Analysis of the Active Center of *Bacillus steaerotherophilus* Neopullulanase

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The active center of the neopullulanase from *Bacillus steaerotherophilus* was analyzed by means of site-directed mutagenesis. The amino acid residues located in the active center of the neopullulanase were tentatively identified according to a molecular model of *Taka-amylase A* and homology analysis of the amino acid sequences of neopullulanase, *Taka-amylase A*, and other amylolytic enzymes. When amino acid residues Glu and Asp, corresponding to the putative catalytic sites, were replaced by the oppositely charged (His) or noncharged (Gln or Asn) amino acid residue, neopullulanase activities toward α-(1→4)- and α-(1→6)-glucosidic linkages disappeared. When the amino acids corresponding to the putative substrate-binding sites were replaced, the specificities of the mutated neopullulanases toward α-(1→4)- and α-(1→6)-glucosidic linkages were obviously different from that of the wild-type enzyme. This finding proves that one active center of neopullulanase participated in the dual activity toward α-(1→4)- and α-(1→6)-glucosidic linkages. Pullulan is a linear glucan of maltotriosyl units linked through α-(1→6)-glucosidic linkages. The production ratio of panose from pullulan was significantly increased by using the mutated neopullulanase which exhibited higher specificity toward the α-(1→4)-glucosidic linkage. In contrast, the production ratio of panose was obviously decreased by using the mutated neopullulanase which exhibited higher activity toward the α-(1→6)-glucosidic linkage.

Neopullulanase has been reported to be a new type of pullulan-hydrolyzing enzyme from *Bacillus steaerotherophilus* TRS40 (8). The enzyme mainly hydrolyzes the α-(1→4)-glucosidic linkages of pullulan to produce panose (6-O-α-glucosyl-maltose). Neopullulanase could hydrolyze pullulan efficiently and hydrolyzed only a small amount of starch (8). Further studies revealed that neopullulanase hydrolyzed not only α-(1→4)-glucosidic linkages but also specific α-(1→6)-glucosidic linkages of several branched oligosaccharides (5). The gene for neopullulanase (npIT) has been cloned (8) and sequenced (7).

We have previously reported the existence of the four highly conserved regions in α-amylases (EC 3.2.1.1), neopullulanase, isoamylase (EC 3.2.1.68), pullulanase (EC 3.2.1.41), and cyclodextrin glucanotransferases (EC 2.4.1.19) (7, 9, 13). These regions are most likely to constitute the active center of each enzyme (7, 10). Among these amylolytic enzymes, only α-amylases from *Aspergillus oryzae* (Taka-amylase A) (10) and from porcine pancreas (1) have been investigated by X-ray crystallographic analysis.

Recently, other pullulan-hydrolyzing enzymes that hydrolyze both α-(1→4)- and α-(1→6)-glucosidic linkages have been reported (2, 15, 16). Melasniemi et al. (11) reported that the primary structure of the α-amylase-pullulanase from *Clostridium thermohydrosulfuricum* closely resembled that of neopullulanase. This α-amylase-pullulanase also has dual activity toward α-(1→4)- and α-(1→6)-glucosidic linkages (11). The authors suggested that two of the four highly conserved regions mentioned above were duplicated in this enzyme (11).

On the other hand, Plant et al. (15) suggested that the same active center participated in the dual activity toward α-(1→4)- and α-(1→6)-glucosidic linkages, as indicated by studies of chemical modification and enzymatic kinetics of *Thermoanaerobium* sp. pullulanase. However, no direct proof that the dual activity of such pullulan-hydrolyzing enzymes toward α-(1→4)- and α-(1→6)-glucosidic linkages exists in the same active center has been reported.

In this report, we present evidence that one active center of *B. steaerotherophilus* neopullulanase participates in the dual activity toward α-(1→4)- and α-(1→6)-glucosidic linkages. The different specificities toward α-(1→4)- and α-(1→6)-glucosidic linkages of some mutated neopullulanases are also described.

MATERIALS AND METHODS

**Media.** L broth (4) was used for culture of *Escherichia coli* and enzyme preparation. 2× YT broth (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl in 1 liter of deionized water) was used for the preparation of phage DNA. Ampicillin was used at a final concentration of 100 μg/ml.

**Bacterial strains, plasmids, and phages.** *E. coli* TG-1 [supE hsdS5 thi Δ(lac-proAB) F' (traD36 proAB*) lacFlacZΔM15)] (17) was used as a host for the site-directed mutagenesis and for preparation of wild-type and mutated neopullulanases. pUC119 (19) was used as a vector plasmid, and phage M13mp18/19 was used for preparation of single-stranded DNA (12). Plasmid pPP10 (Tc' npIT') [structural gene of the neopullulanase from *B. steaerotherophilus* TRS40] was described previously (8).

**Site-directed mutagenesis.** Mutagenesis was done with an oligonucleotide-directed in vitro mutagenesis system (Amer-sham Corp., Amersham, England). Chemically synthesized oligonucleotides (18-mer to 23-mer) were used. The mutations were confirmed by DNA sequencing. DNA sequencing was done by the dideoxy chain-terminating method (18) as described previously (7). The sequence reaction started from the M13 linker region with the universal primer or was

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prised by internally annealing 17-mer synthetic oligonucleotides.

**Transformation.** Transformation of *E. coli* with plasmid DNA was done as described previously (6).

**Preparation of wild-type and mutated neopullulanase.** *E. coli* TG-1 carrying a recombinant plasmid encoding wild-type or mutated *npt* was incubated overnight in 5 ml of L broth at 37°C. Then 100 ml of L broth in a 500-ml Sakaguchi flask was inoculated with 1 ml of the overnight preculture and shaken at 37°C to mid-log phase (optical density at 660 nm = 0.6). Isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration, 1 mM) was added as the inducer of the lac promoter, and cultivation was continued at 30°C for 24 h from the inoculation time. An 80-ml cell culture sample was harvested by centrifugation (8,000 × g, 30 min) and washed with 40 ml of 20 mM sodium phosphate buffer (pH 6.5). The cell pellet was then suspended in the same buffer containing 10 mM 2-mercaptoethanol, and the total volume was adjusted to 20 ml. The cells were disrupted by sonication (19.5 kHz, four times for 30 s each with 30-s intervals) in an ice-ethanol bath, and the remnant was removed by centrifugation (27,000 × g, 30 min). The cell extract from *E. coli* TG-1 carrying a vector plasmid exhibited no activity on pullulan and malto-oligosaccharides. Furthermore, we could find no difference in activity among the neopullulanases purified from *B. stearothermophilus* TRS40, *Bacillus subtilis* NA-1(pPP10), and the crude enzyme prepared from *E. coli* TG-1. Therefore, the cell extract from *E. coli* TG-1 carrying a recombinant plasmid encoding wild-type or mutated *npt* was directly used for the analysis of enzyme activity.

**Assay of neopullulanase activity.** Wild-type and mutated neopullulanase activities were assayed at 40°C as described previously (5).

**Analysis of hydrolysis products.** Analytical paper chromatography was carried out in the ascending mode on Toyo no. 50 filter paper as described previously (5). High-performance liquid chromatography (8) was also used for analysis of the reaction products.

**Preparation of branched oligosaccharides for substrates.** 6'-O-α-Glucosyl-maltotriose was prepared from the hydrolysate of maize amylopectin by saccharifying α-amylase (5). 6'-O-α-Maltosyl-maltose was obtained from panose and starch by the coupling reaction with cyclodextrin glucanotransferase (5). These oligosaccharides were purified by preparative paper chromatography (5).

**Protein assay.** Protein concentration was measured by the Bio-Rad protein assay kit (Bio-Rad, Richmond, Calif.), with bovine γ-globulin as the standard.

**Other procedures.** Plasmid DNA was prepared by either the rapid alkaline extraction method or CsCl-ethidium bromide equilibrium density gradient centrifugation as described previously (4). Treatment of DNA with restriction enzymes or DNA polymerase I (Klenow fragment) and ligation of DNA with T4 ligase were done as recommended by the manufacturer. Cyclodextrin glucanotransferase from *Bacillus macerans* was purchased from Amano Pharmaceutical Co., Ltd., Nagoya, Japan. Unless otherwise specified, all chemicals used were from Wako Pure Chemical Industries, Osaka, Japan.

**RESULTS**

Tentative identification of amino acid residues constituting the active center of the neopullulanase. We previously identified four highly conserved regions in α-amylases, neopullulanase, isoamylase, pullulanase, and cyclodextrin glucanotransferases (7, 9, 13). Using the alignment of amino acid residues in the highly conserved regions, a molecular model and catalytic residues of *Taka*-amylase A (10) were used for analysis of these enzymes. Glu-230 and Asp-297 were proposed as catalytic sites of *Taka*-amylase A, and His-122, Asp-206, Lys-209, His-210, and His-296 were proposed as substrate-binding sites (10). These catalytic and substrate-binding sites were surprisingly conserved in the amylolytic enzymes mentioned above (7).

These facts suggested that Glu-357 and Asp-424 of neopullulanase, which corresponded to Glu-230 and Asp-297 of *Taka*-amylase A, respectively, might act as catalytic sites (7) (Fig. 1). It was also suggested that His-247, Asp-328, Asn-331, Gln-332, and His-423 of neopullulanase, corresponding to His-122, Asp-206, Lys-209, His-210, and His-296 of *Taka*-amylase A, might act as substrate-binding sites or at least constitute the active center (7) (Fig. 1). If the same active center of neopullulanase participated in the dual activity toward α(1→4)- and α(1→6)-glucosidic linkages, we should obtain the following results: (i) neopullulanase simultaneously loses activity toward both glucosidic linkages following the replacement of Glu-357 or Asp-424, and (ii) the enzyme specificity toward each glucosidic linkage can be altered by the replacement of His-247, Asp-328, Asn-331, Glu-332, or His-423.

On the basis of this presumption, we identified amino acid residues Glu-357, Asp-424, His-247, Asp-328, Asn-331, Gln-332, and His-423 as targets for substitutions to analyze and manipulate the active center of neopullulanase (Fig. 1).

**Amino acid substitution by site-directed mutagenesis and preparation of wild-type and mutated neopullulanases.** The four highly conserved regions which are most likely to constitute the active center of neopullulanase are encoded by the 913-bp *SalI-EcoRI* fragment of plasmid pPP10 (7). The *SalI-EcoRI* fragment was cloned into the *SalI-EcoRI* sites of phage M13mp18/19 multiple-cloning sites. The single-stranded DNA was prepared from the phage and used as the template for site-directed mutagenesis (Fig. 2). Sequence analysis of the 913-bp *SalI-EcoRI* fragment verified that site-directed mutagenesis introduced the desired nucleotide change and no second-site mutations. Plasmids were con-
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BamHI, SmalI, were of these

Asterisks denote mutations. HindIII, Sphl, Pstl, SalI, Xbal, BamHI, Smal, KpnI, SacI, and EcoRI cleavage sites are indicated by H, Sp, Ps, Sa, X, B, Sm, K, Sc, and E, respectively.

constructed for easy preparation of wild-type and mutated neopullulanases, as shown in Fig. 2. The SalI-EcoRI fragment in the multiple-cloning site of plasmid pUC119 was removed by digestion of SalI and EcoRI. The cohesive ends of these cleavage sites were filled with DNA polymerase I, and then the blunt ends were ligated with T4 ligase. This deletion plasmid was designated pUC129 (Fig. 2). The whole nptI gene is contained in a 2,154-bp HindIII-Sphl fragment of pPP10 (7). The fragment was cloned in the HindIII-Sphl sites of pUC129. Thus, the recombinant plasmid was designated pUNP129 (Fig. 2). Since both SalI and EcoRI sites were unique in pUNP129, the wild SalI-EcoRI fragment was easily exchanged for the mutated fragment. nptI was located downstream from the lac promoter in pUNP129, and nptI was expressed efficiently in E. coli by the addition of IPTG as an inducer.

E. coli TG-1 carrying a plasmid (pUNP129 or pUNP129*) which encoded wild-type or mutated nptI was cultivated, and a wild-type or mutated enzyme sample, respectively, was prepared.

Activity of mutated neopullulanases. By using the crude enzyme prepared from E. coli, the activities of the 13 mutated neopullulanases were estimated (Table 1). When one of the putative catalytic sites, Glu-357, was replaced with His or Gln (E357H or E357Q mutation), the enzyme activity disappeared. Additionally, neopullulanase lost all activity following the replacement of Asp-328 with His or Asn (D328H or D328N mutation).

When the putative substrate-binding site of neopullulanase, His-247 or His-423, was replaced with Glu (H247E or H423E mutation), we detected 12 or 2.9% of the activity of the wild-type enzyme (Table 1). Alignment of amino acid sequences in the highly conserved region shows that the position of Glu-322 of the neopullulanase corresponds to that of His-210 of Taka-amylase A (Fig. 1) (7). When Glu-332 was replaced with His (E332H mutation), we detected 8.6% of the activity of the wild-type enzyme (Table 1).

We have indicated that the consensus sequence of the last two amino acid residues in the highly conserved region 2 is Lys-His in α-amylases and cyclodextrin glucoamylotransferases (7). These two amino acid residues are different in neopullulanase, isoamylase, and pullulanase (7, 9). Therefore, the two amino acid residues (Asn-331–Glu-332) of neopullulanase were replaced with Lys-His (α-amylase type), Ser-Val (isoamylase type) (7), Gly-Tyr (Klebsiella aerogenes pullulanase type) (7), or Gly-Ile (B. stearothermophilus pullulanase type) (9). We obtained relatively low activities from crude enzyme preparations from E. coli carrying nptI with these mutations (Table 1; N331K-E332H, N331S-E332V, N331G-E332Y, and N331G-G332I, respectively).

**PULLULAN HYDROLYSIS PRODUCTS OF MUTATED NEOPULLULANASES.** Among the mutated neopullulanases indicated in Table 1, H247E, H423E, E332H, and N331S-E332V enzymes, which exhibited enough activity (0.01 U/ml of broth) to enable analysis of their activity patterns, were tested for their products from pullulan. The reaction mixture consisted of 250 μl of 2% pullulan in 0.2 M sodium acetate buffer (pH 6.0) and 250 μl of the enzyme sample. The reaction was stopped by treatment at 100°C for 5 min after the endpoint of each reaction was reached. The hydrolysis products were analyzed by high-performance liquid chromatography. While each of the mutated enzymes produced panose, maltose, and glucose from pullulan, the ratios of the hydrolysis products were obviously different from that of wild-type neopullulanase.

**TABLE 1. Activity yields of various mutated neopullulanases**

| Mutation       | Site and type of mutation (corresponding to amino acid residues of Taka-amylase A) | Activity (U/ml of broth) | Ratio (%) |
|----------------|----------------------------------------------------------------------------------|--------------------------|-----------|
| E357H          | Glu-357 (Glu-230) → His                                                         | ND*                      |           |
| E357Q          | Glu-357 (Glu-230) → Gln                                                         | ND                       |           |
| D424H          | Asp-424 (Asp-297) → His                                                         | ND                       |           |
| D424N          | Asp-424 (Asp-297) → Asn                                                         | ND                       |           |
| D328H          | Asp-328 (Asp-206) → His                                                         | ND                       |           |
| D328N          | Asp-328 (Asp-206) → Asn                                                         | ND                       |           |
| H247E          | His-247 (His-122) → Glu                                                         | 0.051                    | 12        |
| H423E          | His-423 (His-296) → Glu                                                         | 0.012                    | 2.9       |
| E332H          | Glu-322 (His-210) → His                                                         | 0.036                    | 8.6       |
| N331K-E332H    | Asn-331 (Lys-209) → Lys, Glu-322 (His-210) → His                                | 0.003                    | 0.7       |
| N331S-E332V    | Asn-331 (Lys-209) → Ser, Glu-322 (His-210) → Val                               | 0.032                    | 7.6       |
| N331G-E332Y    | Asn-331 (Lys-209) → Gly, Glu-322 (His-210) → Tyr                              | 0.007                    | 1.7       |
| N331G-E332I    | Asn-331 (Lys-209) → Gly, Glu-322 (His-210) → Ile                               | 0.009                    | 2.1       |
| Wild type      |                                                                                  | 0.420                    | 100       |

* ND, not detectable (≤0.001 U/ml).
nase. The results of quantitative analysis are shown in Table 2. The production ratios of panose by H247E and H423E enzymes were significantly higher than that by the wild-type enzyme. On the other hand, the production ratios of panose by E332H and N331S-E332V enzymes were clearly lower than that by the wild-type enzyme.

Hydrolysis activities of wild-type and mutated neopullulanases toward α-(1→4)- and α-(1→6)-glucosidic linkages. As indicated in Table 2, the ratios of products from pullulan by the mutated neopullulanases clearly differed from that by the wild-type enzyme. This finding suggested that each mutation altered the specificity of neopullulanase toward α-(1→4)- and α-(1→6)-glucosidic linkages. We previously showed a model of pullulan hydrolysis by neopullulanase (5). According to the model, when the neopullulanase specificity toward α-(1→4) linkages of the intermediate products from pullulan becomes higher, the production ratio of panose should increase. When the specificity toward α-(1→6) linkages of the intermediate products from pullulan becomes higher, the production ratio of panose should decrease.

We confirmed this hypothesis quantitatively, as described below. We prepared 6°-O-α-glucosyl-maltotriose and 6°-O-α-maltosyl-maltose to analyze the hydrolysis activity toward α-(1→4)- and α-(1→6)-glucosidic linkages individually, as described previously (5). 6°-O-α-glucosyl-maltotriose was the substrate for the analysis of α-(1→4) linkage-hydrolyzing activity, and 6°-O-α-maltosyl-maltose was the substrate for analysis of α-(1→6) linkage-hydrolyzing activity. The structures of both substrates exist in the structure of pullulan. 6°-O-α-Glucosyl-maltotriose is hydrolyzed at the α-(1→4) linkage by neopullulanase, and panose and glucose are produced (5). 6°-O-α-Maltosyl-maltose is hydrolyzed at the α-(1→6) linkage by neopullulanase, and maltose is produced (5). These substrates were hydrolyzed by the wild-type or mutated neopullulanases. Samples of 150 μl of 1% each substrate (50 mM sodium acetate buffer solution, pH 6.0) and 150 μl of the wild-type or mutated enzyme sample were incubated at 40°C; 10 μl of each sample at various incubation times was analyzed by paper chromatography (data not shown). The ratios of the hydrolysis activity of the mutated neopullulanases toward these two substrates were clearly different from that of the wild-type enzyme. Therefore, glucose produced from 6°-O-α-glucosyl-maltotriose and maltose from 6°-O-α-maltosyl-maltose were quantitatively analyzed by high-performance liquid chromatography. The amounts of glucose and maltose were defined as α-(1→4)- and α-(1→6)-hydrolyzing activities, respectively (Table 3). As we expected, the specificities toward the α-(1→4)-glucosidic linkage of H247E and H423E enzymes, which produced more panose from pullulan, were significantly higher than that of the wild-type enzyme (Table 3). On the other hand, the specificities toward the α-(1→6)-glucosidic linkage of E332H and N331S-E332V enzymes, which produced less

### TABLE 2. Sugar compositions of pullulan hydrolysate by wild-type and mutated neopullulanases

| Mutation   | Ratio of products from pullulan (%) | Panose | Maltose | Glucose |
|------------|-------------------------------------|--------|---------|---------|
| H247E      | 81.4                                | 9.9    | 8.9     |
| H423E      | 82.1                                | 9.4    | 8.5     |
| E332H      | 40.4                                | 38.2   | 21.3    |
| N331S-E332V| 44.9                                | 35.6   | 19.5    |
| Wild type  | 77.9                                | 13.4   | 8.7     |

### TABLE 3. Hydrolysis activities of wild-type and mutated neopullulanases toward α-(1→4)- and α-(1→6)-glucosidic linkages

| Mutation   | Activity (U/ml) | Ratio, A/B |
|------------|-----------------|------------|
|            | α-(1→4) hydrolyzing (A) | α-(1→6) hydrolyzing (B) |
| H247E      | 0.095           | 0.014      | 6.8     |
| H423E      | 0.046           | 0.0067     | 6.9     |
| E332H      | 0.020           | 0.0096     | 2.1     |
| N331S-E332V| 0.026           | 0.012      | 2.2     |
| Wild type  | 0.16            | 0.035      | 4.6     |

*One unit of α-(1→4)-hydrolyzing activity is defined as the amount of enzyme which releases 1 μmol of glucose per min from 0.5% 6°-O-α-glucosyl-maltotriose. One unit of α-(1→6)-hydrolyzing activity is defined as the amount of enzyme which releases 2 μmol of maltose per min from 0.5% 6°-O-α-maltosyl-maltose.

### TABLE 4. Activity yields of His-247- or His-423-mutated neopullulanase

| Mutation   | Activity (U/ml of broth) | Ratio (%) |
|------------|--------------------------|-----------|
| H247Q      | 0.017                    | 4.0       |
| H247D      | 0.0082                   | 2.0       |
| H247K      | ND*                      | -         |
| H247R      | ND                       | -         |
| H247G      | ND                       | -         |
| H247A      | 0.0362                   | 8.6       |
| H247E      | 0.051                    | 12        |
| H423Q      | 0.132                    | 31        |
| H423D      | ND                       | -         |
| H423K      | ND                       | -         |
| H423R      | ND                       | -         |
| H423G      | 0.224                    | 53        |
| H423A      | 0.460                    | 110       |
| H423E      | 0.012                    | 2.9       |
| Wild type  | 0.420                    | 100       |

*ND, not detectable (<0.001 U/ml).
nose by the H247A enzyme was clearly lower than that by the wild-type enzyme. On the other hand, the relative production of panose by the H423Q, H423G, and H423A enzymes was higher than that by the wild-type and H423E enzymes.

We also compared specific activities of the wild-type and mutated neopullulanases. The wild-type, H247E, H247Q, H247A, H423E, H423Q, H423G, and H423A enzymes were purified from *E. coli* cell extract as described previously (8). Each enzyme produced a single band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Only H247Q and H423Q enzymes exhibited fairly high specific activities (Table 6).

### DISCUSSION

Glu-357 and Asp-424 of neopullulanase correspond to the catalytic sites, Glu-230 and Asp-297, of Taka-amylase A (7). When these two amino acid residues were replaced with His and Gln, and with His and Asn, respectively, neopullulanase activity disappeared (Table 1). Since the enzyme activity disappeared upon the replacement of Glu-357 and Asp-424 with oppositely charged or noncharged amino acid residues, Glu-357 and Asp-424 are probably part of the catalytic sites of neopullulanase. Asp-328 of neopullulanase corresponds to one of substrate-binding sites, Asp-206, of Taka-amylase A (7). When Asp-328 was replaced with His or Asn, neopullulanase activity vanished (Table 1). The Asp-197 and Asp-300 residues of porcine pancreatic α-amylase have been reported to be catalytic sites, as determined by X-ray crystallographic analysis (1). Asp-197 and Asp-300 correspond to Asp-206 and Asp-297 of Taka-amylase A (1, 7). Therefore, Asp-328 of neopullulanase may also act as a catalytic site.

We obtained several mutated neopullulanases by replacing amino acid residues which corresponded to the substrate-binding sites of Taka-amylase A (Table 2). These enzymes, H247E, H423E, E332H, and N331S-E332V, differed from the wild-type neopullulanase in their specificities toward α-(1→4) and α-(1→6)-glucosidic linkages (Table 3). The H247E and H423E enzymes exhibited higher specificities toward α-(1→4) linkages relative to the wild-type neopullulanase (Table 3) and consequently produced more panose from pullulan (Table 2). In contrast, the E332H and N331S-E332V enzymes exhibited lower specificities toward α-(1→4) linkages relative to the wild-type neopullulanase (Table 3) and consequently produced less panose from pullulan (Table 2). These results suggested that amino acid residues His-247, His-423, Glu-332, and Asn-331 are involved in substrate recognition.

Replacement of only one of the amino acid residues which are most likely to be part of the catalytic sites caused the complete loss of activity simultaneously on α-(1→4)- and α-(1→6)-glucosidic linkages. Furthermore, replacement of the amino acid residues which are most likely to be located near two different catalytic sites changed the specificities toward α-(1→4) and α-(1→6) linkages. These facts prove that one active center of the neopullulanase participates in the dual activity toward α-(1→4)- and α-(1→6)-glucosidic linkages. These facts additionally let us to conclude that the hydrolysis mechanisms of α-(1→4)- and α-(1→6)-glucosidic linkages are basically identical. Therefore, we think that an α-(1→4)-hydrolyzing enzyme (α-amylase, for example) could be altered to an α-(1→6)-hydrolyzing enzyme (isoamylase or pullulanase) by manipulating its substrate-binding specificity toward glucosidic linkages.

Recently, some other pullulan-hydrolyzing enzymes that demonstrate dual specificity toward α-(1→4)- and α-(1→6)-glucosidic linkages have been reported (2, 15, 16). Melasnemi et al. (11) suggested the existence of two active centers in the α-amylase-pullulanase which hydrolyzed both α-(1→4)- and α-(1→6)-glucosidic linkages. They also reported that the primary structure of the enzyme closely resembled that of neopullulanase. Our analysis of neopullulanase by the replacement of amino acid residues represents the first direct proof that one active center participates in the dual activity of such pullulan-hydrolyzing enzymes.

We obtained some mutated neopullulanases which produced more panose from pullulan by the replacement of His-247 and His-423 (Tables 2 and 5); their specific activities were lower than that of wild-type enzymes. When His-247 was replaced with Glu and Gln, the mutated neopullulanases produced relatively more panose than did the wild-type enzyme (Table 5). However, when His-247 was replaced with Ala, the mutated enzyme produced less panose (Table 5). On the other hand, when His-423 was replaced with Glu, Gln, Gly, and Ala, every mutated neopullulanase produced more panose (Table 5). To investigate the role of His-247 and His-423 in neopullulanase, we compared the effects of pH on the activities of these mutated enzymes with the effects on the wild-type neopullulanase activity (Fig. 3). The pH profiles of the mutated enzymes which were replaced at position His-242 were very similar to that of wild-type neopullulanase (data not shown). However, the pH profile of the mutated enzymes which were replaced at position His-247 were clearly different from that of the wild-type enzyme (Fig. 3). This result suggests that His-247 is located close to the catalytic sites of neopullulanase and affects the pKa value of the catalytic sites. Further work is still needed to elucidate the role of these amino acid residues.

Panose is mildly sweet, is nonfermentable by oral bacteria, and inhibits the synthesis of insoluble glucan from
sucrose (3, 14). Therefore, panose might be used as an anticariogenic sweetener in foods. In this context, we are presently attempting to develop a system for continuous production of extremely high panose syrup from pullulan by employing neopullulanase. Further investigations are now in progress to improve neopullulanase for efficient production of panose.

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