Alteration of the Specificity of Neopullulanase by Protein Engineering[†]

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The specificity of the neopullulanase from *Bacillus stearothermophilus* was altered by protein engineering. The amino acid residues constituting 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 one of the putative catalytic sites (Glu-357, Asp-424, and Asp-328) were replaced by the opposite charged (His) or non-charged (Gln or Asn) amino acid residue, neopullulanase activities toward α -(1 $\rightarrow$ 4)- and α -(1 $\rightarrow$ 6)-glucosidic linkages disappeared. When the putative substrate-binding sites were replaced, the specificities of the mutated neopullulanases toward α -(1 $\rightarrow$ 4)- and α -(1 $\rightarrow$ 6)-glucosidic linkages were obviously different from that of the wild-type enzyme. This finding proves that one active center of the neopullulanase participated in the dual activity toward α -(1 $\rightarrow$ 4)- and α -(1 $\rightarrow$ 6)-glucosidic linkages. Some mutated neopullulanases exhibited higher specificities toward the α -(1 $\rightarrow$ 4) linkages relative to the wild-type enzyme. The mutated enzymes exhibiting higher specificities toward the α -(1 $\rightarrow$ 6) linkages were also obtained. These mutated neopullulanases were tested for the production of panose, a branched oligosaccharide which might be used as an anticariogenic sweetener. The production ratio of panose from pullulan was significantly increased by using the mutated neopullulanase which exhibited higher specificity toward the α -(1 $\rightarrow$ 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 $\rightarrow$ 6)-glucosidic linkage.

Pullulan is a linear polymer of α -(1 $\rightarrow$ 6) linked maltotriosyl units.¹⁾ The degradation of pullulan can proceed by cleavage of α -(1 $\rightarrow$ 6) linkages or any of the two α -(1 $\rightarrow$ 4) linkages. Four types of pullulan-hydrolyzing enzymes have been reported: (i) glucoamylase (glucan 1,4- α -glucosidase; EC 3.2.1.3),²⁾ which hydrolyzes pullulan from nonreducing ends to produce glucose; (ii) pullulanase (α -dextrin endo-1,6- α -glucosidase; EC 3.2.1.41),³⁾ from *Klebsiella pneumoniae*, which hydrolyzes α -(1 $\rightarrow$ 6)-glucosidic linkages of pullulan to produce maltotriose; (iii) isopullulanase (EC 3.2.1.57),⁴⁾ from *Aspergillus niger*, which hydrolyzes α -(1 $\rightarrow$ 4)-glucosidic linkages of pullulan to produce isopanose (6-O- α -maltosyl-glucose); and (iv) neo-

pullulanase (described below), which hydrolyzes α -(1 $\rightarrow$ 4)-glucosidic linkages of pullulan to produce panose (6²-O- α -glucosyl-maltose). These action patterns are diagrammatically shown in Fig. 1.

The last enzyme activity has been reported for *Thermoactinomyces vulgaris* α -amylase.⁵⁾ Most of the α -amylases found in nature cannot hydrolyze pullulan. In contrast, *Thermoactinomyces* α -amylase can hydrolyze not only starch but also pullulan. We isolated many pullulan-hydrolyzing thermophilic bacteria and finally found a new type of pullulan-hydrolyzing enzyme in *Bacillus stearothermophilus* that

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Fig. 1. Enzymatic hydrolysis of pullulan.

Symbols: ○, glucose; ●, glucose with reducing end; —, α -(1→4) linkage; ↓, α -(1→6) linkage.

produced panose from pullulan. The enzyme characteristics were clearly distinguished from α -amylase and should be considered a new enzyme. Therefore, we designated the enzyme which produced panose from pullulan as neopullulanase.⁶⁾

In this article, we review the characterization of the neopullulanase and molecular cloning and nucleotide sequence of the gene. Analysis of the active center of the enzyme and alteration of the specificity by protein engineering are highlighted. Our prospects of engineering of amylolytic enzymes are also described.

ISOLATION OF THERMOPHILIC BACTERIA THAT PRODUCE NEOPULLULANASE AND CHARACTERIZATION OF THE ENZYME

Eighteen pullulan-hydrolyzing thermophilic bacteria were isolated at 60°C from samples of compost or soil. The culture supernatants were used for the assay of pullulan-hydrolyzing enzyme. Pullulan was hydrolyzed with these enzymes, and the products were analyzed. Among these strains, thermophilic bacterium TRS40, which produced panose-producing enzyme, was isolated. Strain TRS40 was a strictly aerobic, gram-positive, endospore-forming bacillus. The maximum growth temperature for the strain at pH 7 was 65°C. Hence, strain TRS40 could be classified as *B. stearothermophilus*.⁶⁾

The panose-producing enzyme was purified to homogeneity on sodium dodecyl sulfate-polyacrylamide gel from the culture supernatant of *B. stearothermophilus* TRS40.⁶⁾ Pullulan and starch were hydrolyzed with the enzyme. The enzyme hydrolyzed pullulan efficiently, but hydrolyzed less starch. Therefore, the enzyme should be considered a new type of pullulan-hydrolyzing enzyme rather than an α -amylase. Thus we designated the enzyme as neopullulanase. The sugar composition of pullulan hydrolysate was quantitatively analyzed. The weight percentages of panose, maltose, and glucose were 71.5, 19.1, and 9.4%, respectively.⁶⁾ The production of glucose and maltose in addition to panose from pullulan was seemingly due to two enzymes: (i) the enzyme that produced panose from pullulan and (ii) the enzyme that produced glucose and maltose from panose. In this context, the gene (*nplT*) for the neopullulanase was cloned and expressed in *Bacillus subtilis*⁶⁾ (described later). Highly purified neopullulanase from *B. subtilis* carrying plasmid pPP10 (containing *nplT* in a 3.4 kb *Hind*III fragment from *B. stearothermophilus* TRS40) also produced glucose and maltose in addition to panose from pullulan. Since about 1.8 kb DNA fragment is needed for the neopullulanase, the possibility that two different enzyme genes were cloned simultaneously in a 3.4 kb *Hind*III fragment would be ruled out. Accordingly, we concluded that the single enzyme produced panose, glucose, and maltose from pullulan.⁶⁾ For the production of glucose and maltose in addition to panose from pullulan, the neopullulanase has to hydrolyze α -(1→6)-glucosidic linkages besides α -(1→4)-glucosidic linkages.

PATTERN OF ACTION OF NEOPULLULANASE ON PULLULAN

The action of neopullulanase from *B. stearothermophilus* TRS40 on many oligosaccharides was tested.⁷⁾ Maltose, isomaltose, panose, maltotriose, maltotriitol, and the mixture of isopanose and 6'-*O*- α -glucosyl-maltose were digested with neopullulanase, and the time course of the products was analyzed by paper chromatography. Neopullulanase did not hydrolyze maltose, isomaltose, and panose. Maltotriose and malto-

Fig. 2. Model of pullulan hydrolysis by neopullulanase.

Symbols are as in Fig. 1. The oligosaccharides enclosed by rectangles are the final products.

triitol could be hydrolyzed, and both maltose and glucose were produced, indicating that neopullulanase hydrolyzed α -(1 $\rightarrow$ 4) linkages next to both nonreducing and reducing ends. The production of glucose from maltotriitol was confirmed by the glucose-oxidase peroxidase method (Glucose B-test ; Wako Pure Chemical Industries). The mixture of isopanose and 6¹-O- α -glucosyl-maltose could be also hydrolyzed, and both maltose and glucose were produced. These facts revealed that neopullulanase hydrolyzed not only α -(1 $\rightarrow$ 4) linkages but also α -(1 $\rightarrow$ 6) linkages.

Neopullulanase hydrolyzed maltotetraose and produced maltose and smaller amount of glucose. These results showed that the middle position of the three α -(1 $\rightarrow$ 4) linkages was mainly cleaved. 6³-O- α -Glucosyl-maltotriose was hydrolyzed, and panose and glucose were produced. On the other hand, neopullulanase attacked the

α -(1 $\rightarrow$ 6) linkage of 6²-O- α -maltosyl-maltose and produced maltose.

To investigate the pattern of action of neopullulanase more precisely, we quantitatively analyzed the time course of the sugars making up the pullulan hydrolysate by high-performance liquid chromatography.⁷⁾ Only panose was detected in the early stage of the reaction (0 to 30 min) ; maltose production followed after 30 min. Glucose could not be detected in the early stage at all but could be detected after 60 min.

According to the results mentioned above, a model of the action of neopullulanase on pullulan was proposed (Fig. 2). In the first step, neopullulanase attacks only α -(1 $\rightarrow$ 4)-glucosidic linkages on the nonreducing side of α -(1 $\rightarrow$ 6)-glucosidic linkages of pullulan at random and produces panose and several intermediate products composed of some panose units. In the second step, taking 6²-O- α -(6³-O- α -glucosyl-mal-

totriosyl)-maltose as an example of one of the intermediate products, neopullulanase attacks either α -(1 $\rightarrow$ 4) or α -(1 $\rightarrow$ 6) linkages and produces panose or 6³-*O*- α -glucosyl-maltotriose plus maltose, respectively. In other words, the enzyme can hydrolyze not only α -(1 $\rightarrow$ 4) linkages but also α -(1 $\rightarrow$ 6) linkages of partial hydrolysates of pullulan (intermediate products). In the third step, 6³-*O*- α -glucosyl-maltotriose is hydrolyzed by the enzyme, and glucose is produced together with panose. To confirm the model of pattern of action, we extracted intermediate products produced from pullulan by neopullulanase and analyzed the structures by glucamylase, pullulanase, and neopullulanase analyses. The experimental results supported the abovementioned model (Fig. 2) of the pattern of action of neopullulanase on pullulan.⁷⁾

MOLECULAR CLONING AND NUCLEOTIDE SEQUENCE OF THE NEOPULLULANASE GENE

The neopullulanase gene (*nplT*) was cloned and expressed in *B. subtilis*. A recombinant plasmid pPP10, which obtained from the transformant, contained 3.4 kb *Hind*III fragment from *B. stearothermophilus* TRS40.⁶⁾ *B. subtilis* NA-1 carrying pPP10 produced about 30-fold more neopullulanase than *B. stearothermophilus* TRS40 did. The nucleotide sequence of *nplT* was determined.⁸⁾ Starting from the ATG codon at position +1 and terminating in a TAG non-sense codon at position +1765, the single open reading frame was composed of 1764 nucleotides. The molecular weight of the neopullulanase was calculated as 69,144 from the amino acid sequence (588 amino acid residues).⁸⁾

ANALYSIS OF THE ACTIVE CENTER OF NEOPULLULANASE AND ALTERATION OF THE ENZYME SPECIFICITY BY PROTEIN ENGINEERING

We analyzed the homologous amino acid sequences for α -amylases, neopullulanase, isoamylase, pullulanase, and cyclodextrin glucanotransferases. It was previously demonstrated that four highly conserved regions existed in eleven different α -amylases⁹⁾ and the regions

were most likely to constitute the active center of each enzyme.^{8,10)} These homologous regions have also been discussed for other kinds of amylolytic enzymes.¹¹⁻¹⁴⁾ As the results demonstrate (Fig. 3), the four highly conserved regions previously demonstrated in α -amylases were also found in neopullulanase, isoamylase, pullulanases¹⁵⁾ and cyclodextrin glucanotransferases. Among these amylolytic enzymes, only α -amylases from *Aspergillus oryzae* (Taka-amylase A)¹⁰⁾ and from porcine pancreas¹⁶⁾ have been investigated by X-ray crystallographic analysis. Using the alignment of amino acid residues in the homologous regions, a molecular model and catalytic residues of Taka-amylase A¹⁰⁾ were used for analysis of other α -amylases, neopullulanase, isoamylase, pullulanases, and cyclodextrin glucanotransferases. 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 (Fig. 3).

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 (Fig. 4). Asp-328 of neopullulanase corresponds to one of substrate-binding sites, Asp-206, of Taka-amylase A (Fig. 3). 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.¹⁶⁾ They correspond to Asp-206 and Asp-297 of Taka-amylase A (Fig. 3). Therefore, Asp-328 of neopullulanase may also act as a catalytic site (Fig. 4). Indeed, Matsuura *et al.*¹⁷⁾ recently reported that Asp-206, Glu-230, and Asp-297 must be considered to be involved in the catalytic reaction of Taka-amylase A.

It was also suggested that His-247, Asn-331, Glu-332, and His-423 of neopullulanase, corresponding to His-122, Lys-209, His-210, and His-296 of Taka-amylase A, might act as substrate-binding sites, or at least, constitute the active center (Fig. 4). If the same active center of neopullulanase participated in the dual activity toward α -(1 $\rightarrow$ 4)- and α -(1 $\rightarrow$ 6)-glucosidic link-

		Region 1	Region 2	Region 3	Region 4
AMY Consensus		DAVINH	GFRLDAAKH	EVID	FVDNHD
AMY <i>A.ory.</i>	117	DDVANH	GLRIDTVKH	230EVLQ	292FVENHD
AMY <i>B.stearo.</i>	101	DDVFDH	230GFRLDVVKH	264EYWS	326FVDNHD
AMY <i>B.amylo.</i>	98	DDVVLNH	227GFRIDAAKH	261EYWQ	323FVENHD
AMY <i>B.sub.</i>	97	DAVINH	172GFRFDAAKH	208EILQ	264WVESH
AMY Rat	96	DAVINH	190GFRLDAAKH	230EVID	292FVDNHD
AMY Mouse, s	96	DAVINH	193GFRLDASKH	233EVID	295FVDNHD
AMY Mouse, p	96	DAVINH	190GFRLDAAKH	230EVID	292FVDNHD
AMY Hog	96	DAVINH	193GFRIDASKH	233EVID	295FVDNHD
AMY Human, s	99	DAVINH	196GFRIDASKH	236EVID	298FVDNHD
AMY Human, p	99	DAVINH	196GFRLDASKH	236EVID	298FVDNHD
AMY Barley	101	DIVINH	127DGRLDWGPH	218EVWD	299FVDNHD
NPL <i>B.stearo.</i>	242	DAVFNH	324GWRLDVANE	357EIWH	419LLGSHD
IAM <i>P.amylo.</i>	291	DDVYNH	370GFRFDLASV	454EWSV	502FIDVHD
PUL <i>K.aero.</i>	600	DDVYNH	671GFRFDLMGY	704EGWD	827YVSKHD
PUL <i>B.stearo.</i>	281	DDVYNH	348GFRFDLMGI	381EGWD	464YVESH
CGT <i>K.pne.</i>	130	DYADNH	219AIRIDAIKH	257EWFG	328FMDNHD
CGT <i>B.mace.</i>	135	DFAPNH	225GIRFDVVKH	258EWFL	324FIDNHD
CGT <i>Alk.B.</i>	135	DFAPNH	225GIRVDVVKH	257EWFL	323FIDNHD
CGT <i>B.stearo.</i>	131	DFAPNH	221GIRMDVVKH	253EWFL	319FIDNHD

Fig. 3. Highly conserved regions among α -amylase, neopullulanase, isoamylase, pullulanase, and cyclodextrin glucanotransferase.

The amino acid residues identical with the consensus sequence of α -amylase are shaded. Enzymes are abbreviated as : AMY, α -amylase ; NPL, neopullulanase ; IAM, isoamylase ; PUL, pullulanase ; CGT, cyclodextrin glucanotransferase. Enzyme sources are abbreviated as : *A.ory.*, *Aspergillus oryzae* ; *B.stearo.*, *Bacillus stearothermophilus* ; *B.amylo.*, *Bacillus amyloliquefaciens* ; *B.sub.*, *Bacillus subtilis* ; *P.amylo.*, *Pseudomonas amyloclavata* ; *K.aero.*, *Klebsiella aerogenes* ; *K.pne.*, *Klebsiella pneumoniae* ; *B.mace.*, *Bacillus macerans* ; *Alk.B.*, *Alkalophilic Bacillus* ; *s, saliva* ; *p, pancreas*. Numbering of the amino acid sequences of the enzymes starts at the amino-terminal amino acid of each mature enzyme. Catalytic sites and substrate-binding sites by Matsuura *et al.*¹⁰ for Taka-amylase A from *A.oryzae* are indicated by $\circ$ and $\square$, respectively.

ages, we should obtain the following results : (i) neopullulanase simultaneously loses activity toward both glucosidic linkages following the replacement of Glu-357, Asp-424 or Asp-328, and (ii) the enzyme specificity toward each glucosidic linkage can be altered by the replacement of His-247, Asn-331, Glu-332, or His-423.

On the basis of this presumption, we identified amino acid residues Glu-357, Asp-424, Asp-328, His-247, Asn-331, Glu-332, and His-423 as targets for substitutions to analyze and manipulate the active center of neopullulanase (Fig. 4).

By using pUC119 as a vector plasmid, *nplT* was subcloned in *Escherichia coli*.¹⁸ *nplT* was located downstream from the *lac* promoter in this recombinant plasmid, and *nplT* was expressed efficiently in *E. coli* by the addition

of IPTG as an inducer. Mutagenesis was done with an oligonucleotide-directed mutagenesis system (Amersham Corp., Amersham, England). *E. coli* carrying a plasmid which encoded wild-type or mutated *nplT* was cultivated, and wild or mutated enzyme sample, respectively, was prepared.¹⁸

The activities of the thirteen mutated neopullulanases were estimated (Table 1). As we expected, the enzyme activity disappeared when one of Glu-357, Asp-424, and Asp-328 was replaced with oppositely charged (His) or non-charged (Gln or Asn) amino acid residue. On the bases of these results and the homology analyses with Taka-amylase A and porcine pancreatic α -amylase, described above, it is most likely that Glu-357, Asp-424, and Asp-328 residues of neopullulanase act as catalytic sites.

When the putative substrate-binding site of

Fig. 4. Proposed substrate-binding model of Taka-amylase A¹⁰⁾ 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.

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

^aND, Not detectable (≤ 0.001 U/ml).

neopullulanase, His-247 or His-423, was replaced with Glu, 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. 3). When Glu-332 was replaced with His, 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 (Fig. 3).⁸⁾ These two amino acid residues are different in neopullulanase, isoamylase, and pul-

lulanases (Fig. 3). Therefore, the two amino acid residues (Asn-331-Glu-332) of neopullulanase were replaced with Lys-His (α -amylase type), Ser-Val (isoamylase type), Gly-Tyr (*Klebsiella aerogenes* pullulanase type), or Gly-Ile (*B. stearothermophilus* pullulanase type). We obtained relatively low activities from the enzyme preparations from *E. coli* carrying *nplT* with these mutations (Table 1).

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 specificities. We prepared 6³-O- α -glucosyl-maltotriose and 6²-O- α -maltosyl-maltose to analyze the hydrolysis activity toward α -(1→4)- and α -(1→6)-glucosidic linkages individually, as described previously.⁷⁾ 6³-O- α -Glucosyl-maltotriose was the substrate for the analysis of α -(1→4) linkage-hydrolyzing activity, and 6²-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. 6³-O- α -Glucosyl-maltotriose is hydrolyzed at the α -(1→4) linkage by neopullulanase, and panose and glucose are produced.⁷⁾ 6²-O- α -Maltosyl-maltose is hydrolyzed at the α -(1→6) linkage by neopullulanase, and maltose is produced.⁷⁾ These substrates were hydrolyzed by the wild-type or mutated neopullulanases. Glucose produced from 6³-O- α -glucosyl-maltotriose and maltose from 6²-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 2). The specificities toward the α -(1→4)-glucosidic linkage of H247E and H423E enzymes were significantly higher than that of the wild-type enzyme (Table 2). On the other hand, the specificities toward α -(1→6)-glucosidic linkage of E332H and N331S-E332V enzymes were obviously higher than that of the wild-type enzyme (Table 2).

In this context, E357H, E357Q, D424H, D424N, D328H, and D328N enzymes, which completely lost pullulan-hydrolyzing activity, could not hydrolyze either 6³-O- α -glucosyl-maltotriose or 6²-O- α -maltosyl-maltose. These facts

Table 2. 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 μ mol of glucose per min from 0.5% 6³-O- α -glucosyl-maltotriose. One unit of α -(1→6)-hydrolyzing activity is defined as the amount of enzyme which releases 2 μ mol of maltose per min from 0.5% 6²-O- α -maltosyl-maltose.

proved that these neopullulanase derivatives completely lacked both α -(1→4)- and α -(1→6)-hydrolyzing activities.

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, the replacement of the amino acid residues which are most likely to be involved in substrate recognition 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 lead to the conclusion 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.

We previously showed a model of pullulan hydrolysis by neopullulanase (Fig. 2).⁷⁾ 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 be-

Table 3. 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 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).

comes higher, the production ratio of panose should decrease.

We confirmed this hypothesis quantitatively by the analyses of H247E, H423E, E332H, and N331S-E332V enzymes for their products from pullulan (Table 3). 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. As we expected, the production ratios of panose by H247E and H423E enzymes were significantly higher than that by the wild-type enzyme. The production ratios of panose by E332H and N331S-E332V enzymes were clearly lower than that by the wild-type enzyme.

We obtained two mutated neopullulanases which produced more panose from pullulan by the replacement of His-247 or His-423 (Table

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

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

Mutation	Specific activity (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

3). Panose is mildly sweet, is nonfermentable by oral bacteria, and inhibits the synthesis of insoluble glucan from sucrose.^{19,20} Therefore, panose might be used as an anticariogenic sweetener in foods. 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 of 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 panose 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. Each enzyme produced a single band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Only H247Q and H423Q enzymes exhibited fairly high specific activities (Table 6).

To investigate the role of His-247 and His-423 in neopullulanase, we compared the effect of pH on the activities of these mutated enzymes with the effects on the wild-type neopullulanase activity.¹⁸⁾ The pH profiles of the mutated enzymes which were replaced at position His-423 were very similar to that of wild-type neopullulanase. 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. This result suggests that His-247 is located close to the catalytic sites of neopullulanase and affects the pK_a value of the catalytic site.

Neopullulanase is a good material for the basic research work in the protein engineering of amylolytic enzymes because one active center of the enzyme catalyzes the hydrolysis reactions of not only α -(1 $\rightarrow$ 4)- but also α -(1 $\rightarrow$ 6)-glucosidic linkages. Why does α -amylase exclusively hydrolyze α -(1 $\rightarrow$ 4)-glucosidic linkage? Why does pullulanase exclusively hydrolyze α -(1 $\rightarrow$ 6)-glucosidic linkage²¹⁾? What is the difference between α -(1 $\rightarrow$ 4)-hydrolyzing enzymes and α -(1 $\rightarrow$ 6)-hydrolyzing enzymes? Neopullulanase may lead us to the answers.

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【質問】

福山大 溝上

(1) この新しいアミラーゼは非還元末端より2番目の結合を認識しながら、 α -1,4結合、 α -1,6結合を問わずに加水分解しているようですが、その他の結合に対する作用性として、 G_2 -Phenol、 G_2 -F等に対する作用性はいかがですか。

(2) 反応生成糖のペークロで G_3 とパノースの中間付近に見られたマイナーなスポットは何でしょうか。

(3) この反応は粗酵素を用いた場合でも精製酵素を

使ったときと同様の生成パターンを示すのでしょうか。つまり、他のアミラーゼの存否についてお聞かせください。

【答】

(1) これらの配糖体に対する作用はまだ調べておりません。多分、どちらからもマルトースが遊離してくるものと予想されます。

(2) 高濃度のプルランに本酵素を作用させるとパノース、マルトース、およびグルコースの他にごく少量のイソマルトースが生成物として検出されます⁷⁾ことから、高速液体クロマトグラフィーでは検出不能なレベルのイソマルトースではないかと考えています。

(3) 元株である *B. stearothermophilus* TRS40 に本酵素を生産させた場合は電気泳動的に単一バンドを示すまで精製した酵素を使用して実験を行っております。また、本酵素遺伝子をクローン化した *E. coli* あるいは *B. subtilis* からはもともと他のアミラーゼ活性がまったく検出されず、これから調製したネオプルラナーゼの粗酵素液が元株から精製した酵素とまったく同じ反応をすることを確認しております。すなわち、これらの反応液中にはネオプルラナーゼ以外の他のアミラーゼは存在しないといえます。

【質問】 大阪市工研 北畑

遺伝子工学で変異させました酵素は澱粉に対する活性はどのようになっていますか。

【答】

変異酵素に関しては、澱粉への作用は調べておりません。今後、調べてゆきたいと思っております。

【質問】 東京農工大 坂野

ネオプルラナーゼの触媒部位は、三つのアミノ酸残基

(Asp328, Glu357, Asp424) から成ると推定されましたが、それら三つの残基はどのようなメカニズムで触媒作用をすると考えられていますか。

【答】

触媒機構のメカニズムは α -アミラーゼ、ネオプルラナーゼ、イソアミラーゼ、プルラナーゼ、および CGTase において基本的に同じであると考えております。したがって、松浦先生が最近ご発表になっているタカアミラーゼ A の触媒メカニズム¹⁷⁾が適用できると考えております。

【質問】

東京農工大 殿塚

ペーパークロマトグラフィーの結果を見ると、多少澱粉も切断しているようですが、市販の可溶性澱粉に対しての比活性はプルランに対し、どのくらいの値になるのでしょうか。

【答】

アミロースへの反応性は相当高いようですが、アミロペクチンに対しては明らかにプルランより反応性は劣ります。これらおよび市販の可溶性澱粉に対しての比活性は求めておりません。

【質問】

福山大 山本

パノースが大量に酵素的に生産できることは素晴らしいことと思います。この機会にパノース、イソパノースの旋光性を調べて報告してもらいたいと思います。

【答】

これは興味の持たれる点であり、ぜひ調べたいと思っております。

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