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**Screening for improved activity of a transglutaminase from *Streptomyces mobaraensis*
created by a novel rational mutagenesis and random mutagenesis**

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ABSTRACT

Microbial transglutaminase (MTG) has been used extensively in academic research and the food industries through its cross-linking or posttranslational modification of proteins. To improve MTG, a novel method of **rational** mutagenesis, called WASH-ROM (Water Accessible Surface Hot-space Region Oriented Mutagenesis), was first attempted. Based on the three-dimensional structure of MTG, 151 point mutations were **selected** at 40 different residues bearing high solvent accessibility surface area, within a 15 Å of the active center site nucleophile, Cys64. Among them, 32 mutants showed higher specific activity than the wild type enzyme. We found that beneficial mutations are distributed in two regions and with distinctive amino acid substitutions. Next, random mutagenesis was applied to the entire MTG region by developing a new plate assay-based screening system, using *Corynebacterium glutamicum* as the secretion host strain. This *in vivo* screening system allowed us to readily distinguish the change in enzymatic activity upon mutation by monitoring the intensity of enzymatic reaction-derived color zones which appeared around the recombinant cell colonies on the plate. From the library of 24,000 clones, 10 mutants were finally selected as beneficial enzymes exhibiting higher specific activity than wild type. Notably, most of the mutations differed from those obtained by WASH-ROM, except for H289Y. Beneficial mutations were distributed in two other regions as well. Furthermore, we found that the FRAP-S199A mutant (FRAP: N-terminal four amino acid residues extension) showed the highest specific activity (45 U/mg: 1.7 times higher than the wild type enzyme). Through these different mutation approaches, various beneficial positions leading to increased specific activity of MTG were surveyed.

INTRODUCTION

Transglutaminases (TGases: protein-glutamine γ -glutamyltransferase, EC 2.3.2.13) are a family of enzymes that catalyze an acyl transfer reaction between a γ -carboxyamide group of a glutamine residue in a peptide chain and an ϵ -amino group of lysine residue, resulting in the formation of an ϵ -(γ -glutamyl) lysine cross-linkage. TGases are widely distributed in various cells and tissues of mammals, and their physiological properties have been extensively studied (Folk 1980). The TGases derived from animals are calcium ion-dependent enzymes, and are distributed in most organs, skin, and blood. To date, for example, guinea pig liver TGase (Ikura et al. 1988), human epidermis keratinocyte TGase (Thacher 1989), human blood coagulation factor XIII (Ichinose et al. 1986) and red sea bream TGase (Yasueda et al. 1995) have been reported. However, the industrially most valuable TGase was found in the genus *Streptomyces* (*Actinomycetes*). *Streptomyces mobaraensis* s-8112 TGase (MTG; microbial transglutaminase) is calcium ion-independent, and basic and applied functional studies have demonstrated its industrial utility (Kuraishi et al. 1996; Motoki et al. 1998; Sakamoto et al. 1993; Yokoyama et al. 2004). The enzymatic properties of MTG have been characterized (Ando et al. 1998; Nonaka et al. 1989). Purified MTG has been analyzed, and its complete amino acid sequence identified (Kawajiri et al. 1993). MTG is known to be expressed as a prepro-enzyme which is processed to the mature form (Pasternack et al. 1998; Umezawa et al. 2004; Zotzel et al. 2003a; Zotzel et al. 2003b). Its pro-region is thought to be essential for efficient protein folding, secretion, and suppression of its enzymatic activity.

MTG has been used in the food industry for the modification of proteins. It is used in the binding of meat or fish and gelled food products such as jelly, yogurt or cheese. Moreover, it is an enzyme having a large potential utility in manufacturing the materials used in cosmetics, thermostable microcapsules the carriers for immobilized enzymes. To date,

the enzyme is produced by conventional fermentation and thus, it would be valuable to develop an efficient system for production of MTG., and various studies of this sort have been reported (Washizu et al. 1994; Yokoyama et al. 2000, 2002). Recently, pro-MTG has reportedly been efficiently secreted by *Corynebacterium glutamicum*, and subsequently activated by the co-expressed protease (Date et al. 2003; Kikuchi et al. 2003). This strain is used for the industrial production of amino acids such as glutamate, lysine, and others, which have been used in food, animal feed and pharmaceuticals for several decades. It is non-pathogenic and produces no hazardous toxins. Furthermore, considerable experience has been accumulated concerning the appropriate fermentation conditions. In these respects, it is likely that *C. glutamicum* is suitable for industrial-scale protein production.

In addition to food applications, novel potential applications have emerged in non-food areas. These applications cover a range of areas, including medical engineering, material science, textiles and leather processing (Fontana et al. 2008; Zhu et al. 2008). There are many promising applications beyond the food area, but it was necessary to improve MTG to fit such applications. For this purpose, C. K. Marx developed a screening method, including a proteolytic activation step of the expressed soluble pro-enzyme, and selected improved thermostability variants by random mutagenesis (Marx et al. 2008).

In the present study, to improve the specific activity of MTG, we carried out two engineering approaches, rational mutagenesis targeting the immediately surrounding area of the active center site, Cys64, and random mutagenesis targeting the entire MTG region. This rational approach is based on a novel region-directed mutagenesis, called WASH-ROM (Water Accessible Surface Hot-space Region Oriented Mutagenesis). Also, a bacterial enzyme secretion-based plate assay system was developed to efficiently select positive candidates through the screening process. Most of the beneficial mutations with higher specific activities than the wild type enzyme were different between the two approaches, and

the activity-increase related positions and regions were identified in the three dimensional-structure of MTG. Thus, by a combination of different mutagenesis approaches, we found that there are multiple pathways to access beneficial mutations for the acquisition of higher specific activity.

MATERIALS AND METHODS

Bacterial strains, culture medium, and plasmids.

E.coli JM109, *E.coli* DH5 α and *Corynebacterium glutamicum* YDK010 (Kikuchi et al. 2002) were used in this study. *E. coli* JM109 were grown in Luria broth and used as an intermediate host for various plasmid constructions. *E. coli* DH5 α were used as a host for a random mutagenesis library. *C. glutamicum* was grown in CM2G medium [5 g of glucose, 10 g of tryptone, 10 g of yeast extract, 5 g of NaCl, 0.2 g of DL-methionine per liter of distilled water, adjusted to pH 7.2] at 30 °C. A MTG-production medium for *C. glutamicum* was used with MMTG medium [60 g of glucose, 1 g of MgSO₄, 30 g of (NH₄)₂SO₄, 1.5 g of KH₂PO₄, 0.01 g of FeSO₄·7H₂O, 0.01 g of MnSO₄·4H₂O, 450 µg of thiamine hydrochloride, 450 µg of biotin, 0.15 g of DL-methionine, 50 g of CaCO₃ per liter of distilled water, adjusted to pH 7.5] at 30 °C. *C. glutamicum* was transformed by electroporation as described previously (Lieble et al. 1989). Antibiotics were added to final concentrations of 25 mg/l for kanamycin (*C. glutamicum* and *E. coli*), 5 mg/l (*C. glutamicum*) or 30 mg/l (*E. coli*) for chloramphenicol, and 50 mg/l for ampicillin (*E. coli*).

DNA manipulations.

All DNA manipulations followed standard procedures (Sambrook et al. 1989). PCR with Pyrobest DNA polymerase (Takara Shuzo, Kyoto, Japan) was performed in 50 µl reaction mixtures for 5 min at 94 °C, and for 25 cycles of 10 sec. at 98 °C, and 30 sec. at 55 °C, and 3 min at 72 °C. Nucleotide sequences were determined by using the BigDye terminator cycle sequencing FS ready reaction kit (Applied Biosystems) and DNA sequencer (model 377; Applied Biosystems).

Rational mutagenesis of MTG.

Mutagenesis of the MTG gene was carried out, using QuickChange® II Site-Directed Mutagenesis Kit (STRATAGENE®) according to the manufacturer's manual. Pro-MTG expression vector pPSPTG11 (Date et al. 2003) was used for template DNA. Mutagenesis primers were synthesized in reference to the codons of *C. glutamicum*. After digestion with DpnI, the resulting plasmids containing the mutated MTG gene were transformed into *E. coli* JM109. Mutations were confirmed by DNA sequencing, using an ABI DNA sequencer, according to the manufacturer's manual. The mutated plasmids were transformed into *C. glutamicum* for rational screening.

Construction of a random mutation library based on error-prone PCR.

For random mutagenesis, 10 ng template DNA (Wild type pro-MTG expression plasmid pPSPTG1(Kikuchi et al. 2003)) were applied in the error-prone PCR reaction (10 mM Tris-HCl, 50 mM KCl, 0.1 mg/ml BSA, 5 mM MgCl₂, 0.25 mM dNTPmix, Forward primer (25 pmol/ml), Reverse primer (25 pmol/ml), 10% DMSO and 0.5 % rTaq polymerase (Takara Shuzo, Kyoto, Japan)). PCR was performed in 100 µl reaction mixtures for 3 min at 94 °C, and for 25 cycles of 2 min at 94 °C, and 30 sec. at 58 °C, and 3 min at 72 °C. The PCR primers were derived from sequences at the beginning and at the end of the MTG gene without the pro-sequence (forward-primer: 5'- GCCCCCGACTCCGACGACAG-3', reverse-primer: 5'-TCGCTGCAGGTCGACTCTAG-3'). The PCR fragment and pPSPTG1 were successively digested by *Bam*HI and *Bst*EII. The digested PCR fragment and a large fragment of pPSPTG1 were ligated and transformed into *E. coli* DH5α. Plasmids extracted from 100 mixed *E. coli* clones were transformed into *C. glutamicum* YDK010, which carried the SAMP-45 expression vector pVSS1 (Kikuchi et al. 2003), and 300 *C. glutamicum* clones were selected. A total of 24,000 *C. glutamicum* clones were obtained from 8,000 *E. coli* clones for random screening.

Cultivation and activation of the rational mutant clones.

Deepwell microtiter plates (Flat-Bottom Block, QIAGEN K.K.) were filled with 1ml CM2G medium per well. Each well was inoculated with a single colony of transformed *C. glutamicum* cells using a sterile toothpick and sealed with a gas permeable adhesive seal (AirPore™ Tape Sheets, QIAGEN K.K.). They were incubated in a shaking incubator (BioShaker M·BR-022 TAITEC) at 1500 rpm, 30 °C for 16-20h. For MTG expression, 50 µl of the culture were used to inoculate a new deepwell microtiter plate containing 1ml of MMTG medium. It was sealed as described above and incubated in the same shaking incubator at 1500 rpm and 30 °C for 48h, followed by centrifugation (3000 rpm, 20 min.). 200 µl of the supernatant were inoculated onto a new deepwell microtiter plate, containing 800µl of 50 mM Sodium Phosphate buffer and 0.0125 % Protease from *Bacillus licheniformis* (P4860, Sigma-Aldrich Japan). It was incubated at 30 °C for 16h for activation of MTG. In the case of test tube culture, proteolytic activation was carried out in the same method in a new test tube.

Assay of MTG activity.

MTG activity was measured by the calorimetric hydroxamate procedure using *N*-carbobenzoxy-L-glutaminyglycine (Z-Gln-Gly) (Yokoyama et al. 2002). Buffer A consisted of 0.03 M CBZ-Gln-Gly, 0.1 M NH₂OH, 0.01 M glutathione (reduced form) and 50 mM MES, pH 6.0. Buffer B consisted of 1 N HCl, 4% TCA and 5% FeCl₃·6H₂O. One hundred µl of the test sample was incubated in 1 ml of buffer A at 37 °C, and the reaction was terminated by the addition of 1 ml buffer B. The absorbance at 525 nm was measured. One unit was defined as the formation of one micromole of hydroxamic acid per min. L-glutamic acid γ-monohydroxamate was used as the standard. To adapt the microtiter plate

assay for mutant screening, microtiter plates were filled with 30µl sample and 50µl buffer A per well, then incubated at 37 °C for 20 min. The reaction was terminated by the addition of 50 µl of buffer B. The absorbance at 525 nm was measured by a plate reader (SpectraMaxM2, Molecular Devices).

Plate assay.

C. glutamicum, carrying the random mutated pro-MTG expression vector pPSPTG1 and SAM-P45 expression vector pVSS1 (Kikuchi et al 2003), was incubated on a CM2G plate, containing 25 mg/l kanamycin and 5 mg/l chloramphenicol at 30 °C for 24 hours, then transferred onto a nitrocellulose membrane (ADVANTEC MFS, INC.). This membrane was incubated on the MMTG medium plate, containing 25 mg/l kanamycin and 5 mg/l chloramphenicol at 30 °C for 24 hours. After incubation, this membrane was transferred onto the metal plate. 6 ml substrate solution, consisting of 0.12 M CBZ-Gln-Gly, 0.4 M NH₂OH, 0.04 M glutathione (reduced form) and 0.2 M MES, pH 6.0, were sprayed on the membrane, and this sprayed form was incubated at 37 °C for 45min. Reaction was stopped by the spraying on Buffer B, which consisted of 1 N HCl, 4% TCA and 5% FeCl₃·6H₂O. **Yellow** around a colony was taken to be inactive MTG, while red was judged to be active MTG.

Determination of protein concentration.

Analytical reverse-phase HPLC was performed using a Vydac C4 column (214TP5410, 4.6-mm internal diameter x 10 cm, Grace Davison Discovery ScienceTM). The column was equilibrated with 24% acetonitrile and 0.1% trifluoroacetic acid / water, and the proteins were eluted with a linear acetonitrile gradient from 24 to 40% in 0.1% trifluoroacetic acid / water over 20 min at a flow rate of 1 ml/min at ambient temperature. The eluent was monitored by

UV absorbance at 280 nm. The concentration of MTG was determined by reverse-phase HPLC, using purified wild type enzyme as a protein standard. Wild type MTG was prepared from the culture supernatant of *Streptoverticillium* sp. S-8112 as described previously (Ando et al. 1989).

Total protein concentration was measured by Bradford method, using Bio-Rad Protein Assay Kit.

Purification of MTG.

Activated MTG solution was diafiltrated 10-fold with 20 mM sodium acetate, pH 5.5, and applied to a cation-exchange column (RESOURCE S 6ml; GE Healthcare UK Ltd.) equilibrated with 20 mM sodium acetate, pH 5.5. After the column was washed with 1 column-volume of the same buffer, MTG was eluted with a linear gradient of sodium chloride from 0 to 500 mM over 10 column volumes at a flow-rate of 6 ml/min. Fractions were assayed by the hydroxamate method and analytical reverse-phase HPLC. Fractions at the top with the same specific activity were collected.

For buffer-exchange, the collected fractions were applied to a gel filtration column (Sephadex G25 M, GE Healthcare UK Ltd.) equilibrated with 20 mM sodium phosphate, pH 6.0, and the proteins were eluted with the same buffer. Purified MTG fractions were freeze-dried at -80 °C before use.

RESULTS

Design of the rational mutation of MTG.

Rational mutagenesis was first attempted. We calculated the **percent SAS** (Solvent Accessibility Surface area) **of each amino acid residue in the mature MTG region**. The **percent SAS** is obtained by dividing the side chain SAS by the total SAS. The three dimensional structure of MTG was determined (Kashiwagi et al. 2002). **We considered** amino acid residues, **with high value of percent SAS, had a possibility to** interact with substrates. **Among 90 amino acid residues within 15 Å from the active center site Cys64, we selected 40 amino acid residues with high value of percent SAS (Table 1.).** We have called this novel methodology WASH-ROM (Water Accessible Surface Hot-space Region Oriented Mutagenesis). **We determined one to six (mainly four or five) replacement amino acid residues for each amino acid, considering the type of amino acid in each case.** Adequate polar and non-polar amino acid residues were occupied at the positions selected in this rational approach.

Preparation and screening rationally designed mutants.

Rationally designed mutant expression vectors were constructed using the QuickChange® II Site-Directed Mutagenesis Kit (STRATAGENE®) (See Material & Methods). **pPSPTG11 (Date et al. 2003) was used for the template DNA. pPSPTG1 expresses wild type pro-MTG, however, N-terminal amino acid sequence of MTG was differed from that of wild type MTG after proteolytical activation (Date et al. 2003).** **pPSPTG11 expresses altered Pro-MTG, whose C-terminal of pro-peptide was changed from Arg-Ala-Pro to Gly-Pro-Lys. N-terminal amino acid sequence of MTG was equal to that of natural MTG after proteolytical activation (Kikuchi et al. 2003).** 151 mutant vectors were

constructed in *E.coli* and transformed into *C. glutamicum*. The clones constructed above were cultivated in a deepwell microtiter plate and proteolytically activated in another deepwell microtiter plate (See Materials & Methods). Enzyme activity was measured in the microtiter plates as well as the total protein concentration. Of the initially screened clones, 84 clones showed a higher or equal specific activity as the wild type MTG. These clones were cultivated in one test tube and proteolytically activated in another test tube (See Materials & Methods). Finally, the specific activities of the screened enzymes were precisely calculated by measuring their protein concentrations with RP-HPLC. Of the second group of screened clones, 32 clones showed a higher specific activity (Table 2.). Wild type MTG exhibited 27.2 U/mg (This is a mean value and width is up to 7%), and 8 mutants (D3F, R26F, W59F, V65I, Q74A, Y75F, Y75A, and T77S) with more than 1.3 times the specific activity of the wild type enzyme. Among them, Y75F exhibited the highest activity (41.7 U/mg), corresponding to 1.5 times that of the wild type enzyme.

Assignment of the beneficial positions of rational mutation on the three-dimensional structure.

The MTG molecule forms a single, compact domain and adopts a disk-like shape with a deep cleft at the edge of the disk. Cys64, the residue essential for the catalytic activity, exists at the bottom of the cleft. The mutations responsible for the increased specific activity were assigned on the three-dimensional (3D) structure (Fig. 1.). First, V65, located next to the active center site comprises the internal residues of MTG (percent SAS of V65 is 3.0). **Minor change (replacement Val with Ile) led to higher specific activity.** Next, we found two regions related to increased activity, which were both localized in a symmetric relation to each other in the disk-like structure of MTG. One region, including Y75, Q74, T77, and R238, was located on the surface of the front side of the MTG. In particular, Y75 is located

within 10 Å of Cys64, and its side chain faces Cys64. The highest activity was gained by a replacement with Phe at this position. **This region may be related to substrate recognition.** The other region, including H289, E28, R26, S303, and S299, was located on the back side of the MTG, as reported in the literature (Kashiwagi et al. 2002). Except for E28, replacement with hydrophobic amino acids led to increased specific activity. These residues may be also related to substrate recognition.

Construction of a novel plate assay for efficient screening

Next, to explore beneficial mutations by random mutagenesis, we have attempted to establish a novel plate assay to readily screen positive candidates with higher activity compared to the wild type MTG. Previously, we demonstrated that active MTG can efficiently secrete in *C. glutamicum* by using the wild type pro-MTG expression plasmid pPSPTG1 and the activation protease SAM-P45 expression plasmid pVSS1 (Kikuchi et al. 2003). Based on the findings, this liquid assay system was transferred to the plate assay system. Figure 2 shows the typical pattern. When *C. glutamicum* is grown on the membrane, the secreted active form of MTG can be detected by means of sprayed substrate solution. *C. glutamicum* YDK010, carrying pPSPTG1 and pVSS1, exhibited red (Fig. 2. A), while *C. glutamicum* YDK010, carrying pPK4 (Kikuchi et al. 2003), and pVSS1 exhibited yellow (Fig. 2. B). It was thus possible to efficiently distinguish the red active mutants from the inactive mutants, which were yellow.

Screening for random mutation library.

Figure 3 summarizes the screening process. The mutant library was initially constructed using error-prone PCR, as described in the Materials and Methods. The mutation rate was controlled so as to be 2 or 3 base pairs in the MTG-encoding region (Taguchi et al. 1998).

Approximately 24,000 clones were obtained as a bacterial colony formation on the plate. All of the clones were subjected to the plate assay. Approximately 5,000 clones showed activity. At this step, mutants which had lost activity were efficiently eliminated using the convenient plate assay. These positive clones were sequentially subjected to the second and third screening. In the second screening, all of the 5,000 clones were cultivated using the deepwell microtiter plate, followed by hydroxamate assay with the microtiter plate. In the third screening, the selected 200 clones were cultivated using the test tube, followed by hydroxamate assay with the test tube, and finally the 78 clones were obtained as positive candidates. DNA sequencing of the 78 clones revealed that 32 clones had single to triple mutations, while 46 clones had no mutations. This suggested that bacterial cell protein secretion efficiency may be involved to some extent in this assay. After removing overlapping clones from the 32 clones with mutations, the specific activities of the selected 27 clones were precisely calculated by measuring their protein concentrations with RP-HPLC. In these 27 clones, 11 clones clearly showed higher specific activity than wild type MTG. These clones include 15 point mutations, including H289Y, which was also selected by rational mutation (Table 3.).

Analysis of selected mutants.

To evaluate the effect of single mutations, we constructed 14 mutants, except H289Y, using the QuickChange® II Site-Directed Mutagenesis Kit (STRATAGENE®) (See Material & Methods). Purified wild type MTG shows 27 U/mg (This is mean value and width is up to 4%). Ten of the 14 purified mutated proteins exhibited higher specific activities than the wild type enzyme (Table 4.). M16T exhibited the highest (42.5 U/mg), 1.6 times higher specific activity than the wild type enzyme. S199A exhibited 30.0 U/mg, but this mutant had more than 40 U/mg before evaluation of the single mutation. We found that N18 mutant

(Table 3.) had N-terminal four amino acid residues extension (FRAP-extension). The wild type pro-transglutaminase is proteolytically activated to yield FRAP-MTG by the activation protease SAM-P45. Purified FRAP-S199A was determined to be 45.0 U/mg. This value was the highest among all of the mutants. The specific activities of FRAP-MTG and MTG were approximately the same. Both mutation (S199A) and FRAP-extension evidently cooperate with each other in the higher specific activity of MTG.

Assignment of beneficial positions at random mutation on the three-dimensional structure.

The mutations responsible for the increased specific activity, which were assigned to the 3D structure and the two regions were identified (Fig. 4.). One region, including A10, P12, D14, M16, V30, and N32, was located at the outer surface of the protrusion that contains N-terminus (on the left side surface of the MTG, as defined in the literature (Kashiwagi et al. 2002)). Except for D14, replacement of the hydrophilic amino acids led to increased specific activity. Marx *et al.* reported that all single point mutations related to changed thermal properties of MTG are located in the N-terminal domain (Marx et al. 2008). Further, combination of FRAP-extension with the S199A mutant led to the highest specific activity. This region may be related to the stability of MTG and is an important region for further improvement in the future. The other region included Y34, Y42, and S199, which are internal residues of the MTG. The SAS percentages of Y34, Y42, and S199 are 2.0, 5.7 and 0.0, respectively. These replacements may be a good fit the surrounding amino acid residues.

DISSCUSSION

To improve the activity of MTG, we have applied two different approaches. **Rational**

mutagenesis, WASH-ROM, was adopted to survey the beneficial positions that can lead to the improvement of MTG activity. The region for mutation was targeted to the surrounding area of the active center Cys64, which is located at the hydrophobic core of MTG. Beneficial positions were addressed evenly on the two opposite sides. One region corresponds to the surface of the front of the MTG, while the other region is located on the back side of the MTG. This result suggests that these two regions may interact with two types of substrates, an acyl donor and acyl acceptor. In terms of the identification of beneficial positions, this distance-based approach would be applicable to survey regions further away from the active center site. It would be an efficient approach to obtain mutants with improved activity, because interactions between enzymes and substrates are relatively weak enough in comparison with those of protein-protein or protein-peptide, and they have potential to improve by modifying their binding surface due to amino acid substitutions in enzymes. On the other hand, random mutagenesis was expected to be effective in surveying entire region of MTG. The novel plate assay facilitated the screening process. In fact, beneficial mutations which differed from those identified by a rational approach were obtained by a random approach. It should be noted that the N-terminal domain, which is far from active center Cys 64, was important. In particular, a synergistic activity increase was obtained by a combination of the N-terminal four residues extension, FRAP, with the S199A mutation. The N-terminal domain is reported to also have a potential for acquiring thermal stability (9). Taken together with these findings, the N-terminal region is an attractive target for mutagenesis performed to gain activity-increased and/or stability-increased mutants by multiply combining beneficial mutations, as shown by the mutants obtained here.

In this study, primarily surveying beneficial positions related to activity-increase has been efficiently performed by the two different approaches, but fine-tuned amino acid substitutions at individual positions could not be explored at the present. Therefore, the next generation

enzyme evolution is underway to gain further attractive evolved enzymes with highly improved activity by saturation mutagenesis and mutation scrambling.

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FIGURE LEGENDS

TABLE 1. Amino acid residues selected for rational mutagenesis (WASH-ROM)

Amino acid residues with a high percentage of SAS (Solvent Accessibility Surface area), within 15 Å of the active center Cys64 are shown. The percentage of SAS is obtained by dividing the side chain SAS by the total SAS.

TABLE 2. The specific activities of rationally designed mutants.

The specific activities of 32 clones selected by rational mutagenesis are shown. The enzyme activity assay and protein concentration measurement were carried out as described in Materials and Methods.

FIG. 1. Rationally designed mutation points assigned on the three-dimensional structure.

Schematic ribbon drawings of the MTG molecule viewed from above the plate face (5). Cys64 and the mutated amino acid residues are indicated by the combination of one character notation and a number. Side chains are represented by a ball-and-stick model. The illustration was drawn with the program MolFeat v3.6 (FiatLux Corporation).

FIG. 2. Typical pattern of plate assay.

A: *C.glutamicum* YDK010, carrying pPSPTG and pVSS1 (7), displaying the positive, red zone, which appeared around the colony. B: *C.glutamicum* YDK010, carrying pPK4 and pVSS1 (7), displaying the negative, yellow zone, which appeared around the colony.

FIG. 3. The screening process of random mutagenesis.

This is a flowchart showing the screening process of the random mutagenesis. The numbers in the square boxed indicate the colony numbers at each of the screening step.

Table 3. Specific activities of the mutants obtained by random mutagenesis.

Specific activities of 11 clones, selected by the screening process performed by random mutagenesis (Fig. 3.), are shown. The enzyme activity assay and protein concentration measurement were carried out as described in Materials and Methods.

Table 4. Specific activities of the individually mutated enzymes obtained by random mutagenesis.

Specific activities of 10 single mutants are shown. The enzyme activity assay and protein concentration measurement were carried out as described in Materials and Methods.

FIG. 4. Randomly screened mutation points assigned on the three-dimensional structure.

Schematic ribbon drawings of the MTG molecule viewed from above the plate face (5). Cys64 and the mutated amino acid residues are indicated by the combination of one character notation and a number. The side chains are represented by a ball-and-stick model. The illustration was drawn with the program MolFeat v3.6 (FiatLux Corporation).

TABLE 1.

5 Å from Cys64			10~15 Å from Cys64		
Residual number		Percent SAS	Residual number		Percent SAS
62	Tyr	43.4	284	Ser	77.0
276	Asn	33.7	250	Gly	70.7
277	His	18.1	77	Thr	70.6
254	Phe	8.8	238	Arg	57.5
65	Val	3.0	4	Asp	50.5
275	Gly	1.9	303	Ser	48.3
5~10 Å from Cys64			74	Gln	44.1
Residual number		Percent SAS	26	Arg	42.1
252	Val	47.5	281	Pro	41.9
75	Tyr	42.1	28	Glu	38.8
285	Leu	41.9	5	Arg	37.3
302	Tyr	39.0	301	Gly	36.9
239	Asn	33.1	58	Glu	32.7
278	Tyr	30.9	241	Pro	31.0
253	Asn	28.7	299	Ser	29.4
289	His	26.8	3	Asp	29.2
251	Phe	19.6	30	Val	28.9
59	Trp	19.0	297	Asn	25.3
304	Asp	13.7	6	Val	24.2
305	Phe	6.5	287	Ala	22.9
273	Thr	4.8	291	Tyr	21.3

TABLE 2.

Mutation	Specific activity (U/mg)	Mutation	Specific activity (U/mg)	Mutation	Specific activity (U/mg)
D3F	35.8	W59F	35.9	T77L	31.4
D3N	33.5	V65I	36.0	R238L	30.7
D3L	32.4	Q74A	37.4	R238F	30.3
R5K	33.4	Q74N	34.4	S284T	30.9
V6T	30.2	Q74L	30.1	H289F	34.9
R26F	35.5	Y75F	41.7	H289Y	33.0
R26L	30.4	Y75A	35.5	S299L	32.1
E28D	32.3	Y75H	31.6	S303T	32.4
V30I	33.8	T77S	36.6	S303F	30.6
V30T	30.5	T77F	34.1	S303A	30.6
E58D	32.7	T77A	33.6	–	27.2

Fig. 1.

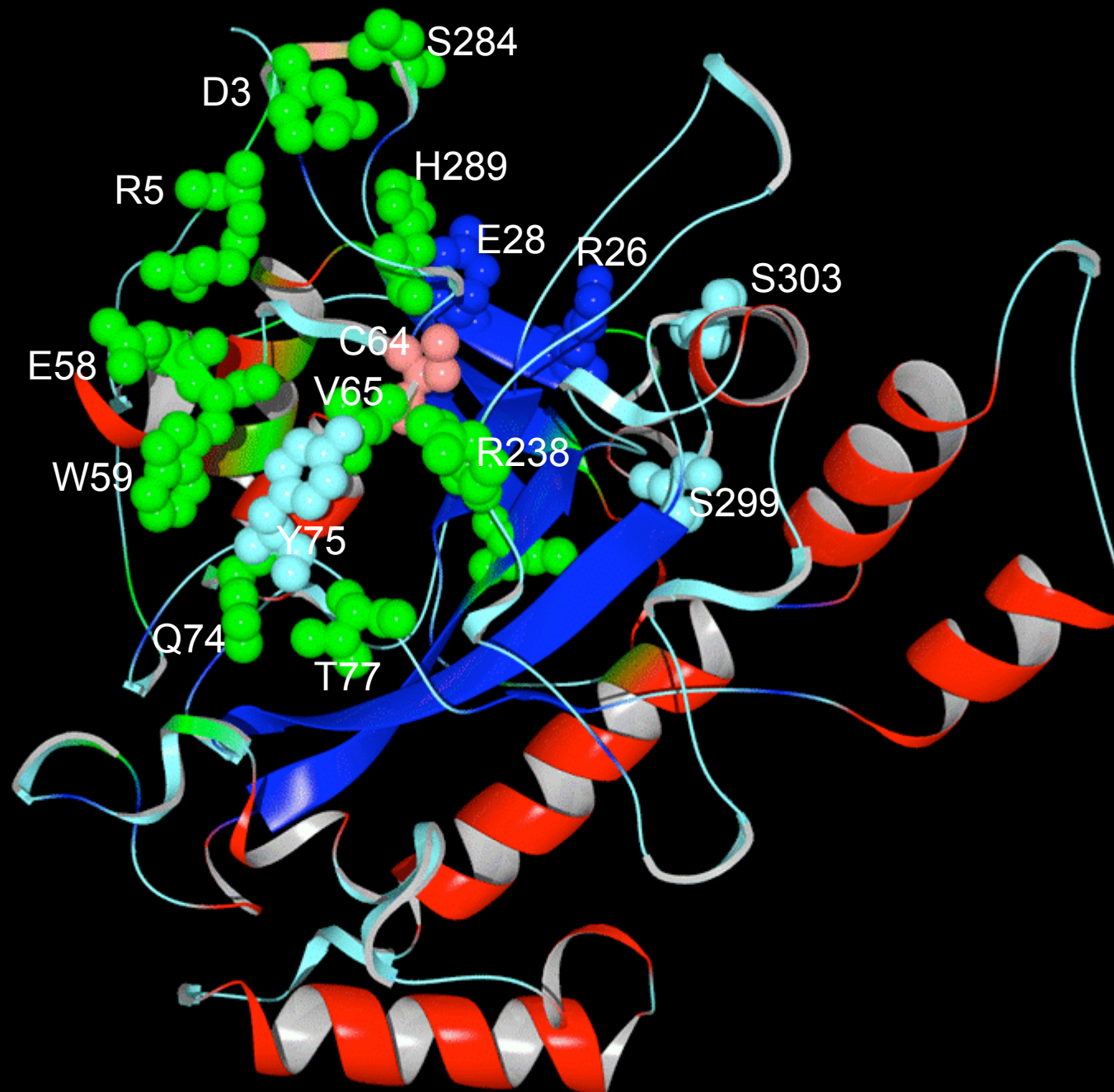


Fig. 2.

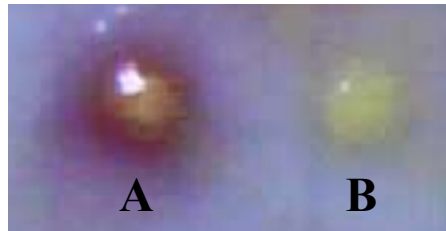


Fig. 3.

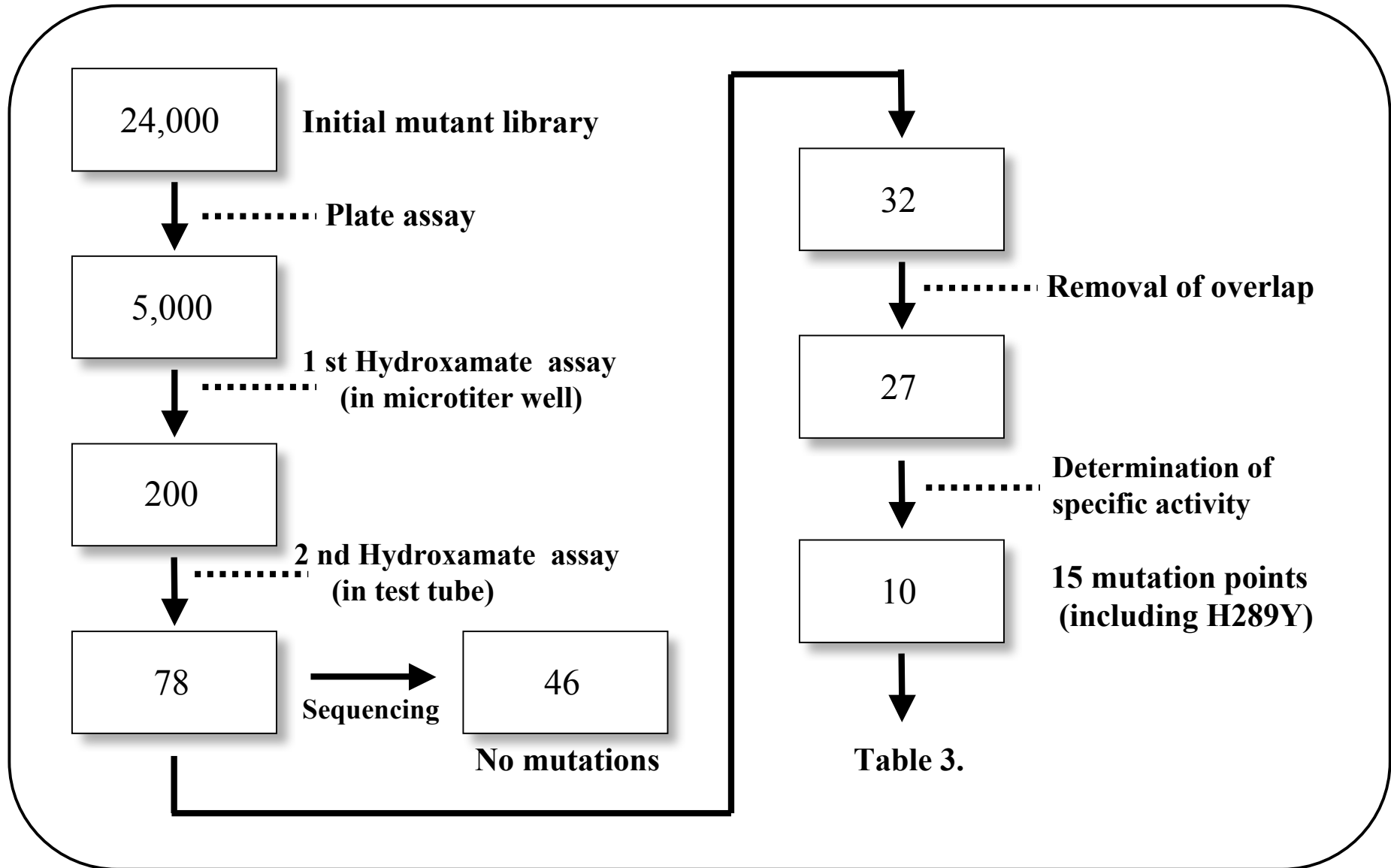


TABLE 3.

Mutant No.	Mutation	Specific activity (U/mg)
N18	199S→A	42.9
N66	14D→N	26.4
N73	12P→S	28.6
N106	42Y→H	27.1
N125	32N→D 264E→D 320N→T	28.7
N126	320N→D	27.4
N137	30V→D	27.2
N134	16M→T 283G→S	29.8
N140	10A→S	27.2
N149	289H→Y	27.4
N182	34Y→F 268D→N	28.6

TABLE 4.

Mutation	Specific activity (U/mg)	Mutation	Specific activity (U/mg)
10A→S	29.7	32N→D	28.9
12P→S	32.3	34Y→F	34.3
14D→N	30.5	42Y→H	28.5
16M→T	42.5	199S→A	30.0
30V→D	29.1	320N→D	29.2

Fig. 4.

