Analysis of the Active Center of *Bacillus stearothermophilus* Neopullulanase

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The active center of the neopullulanase from *Bacillus stearothermophilus* 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 specificity toward the α-(1→6)-glucosidic linkage.

Neopullulanase has been reported to be a new type of pullulan-hydrolyzing enzyme from *Bacillus stearothermophilus* TRS40 (8). The enzyme mainly hydrolyzes the α-(1→4)-glucosidic linkages of pullulan to produce panose (6^2-*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 (*nplT*) 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. stearothermophilus* 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 hsdΔ5 thi Δ(lac-proAB)* F′(*traD36 proAB⁺ lacI^qlacZΔM15*)] (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^r *nplT⁺* [structural gene of the neopullulanase from *B. stearothermophilus* TRS40]) was described previously (8).

Site-directed mutagenesis. Mutagenesis was done with an oligonucleotide-directed in vitro mutagenesis system (Amersham 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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primed by internally annealing 17-mer synthetic oligonucle-
otides.

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 *nplT* 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) (fi-
nal 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*, 5 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 ad-
justed 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 centrif-
ugation (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 *nplT*
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 chroma-
tography 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.
6^3-*O*-α-Glucosyl-maltotriose was prepared from the hydroly-
sate of waxy maize amylopectin by saccharifying α-amylase
(5). 6^2-*O*-α-Maltosyl-maltose was obtained from panose and
starch by the coupling reaction with cyclodextrin glucan-
otransferase (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 bro-
mide equilibrium density gradient centrifugation as de-
scribed 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 Pharmaceu-
tical Co., Ltd., Nagoya, Japan. Unless otherwise specified,
all chemicals used were from Wako Pure Chemical Indus-
tries, Osaka, Japan.

RESULTS

**Tentative identification of amino acid residues constituting
the active center of the neopullulanase.** We previously iden-
tified four highly conserved regions in α-amylases, neopul-
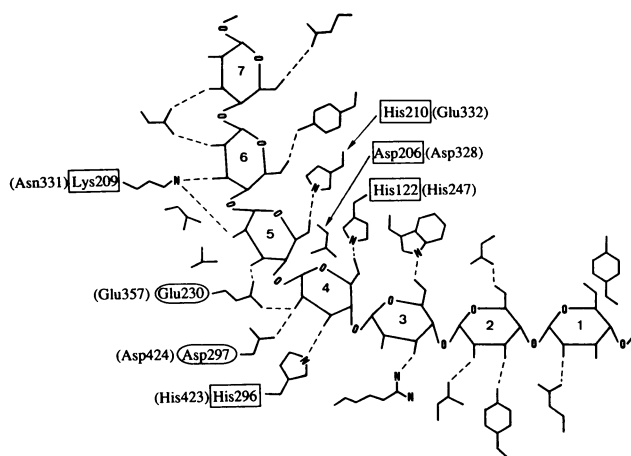lulanase, isoamylase, pullulanase, and cyclodextrin glucan-

FIG. 1. Proposed substrate-binding model of Taka-amylase A
(10) and the corresponding amino acid residues of neopullulanase.
Catalytic sites and substrate-binding sites for Taka-amylase A are
indicated by oblongs and rectangles, respectively. Corresponding
amino acid residues of neopullulanase are shown in parentheses.

otransferases (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 pro-
posed 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 neo-
pullulanase, 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 simulta-
neously loses activity toward both glucosidic linkages fol-
lowing 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,
Gln-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 *Sal*I-*Eco*RI fragment of plasmid pPP10 (7).
The *Sal*I-*Eco*RI fragment was cloned into the *Sal*I-*Eco*RI
sites of phage M13mp18/19 multiple-cloning sites. The sin-
gle-stranded DNA was prepared from the phage and used as
the template for site-directed mutagenesis (Fig. 2). Sequence
analysis of the 913-bp *Sal*I-*Eco*RI fragment verified that
site-directed mutagenesis introduced the desired nucleotide
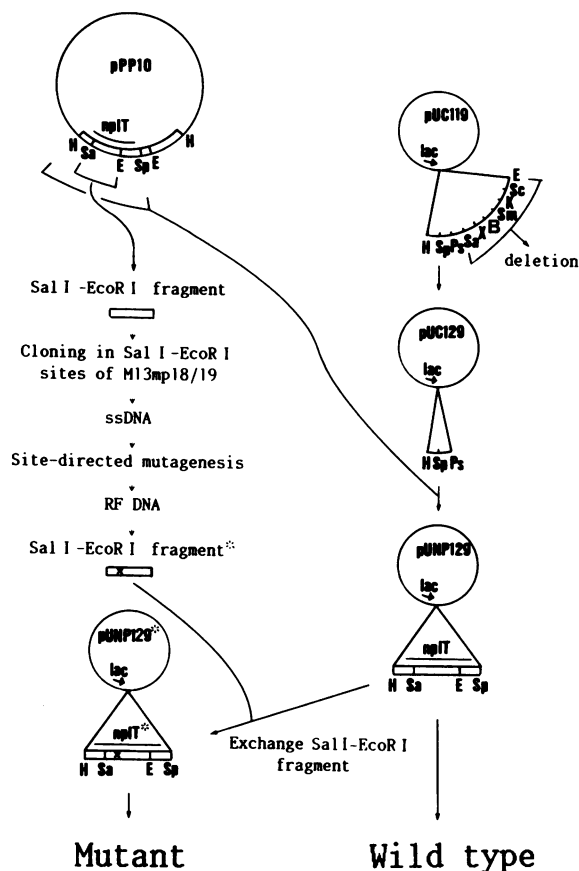change and no second-site mutations. Plasmids were con-

FIG. 2. Schemes of DNA construction and mutation in *nplT*. Asterisks denote mutations. *Hind*III, *Sph*I, *Pst*I, *Sal*I, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, *Sac*I, and *Eco*RI cleavage sites are indicated by H, Sp, Ps, Sa, X, B, Sm, K, Sc, and E, respectively.

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[a]	
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

[a] ND, not detectable (≤0.001 U/ml).

structed for easy preparation of wild-type and mutated neopullulanases, as shown in Fig. 2. The *Sal*I-*Eco*RI fragment in the multiple-cloning site of plasmid pUC119 was removed by digestion of *Sal*I and *Eco*RI. 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 *nplT* gene is contained in a 2,154-bp *Hind*III-*Sph*I fragment of pPP10 (7). The fragment was cloned in the *Hind*III-*Sph*I sites of pUC129. Thus, the recombinant plasmid was designated pUNP129 (Fig. 2). Since both *Sal*I and *Eco*RI sites were unique in pUNP129, the wild *Sal*I-*Eco*RI fragment was easily exchanged for the mutated fragment. *nplT* was located downstream from the *lac* promoter in pUNP129, and *nplT* 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 *nplT* 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. Similarly, when the other putative catalytic site, Asp-424, was replaced with His or Asn

(D424H or D424N 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 glucanotransferases (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 *nplT* with these mutations (Table 1; N331K-E332H, N331S-E332V, N331G-E332Y, or N331G-G332I mutation, 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 neopullula-

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	9.6	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[a]

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

[a] 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% 6^3-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% 6^2-O-α-maltosyl-maltose.

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 6^3-O-α-glucosyl-maltotriose and 6^2-O-α-maltosyl-maltose to analyze the hydrolysis activity toward α-(1→4)- and α-(1→6)-glucosidic linkages individually, as described previously (5). 6^3-O-α-glucosyl-maltotriose was the substrate for the analysis of α-(1→4) linkage-hydrolyzing activity, and 6^2-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. 6^3-O-α-Glucosyl-maltotriose is hydrolyzed at the α-(1→4) linkage by neopullulanase, and panose and glucose are produced (5). 6^2-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 6^3-O-α-glucosyl-maltose and maltose from 6^2-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

panose from pullulan, were obviously higher than that of the wild-type enzyme (Table 3).

In this context, E357H, E357Q, D424H, D424N, D328H, and D328N enzymes, which completely lost pullulan-hydrolyzing activity, could not hydrolyze either 6^3-O-α-glucosyl-maltotriose or 6^2-O-α-maltosyl-maltose (data not shown). These facts proved that these neopullulanase derivatives completely lacked both α-(1→4)- and α-(1→6)-hydrolyzing activities.

Attempt to improve neopullulanase for efficient production of panose. As described above, we obtained two mutated neopullulanases which produced more panose from pullulan by the replacement of His-247 or His-423 (Table 2). In an attempt to further improve neopullulanase for efficient production of panose, we replaced His-247 and His-423 with Gln, Asp, Lys, Arg, Gly, and Ala (Table 4). The H247Q, H247D, H247A, H423Q, H423G, and H423A enzymes exhibited pullulan-hydrolyzing activity, while the H247K, H247R, H247G, H423D, H423K, and H423R enzymes did not.

We analyzed pullulan hydrolysis products by those mutated neopullulanases which exhibited enough activity (0.01 U/ml broth) for further experiments (Table 5). The relative amount of panose produced by the H247Q enzyme was significantly higher than that produced by the wild-type and H247E enzymes. However, the relative production of pa-

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[a]	–
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

[a] ND, not detectable (≤0.001 U/ml).

TABLE 5. Sugar compositions of pullulan hydrolysate by
His-247- or His-423-mutated neopullulanase

Mutation	Ratio of products from pullulan (%)		
	Panose	Maltose	Glucose
H247Q	84.5	7.7	7.8
H247A	64.6	21.0	14.4
H247E	81.4	9.6	8.9
H423Q	87.6	6.4	6.0
H423G	85.8	7.0	7.2
H423A	86.4	7.1	6.5
H423E	82.1	9.4	8.5
Wild type	77.9	13.4	8.7

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, neopullu-
lanase 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,

TABLE 6. Specific activities of wild-type and His-247- or
His-423-mutated neopullulanases

Mutation	Sp act (U/mg)	Ratio (%)
H247E	1.73	8.0
H247Q	3.83	18
H247A	1.19	5.5
H423E	0.188	0.87
H423Q	6.23	29
H423G	1.38	6.4
H423A	3.10	14
Wild type	21.7	100

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 neopullu-
lanase (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 in-
volved 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 (isoamy-
lase 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). Melasni-
emi 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 re-
ported that the primary structure of the enzyme closely
resembled that of neopullulanase. Our analysis of neopullu-
lanase 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 pro-
duced 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 enzyme (Table 6). When
His-247 was replaced with Glu and Gln, the mutated neo-
pullulanases 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 pa-
nose (Table 5). On the other hand, when His423 was
replaced with Glu, Gln, Gly, and Ala, every mutated neop-
ullulanase 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-423 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 pK_a value of the catalytic sites. Further work is
still needed to elucidate the role of these amino acid resi-
dues.

Panose is mildly sweet, is nonfermentable by oral bacte-
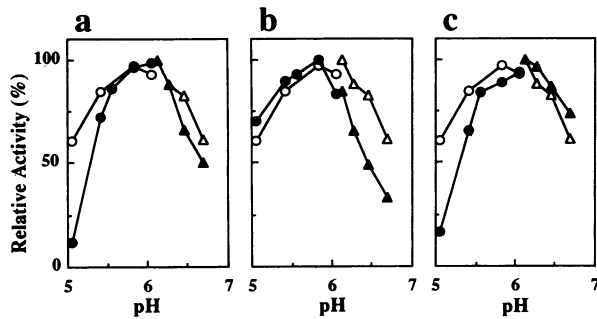ria, and inhibits the synthesis of insoluble glucan from

FIG. 3. Effects of pH on activities of wild-type (open symbols) and mutated (closed symbols; H247E [a], H247Q [b], and H247A [c]) neopullulanases. Reaction mixture (200 μl) consisting of 50 μl of 4% pullulan, 50 μl of 0.4 M sodium acetate buffer (O, ●) or 0.4 M sodium phosphate buffer (△, ▲), and 100 μl of enzyme solution was incubated at various pHs at 40°C for 30 min. The highest activity is designated 100%.

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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