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Construction of combinatorial library of starch-binding domain of *Rhizopus oryzae* glucoamylase and screening of clones with enhanced activity by yeast display method

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Abstract

Most of the glucoamylases (GA), which catalyze the hydrolysis of α-1,4 and α-1,6 glycosidic linkages, have a distinct region called a starch-binding domain (SBD). We have developed a powerful method for screening a library of GA mutants by a combination of GA display and SBD mutagenesis on the yeast-cell surface. In the case of *Rhizopus oryzae* glucoamylase (RoGA), three amino acids (63S, 71T, 73S) of the SBD were combinatorially mutated to enhance the degradation activity toward cooked corn starch and the mutated RoGAs were displayed on yeast-cell surface by cell-surface engineering. After the first screening by halo assay using an iodine-starch reaction, about 200 of the 8000 colonies formed clear halos. Incubation of the yeast with the mutated and displayed RoGAs caused direct degradation of cooked corn starch. Repeated screening revealed that some of the mutants produced a degradation rate around ∼1.4-fold higher than did wild type. The results obtained from the DNA sequences of the mutated SBDs indicated that amino-acid residues with a carbonyl group (D, E, Q, N) in the SBD enhance the degradation ability of the GA by enhancing the binding activity of the SBD.

Keywords: Cell-surface engineering; Glucoamylase; Starch-binding domain (SBD); Combinatorial mutagenesis; Molecular display: yeast-cell surface

1. Introduction

Construction of a large gene library and conversion to a protein library following rapid screening for function has become a novel strategy in protein engineering. Recent advances in DNA manipulation enable us to construct large DNA libraries easily by error-prone PCR and DNA shuffling [1,2]. Nowadays, one of the most crucial procedures in protein engineering is the rapid screening of protein libraries to obtain a clone with the desired function. Phage display is a powerful screening method for libraries of mutated proteins [3,4] and can be applied to a large set of gene libraries (genotype) with externally displayed peptides (phenotype) because of its high transformation efficiency. Phages cannot however express proteins that require post-translational glycosylation or proteolytic modification to attain their active forms. Furthermore, in the phage-display system, it can be difficult for mutant proteins other than peptides to attain an active form with catalytic activity.

We have developed a yeast display system using cell-surface engineering [5,6], which allows the display of proteins and peptides on the yeast-cell surface as fusion proteins linked to the C-terminal half of α-agglutinin. As yeast can express many proteins with post-translational modification in their active forms, the yeast display system promises to become a powerful screening method able to lead from gene libraries directly to the selection of clones with enzymatic activities. Previously, we have demonstrated the utility of the yeast display system in changing the substrate specificity of lipase [7].

In the present study we further applied this system to the enhancement of *Rhizopus oryzae* glucoamylase (RoGA) activity. Glucoamylase (GA) [EC 3.2.1.3] catalyzes the hydrolysis of α-1,4 and α-1,6 glycosidic linkages to release β-D-glucose from the non-reducing ends of starch. RoGA fused with the C-terminal half of α-agglutinin and

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displayed on the cell surface of yeasts can degrade corn starch and produce ethanol [8]. The tertiary structure of GA consists of a catalytic domain connected to a starch-binding domain (SBD) with a linker region. The structure of the SBD of *Aspergillus niger* GA bound to β-cyclodextrin, a starch-mimic compound, has been determined by NMR [9,10]; there are two binding sites: Site 1 is a rigid and compact binding site where hydrophobic-ring interaction between the sugar rings and aromatic rings of the two tryptophans has been reported to play an important role for binding to substrates [10]; Site 2 is a longer and more flexible binding site where not only the hydrophobic-ring interaction of the two tyrosines but also some hydrogen bonds between the substrates and amino acids adjacent to the two tyrosines contribute to binding. While most SBDs are located in the C-terminal region, the SBD of RoGA is in the N-terminal region and has a low degree of homology with the SBDs of the rest of the fungal GA family, and seems thus to have undergone a solitary molecular evolution [11].

We have attempted to enhance the activity of RoGA by saturated (combinatorial) mutation of this unique SBD. In the present study, we mutated three amino acids combinatorially to enhance the activity of RoGA. Combinatorial mutation can create 20^3 mutants, which contain all possible combinations of characteristic positions. Data on the clones gained from the library may contribute to an understanding of the structure–function relationship of the SBD. We screened the combinatorial library for catalytic activity using the yeast display system and isolated clones with enhanced activity. We also discuss here the characteristics of the mutated amino-acid residues.

2. Experimental

2.1. Strains and media

Escherichia coli DH5α [*F-, endAl, hsdR17*(r_K^+, m_K^+)*, supE44, thi-1,* λ$^-$*, recA1, gyrA96, ΔlacU196, ϕ80dlacZΔM1*] was used as a host for DNA manipulation and *Saccharomyces cerevisiae* strain MT8-1 (*MATa, ade, his3, leu2, trp1, ura3*) [12] as the host for protein expression. *E. coli* was grown in LB medium [1% (w/w) tryptone, 0.5% yeast extract, 0.5% sodium chloride] containing 50 μg/ml ampicillin. YPD medium (1% yeast extract, 2% peptone, 2% glucose) was used to cultivate the yeast host cells. The yeast transformants were cultivated in SD-W medium [2% glucose and 0.7% yeast nitrogen base without amino acids (Difco, MI, USA) with appropriate supplements] containing 2% casamino acids.

2.2. Construction of plasmids

pGA11 [8] is a multicopy plasmid for the expression of the RoGA/α-agglutinin fusion gene containing the secretory signal sequence of RoGA under the control of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter. A DNA fragment containing *R. oryzae* GA and isolated from the plasmid pGA11 by *Eco*RI and *Xho*I digestion, was introduced into the *Eco*RI-*Xho*I site of pBluescript II SK(+) (Toyobo, Osaka, Japan). A *Ban*III site was generated at the Ala-Ser-Ile-encoding region of SBD by site-directed mutagenesis using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, CA, USA) and the two primers 5′-CTAATACTGGACATTCTCTGCATCGATTAATGGTATCAAGGAG-3′ and 5′-CTCCTTGATACCATTAATCGATCGAGAGAATGTAACGTAT-TCG-3′ (*Ban*III site underlined). A DNA fragment containing the RoGA with the generated *Ban*III site was isolated from the resulting plasmid by *Eco*RI and *Xho*I digestion and introduced into the plasmid pGA11, which was re-named pGA11 (BanIII). The plasmid for the expression of RoGA with mutated SBD was constructed as follows: a DNA fragment containing the SBD-coding region and *Eco*RI and *Ban*III sites of pGA11B was amplified by PCR using the primers 5′-TCTTGAATTCATGCAACTGTTCAAATTTGC-3′ and 5′-CATTATCGATGCAGAGAATGTCCAGTATTCGTAATTTGAMNNAGAMNNMNNAGCAGAGTAAGAAGCAGCAATGGTGTTTCCATTATTATTCCAGTTG-3′ (N, mixture of A, T, G and C; M, mixture of A and C). The amplified fragment was digested by *Eco*RI and *Ban*III, and introduced into the larger product of pGA11B after digestion with *Eco*RI and *Ban*III. The resulting expression plasmid for mutated RoGAs (SBD combinatorial library) was used to transform the yeast.

2.3. Screening by halo assay

Transformants with mutated RoGA displayed on yeast-cell surface were spread on SD-W + 1% casamino acid agar plates containing 0.2% cooked corn starch. After exposure of the plates to iodine vapor, colonies able to degrade the corn starch were identified by clear halos.

2.4. Assay of glucoamylase activity toward cooked corn starch

The substrate for glucoamylase reaction was prepared by adding cooked corn starch to 20 mM sodium acetate buffer (pH 4.6) to give a concentration of 0.04%. Yeast cells were cultivated in 10 ml of SD-W medium containing 2% casamino acids and collected by centrifuging at 3000 × *g* for 10 min. After the cells (A600 = 2.0) had been washed with sodium acetate buffer, the reaction was started by adding 1 ml of the substrate solution. After 12 h incubation at 30 °C, 100 μl of the reaction solution was treated with 25 μl of 5N HCl at 100 °C for 2 h to degrade all the residual corn starch to glucose. The solution was then neutralized with 10 μl of 5N NaOH and the residual content of corn starch was assayed by Glucose CII-Test Wako kit (Wako Pure Chemical, Osaka, Japan) as residual glucose content.

2.5. DNA sequencing

DNA fragments of around 400 bp encoding the mutated portion of the SBD were amplified from yeast colonies by PCR. The amplified fragments were purified by quantum prep PCR kleen spin columns (Bio-Rad, CA, USA) and sequenced using a Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 373A DNA Sequencer (Perkin-Elmer/Applied Biosystems, CA, USA).

3. Results

3.1. Strategy for construction of combinatorial library of RoGA SBD

Homology data on RoGA SBD and the SBD of *A. niger* GA shows that the two important aromatic residues are conserved in both sequences in Site 1 but not in Site 2 (Fig. 1a). On the basis of homology with *A. niger* GA, we focused on the putative residues in Site 2 and the adjacent amino acids (63S, 71T, 73S). The position of 63S is Y in *A. niger* GA and contributes to hydrophobic-ring interaction with substrates. The position of 71T is Y in *A. niger* GA. Although this Y shows no clear interaction with cyclodextrin in NMR data, in other GA SBDs this position is mainly Y. The position of 73S is T in *A. niger* GA. This T also shows no clear binding interaction in NMR data, but in other SBDs this position is mainly T or D [11]. We therefore constructed the combinatorially mutated library of RoGA in 63S, 71T, and 73S (Fig. 1b).

3.2. Halo assay of yeast cells displaying combinatorially mutated RoGAs

To introduce combinatorial mutation directly using oligonucleotide-directed mutagenesis, it is necessary to generate unique restriction sites near the target DNA sequences. *Ban*III was generated near the (63S, 71T, 73S)-coding DNA sequence in pGA11 (Fig. 2) by site-directed mutagenesis as described in Experimental.

The plasmids containing the combinatorial library of RoGA SBD, constructed by combinatorial mutagenesis (Fig. 2) were transformed into yeast cells. The transformants were spread on SD-W + 1% casamino acid medium agar plates containing 0.2% cooked corn starch. Altogether around 8.0×10^3 colonies were obtained. The plates were exposed to iodine vapour for 10 min, and around 200 of the 8.0×10^3 colonies produced clear halos. Colonies which formed clear halos were picked out, re-spread on plates, and re-screened, and the 48 colonies with the clearest halos were finally selected.

3.3. Evaluation of amylase activity of yeast cells displaying combinatorially mutated RoGA on their surfaces

The relative amylase activity of the 48 colonies obtained and of other colonies with no halo were assayed with cooked corn starch. Most of the 48 colonies exhibited 70–140% of the activity of the wild-type RoGA displayed, while colonies with no halo exhibited 10–30% (data not shown). Eleven of the 48 colonies exhibited higher activity than the wild-type RoGA displayed on the cell surface (Table 1). These results

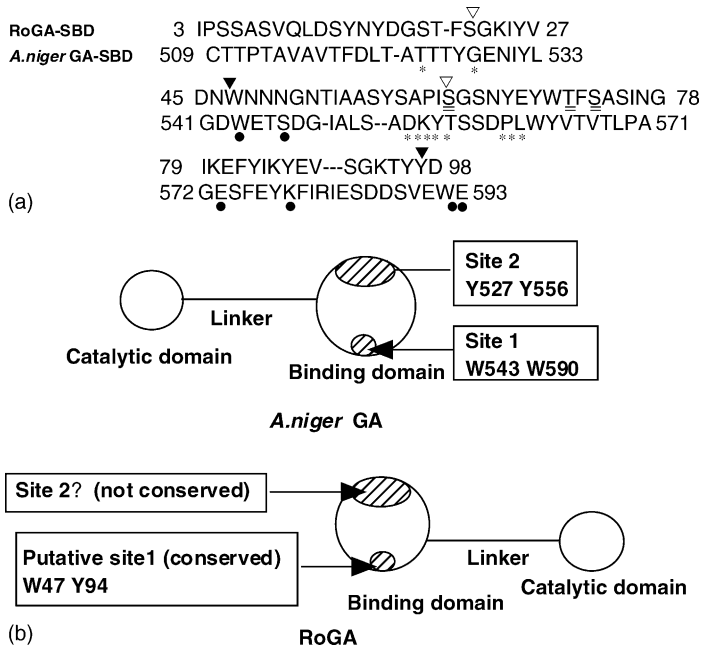

Fig. 1. Comparison of SBD sequences from RoGA and *A. niger* GA. (a) Homology of SBD sequences. Amino acids involved in hydrophobic-ring-ring interactions at Site 1 or Site 2 of *A. niger* GA are represented by (*) Site 1 or (●) Site 2. Combinatorially mutated positions are represented by double underlines. The amino-acid residues discussed in the text are indicated by the symbols of open and closed reverse triangles. (b) Models of structure of two GAs. RoGA has SBD at the N-terminal but *A. niger* GA has SBD at the C-terminal. Site 1 is conserved in both sequences but Site 2 is not.

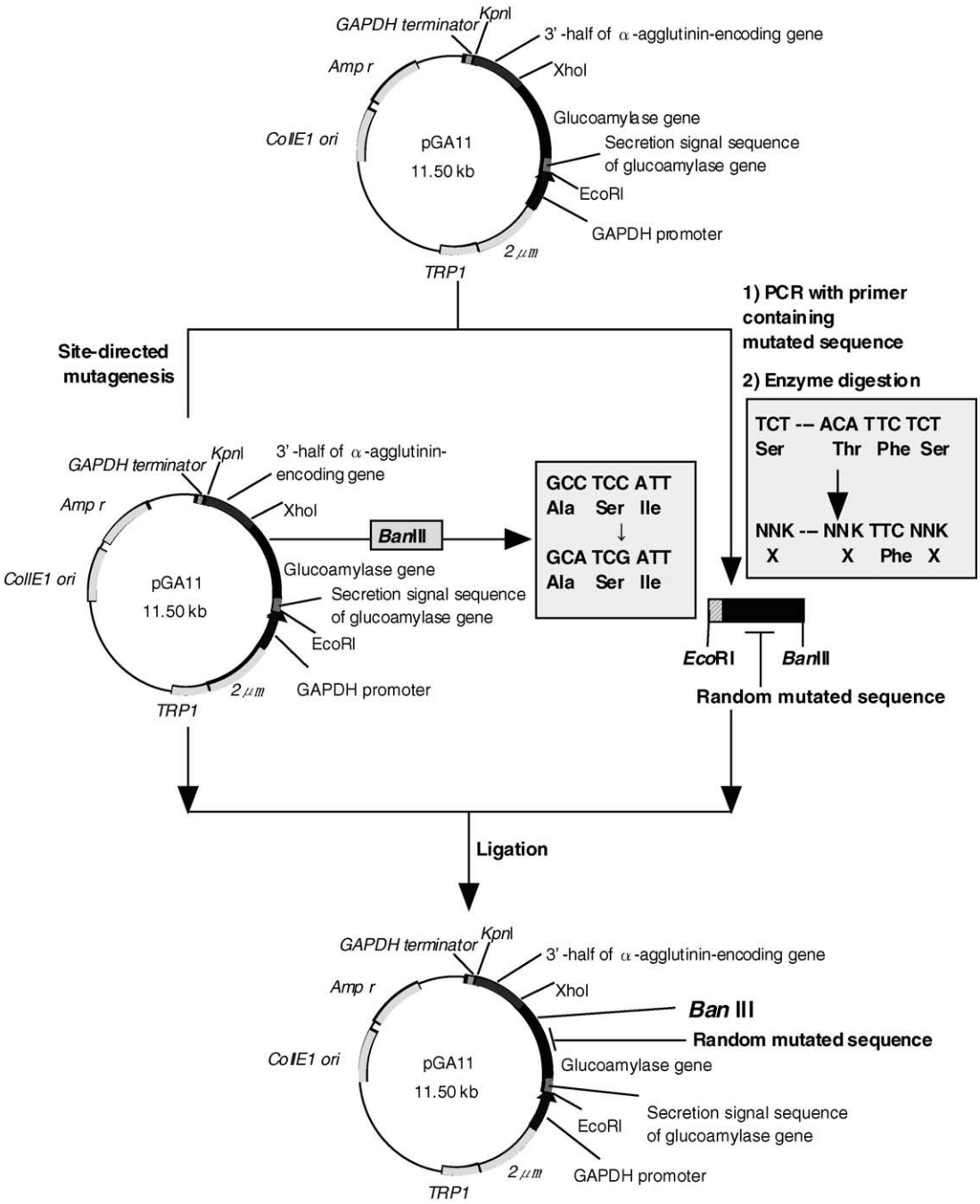

Fig. 2. Construction of plasmids for expression of SBD-combinatorially mutated RoGA on yeast-cell surface.

demonstrate that combinatorial mutagenesis of SBD with a yeast display system produces saturated mutant proteins easily and allows rapid screening for RoGA mutants with enhanced catalytic activity.

3.4. Analysis of combinatorially mutated amino-acid residues of SBD

The DNA sequences of the combinatorially mutated positions of SBD in the mutants and their deduced amino-acid residues were determined as described in Section 2 (Table 1). The amino-acid sequences of the SBD of mutants with high catalytic activity showed a pronounced feature: they mostly contained carbonyl-group-containing amino acids (D, E, N, Q).

3.5. Evaluation of amylase activity of mutants containing Tyr in mutated position

While Tyrs have been found in other amylase SBDs, there have been no mutants with Tyr in the mutated position in our previous combinatorial libraries. We constructed four

Table 1
Relative activity of mutants toward cooked corn starch and sequences of combinatorially mutated positions

	Sequence			Relative activity
	63	71	73	
Wild type	S	T	S	1.00
M-1	L	E	N	1.29
M-2	P	Q	E	1.46
M-3	M	H	Q	1.45
M-4	M	N	T	1.06
M-5	E	Q	R	1.15
M-6	L	T	A	1.02
M-7	D	E	E	1.25
M-8	R	D	H	1.25
M-9	Q	S	K	1.36
M-10	P	Q	R	1.20
M-11	V	E	Q	1.15
Y-1	Y	T	S	0.17
Y-2	S	Y	S	0.24
Y-3	S	T	Y	0.25
Y-4	Y	Y	Y	0.39

Wild type, pGA11B harboring yeasts; M-1 to M-11, selected mutants; Y–1 to Y–4, tyrosin-containing mutants. Data are means based on five independent measurements. Deviations are all less than 10%.

mutants containing Tyr in mutated positions by site-directed mutagenesis and assayed their catalytic activity by the same method as in Section 3.2. All four mutants [Y-1(63Y, 71T, 73S), Y-2(63S, 71Y, 73S), Y-3(63S, 71T, 73Y), Y-4(63Y, 71Y, 73Y)] had poor catalytic activity toward cooked corn starch (Table 1). For this reason, we were unable to screen mutant proteins with Tyr.

4. Discussion

In the yeast display system, the catalytic activity of the mutants is directly assayed on yeast-cell surface without purification of the mutated enzymes [7]. The yeast display system is an attractive method of enzyme engineering because it allows rapid screening for catalytic activity and the ready expression and isolation of many industrially applicable enzymes.

In the present study, we constructed a combinatorial library of RoGA SBD using the yeast display system and screened for activity toward cooked corn starch. We obtained a number of mutants exhibiting higher activity (>1.4-fold) than wild type in the first screening by halo assay and the relative assay using cooked corn starch.

There are several reports on the study of SBD by site-directed mutagenesis which provide information on the binding mechanism of SBD to starch [13]. However, there are few reports on the enhancement of the catalytic activity of GA by the SBD mutation. The SBD of RoGA is located in the N-terminal region and displays low homology with other SBDs [11]. We therefore considered that the SBD of RoGA had potential for improvement of its binding activity by mutation. In structure analysis of the SBD of *A. niger* GA bound to β-cyclodextrin, two binding sites (Sites 1 and 2) have been identified [10]. Data on homology with other GAs shows that, in the SBD of RoGAs, Site 1 is somewhat conserved but Site 2 is not. It is considered that the configuration of Site 2 is flexible and that many more amino acids contribute to direct or indirect substrate binding at Site 2 than at Site 1 [14]. We therefore combinatorially mutated at three residues in the putative Site 2 region and adjacent to it (63S, 71T, 73S). In other GAs, these sites are composed of Y or D. Data on the screening of the combinatorial library indicate that mutations to amino-acid residues containing a carbonyl group (D, E, Q, N) enhance the catalytic activity of RoGA. Newly generated hydrogen bondings between the carbonyl groups of the amino-acid residues and the substrate seem to enhance the binding ability of SBD to starch and thereby enhance the catalytic activity of RoGA. While mutations to Tyr were expected from homology research to enhance the binding ability of SBD, there were no active mutants with Tyr in the mutated positions in our combinatorial library. Tyr-containing mutants in RoGA prepared by point mutation exhibited poor activity. Hydrophobic-ring interaction between the sugar rings of starch and the aromatic rings of amino acids requires that the two rings be parallel. It may be difficult for the aromatic rings of Tyr in the position (63, 71, 73) to achieve a parallel position with the sugar rings of starch.

In combinatorial mutagenesis, the 3D structural data can be considered to determine the mutated regions and plural positions, and will allow easier determination of the mutated amino acids than error-prone PCR or DNA shuffling. The combination of combinatorial mutagenesis and rapid selection for function using the yeast display system can generate large sets of data in correlations of mutated amino acids to protein functions which can be utilized to progress research into enzyme engineering.

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