mutation introduction and the use of the molecular display system opens a new door to obtaining proteins or enzymes with higher activities.

2-2. High-throughput screening system of modified enzymes

In recent years, as material technologies including nano- and micro-technologies have progressively advanced, some high-throughput screening systems have been developed. In the conventional screening method in which agar plates are used to select colonies on the basis of halo formation, colonies forming a halo are recultivated and then their functions and biochemical properties are analyzed in detail. Such a method requires large amounts of medium and considerable effort and time, particularly in the screening of numerous mutants.

Furthermore, the fluorescence-activated cell sorting (FACS) system, which is used for the screening of clones with high activities, permits the rapid quantitative and selective isolation and evaluation of significant clones. By using FACS, target cells are detected only by fluorescence of labeled chemicals bound to target cells, and then can be separated. To screen easily and to obtain a desired single cell, a novel screening system for a single cell was constructed using a yeast cell microchamber chip in combination with the

molecular display system (39).

It was possible to place a single yeast cell into each microchamber, to observe the behavior of a single cell, to select the target cell, and to cultivate a single cell in each microchamber, which was made possible by the prevention of the rapid drying of the medium (40). Using a ROL-displaying yeast, the hydrolytic reaction of a single cell could be detected in a microchamber. Furthermore, the ROL-encoding gene was amplified by PCR and then its sequence was confirmed using a DNA sequencer. These results demonstrate that this yeast cell chip in combination with the molecular display system may be used as a tool in a high-throughput screening system not only for a single living cell and a whole-cell catalyst but also for functional protein molecules from protein libraries displayed on the cell surface (39).

Further directions

A molecular display system using yeast *S. cerevisiae* strain enables the display of large protein molecules on the cell surface, in comparison with systems using bacterial cells and phages. Yeasts can produce foreign

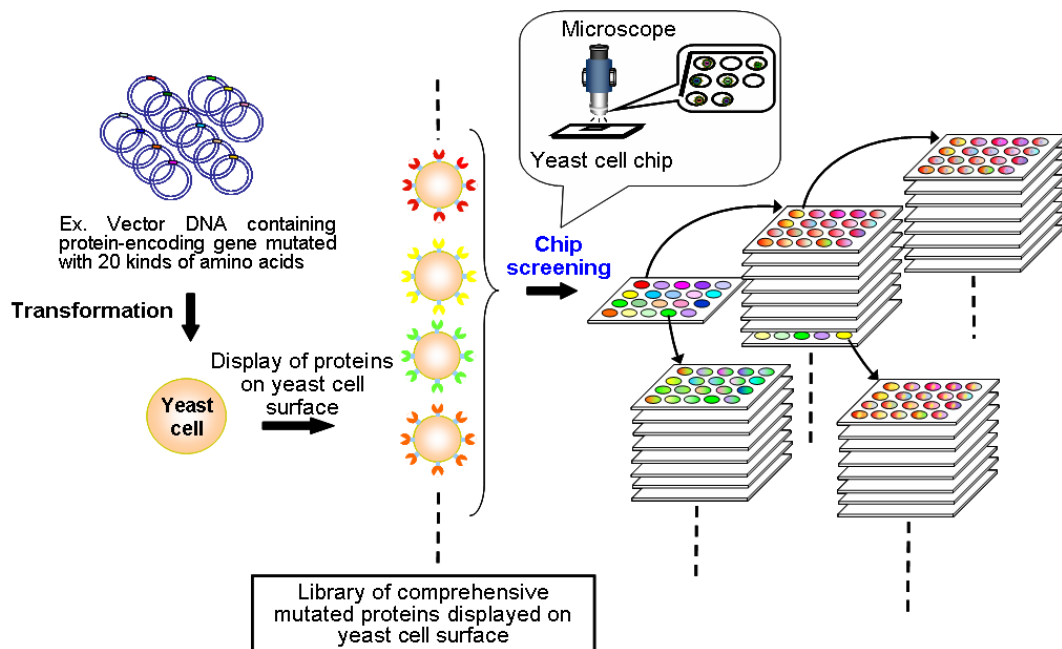


Fig. 7. Comprehensive analysis and screening of displaying-proteins using yeast cell microchamber chip.

proteins with correct conformations in the form of undergoing the post-translational modification. The display of peptides or proteins on the yeast cell surface endows yeasts with novel functions.

With the development of genome analysis, numerous single-nucleotide polymorphisms (SNPs) have been found in a genome sequence. These SNPs seem to be related to individual differences, such as responsiveness to medicine and susceptibility to a disease. By examining the structure and function of mutant or modified proteins generated from protein-encoding genes containing SNPs, the use of a “tailor-made medicine” suitable for a specific person or patient will become possible. The development of methods of analyzing SNPs rapidly will be important to realize this goal.

To analyze protein structure-function relationships, it is required to prepare various mutant proteins. By using the molecular display system, the preparation of many protein libraries becomes easy and a rapid functional analysis of proteins becomes possible because protein-displaying yeasts are used as single protein clusters without requiring conventional protein purification procedures. Furthermore, the high-throughput system coupled with the yeast molecular display system permits the screening for target cells with higher activities or novel functions (Fig. 7). This yeast molecular display system will be widely applicable in scientific fields as a molecular tool to prepare various proteins, easily, and rapidly.

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