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Review

Future direction of molecular display by yeast-cell surface engineering

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Abstract

Cell surface engineering using yeast cells has developed in many fields of biotechnology. Active peptides and proteins with larger size of molecules can be displayed on the yeast-cell surface than with the phage display system, although the latter has greater transformation efficiency. Because of these aspects, the yeast-cell surface engineering system represents a novel field of protein-engineering and protein creation. In this review, its future directions are described and discussed.

Keywords: Yeast; Cell surface; Cell surface engineering; Molecular display; Protein engineering

1. Introduction

As the yeast display system allows active enzymes with various sizes and forms to be displayed [1], it is expected that a combination with crystallization analysis and computerized modeling will facilitate combinatorial analysis of the structure–function relationship of proteins and the construction of a practical protein-engineering system. Futhermore, the possibility of creating completely novel and functional proteins from random DNA alignments has been demonstrated. In conjunction with molecular display systems and high-throughput systems for combinatorial and speedy analysis of the functions of proteins derived from many genes and artificially synthesized DNA, methods of proteome analysis and protein library construction have been also developed (Fig. 1). The combination of these systems is expected to make possible easy and simultaneous analysis of DNA data and protein function and to greatly support the combination of genomics with proteomics. This review concentrates, among these developments, on innovation in protein-engineering and on the creation of novel proteins.

2. Contribution to innovation in protein-engineering

Previous research into protein-engineering has concentrated on: (i) isolation of the gene encoding a target protein; (ii) construction of systems for overexpression of the target gene; (iii) purification of the protein expressed; (iv) crystallization of the protein and analysis of its structure by X-ray diffraction; (v) modeling by computer; (vi) analysis of random mutagenesis and site-directed mutagenesis; and (vii) improvement of activity and functions for practical application.

To further advance understanding of protein functions, further innovation in methodology has become necessary. Based on the molecular display system described in "combinatorial bioengineering" [1], previous methods of protein-engineering have changed. The novel method has led to the improvement of protein-engineering research strategies from mutagenesis of individual points to mutagenesis of multiple and combinatorial points in the combination of structural information. This method begins with the construction of a protein library with continuous or non-continuous combinatorial mutation of target domains and regions. Next, direct screening of target clones with a high-throughput system becomes possible. In the case of the yeast display system, the correspondence between the genotype (introducing the gene) and the phenotype

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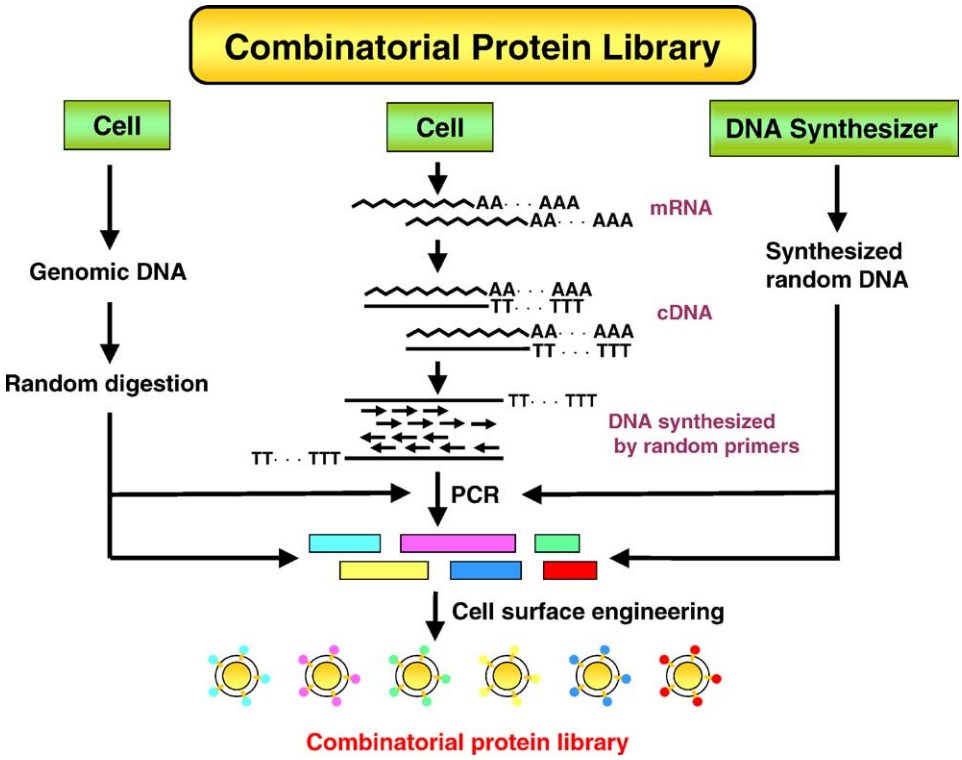

Fig. 1. Construction of a random combinatorial protein library for yeast-cell surface display using three different methods.

(expressing the gene) becomes clear by the determination of the DNA sequence encoding the displayed proteins by simply providing primers on either side of the introduced gene. Furthermore, it is not necessary to purify the mutated proteins individually. Whole-cell biocatalysts with mutated proteins can thus be prepared easily after cultivation. These innovative methods are expected to lead breakthroughs in protein-engineering in the future [1].

Systems in which libraries of peptides are displayed on phage or another biological resource have proved useful in the assay and analysis of large numbers of mutated peptides for the purpose of protein and enzyme improvement. Phage display is the most popular method at present because of its high transformation efficiency ($<10^{11}$), but does not permit post-translational glycosylation or proteolytic modification. Moreover, the size of the molecules that can be displayed on the phage surface is limited and clones can be screened only when they have improved affinity for substrates or ligands. On the other hand, as the yeast-cell surface engineering system allows the expression of many of the functional proteins as well as peptides, necessary for post-translational modification, it would seem to be uniquely useful among the various display systems reported hitherto. One example is the improvement of lipase by the creation of a combinatorial library of the domain that determines substrate specificity [2,3]. *Rhizopus oryzae* lipase (ROL), which has been used to produce diesel fuel from vegetable oil requires modification to attain a mature form and has substrate specificity to long-chain substrates. Its three-dimensional structure can be predicted from that of *Rhizopus niveus* lipase, which is 99% identical to ROL in its amino acid composition. There have been many reports on the structure of microbial lipases; almost all, including ROL, have a lid domain in the active site, the movement of which occurs at substrate concentration above the critical micellar level and is necessary for activation. The lid domain, which directly contacts the substrate, is an interesting domain with great influence on the substrate specificity of the lipase.

In the display of ROL, the first success was spacer-mediated display of ROL on the yeast-cell surface [2]. As expected, the ROL-displaying cells exhibited a high lipase activity towards triolein. The insertion of the spacer, composed of a Ser/Gly repeat sequence of different lengths, between ROL and the C-terminal half of α-agglutinin was demonstrated to contribute to the increase in lipase activity on the cell surface. Linker peptides had a significant enhancing effect on hydrolyzing activity. The spacer on the cell surface of the ROL-displaying cells seemed to contribute to the separation of the active moiety and the cell-wall binding moiety. As the fatty-acid binding site of ROL is located near the C-terminal, insertion of the spacer may help the ROL-α-agglutinin fusion protein to create space for access to substrates. It should be noted that longer linker peptides yielded higher activity towards triolein. Cell surface-displayed ROL exhibited the same or higher levels of enzyme activity towards triolein compared to extracellularly produced ROL.

To investigate the relationship between the amino acid sequence of the lid domain of ROL and its substrate specificity, the six amino acids (Phe88–Arg89–Ser90–Ala91–Ile92–Thr93) constituting the lid domain were combinatorially changed and the mutated ROLs displayed on the yeast-cell surface by cell surface engineering [3]. Clones exhibiting halos around plate colonies containing tributyrin or soybean oil were screened. Several clones showed clear halos on tributyrin-containing plates. Assays using fluorescent substrates (fluorescein dibutyrate and fluorescein dilaurate) indicated that cells displaying mutated enzymes had a lower activity than cells displaying the wild-type enzymes, but there were several cells which exhibited a unique substrate specificity. In *Rhizomucor miehei* lipase [4], the 'Arg' of the lid domain is important to stabilize the open conformation of the lid. In the mutated ROL library examined, the three mutants that formed clear halos had a conserved sequence of [basic amino acid]–[polar amino acid]–[non polar amino acid]. This indicates that this sequential alignment is important in allowing the lid to activate ROL. When the position of the sequence of [based amino acid]–[polar amino acid]–[non polar amino acid] was shifted to the left by one amino acid, the enzyme exhibited that the ratio of activity toward dibutyrate/dilaurate was considerably different to that of the wild-type. These results indicate that not only the position of the basic amino acid but also the sequence of [basic amino acid]–[polar amino acid]–[non polar amino acid] is essential for the determination of substrate specificity by the lid domain.

Alteration of the chain-length specificity of ROL was produced by combinatorial mutagenesis of the lid domain. As shown, the yeast display method is an effective technique for obtaining interesting clones from functional protein libraries by introduction of high-throughput screening. There have been many reports on the study of microbial lipases by site-directed mutation, which requires information on conformation and electrostatic properties. However, not all studies using site-directed mutations have led to the desired results. Recently, the combinatorial mutation of Phe94 in the groove of *R. miehei* lipase was undertaken to alter the chain-length specificity [5]. Fourteen of nineteen possible mutants were purified from the culture supernatants of *Pichia pastoris* and several mutants with high activity to short-chain substrates were obtained. Because of its applicability to the assay of mutants, the yeast display method allows combinatorial mutagenesis of multi-point amino acids. Combinatorial mutagenesis of the six amino acid residues of the lid domain of ROL readily supplied several mutant enzymes with altered chain-length specificity but conserved amino acid sequence [3]. Further studies, including combinatorial mutagenesis of the alignment of two or three amino acids in the lid domain, should produce further interesting mutant enzymes.

These results obtained in combinatorial mutagenesis by yeast-cell surface display may prove instructive in the development of protein-engineering strategies.

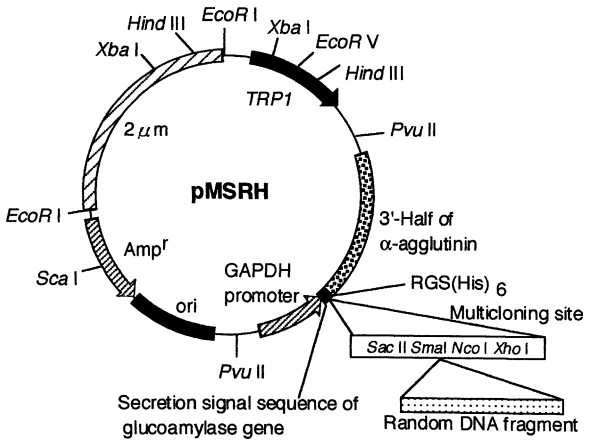

Fig. 2. Multi-copy cassette vector pMSRH for yeast-cell surface display of a random combinatorial protein library.

3. Contribution to the creation of novel and functional proteins

The creation of novel and functional proteins requires the construction of a combinatorial protein library which can be displayed on the cell surface of *Saccharomyces cerevisiae* using a multi-copy cassette vector (Fig. 2). The newly displayed proteins will, it is expected, renovate the yeast-cell structures and possibly endow the yeast host cells with new functions and phenotypes. In the absence of quantitative and computational data on the structure–function relationships of proteins, rational approaches to mutagenesis have limited potential for success in rapidly altering protein molecular properties to meet predefined criteria. An alternative strategy, construction and selection of random-mutated combinatorial libraries, has yielded numerous successes. In vitro selection from molecular libraries has rapidly come of age as a protein-engineering tool. In a library, the massive number of variants that can be simultaneously surveyed using either chemical or biological approaches has a key consequence for protein-engineering. It makes it practical to mutate multiple residues of a protein simultaneously, enabling complex, non-additive combinatorial effects to be obtained. Thus, changing proteins' properties may not rely mostly on a detailed molecular understanding of their functions, and biological libraries can offer a powerful tool for improving protein function.

For the creation of novel and functional proteins, the attractive and practical property of organic-solvent-tolerance was selected. Expected to find wide application in bioprocesses, organic-solvent-tolerance is a cell surface-related function on the mechanism of which, however, there has until now been no genetic information available in eukaryotic organisms. In such cases, a random combinatorial protein library which can be displayed on the yeast-cell surface is helpful, as renovating the cell surface may create new phenotype strains. A random combinatorial protein library that could be displayed on the yeast-cell surface using cell

surface engineering technology was therefore constructed (Fig. 1).

As a combination of both the library method and the yeast-cell surface display technique, a combinatorial protein library that could be displayed on the yeast-cell surface was constructed. A multi-copy plasmid, designated pMSRH (Fig. 2), was constructed to create a random combinatorial protein library that could be displayed on the yeast-cell surface using cell surface engineering technology. The random protein-encoding DNA fragments were fused to the gene encoding the C-terminal 320 amino acids of α-agglutinin, which has a GPI anchor attachment signal sequence required for anchorage on the yeast-cell surface. Here, the RGS $(His)_6$ short DNA fragment was used as an epitope to check the subcellular location of the displayed random proteins by fluorescent labeling of the immuno-reaction.

The cDNA obtained from the mRNA of *S. cerevisiae* was used as the template, following which the method of DNA random-priming was applied to generate a pool of random fragments for library construction [6]. These DNA fragments were fused. After transformation into yeast cells, the inserted proteins were transferred to the outmost surface of the yeast-cell-wall by anchorage with α-agglutinin if they were fused in-frame.

The method of DNA random-priming is an improved method of random-priming recombination (RPR). RPR is a simple and efficient method for in vitro mutagenesis and can recombine polynucleotide sequences. It is therefore, considered a very flexible and easy way to generate mutant libraries for directed evolution. Unlike RPR, DNA random-priming produces many fragments of different length and different recombination frequency. The reaction conditions, such as length and concentration of random primers, can be manipulated to achieve the desired mutagenic rate and recombination frequency.

From the library [7], randomly selected colonies were isolated and the inserted random fragments sequenced and compared with the *S. cerevisiae* genome database (data not shown). It was found that the recombination frequency varied between 0 and 1% depending on the experimental conditions.

In order to confirm cell surface display, a number of colonies were randomly selected from the constructed library and immunofluorescence-labeled with the antibody against $RGS(His)_4$ as the primary antibody and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody as the secondary antibody. It was confirmed that the inserted in-frame random proteins were successfully displayed on the yeast-cell surface. Using the method of DNA random-priming, a vast pool of DNA fragments with different lengths and various structures was generated for the combinatorial library. Proteins displayed on the yeast-cell surface made a great contribution to yeast breeding. When these fused random proteins are displayed on the yeast-cell surface, their intrinsic localization, composition, and conformation may be somewhat altered, and these changes will increase the variability of the library and enrich the selectable phenotypes.

From this random combinatorial protein library, an organic-solvent tolerant yeast strain, as one of examples, was obtained by screening with *n*-nonane. Many organic solvents are highly toxic to living organisms because they accumulate in and disrupt cell membranes. There are few reports on eukaryotic organisms that can adapt to and survive these antimicrobial agents. The acquisition of *n*-nonane-tolerance was proved to be due to the presence of transformed plasmids.

Organic solvents are already used widely in bio-transformations with enzymes. The use of organic solvents also has several advantages in the application of whole-cell systems: the solvents can increase the solubility of poorly water-soluble substrates or products; using the second phase of an organic-solvent, products can be extracted continuously from an aqueous reaction system, enabling not only the reduction of the inhibitory effects of the product but also much easier recovery, with positive effects on the cost of downstream processing. However, organic solvents, like alcohol, aromatics, and phenols, are classic antimicrobial agents. The problems resulting from the toxicity of organic solvents to whole cells are still severe drawbacks in biocatalysis.

Micro-organisms that can adapt and survive in the presence of organic solvents are therefore of great interest. Recently, some strains of *Pseudomonas aeruginosa*, *P. fluorescens*, *P. putida*, and *Escherichia coli* have been found to have such tolerance [7], but there have so far been few reports on eukaryotic micro-organisms with organic-solvent-tolerance and no related genetic information.

Since the first solvent tolerant strain was isolated, research has begun to uncover the mechanisms responsible for this unique property. Different mechanisms have been proposed in prokaryotic cells, including the presence of an efflux system localized in the cell envelope, which actively decreases the amount of solvent in the cell and alters the composition of the outer membrane. In bacteria, as the membrane is the main target of the toxic action of solvents, research is concentrated on changes in the composition and structure of the membrane. In eukaryotic cells, such as yeast cells, things become complicated because of the different cell structure, especially the different composition of the cell envelope. Although there has been no report on the mechanism of organic-solvent-tolerance in yeast, the complete genome sequence of *S. cerevisiae* reveals the presence of numerous ABC protein-encoding genes whose products are located mostly on the plasma membrane and are implicated in drug, metal, and stress resistance. The yeast-cell surface is thus thought to be involved organic-solvent-tolerance.

Cell surface affinity to hydrophobic organic-solvent was investigated by observing the adherence of cells to isooctane droplets in the aqueous phase, as previously performed by Aono et al. [8]. Isooctane was emulsified by vigorous mixing in a suspension of yeast cells. Most wild-type cells adhered

to the isooctane droplets, suggesting that the yeast-cell surface is closely involved in organic-solvent-tolerance. Therefore, in order to identify the relevant mechanism and to find improved applications for yeast in industrial processes, screening for the protein responsible for the tolerance was attempted from a random combinatorial protein library.

After successive cultivation, one *n*-nonane-tolerant colony was finally obtained. The isolated clone grew very well, but the parent strain *S. cerevisiae* did not. The fact that the selected clone did not grow in the medium containing *n*-nonane as sole carbon source indicates that this strain was not *n*-nonane-trophic, but *n*-nonane-tolerant [7]. The *n*-nonane-tolerance was plasmid-dependent. The plasmid-borne protein was confirmed to be displayed on the yeast-cell surface by immunofluorescent-labeling of the RGS$(His)_4$ epitope. Papain treatment was also applied to further investigate whether the protein displayed on the cell surface was responsible for the *n*-nonane-tolerance. Papain hydrolysis might only destroy the tolerance of cells to *n*-nonane. It therefore seemed probable that the *n*-nonane-tolerance was derived from the random protein displayed on the cell surface.

In conclusion, the selected clone is the first genetically constructed recombinant yeast strain that can tolerate the organic-solvent *n*-nonane. It should provide important information about the organic-solvent-tolerance of eukaryotes, which may lead to a much wider application of yeast in industrial bioprocesses in general. At the same time, the clone can serve as a model for the screening of other new phenotypes and novel functional proteins from combinatorial protein libraries.

As described in this review, random combinatorial random protein libraries by molecular display with yeast-cell surface engineering have a wide range of applications in renovation of the cell surface, improvement of protein functions, and discovery of new functional proteins and living cells.

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