Controlling Substrate Preference and Transglycosylation Activity of Neopullulanase by Manipulating Steric Constraint and Hydrophobicity in Active Center*

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The substrate specificity and the transglycosylation activity of neopullulanase was altered by site-directed mutagenesis on the basis of information from a three-dimensional structure predicted by computer-aided molecular modeling. According to the predicted three-dimensional structure of neopullulanase, the amino acid residue which was most likely to affect the substrate specificity was predicted to be Ile-358. Replacing Ile-358 with Trp, which has a bulky side chain, reduced the accessibility of α-(1→6)-branched oligosaccharides and pullulan as substrates. The transglycosylation activity of neopullulanase appeared to be controlled by manipulating the hydrophobicity around the attacking water molecule, which is most likely used to cleave the glucosidic linkage in the hydrolysis reaction. We predicted three residues, Tyr-377, Met-375, and Ser-422, which were located on the entrance path of the water molecule, might be involved. The transglycosylation activity of neopullulanase was increased by replacing one of the three residues with more hydrophobic amino acid residues; Y377F, M375L, and S422V. In contrast, the transglycosylation activity of the enzyme was decreased by replacing Tyr-377 with hydrophilic amino acid residues, Asp or Ser.

Starch is a polysaccharide that is a mixture of amylose, composed of glucose-polymer linked by α-(1→4)-linkages, and amylpectin, composed of α-(1→4)-linked glucose-polymer branched by α-(1→6)-linkages. Most starch hydrolases and related enzymes catalyze only one of the following four types of reactions (1): hydrolysis of α-(1→4)-glucosidic linkages, hydrolysis of α-(1→6)-glucosidic linkages, transglycosylation to form α-(1→4)-glucosidic linkages, or transglycosylation to form α-(1→6)-glucosidic linkages. Each reaction is typically catalyzed by α-amylase (EC 3.2.1.1), pullulanase (EC 3.2.1.41), isomaltase (EC 3.2.1.68), and gluconotransferase (EC 2.4.1.19), and 1,4-α-D-glcan branching enzyme (EC 2.4.1.18), respectively (1). These reactions and the classification of the enzymes that catalyze them have been clearly distinguished and are represented by the four individual enzymes described above.

However, some exceptions have been reported in which one enzyme catalyzes not only one main reaction but also another side reaction. Bacterial saccharifying α-amylase catalyzes α-(1→4) transglycosylation in addition to the main reaction, hydrolysis of α-(1→4)-glucosidic linkages (2). Cyclomaltooltrixin gluconotransferases feebly hydrolyze α-(1→4)-glucosidic linkages in addition to the main reaction, α-(1→4) transglycosylation (3). Some pullulanases from thermophiles have recently been reported to hydrolyze not only α-(1→6)- but also α-(1→4)-glucosidic linkages (4, 5).

Progress in x-ray crystallographic analysis has allowed researchers to note the structural similarity between α-amylases (6–8) and cyclomaltooltrixin gluconotransferases (9, 10). The structures of both enzymes are based on a similar (α/β) β barrel as their main domain, and they have common amino acid residues which may act as catalytic sites (6, 7, 10). The primary structural analyses (1, 11–14) and the secondary structural predictions (15, 16) suggest a close relationship of the three-dimensional structures for the enzymes which catalyze all of the above mentioned reactions.

We have been studying a new type of pullulan-hydrolyzing enzyme, neopullulanase from Bacillus steaerothermophilus (11, 17, 18). Neopullulanase catalyzes both hydrolysis and transglycosylation of α-(1→4)- and α-(1→6)-glucosidic linkages by one active center (1, 19).

On the basis of a series of experimental results using neopolllulanase and the structural similarities among the enzymes which catalyze these four reactions, it has been proposed that they constitute an α-amylase family (1). We have formulated a number of hypotheses regarding the mechanisms of polymer hydrolysis and transglycosylation reactions (20). Among these is that the difference of specificities toward α-(1→4)- and α-(1→6)-glucosidic linkages is caused solely by the difference in the binding modes of substrate and enzymes. We have obtained strong evidence to prove this, because we have observed that the specificities of neopullulanase toward α-(1→4)- and α-(1→6)-glucosidic linkages can be switched by replacing amino acid residues which might be involved in substrate recognition (19).

The purpose of this paper is to explore in more depth these hypotheses. Using the computer-aided model of the three-dimensional structure of neopullulanase, we predicted the amino acid residue which was most likely to affect the substrate preference. By manipulating this amino acid residue, the en-
zyme specificities toward α-(1→6)-branched oligosaccharides and pullulan were clearly reduced or increased, and the characteristics of one of the mutated neopullulanases were similar to those of α-amylase. In this paper we describe the characteristics of the mutated enzymes which were manipulated with respect to the hydrophobicity of their active center. We also discuss our strategies to identify the amino acid residues which affect the transglycosylation activity of neopullulanase.

MATERIALS AND METHODS

Media

Luria broth (21) was used for the culture of Escherichia coli and enzyme preparation. 2 × YT broth (19) was used to prepare phage DNA. Ampicillin was used at a concentration of 100 mg/ml.

Bacterial Strain, Plasmid, and Phages

E. coli TG-1 (supE hisD5 thi Δlac-proAB- lac ΔlacIΔM15) (22) was used as a host for site-directed mutagenesis and for preparation of wild-type and mutated neopullulanase. Plasmids pPP10 (Tc proAB- lac IΔM15) and pUNP129 (Ap proAB- lac IΔM15) were described previously (19). M13mp19 was used to prepare single-stranded DNA (23).

**TABLE I**

| Substrate                      | Hydrolysis by α-Amylase° | Neopullulanase |
|--------------------------------|--------------------------|----------------|
| Starch                         | ++                       | +              |
| Pullulan                       | –                        | +              |
| 6°-O-α-Glucosyl-maltotriose    | –                        | +              |
| 6°-O-α-Maltosyl-maltose        | –                        | +              |
| The smallest limit dextrin     | –                        | +              |
| from starch                    |                          | Panose         |
| Glucosylmaltotriose            |                          | Panose         |

° Taka-amylase A. Data are from Ref. 44.

DNA Manipulation and Site-directed Mutagenesis

Plasmid DNA was prepared by either the rapid alkaline extraction method (24) or the QIAGEN plasmid kit (QIAGEN Inc., Chatsworth, CA). Treatment of DNA with restriction enzyme and ligation of DNA with T4 ligase were done as recommended by the manufacturer. To isolate the DNA fragment from agarose gels after electrophoresis, a Gene Clean kit (Qiagen) was used. Site-directed mutagenesis was done with an oligonucleotide-directed in vitro mutagenesis system (Amersham Corp.). Chemically synthesized oligonucleotides (19–30-mer) were used to generate site-directed mutants. The mutations were confirmed by the dideoxy chain terminating method.
as described previously (11). The sequence reaction started from the M13 linker region with the universal primer or was primed by internal annealing 17-mer synthetic oligonucleotides. The 913-base pair SalI-EcoRI fragment of plasmid pPP10 was cloned into the SalI-EcoRI sites of phage M13mp19 multiple-cloning sites. The single-stranded DNA was prepared from the phage and used as the template for site-directed mutagenesis. Sequence analysis of the 913-base pair SalI-EcoRI fragment verified that site-directed mutagenesis introduced the desired nucleotide change without second-site mutations. The 913-base pair SalI-EcoRI fragment containing the mutation was excised from M13mp19 double-stranded replicative form DNA. Then the wild SalI-EcoRI fragment in pUNP129 was replaced by this fragment with mutation, described previously (19). The wild-type and mutated nplT genes were expressed in E. coli TG-1 under control of the lac promoter on pUNP129 by induction with 1 mM isopropyl-β-D-thiogalactopyranoside.

FIG. 2. Models of the enzyme-substrate complex for wild-type and mutated neopullulanases. The complexes for wild-type (a and b) and I358W (c and d) enzymes with maltotetraose (a and c) and 6'-O-α-maltosyl-maltose (b and d) are exhibited. The main chain and the side chain of the catalytic residues (E357, Glu357; D328, Asp-328; D424, Asp-424) are, respectively, indicated by a yellow tube and red sticks. The side chain of the amino acid residue at position 358 is emphasized by space filling model (green). The substrate molecules are exhibited by stick models: carbon (gray) and oxygen (red). The reducing end of each substrate is located on the upper side of the figures. The glucosidic linkage cleaved by hydrol ysis was pointed by a black arrow.
### Table II

| Specific activity (units/mg) | Pululan (P) | Starch (S) | Ratio, P/S |
|-----------------------------|------------|------------|------------|
| Wild-type                   | 28.6 ± 1.3* | 13.7 ± 0.6 | 2.09       |
| 1358W                      | (2.77 ± 0.17) × 10⁻³ | (6.74 ± 0.36) × 10⁻² | 4.11 × 10⁻² |
| 1358V                      | 18.2 ± 0.2  | 7.03 ± 0.12 | 2.59       |

*Values represent means of four independent experiments ± S.D.

### Preparation and Purification of Wild-type and Mutated Neopullulanase

The crude enzyme was prepared from E. coli TG-1 carrying a recombinant plasmid encoding wild-type or mutated nptI, as described previously (19). The crude enzyme was applied to a column (1.6 cm × 10 cm) of Q-Sepharose (Pharmacia, Uppsala, Sweden) that had been equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol. The active enzyme was eluted with a 0–0.4 M NaCl gradient. Ammonium sulfate was added to the enzyme fraction to a final concentration of 0.85 M, and the mixture was applied to a column (1.5 × 10 cm) of phenyl-Toyopearl 650 M (Tosoh, Tokyo, Japan) that had been equilibrated with the same buffer containing 10 mM 2-mercaptoethanol and 0.85 M ammonium sulfate. The active fraction was eluted with a 0.85–0 M ammonium sulfate gradient. The wild-type and mutated neopullulanases were finally purified to homogeneity using a PL-SAX HPLC column (0.46 × 20 cm; Polymer Laboratories, UK).

### Assay of Neopullulanase Activity

Pullulan-hydrolyzing or starch-hydrolyzing activities of the wild-type and mutated neopullulanases were assayed by measuring the amount of reducing sugars released from each substrate. The reaction mixture contained 0.5% pullulan or starch in 0.1 M sodium phosphate buffer (pH 6.0) and the enzyme. The reaction was stopped after an appropriate incubation period at 40 °C by the addition of 3,5-dinitrosalicylaldehyde (18); 1 unit of enzyme activity was defined as the amount of enzyme which released 1 μmol of reducing sugar as glucose/min under the standard assay conditions described above. The enzyme activity was also assayed by measuring the amount of glucose obtained from maltotriose as a substrate. The reaction mixture consisted of 1% maltotriose in 50 mM sodium phosphate buffer (pH 6.0) and the enzyme. The reaction was stopped by boiling for 5 min, and the amount of glucose was measured by the glucose-oxidase/peroxidase method (Gluco B-test; Wako Pure Chemical Industries, Osaka, Japan). 1 unit of enzyme activity was defined as the amount of enzyme which released 1 μmol of glucose per min.

### Analysis of Reaction Products

The sugar composition of the reaction products was quantitatively analyzed by high-performance liquid chromatography with a LiChrospher NH₄ column (Merck AG, Darmstadt, Germany) and an Aminex HPX-42A column (Bio-Rad) as described previously (1, 26).

### Preparation of Branched Oligosaccharides for Substrates

6-O-α-Maltosyl-maltose was obtained from panose and starch by the coupling reaction with cyclomaltodextrin glucanotransferase (18). The oligosaccharide was purified by preparative paper chromatography (18). 6-O-α-Glucosyl-maltotriose was purchased from Sigma.

### Computer-aided Molecular Modeling

Homology Modeling of Neopullulanase—The strategy of our homology modeling is as follows: 1) homology search from Protein Data Bank (PDB) and 2) alignment. The algorithm (27) we used is a variant of the Needleman and Wunsch algorithm (28), but it improves the accuracy of the alignment owing to the introduction of the concept of hydrophobic core scores (29). 3) Loop searching and loop replacement when insertion/deletion sites are required. 4) Side chain replacement. 5) Removal of the steric hindrances between side chains. Here, 1,000 random conformational searches for each side chain, and 1,000,000 for whole side chains were carried out by using random numbers which reproduce normal distribution of dihedral angles, experimentally found in crystals (30). 6) Energy minimization. The force field parameters of AMBER version 3.0 revision A (31) were used with cut-off distance of 10 Å for non-bonded interactions and distance-dependent dielectric constant. Conjugate gradient optimization was carried out with a termination threshold of the maximum component of the energy gradients of 0.1 kcal/mol Å⁻¹.

Modeling of Substrates—The model structure of maltotetraose was built by linking α-(1→4)-glucosidic bonds, based on the crystal structure (32) of α-α-glucose which has the C₆, C₁, chain form. Here, any oligosaccharide or its fragments are assumed to have a left-handed regular helical structure with 6.6 glucoses per turn, using two dihedral angles (33) of φ(H₁-C₁-Oglyc-C₄') = −15° and ψ(C₁-Oglyc-C₄'-C₃') = −15° for the glucosidic linkages. Another substrate, 6-O-α-maltosyl-maltose, was also built using the crystal structure of α-panose (34) for its α-(1→6)-glucosidic linkage conformational analysis.

Docking of Substrates and Mutagenesis in Model—Aspergillus oryzae α-amylase (Taka-α-amylase A)-maltotetraose complex structure, which we had already predicted (2), was used as a template. The structure for the neopullulanase-maltotetraose complex was obtained by a least-squares fit of the neopullulanase modeled above to this template, where the relatively smaller maltotetraose was created from the 3rd to 6th units of the maltotetraose of the template. Another substrate, 6-O-α-maltosyl-maltose, was docked into the cleft, on the assumption that the binding mode of its 1st and 2nd residues is the same as that of the 1st and 2nd of the maltotetraose. In this way, we obtained structures of the wild-type enzyme complexed with maltotetraose or 6-O-α-maltosylmaltose. Basium sulfate was used and the cleft for the amino acids which were likely to affect the substrate specificity, and finally we focused on position 358 (see "Results"). Structural models for the mutants I358W and I358V at this position were constructed in the following way. First, the side chain of Ile-358 was changed into that of Trp (or Val) by fitting their side chain heavy atoms as well as possible. Then all dihedral angles in the side chain of the substituted residue Trp (or Val) were rotated so that it had the most preferable van der Waals contacts with other side chains and the substrate.

A program package for protein engineering and drug design, BIO-CES[E] (NEC Corp., Japan), was used for all the above molecular modeling and docking studies. This package runs on an NEC workstation EWS4800 with a graphic display of PS-390, or on an IRIS Indigo.

### Molecular Dynamics Simulation of the Complex Model—Neopullulanase was enclosed with the crystallographically determined waters (35) of Taka-α-amylase A, and the overlapping waters were removed, so that 177 waters were retained. Terminal and charged residues were assumed to be in their normal states, except that Glu-357 was assumed to be in an ionized state. We used OPLS (36)/AMBER (31) force field for protein, and GROMOS field (37) for glucose chains, and TIP3P for waters. The waters were constrained to their initial positions, although very weakly, with a force constant of 0.1 kcal/mol Å⁻². Additional restraints were imposed on the following hydrogen-bonded atomic pairs between enzyme-substrate: Asp-328:OD1-Gic-4C1, Asp-328:OD2-Glic-4C1, Glu-357:OE2-Glic-504, His-423:NE2-Gic-402, His-247:NE2-Gic-406, Asp-472:NH1-Gic-302, Asp-468:OD1-Gic-303. These pairs were chosen on the basis of the x-ray structure (38) of the complex between maltose and cyclomaltodextrin glucanotransferase from Bacillus circulans whose structure is considered to be similar to neopullulanase. Minimization were carried out until the root mean square gradient dropped to about 0.02 kcal/mol Å⁻¹, and then 30 ps molecular dynamics simulation was performed at 300 K with a time step of 1 fs. All calculations were carried out with a newly developed molecular dynamics program (39) by the NEC members of the authors.

### Other Procedures

The protein concentration was measured by the Bio-Rad protein assay kit (Bio-Rad) with bovine γ-globulin as the standard. Agarose gel electrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were done under standard conditions (22). Transformation of E. coli with plasmid DNA was done as described elsewhere (40). Cyclomaltodextrin glucanotransferase from Bacillus macerans was purchased from Amano Pharmaceutical Co., Nagoya, Japan. Unless otherwise specified, the sources of all other reagents have been described previously (41, 42).

### RESULTS

Computer-aided Model of the Three-dimensional Structure of Neopullulanase and Prediction of Amino Acid Residue Which...
May Affect the Substrate Preference—From the result of homology search, Taka-amylase A was selected as the most suitable template for homology modeling. The coordinates of the atoms for Taka-amylase A (entry ID, 2TAA (6) and 6TAA (35)), were obtained from the Protein Data Bank at the Brookhaven National Laboratory. The final alignment between neopullulanase and Taka-amylase A amino acids sequences is shown in Fig. 1.

Although there were several insertions and deletions in the primary structure of neopullulanase, we concluded that the secondary structure of neopullulanase, which had a structure basically the same as that of Taka-amylase A (6), composed of (αβ)8 barrel and antiparallel β-sandwich. This is because most of the secondary structures corresponding to Taka-amylase A were found not to be destroyed by such insertions and deletions, and all the catalytic sites and main substrate-binding sites were preserved. The differences in specificities between Taka-amylase A, as a typical starch-saccharifying α-amylase, and neopullulanase are summarized in Table 1.

Ile-358 is located just next to the catalytic site Glu-357. The residue corresponding to this position 358 varies among the α-amylase family (1), but its hydrophobicity is consistently well conserved. Its position shown in Fig. 2, a and b, is also suggestive of its important role in controlling substrate specificity. For these reasons, we identified Ile-358 as a target for substitution to alter the neopullulanase specificity.

Model of the Enzyme-Substrate Complex for the Mutated Neopullulanase—According to the model for the enzyme-substrate complex, maltotetraose fit well into the active cleft of the wild-type neopullulanase (Fig. 2a). 6′′-O-α-Maltosyl-maltose also fit well into the cleft (Fig. 2b). However, their fitting patterns with residue 358 are considerably different from each other because the α-(1→6)-branching pushes Glc-3 of the substrate chain slightly to the side chain of residue 358. For this reason, it was most likely that the acceptability of 6′′-O-α-maltosyl-maltose as a substrate would be increased by the replacement of Ile-358 with Val (I358V mutation) which was functionally similar to Ile and had a smaller side chain. In contrast, the acceptability of 6′′-O-α-maltosyl-maltose would be seriously reduced by the replacement of Ile-358 with Trp (I358W mutation), causing a steric hindrance between O-3 of Glc-3 and the side chain of Trp-358 (Fig. 2d). In our models, the distance between Trp-358C23 and Glc-3O3 in 6′′-O-α-maltosyl-maltose was 1.6 Å. It is much shorter than the shortest distance between Trp-358 and maltotetraose (Trp-358C23 and Glc-3O2), which is 2.5 Å. In this context, it was likely that I358V and I358W (Fig. 2c) mutations would not seriously affect the enzyme-substrate complex when maltotetraose was used as the substrate.

Because Ile, Trp, and Val are all nonpolar hydrophobic amino acids, we expected that the alteration of enzyme characteristics would probably be caused by only the volume of the side chain of the amino acid residue at position 358.

Preparation and Characterization of Wild-type, I358W, and I358V Mutated Neopullulanases—Wild-type and mutated neopullulanase were purified from the cell extract of E. coli TG-1 carrying a plasmid which encoded wild-type or mutated nplT, respectively (figure not shown). Western immunoblot analysis (43) showed that I358V and I358W mutated enzymes reacted against anti-neopullulanase antiserum to the same degree as the wild-type enzyme (figure not shown). Because I358W enzyme barely hydrolyzed pullulan, but hydrolyzed starch, we evaluated enzyme activity for starch as a substrate. The specific activity of I358W enzyme for pullulan was less than 1/24 of that for starch (Table II). When we used the same amount of enzymes on a starch-hydrolyzing activity basis, wild-type, I358W, and I358V enzymes exhibited almost the same.

Fig. 3. Time course of hydrolyses of maltotetraose (a), 6′′-O-α-maltosyl-maltose (b), and 6′′-O-α-glucosyl-maltotriose (c). The schematic representation of the reaction is shown above the results. The reaction mixture, consisting of 0.5% substrate in 0.1 M sodium phosphate buffer (pH 6.0) and wild-type (○), I358V (△), or I358W (●) enzyme (4 units/g of substrate). The remaining substrates were quantitatively analyzed by high-performance liquid chromatography. The activities of the wild-type and the mutated neopullulanase were evaluated using starch as a substrate. Symbols: ○, glucose; △, glucose with a reducing end; ●, α(1→4)-linkage; △, α(1→6)-linkage; ●, enzyme reaction. Each point represents the mean value of two independent experiments. Similar data were obtained in each experiment.
activity on maltotetraose (Fig. 3a). However, the activities of these three enzymes on 6\(^2\)-O-\(\alpha\)-maltosyl-maltose and 6\(^2\)-O-\(\alpha\)-glucosyl-maltotriose were quite different (Fig. 3, b and c). The activities of the I358W enzyme were less than 30 and 15% of those of the wild-type neopullulanase when 6\(^2\)-O-\(\alpha\)-maltosyl-maltose and 6\(^3\)-O-\(\alpha\)-glucosyl-maltotriose were used as substrates, respectively (Fig. 3, b and c). In contrast, the activities of the I358V enzyme on these \(\alpha\)-(1→6)-branched oligosaccharides were significantly higher than that of the wild-type enzyme (Fig. 3, b and c). These results are consistent with the model of the enzyme-substrate complex, described above (Fig. 2).

Pullulan and Starch Hydrolyses by Wild-type and Mutated Neopullulanases—The specificities of mutated neopullulanase toward pullulan and starch were compared with those of wild-type neopullulanase. The specific activities of wild-type neopullulanase for pullulan and starch were 28.6 and 13.7 units/mg, respectively. Although the specific activity of the I358W enzyme was very low, that of the I358V enzyme was retained at more than 50% of that of the wild-type neopullulanase (Table II). When the preference for pullulan was expressed as the ratio of the specific activities for pullulan to starch, the value of wild-type neopullulanase was 2.09. The value of I358W and I358V mutated enzymes were 0.0011 and 2.59, respectively (Table II).

We analyzed the smallest limit of dextrin from starch produced by wild-type and mutated neopullulanase. The reaction mixture (500 \(\mu\)l) consisted of 0.5% starch in 100 mM sodium phosphate buffer (pH 6.0) and 0.04 unit of enzyme was incubated at 40 °C. The reaction was stopped by treatment at 100 °C for 5 min after the end point of each reaction was reached (140 h). The hydrolysis products were analyzed by high-performance liquid chromatography. While wild-type neopullulanase produced panose as the smallest limit dextrin (Fig. 4a), the I358W enzyme produced 6\(^2\)-O-\(\alpha\)-glucosyl-maltotriose (Fig. 4b). The I358V enzyme produced panose, the same smallest dextrin as the wild-type neopullulanase (data not shown).

The results described above can be thus summarized. The I358W mutation reduced the specificity of neopullulanase toward \(\alpha\)-(1→6)-branched oligosaccharides such as 6\(^2\)-O-\(\alpha\)-maltosyl-maltose and 6\(^3\)-O-\(\alpha\)-glucosyl-maltotriose. The I358W enzyme barely hydrolyzed pullulan, whereas it did hydrolyze starch. The I358W enzyme produced 6\(^2\)-O-\(\alpha\)-glucosyl-maltotriose as the smallest dextrin from starch. These characteristics of the I358W enzyme were quite different from those of wild-type neopullulanase and rather similar to those of typical saccharifying \(\alpha\)-amylase, such as Taka-amylase A (44) (Table I). In contrast, the I358V mutation increased the specificity of neopullulanase toward \(\alpha\)-(1→6)-branched oligosaccharides such as 6\(^2\)-O-\(\alpha\)-maltosyl-maltose and 6\(^3\)-O-\(\alpha\)-glucosyl-maltotriose. The specificity of neopullulanase toward pullulan was consequently increased by the I358V mutation.

A Possible Reaction Mechanism of Neopullulanase and Strategies to Control the Transglycosylation Activity—Based on our complex model, a possible mechanism to explain both the hydrolysis and transglycosylation by neopullulanase was proposed as shown in Fig. 5. The hydrolysis reaction model of Taka-amylase A, which we had previously proposed,\(^2\) was used here to reveal this mechanism. The coordinates of the water molecule which was used for hydrolysis were predicted from a refined structure of Taka-amylase A (6TAA, Protein Data Bank at Brookhaven National Laboratory) (35). Maltotriose was used as a substrate in Fig. 5. The hydrogen atom of un-ionized Glu-357 is added to the oxygen atom to cleave the glucosidic linkage (Fig. 5a). After releasing the reducing-end glucose (Fig. 5b), a water molecule or another maltotriose molecule is introduced to the carbonium cation intermediate (Fig. 5c). The mechanisms for transglycosylation have been explained by bisubstrate processes (1, 45): two molecules of substrate enter the active center in rapid succession. In the case of hydrolysis, the water molecule, located near the glucosidic linkage which should be cleaved, attacks the carbonium cation intermediate (46). Based on the reaction model, we predicted that the transglycosylation activity could be increased by inhibiting the incoming of the attacking water molecule (Fig. 5d).

As shown in Fig. 5, there are three residues on the entrance path of the attacking water molecule: Tyr-377, Met-375, and Ser-422. Therefore, we tried to increase the hydrophobic environment on the path of the water molecule by replacing these three residues. To increase the hydrophobicity, Tyr-377, Met-375, and Ser-422 were replaced by Phe, Leu, and Val, respectively. We employed the following two criteria to determine the amino acid residues to be introduced. The side chain of the amino acid residue should be smaller than or comparable to that of the original amino acid residue. Amino acid residues which might destroy the hydrogen bond network between the enzyme, the substrate, and the water molecule should not be used. In this context, Tyr-377 was also replaced with Asp or Ser to ensure that the hydrophilic residue would introduce the water molecule and promote hydrolysis.

Preparation and Characterization of Y337F, M375L, S422V, Y377D, and Y377S Mutated Neopullulanases—Wild-type and mutated neopullulanases were purified from the cell extract of E. coli TG-1 carrying a plasmid which encoded wild-type or mutated npT, respectively. Western immunoblot analysis (43) showed that all mutated enzymes were immunologically indistinguishable from the wild-type neopullulanase (figure not shown). The specific activity of the mutated neopullulanases varied depending on the mutation and the substrate (Table III).

Transglycosylation Activity of Wild-type and Mutated Neopullulanase—Maltotriose is the most simple substrate for
Controlling Specificity of Neopullulanase

Fig. 5. A possible mechanism of hydrolysis and transglycosylation by neopullulanase. The progress of \(\alpha-(1\rightarrow4)\) transglycosylation reaction is shown. a, binding of maltotriose to the active center. The attack of the proton of Glu-357 (in un-ionized form) on the glucosidic linkage releases the reducing end glucose (transparent ball and stick). b, cleavage of glucosidic linkage and formation of carbonium cation intermediate. The intermediate is stabilized by Asp-328. c, incoming of water molecule or another maltotriose molecule into the active cleft. d, formation of maltopenaose as the result of the attack of the maltotriose molecule on the intermediate. This completes the \(\alpha-(1\rightarrow4)\) transglycosylation reaction. If the water molecule attacks the intermediate, the hydrolysis reaction would be completed with formation of maltose. The main chain of neopullulanase is indicated by a yellow tube. The side chain of catalytic residue (E 357, Glu-357; D 328, Asp-328; D 424, Asp-424) are shown by red sticks. The side chains of the target amino acid (Y 377, Tyr-377; M 375, Met-375; S 422, Ser-422) selected for mutagenesis are shown by green sticks. Maltooligosaccharides are illustrated by a ball and stick model where carbons and oxygen are gray and red, respectively. The water molecule is indicated by a blue sphere of 1.4 Å radius. The reducing end of maltooligosaccharides is located in the upper part of the figure.

Table III Specific activities of wild-type and mutated neopullulanases for three substrates

| Mutation | Pullulan | Specific activity (units/mg) | Starch | Maltotriose |
|----------|----------|----------------------------|--------|-------------|
| Y377F   | 2.47 ± 0.10a | 1.96 ± 0.16 | 0.668 ± 0.028 |
| M375L   | 3.38 ± 0.06 | 3.60 ± 0.08 | 2.60 ± 0.11 |
| S422V   | 0.495 ± 0.043 | 1.48 ± 0.20 | 0.0406 ± 0.0042 |
| Y377D   | 0.126 ± 0.005 | 0.257 ± 0.010 | 0.0660 ± 0.0025 |
| Y377S   | 0.136 ± 0.004 | 0.302 ± 0.011 | 0.0762 ± 0.0029 |
| Wild-type | 28.6 ± 1.3 | 13.7 ± 0.6 | 16.6 ± 0.7 |

a Values represent means of four independent experiments ± S.D.

neopullulanase (1, 18). Neopullulanase hydrolyzes maltotriose to produce glucose and maltose. The enzyme also produces maltotetraose and \(\alpha-(1\rightarrow4)\)-linked oligosaccharides with degree of polymerization \(\geq 4\) by \(\alpha-(1\rightarrow4)\) transglycosylation and isomalto, panose, isopanose, and \(\alpha-(1\rightarrow6)\)-branched oligosaccharides with degree of polymerization \(\leq 4\) by \(\alpha-(1\rightarrow6)\) transglycosylation from maltotriose (Fig. 6) (1, 47). Therefore, we used maltotriose as a substrate and evaluated hydrolysis and transglycosylation activities by measuring the amount of glucose plus maltose and maltotetraose, \(\alpha-(1\rightarrow4)\)-linked oligosaccharides with degree of polymerization \(\geq 4\), isomalto, panose, isopanose, plus \(\alpha-(1\rightarrow6)\)-branched oligosaccharides with degree of polymerization \(\leq 4\), respectively. The reaction mixture (200 \(\mu\)l) contained 10% maltotriose and 0.025 unit of wild-type or mutated neopullulanase in 10 \(mM\) sodium phosphate buffer (pH 6.0). Fig. 7 shows the time course of the composition of maltotriose, hydrolysis products, and transglycosylation products. The amount of the hydrolysis products and transglycosylation products were compared at the point where the substrate (maltotriose) was digested by 40% (Fig. 7). Wild-type neopullulanase produced 22.9% of hydrolysis products and 17.1% of transglycosylation products when 60% of maltotriose remained in the reaction mixture (Fig. 7a). Y377F mutated enzyme produced 19.1% of hydrolysis products and 20.9% of transglycosylation products (Fig. 7b). The ratio of products was inverted compared with that of the wild-type. In contrast, Y377D mutated enzyme produced 23.8% of hydrolysis products and 16.2% of transglycosylation products (Fig. 7c). More hydrolysis products were obtained by the mutant than by the wild-type and transglycosylation was suppressed. These data and the data for other mutated enzyme are presented in Table IV. All the mutants shown in this table behaved according to our expectations.

Discussion

We succeeded in controlling the specificity of neopullulanase toward \(\alpha-(1\rightarrow6)\)-branched oligosaccharides and pullulan. Although we could obtain mutated neopullulanase exhibiting similar characteristics of \(\alpha\)-amylase, the specific activity decreased significantly. This fact may mean that the bulky side chain of Trp perturbed even the backbone conformation of its nearby sites and subsequently influenced the catalytic sites. Whether the perturbation is large or small will be examined by more realistic model structures of the complexes; we are now planning to refine the present structures by more detailed molecular dynamics simulations. The enzyme-substrate complex also needs further consideration to explain the different specificities of wild-type, I358W, and I358V enzymes toward \(6^\circ\)-\(\alpha\)-glucosyl-maltotriose.

We also succeeded in increasing the transglycosylation activity of neopullulanase by increasing the hydrophobicity on the entrance path of the attacking water molecule, which is
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![Diagram showing the reaction of maltotriose with different enzymes](image)

**Fig. 6. Products of hydrolysis and transglycosylation reaction from maltotriose.** When neopullulanase acts on maltotriose, the enzyme catalyzes one of the following three reactions: hydrolysis, α-(1→4) transglycosylation, and α-(1→6) transglycosylation. The products from each reaction are shown. Symbols are as in Fig. 3.

![Graphs showing the time course of the composition of reaction products with wild-type and mutated neopullulanases](image)

**Fig. 7. Time course of the composition of reaction products with wild-type and mutated neopullulanases.** The sugar compositions in the reaction mixtures with wild-type (a), Y377F (b), and Y377D (c) neopullulanases at various incubation times are shown. ●, maltotriose (G3); □, hydrolysis products (H); ○, transglycosylation products (T).

| Mutation | Hydrolysis products (%) | Transglycosylation products (%) | Ratio of products (T/H) |
|----------|-------------------------|-------------------------------|------------------------|
| Y377F    | 19.1 ± 0.4              | 20.9 ± 0.4                   | 1.09                   |
| M375L    | 20.5 ± 0.3              | 19.0 ± 0.3                   | 0.95                   |
| S422V    | 20.0 ± 0.4              | 20.0 ± 0.4                   | 1.00                   |
| Y377D    | 23.8 ± 0.3              | 19.0 ± 0.3                   | 0.88                   |
| Y377S    | 23.2 ± 0.2              | 16.8 ± 0.2                   | 0.72                   |
| Wild-type| 22.9 ± 0.2              | 17.1 ± 0.2                   | 0.75                   |

a The sum of glucose and maltose.

b The sum of isomaltose, panose, isopanose, and dixigosaccharides with degree of polymerization > 4.

c Values represent means of four independent experiments ± S.D.

According to the figure, the transglycosylation activity of *Saccharomycopsis* α-amylase could be increased by the replacement of Trp-84 with Leu. They suggested that the Trp residue might play an important role not only in holding the substrate, but also in liberating the hydrolysis products from the substrate binding pocket. However, it was not clear in their paper why the transglycosylation activity could be increased. From the results described in this paper, one general point becomes very clear: the environment of the active center is important in determining the reaction mode, either hydrolysis or transglycosylation.

Further work is now in progress to convert neopullulanase to glucanotransferase without hydrolysis activity or to glucohydrolase without transglycosylation activity.

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