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Conversion of Neopullulanase-α-Amylase from *Thermoactinomyces vulgaris* R-47 into an Amylopullulanase-Type Enzyme[1]

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TVA I, an α-amylase from *Thermoactinomyces vulgaris* R-47, is a versatile enzyme which hydrolyzes the α-(1→4)-glucosidic linkages of pullulan to produce panose, known as neopullulanase activity, and the α-(1→6)-glucosidic linkages of certain oligosaccharides. We modified the Ala-357, Gln-359, and Tyr-360 residues located in region II, one of the four regions conserved in α-amylase family enzymes, and deleted 11 consecutive amino acid residues located after the C-terminus of region II of the TVA I sequence by means of site-directed mutagenesis. The action pattern of the mutated enzyme for pullulan was greatly altered and it hydrolyzed mainly the α-(1→6)-glucosidic linkages of pullulan to produce maltotriose, while the action patterns for starch and maltooligosaccharides were almost identical to those of the wild-type enzyme. This means that the mutated TVA I has lost the neopullulanase activity, and thus can be designated as an amylopullulanase-type enzyme. The k_{cat}/K_m value of the mutated enzyme for α-(1→6)-glucosidic linkages was virtually unaltered, while that for α-(1→4)-glucosidic linkages was about 100 times smaller than that of the wild-type enzyme.

Key words: α-amylase, amylopullulanase, neopullulanase-α-amylase, pullulanase, site-directed mutagenesis.

Enzymes which hydrolyze specific glucosidic linkages of pullulan are classified as follows.

(i) Enzymes which hydrolyze the α-(1→6)-glucosidic linkages of pullulan (denoted as linkage 1 in Fig. 1) to produce maltotriose: (i-i) pullulanase (α-dextrin endo-1,6-α-glucosidase; EC 3.2.1.41), which also hydrolyzes the α-(1→6)-glucosidic linkages of starch (*1, 2*); (i-ii) amylopullulanase (also called α-amylase-pullulanase; EC 3.2.1.1/41), which also hydrolyzes the α-(1→4)-glucosidic linkages of starch to produce mainly maltose (*3-5*).

(ii) Enzymes which hydrolyze the α-(1→4)-glucosidic linkages of pullulan (denoted as linkage 2 in Fig. 1) to produce panose: (ii-i) α-amylases from *Thermoactinomyces vulgaris* R-47, TVA I (*6*) and TVA II (*7*), and from *Bacillus licheniformis* (*8*), which hydrolyze starch more effectively than pullulan, to produce mainly maltose [designated by Svensson as neopullulanase-α-amylase (*9*); EC 3.2.1.1/135]; (ii-ii) neopullulanase [EC 3.2.1.135], which hydrolyzes starch less efficiently (*10*).

(iii) An enzyme which hydrolyzes the α-(1→4)-glucosidic linkages of pullulan (denoted as linkage 3 in Fig. 1) to produce isopanose: isopullulanase [EC 3.2.1.57], which does not hydrolyze starch (*11*).

The genes for numerous amylolytic enzymes have been cloned recently, and α-amylases that do not hydrolyze pullulan and cyclodextrin glucanotransferases (designated as group I enzymes in this paper) have four highly conserved regions (I-IV) in their primary structures, and three acidic residues, two Asp and one Glu, located in the conserved regions function as catalytic sites (*12*) (Fig. 2A). Except for in isopullulanase (*11*), the four conserved regions have been observed in the primary structures of all the pullulan-hydrolyzing enzymes (designated as group II enzymes in this paper) as well, indicating that both group I and II enzymes can be classified as one protein family, designated as the α-amylase family (*9*). Kinetic studies showed that TVA I hydrolyzes both α-(1→4)- and α-(1→6)-glucosidic linkages at the same active site (*13*). A site-directed mutagenic study carried out by Kuriki *et al.* showed that the three acidic residues, two Asp and one Glu, of neopullulanase participate in the dual activity toward α-(1→4)- and α-(1→6)-glucosidic linkages (*14*). These data suggest that the three conserved acidic residues of group II enzymes function as catalytic sites for the hydrolysis of both α-(1→4)- and α-(1→6)-glucosidic linkages (*15*).

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Abbreviations: IMM, 4[2] α-isomaltosylmaltose; PAPA, 4[3] α-panosylpanose; PNPG2, *p*-nitrophenyl α-D-maltoside; TVA, α-amylase from *Thermoactinomyces vulgaris* R-47.

Several three-dimensional structures for group I enzymes have been reported, and these enzymes have a $(\beta/\alpha)_8$-barrel catalytic domain with a highly symmetrical fold (*16-18*).

The four regions conserved in the α-amylase family are shown in Fig. 2A. In this study we focused on region II. In the group I enzymes, the amino acid sequence is highly conserved, whereas this KH sequence is not conserved in the group II enzymes. The pullulan-hydrolyzing enzymes, particularly TVAs, neopullulanases and amylopullulanases, have very similar $(\beta/\alpha)_8$-barrel catalytic domains (*3-5*). Thus we hypothesize that the latter part of region II plays a role in the hydrolysis of pullulan, and that the conversion of TVAs to neopullulanase or amylopullulanase is possible using the site-directed mutagenic technique.

Thermoactinomyces vulgaris R-47 produces two α-amylases, TVA I and TVA II, both of which efficiently hydrolyze the α-(1→4)-glucosidic linkages of pullulan to produce panose. TVAs also hydrolyze the α-(1→6)-glucosidic linkages of certain oligosaccharides such as isopanose and 4^2 α-isomaltosylisomaltose (*19, 20*). Compared to TVA II, TVA I prefers polysaccharides, *i.e.* starch and pullulan, while TVA II shows outstanding kinetic values for small oligosaccharides (*6*). Therefore, the properties of TVA II closely resemble those of neopullulanase, which scarcely hydrolyzes starch. We aligned the sequences of regions II and III of TVA I, TVA II, and amylopullulanase (Fig. 2B). Although in properties, TVA II seems closer to TVA I than amylopullulanase, in primary structural identity, TVA II is closer to amylopullulanase than TVA I, and TVA I has the following unique features: (i) the latter part of regions II of TVA II and amylopullulanase is VANE, whereas that of TVA I is AAQY; (ii) TVA I has an extra 11 amino acid sequence, ANGNNGSDVTN, located after the C-terminus of region II. We also investigated the sequences of other amylopullulanases and neopullulanases, and these enzymes had the TVA II-type sequence, namely, the VANE sequence (Fig. 2A), but no ANGNNGSDVTN sequence.

In this study, to elucidate the roles of residues of TVA I, three mutants of TVA I were constructed: (i) Del11, in which the 11 amino acid sequence, ANGNNGSDVTN, was deleted; (ii) AQY/VNE, in which Ala-357, Gln-359, and Tyr-360 in the latter part of region II, AAQY, were replaced by Val, Asn, and Glu respectively, giving the VANE sequence; (iii) Del11+AQY/VNE, in which the sites of mutation introduced were a combination of (i) and (ii). We found that the mutated enzymes had lost the activity toward α-(1→4)-glucosidic linkages but showed the amylopullulanase activity.

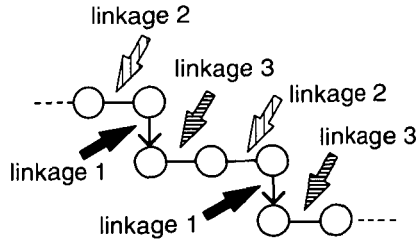

Fig. 1. **Schematic action pattern of pullulan-hydrolyzing enzymes for pullulan.** Symbols: circles, glucose; —, α-(1→4)-glucosidic linkage; ↓, α-(1→6)-glucosidic linkage; bold arrows, enzymatic cleavage points.

MATERIALS AND METHODS

Substrates of the Enzymes—Pullulan (M_r=64,800), α-cyclodextrin, and maltooligosaccharides, from maltose to maltopentaose, were purchased from Wako Pure Chemicals, Japan. Amylose AS-30 (M_r=26,700) and AS-70 (M_r=70,900) were purchased from Nakano Vinegar, Japan. Amylose (DP, degree of polymerization, =17 and 80) was from Hayashibara Biochemical Laboratories, Japan. Soluble starch was from Merck, Germany, and *p*-nitrophenyl α-D-maltoside (PNPG2) was from Sigma, USA. Isopanose [Glc-α-(1→4)-Glc-α-(1→6)-Glc] and panose [Glc-α-(1→6)-Glc-α-(1→4)-Glc] were prepared as

A

	Enzyme	Source	Region I	Region II *	Region III *	Region IV *
Group I	AAM	*A. oryzae*	117 DVVANH	202 GLRIDTVKH	230 EVLD	292 FVENHD
	AAM	*B. stearo*	101 DVVFDH	230 GFRLDAVKH	264 EYWS	326 FVDNHD
	AAM	*B. amylo*	98 DVVLNH	227 GFRIDAAKH	261 EYWQ	323 FVENHD
	AAM	*B. sub.*	97 DVVINH	172 GFRFDAAKH	208 EILQ	264 WVESHD
	AAM	*K. pne.*	130 DYADNH	219 AIRIDAIKH	257 EWFG	328 FMDNHD
	CGT	*B mace.*	135 DFAPNH	225 GIRFDAVKH	258 EWFL	324 FIDNHD
	CGT	*B. stearo.*	131 DFAPNH	221 GIRMDAVKH	253 EWFL	319 FIDNHD
Group II	NAA	TVA I	262 DGVFNH	352 GWRLDAAQY	396 EYWG	467 FLSNHD
	NAA	TVA II	239 DAVFNH	321 GWRLDVANE	354 EIWH	416 LLDSHD
	NPL	*B. stearo.*	242 DAVFNH	324 GWRLDVANE	357 EIWH	419 LLGSHD
	APL	*T. ethano.*	488 DGVFNH	593 GWRLDVANE	626 ELWG	698 LLGSHD
	APL	*T. thermo*	486 DGVFNH	589 GWRLDVANE	622 ENWG	694 LLGSHD
	PUL	*K. aero.*	600 DVVYNH	671 GFRFDLMGY	704 EGWD	827 YVSKHD

B

TVAI	351	DGWRLDAAQYVDANGNNGSDVTNHQIWSEFRNAVKGVNSNAAIIGEYWG
TVAII	320	DGWRLDVANEVD----------HAFWREFRRLVKSLNPHALIVGEIWH
APL *T. ethano*	592	DGWRLDVANEIA----------HDFWVHFRAAINTVKPNAPMIAELWG

Region II Extra sequence of TVA I Region III

Fig. 2. **Comparison of conserved regions of TVA I and related enzymes.** (A) Comparison of the four conserved regions (I-IV) of TVA I and related enzymes. The abbreviations are as follows: AAM, α-amylase; CGT, cyclodextrin glucanotransferase; NAA, neopullulanase-α-amylase; NPL, neopullulanase; APL, amylopullulanase; PUL, pullulanase. The enzyme sources are abbreviated as follows: *A. oryzae, Aspergillus oryzae; B. amylo., Bacillus amyloliquefaciens; B. sub., Bacillus subtilis; K. pne., Klebsiella pneumoniae; B. mace., Bacillus macerans; B. stearo., Bacillus stearothermophilus; T. ethano., Thermoanaerobacter ethanolicus; T. thermo., Thermoanaerobacter thermohydrosulfuricum; K. aero., Klebsiella aerogenes.* The three catalytic amino acid residues of the α-amylase family, two Asp and one Glu, are indicated by asterisks. The numbering of the sequences of the enzymes starts at the N-terminal amino acid of each mature enzyme. The group I enzymes are pullulan-hydrolyzing enzymes, whereas the group II enzymes are enzymes that do not hydrolyze pullulan. (B) Comparison of amino acid sequences of regions II and III of TVA I, TVA II and *Thermoanaerobacter ethanolicus* amylopullulanase. Identical residues are presented in white on black. Bars represent gaps introduced during the alignment process. The conserved regions, II and III, and the extra sequence of TVA I are underlined. Ala-357, Gln-359, and Tyr-360 of TVA I are indicated.

described (*21*). IMM [4^2 α-isomaltosylmaltose; Glc-α-(1→6)-Glc-α-(1→4)-Glc-α-(1→4)-Glc] was also prepared as described (*19*). PAPA [4^3 α-panosylpanose; Glc-α-(1→6)-Glc-α-(1→4)-Glc-α-(1→4)-Glc-α-(1→6)-Glc-α-(1→4)-Glc] was prepared by partial hydrolysis of pullulan with wild-type TVA I and purified on a Toyopearl HW-40S gel filtration column (10×100×2 columns) with water. A series of reducing end-labeled maltooligosaccharides was generously supplied by Dr. M. Tobita (*6*).

Site-Directed Mutagenesis and Expression of Mutated Enzymes—The gene manipulation methods were based on those of Sambrook *et al.* (*22*). The enzymes were prepared from recombinant *Escherichia coli* MV1184 cells (*6*). Oligonucleotide-directed mutagenesis was carried out using a MUTA-GENE *in vitro* mutagenesis kit (Bio-Rad, USA) according to the method of Kunkel (*23*) for the construction of an expression plasmid of TVA II, pTN302-10. Because no signal peptide region was found in the TVA II sequence (*6*), a plasmid, pTN1 (*6*), was modified with an oligonucleotide, 5′-GGA CTC TTC CCC CTT CAA ATT GTT ATC CGC TCA CAA-3′, to connect the N-terminal methionine codon for TVA II with the initiation codon of the *lacZ* gene derived from pUC119, resulting in plasmid pTN302-10. For production of the mature wild-type TVA I, we used plasmid pTV93, in which the initiation codon was linked to the N-terminal alanine codon for the mature TVA I (*7*). The sites where the mutations were introduced are located in the *Eco*RI-*Spl*I fragment (0.18 kb) of the TVA I gene. To construct reliable mutated enzymes, mutated *Eco*RI-*Spl*I fragments were sequenced to confirm that only the expected mutations had been introduced, and the original *Eco*RI-*Spl*I fragment of pTV93 were replaced with the mutated *Eco*RI-*Spl*I fragment. Oligonucleotide-directed mutagenesis was carried out for modification of the Ala-357, Gln-359, and Tyr-360 residues of TVA I. Oligonucleotides 5′-GCC ATT TGC GTC GAC TTC GTT TGC GAC GTC GAG CCG CCA A-3′, 5′-GCC ATT TGC GTC AAC ATG TTT TGC TGC ATC GAG -3′, and 5′-GCC ATT TGC GTC GAC TTC GTT TGC TGC ATC GAG-3′ were used for the construction of AQY/VNE, QY/KH, and QY/NE TVA I mutants, respectively. A PCR mutagenesis method was also employed. Oligonucleotides 5′-GCC CAA ACT AAA CTA-3′ and 5′-AAT ACT CCC CGA TGA-3′ were complimentary to the ends of the amplified region. Oligonucleotides 5′-AGC ACA ATA TGT TGA CCA CCA GAT TTG GAG C-3′ and 5′-TCG CTC CAA ATC TGG TGG TCA ACA TAT TGT GC-3′ were used to delete the 11 amino acid residues from Ala-363 to Asn-373 (Del11). Oligonucleotides 5′-ATC CAC CTC ATT GGC CAC ATC GAG CCG CCA ACC AT-3′ and 5′-GTG GCC AAT GAG GTG GAT CAC CAG ATT TGG AGC GA-3′ were used to replace Ala-357, Gln-359, and Tyr-360 with Val, Asn, and Glu, and to delete the 11 amino acid residues from Ala-363 to Asn-373 simultaneously (Del11+AQY/VNE).

Preparation of TVA I, TVA II, and TVA I Mutants—Wild-type TVA I and TVA II were purified as described (*6*), and the AQY/VNE mutant was purified by the same procedure as for the wild-type TVA I. The Del11 and Del11+AQY/VNE mutants were purified by a different method since the enzymes are not eluted from a Butyl-Toyopearl hydrophobic column (Tosoh, Japan) under acidic conditions; 50 mM sodium acetate buffer, pH 5.0, was changed to 20 mM Tris-Cl buffer, pH 8.0. SDS-PAGE gave a single band for each of the purified enzymes. The protein concentrations were determined by the method of Lowry *et al.* (*24*) using bovine serum albumin (Pierce, USA) as a standard.

Analysis of Hydrolysis Products—The reaction samples were incubated at 40°C in 50 mM sodium acetate buffer, pH 5.0 (for wild-type and mutated TVA I), or 50 mM sodium phosphate buffer, pH 6.0 (for TVA II). The products were separated by thin layer chromatography (TLC) on Kiselgel 60 (Merck) with 1-butanol/ethanol/water (5:5:2 or 5:5:3), and the spots were detected by charring with H_2SO_4. When reducing end-*U*-^{14}C-labeled oligosaccharides were used, the reaction was carried out at 40°C in 30 ml of 50 mM sodium acetate buffer (pH 5.0) for wild-type TVA I and its mutants, and in 30 ml of 50 mM sodium phosphate buffer (pH 6.0) for TVA II. The products were separated by TLC and then autoradiographically quantified with a radioactive scanner, Fuji Film BAS2000 Image Analyzer.

Enzymic Assays—The activities were assayed as described (*6, 7*). The enzymic reaction was carried out in 80 mM sodium acetate buffer (pH 5.0) for TVA I and its mutants, and in 80 mM sodium citrate buffer (pH 6.0) for TVA II at 40°C. The products were analyzed by the Nelson-Somogyi method for pullulan, starch, α-cyclodextrin, maltooligosaccharides, from maltotetraose to maltoheptaose, and various amyloses by monitoring the reducing power of the hydrolysate. One unit of 1% pullulan-hydrolyzing activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol of glucosidic linkages per min. For maltotriose and isopanose, the glucose produced was quantified by the glucose-oxidase/peroxidase methods. To detect the cleavage of PNPG2, the absorbance at 400 nm with an alkaline solution was measured to elucidate the amount of released *p*-nitrophenol.

RESULTS

Actions of Wild-Type and Mutated TVA I on Pullulan and Starch—As the optimal pH of the mutants was 5.0, which did not differ from that of the wild-type enzyme (data not shown), all the assays were carried out at pH 5.0.

One percent pullulan (500 μl) was incubated with 0.65 unit of the wild-type and mutated TVA I at 40°C. For 1% pullulan, the specific activities of the wild-type, Del11, AQY/VNE, and Del11+AQY/VNE enzymes were 240, 150, 15, and 3.7 units/mg respectively, thus the ratio of the concentrations of the enzymes used under these conditions was 1:1.6:16:65. The wild-type enzyme liberated panose from pullulan. However, the wild-type enzyme also liberated glucose and maltose, although the amounts were very low (Fig. 3A, lanes 1-3), suggesting that the wild-type enzyme exhibits weak hydrolytic activity toward linkage 1 or linkage 3, as shown in Fig. 1, in addition to the activity toward linkage 2. The Del11 enzyme liberated mainly panose, but spots of glucose, maltose and maltotriose were clearly detected (Fig. 3A, lanes 4-6). The action pattern of the AQY/VNE enzyme was markedly different from that of the wild-type enzyme, and the production of glucose, maltose, maltotriose, and IMM was increased (Fig. 3A, lanes 7-9). The Del11+AQY/VNE enzyme mainly produced maltotriose from pullulan and the spot of panose was decreased (Fig. 3A, lanes 10-12), namely, the Del11+AQY/VNE enzyme mainly hydrolyzed α-(1→6)-glucosidic

linkages, and linkage 1, of pullulan.

We also incubated 500 μl of 1% starch with 0.65 unit of the wild-type and mutated enzymes at 40°C (Fig. 3B). The action patterns of the wild-type and mutated enzymes were almost identical. These findings indicated that the Del11+AQY/VNE enzyme no longer has neopullulanase activity, and thus it could even be regarded as an amylopullulanase.

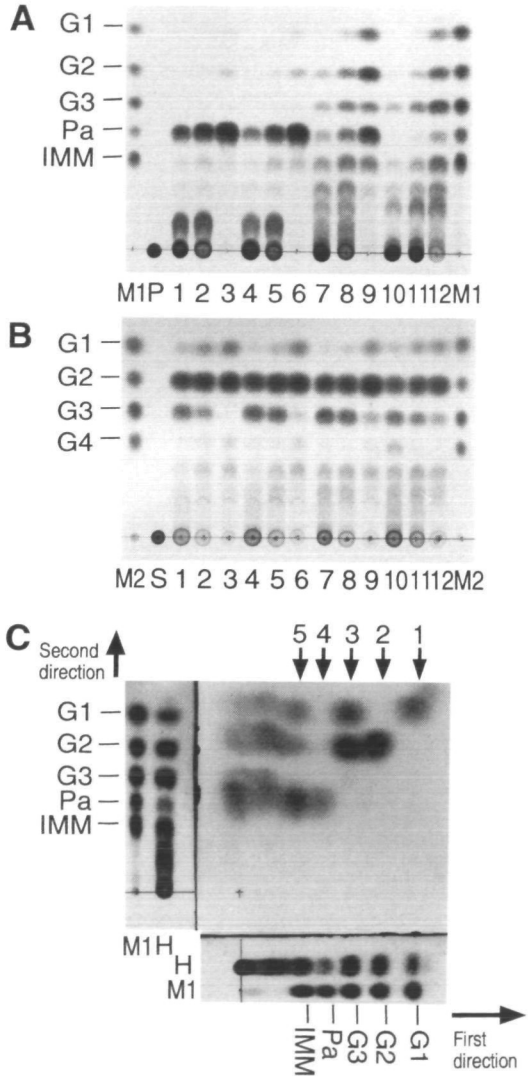

Fig. 3. **TLC showing the actions of wild and mutated TVA I on pullulan and starch.** The reaction mixtures contained 500 μl of 1% pullulan or starch and 0.65 unit of wild-type or mutated TVA I. (A) Time-course of pullulan hydrolysis. Lane M1, oligosaccharide markers: G1, glucose; G2, maltose; G3, maltotriose; Pa, panose; IMM, $4^2\alpha$-isomaltosylmaltose. Lane P, pullulan. Lanes 1-3, 4-6, 7-9, and 10-12 are samples incubated with the wild-type, Del11, AQY/VNE, and Del11+AQY/VNE enzymes, respectively. Lanes 1, 4, 7, 10 are samples at 1 h, lanes 2, 5, 8, 11 are samples at 3 h, and lanes 3, 6, 9, 12 are samples at 20 h. (B) Time-course of starch hydrolysis. M1, oligosaccharide markers: G4, maltotetraose. Lane S, starch. Other abbreviations and the numbers of the lanes are as in (A). (C) Two-dimensional TLC showing the action of the Del11+AQY/VNE enzyme on pullulan. Lanes M1, oligosaccharide markers. The abbreviations are as in (A). Lanes H, hydrolysate of pullulan incubated with the Del11+AQY/VNE enzyme for 20 h. After the major products, denoted as 1-5, had been separated, wild-type TVA I was sprayed and incubated. The TLC was then performed in the second direction.

To confirm the products of the mutants were glucose, maltose, maltotriose, panose, and IMM, two-dimensional TLC was carried out. After the Del11+AQY/VNE enzyme hydrolysate (Fig. 3A, lane 12) had been developed in the first direction, the wild-type enzyme (250 μl of 28 units/ml TVA I in 80 mM sodium acetate buffer, pH 5.0) was sprayed and incubated for 1 h at 50°C, and then redeveloped in the second direction (Fig. 3C). It is known that the wild-type enzyme does not hydrolyze glucose, maltose, or panose, but that it does hydrolyze maltotriose to produce glucose and maltose (*25*). Therefore the spots in lanes 1-4 in Fig. 3C were identified as glucose, maltose, maltotriose, and panose. The major spots in lane 5 generated on the second digestion were glucose and panose. It is also known that the wild-type TVA I hydrolyzes IMM to produce glucose and panose (*19*), thus the major oligosaccharide in lane 5 before the second digestion was identified as IMM. A weak spot of maltose was also detected in lane 5, probably because the strong transglycosylation activity of TVA (*19, 20*) produced several minor oligosaccharides which could not be separated from the spot of IMM during the first development on TLC. The two-dimensional TLC pattern of the AQY/VNE enzyme hydrolysate (Fig. 3A, lane 9) was identical to that of the Del11+AQY/VNE enzyme (data not shown).

Actions of Wild-Type and Mutated TVA I on Oligosaccharides—By introducing the mutation into TVA I, the cleavage point of pullulan was changed from linkage 2 to linkage 1. To confirm this, its action on various oligosaccharides were examined. Each 1% oligosaccharide solution (100 μl) was incubated with 0.085 unit of the wild-type, AQY/VNE or Del11+AQY/VNE enzymes at 40°C for 20 h.

None of the enzymes hydrolyzed maltose and panose (Fig. 4, A and B), suggesting the spots of glucose and maltose on the TLC in Fig. 3A were not derived from maltose or panose. For isopanose, the products of both the wild-type and mutated enzymes were glucose and maltose, but did not include isomaltose, indicating that both the wild-type and mutated enzymes hydrolyzed the α-(1→6)-glucosidic linkages of isopanose (Fig. 4D). For maltotriose and IMM, the action patterns of the mutated enzymes were almost identical to that of the wild-type enzyme, but loss of activity was observed for the mutated enzymes (Fig. 4, C and E). In particular, the Del11+AQY/VNE enzyme hardly hydrolyzed IMM (Fig. 4E, lane 4).

The wild-type enzyme produced only panose from PAPA, a partial hydrolysate from pullulan, whereas the mutated enzymes liberated maltose and IMM in addition to panose (Fig. 4F). If the cleavage point was shifted toward the non-reducing end of PAPA, the products should be isomaltose and 6^2 α-maltosylmaltose. However, no isomaltose spot in lanes 3 and 4 in Fig. 4F, thus the cleavage point changed to the α-(1→6)-glucosidic linkage of PAPA. This finding supports that the cleavage point of the mutated enzymes for pullulan changed to linkage 1.

Kinetic Studies on Oligosaccharides and Pullulan—Why did the cleavage point of the mutated enzymes for pullulan change from linkage 2 to linkage 1? A probable explanation is as follows. The concentrations of the enzymes were different under the conditions in Fig. 3A, namely, the concentration of the Del11+AQY/VNE enzyme was 65 times higher than that of the wild-type enzyme. Because of

the slight activity toward linkage 1 of the wild-type enzyme (Fig. 3A, lanes 1-3), if the mutations reduced the activity toward only the α-(1→4)-glucosidic linkage (for linkage 2), *i.e.* they did not affect the activity toward the α-(1→6)-glucosidic linkage (linkage 1), the activity toward linkage 1 alone would be clearly detectable on TLC with high concentrations of the mutated enzymes.

To assess this possibility, we measured the kinetic parameters for substrates whose cleavage points are only α-(1→4)- or α-(1→6)-glucosidic linkage. Maltotriose and α-cyclodextrin are composed of only α-(1→4)-glucosidic linkages. The wild-type and mutated enzymes hydrolyzed the α-(1→6)-glucosidic linkages of isopanose (Fig. 4D). When *p*-nitrophenyl α-D-maltoside (PNPG2) was used as the substrate, the hydrolysis of neither α-(1→4)- nor α-(1→6)-glucosidic linkages was detected. The kinetic parameters for pullulan, maltotriose, α-cyclodextrin, isopanose, and PNPG2 are summarized in Table I, and the ratio of the k_{cat}/K_m values of the wild-type and mutated enzymes for maltotriose, isopanose, α-cyclodextrin, and PNPG2 are shown in Fig. 5. For maltotriose and α-cyclodextrin whose cleavage points are α-(1→4)-glucosidic linkages, the k_{cat}/K_m values of the mutants were decreased, while for isopanose and PNPG2, whose cleavage points are not the α-(1→4)-glucosidic linkages, the k_{cat}/K_m values were almost identical for the wild-type and mutated enzymes, as we expected.

Hydrolysis of a Series of Maltooligosaccharides, from Maltotriose to Maltoheptaose—At the start of this study, we planned to replace the amino acid residues of TVA I by TVA II-type residues. Both TVA I and TVA II hydrolyze pullulan to produce panose. However, we have already reported that TVA II showed higher hydrolysis efficiency for small oligosaccharides such as maltotriose. To determine whether the action patterns for maltooligosaccharides of the mutated TVA I are like those of wild-type TVA I or TVA II, we determined the kinetic values for a series of maltooligosaccharides, from maltotriose to maltoheptaose (Table II). Although the hydrolytic activity, k_{cat}/K_m, of the Del11+AQY/VNE uniformly decreased for each of the oligosaccharides, the k_{cat}/K_m ratio patterns were almost identical for the wild-type TVA I and Del11+AQY/VNE enzymes, whereas the pattern of TVA II was different (Fig. 6A).

We also investigated the bond-cleavage patterns for a series of reducing end-^{14}C-labeled maltooligosaccharides of the wild-type TVA I, Del11+AQY/VNE, and TVA II enzymes (Fig. 6B). Although wild-type TVA I and TVA II showed similar cleavage patterns for maltotriose and maltotetraose, there was some difference in the cleavage patterns for the hydrolysis of maltopentaose and longer substrates. TVA I has one major cleavage point and another minor point for these substrates, while TVA II has two major cleavage points. The bond-cleavage pattern of the Del11+AQY/VNE enzyme was almost identical to that of wild-type TVA I, but dissimilar to that of TVA II.

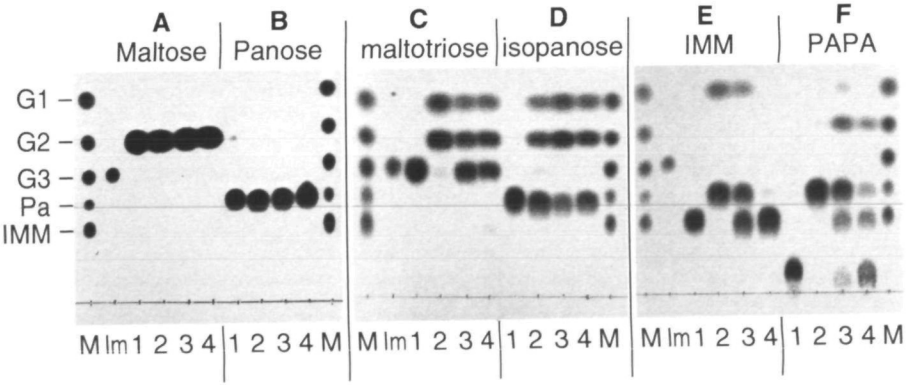

Fig. 4. **TLC showing the actions of the wild-type and mutated TVA I on oligosaccharides.** The reaction mixtures contained 100 μl of 1% oligosaccharide and 0.085 unit of wild-type or mutated TVA I. Lane M, oligosaccharide markers, the abbreviations being as in Fig. 3; lane Im, isomaltose; lane 1, samples with no enzyme added: lanes 2-4, samples incubated for 20 h with the wild-type, AQY/VNE, and Del11+AQY/VNE enzymes, respectively.

TABLE I. **Kinetic parameters of wild-type TVA I, mutated TVA I, and TVA II.** [a] K_m values were calculated using the number-average molecular weights.

	Pullulan[a]			Maltotriose		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)	k_{cat} (s^{-1})	K_m(mM)	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
Wild-type TVA I	403±1	0.131±0.016	3,077	38.0±0.7	6.00±0.25	6.33
Del11	284±14	0.0827±0.0124	3,439	7.60±0.22	4.46±0.34	1.70
AQY/VNE	14.5±0.7	0.0285±0.0039	509	0.369±0.004	1.44±0.06	0.257
Del11+AQY/VNE	4.41±0.16	0.0378±0.0049	117	0.00688±0.00012	0.131±0.014	0.0525
TVA II	64.6±1.0	0.135±0.005	479	12.2±0.5	0.619±0.083	19.7

	α-Cyclodextrin			Isopanose			PNPG2		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
Wild-type TVA I	10.3±0.5	81.3±6.8	0.127	6.72±0.14	28.7±1.3	0.234	318±5	6.71±0.32	47.4
Del11	4.55±0.49	68.2±13.1	0.0667	4.81±0.25	44.3±4.1	0.109	172±7	11.9±1.4	14.5
AQY/VNE	0.211±0.050	52.4±4.3	0.00402	4.82±0.22	37.4±3.2	0.129	732±12	14.3±0.6	51.2
Del11+AQY/VNE	0.0164±0.0014	24.2±5.3	0.000678	1.78±0.09	30.5±3.1	0.0583	242±10	12.4±1.3	19.5
TVA II	34.8±0.8	0.392±0.034	88.8	2.17±0.06	9.68±0.62	0.224	5.05±0.14	0.440±0.053	11.4

DISCUSSION

Site-directed mutagenic studies of the α-amylase family are useful for the design of new industrial amylolytic enzymes. In this study, we successfully manipulated only the activity toward α-(1→4)-glucosidic linkage of TVA I. Because the mutated enzymes still retained slight activity toward α-(1→4)-glucosidic linkages, these enzymes can be designated as amylopullanase-type enzymes. The results indicated that the latter part of region II of pullulan-hydrolyzing enzymes plays a significant role in the hydrolysis of α-(1→4)-glucosidic linkages, and also that independent manipulations of the activity toward the α-(1→4)- and α-(1→6)-glucosidic linkages of pullulan-hydrolyzing enzymes are possible.

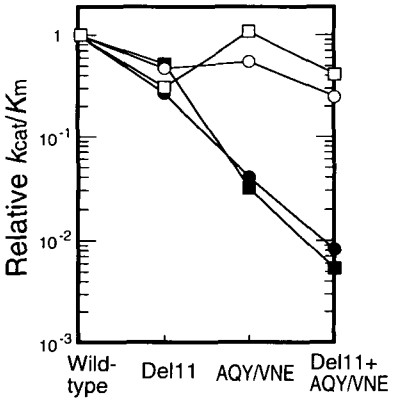

Fig. 5. **Patterns of the ratio of the k_{cat}/K_m values of the wild-type and mutated TVA I for maltotriose α-cyclodextrin, isopanose, and PNPG2.** The values were calculated so that those for the wild-type enzyme = 1, and are plotted on a logarithmic scale. Closed circles, maltotriose; closed squares, α-cyclodextrin; open circles, isopanose; open squares, PNPG2.

This study, however, raises several new questions. Which residues function and how do they hydrolyze the α-(1→4)-glucosidic linkages of pullulan? Why did the Del11+AQY/VNE enzyme behave like an amylopullulanase, not TVA II (or a neopullulanase), although the mutated residues are commonly conserved in not only amylopullulanases but also in TVA II and neopullulanases?

To address the first question, we further investigated the functions of the Ala-357, Gln-359, and Tyr-360 residues by means of site-directed mutagenesis. The sequence, Gln-359 to Tyr-360, of TVA I was replaced by (i) Lys-His, which is the sequence of non-pullulan-hydrolyzing α-amylases (QY/KH mutant), and (ii) Asn-Gln, which is the sequence of TVA II/neopullulanase/amylopullulanase (QY/NE mutant). The action pattern of the QY/KH enzyme for pullulan was almost identical to that of the wild-type enzyme, or rather production of glucose and maltose by the QY/KH enzyme seemed to be decreased (Fig. 7, lanes 1-6). Kuriki *et al.* replaced Glu, which corresponds to Tyr-360 of TVA I, by the non-pullulan-hydrolyzing α-amylases-type residue, His, and observed a similar effect, *i.e.*, loss of activity toward the α-(1→6)-glucosidic linkages (*14*). In contrast, the production of glucose and maltose by the QY/NE enzyme was greater than that by the wild-type enzyme (Fig. 7, lanes 7-9). This alteration of the action pattern for pullulan was, however, less marked than that of the AQY/VNE enzyme (Fig. 3A, lanes 7-9), suggesting that both the Ala residue and the Gln-Tyr sequence affect the hydrolysis of α-(1→4)-glucosidic linkages cooperatively. The Lys-His sequence consists of basic amino acids, while the Asn-Glu sequence includes an acidic amino acid, thus the polar amino acids in this region may determine the activity toward α-(1→4)-glucosidic linkages. On the other hand, we believe that the replacement of Ala-357 of TVA I by Val was the main cause of the steric constraint. The X-ray structures of several α-amylase family enzymes have been reported, and, for example, according to the X-ray structure of the cyclodex-

TABLE II. **Kinetic parameters for maltooligosaccharides of various chain lengths.** [a] K_m values were calculated using the number-average molecular weights.

	Wild-type TVA I			Del11+AQY/VNE		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
Maltotriose (G3)	37.3±1.3	6.63±0.62	5.63	0.01±0.00	0.213±0.026	0.0469
Maltotetraose (G4)	985±103	3.54±0.72	278	1.22±0.08	0.429±0.087	2.84
Maltopentaose (G5)	1,080±180	2.45±0.86	441	2.02±0.10	0.369±0.054	5.47
Maltohexaose (G6)	1,620±240	1.53±0.41	1,060	7.46±0.67	0.876±0.171	8.52
Maltoheptaose (G7)	1,660±190	1.50±0.38	1,110	6.96±0.60	1.12±0.19	6.21
Amylose (DP17)[a]	1,280±210	0.768±0.26	1,670	5.38±0.67	0.160±0.053	33.6
Amylose (DP80)[a]	1,130±70	0.121±0.026	9,340	16.1±1.4	0.133±0.030	121
Amylose AS-30[a]	1,860±100	0.0806±0.093	23,100	22.4±2.8	0.0636±0.0177	352
Amylose AS-70[a]	1,890±60	0.0247±0.0021	76,518	29.9±3.6	0.0491±0.0080	608

	TVA II		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
Maltotriose (G3)	12.2±0.5	0.619±0.083	19.7
Maltotetraose (G4)	42.3±1.9	0.627±0.050	67.5
Maltopentaose (G5)	60.0±9.7	0.215±0.077	279
Maltohexaose (G6)	63.9±6.1	0.232±0.072	275
Maltoheptaose (G7)	64.0±3.6	0.149±0.033	430
Amylose (DP17)[a]	51.3±2.7	0.103±0.017	498
Amylose (DP80)[a]	57.0±2.5	0.0549±0.072	1,040
Amylose AS-30[a]	64.4±5.8	0.0288±0.0061	2,240
Amylose AS-70[a]	57.5±5.7	0.0129±0.0035	4,460

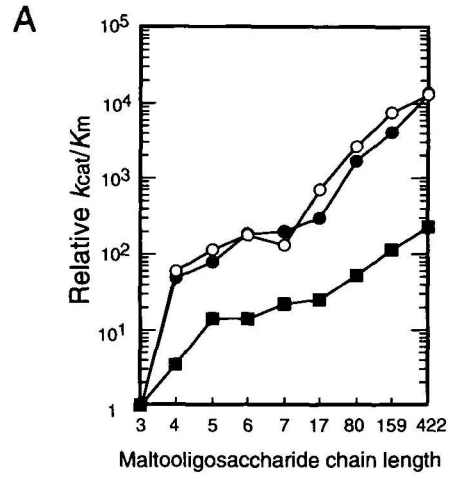

Fig. 6. **Actions of wild-type TVA I, Del11+AQY/VNE TVA I, and wild-type TVA II for maltooligosaccharides.** (A) Patterns of the ratio of the k_{cat}/K_m values. The values were calculated so that those for maltotriose=1, and are plotted on a logarithmic scale. Closed circles, wild-type TVA I; open circles, Del11+AQY/VNE TVA I; closed squares, TVA II. (B) Cleavage point-distribution. Reducing end-^{14}C-labeled oligosaccharides were incubated with the enzymes. The reaction samples were separated by TLC, followed by autographic quantification. Circles represent glucose units and circles with a slash indicate radioactive glucose units. The cleavage-frequencies for the bonds are indicated. The concentrations of the substrates were: maltotriose, 0.991 nM; maltotetraose, 0.750 nM; maltopentaose, 0.603 nM; maltohexaose, 0.505 nM; maltoheptaose, 0.434 nM. The major cleavage points are boxed with bold lines and the minor ones are boxed with thin lines.

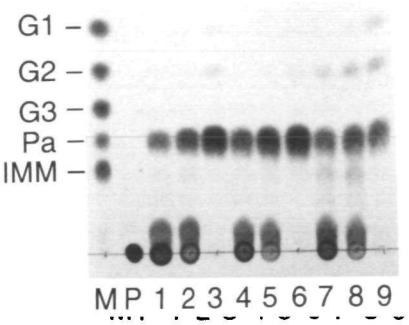

Fig. 7. **TLC showing the actions of the wild-type, QY/KH, and QY/NE TVA I on pullulan.** Lanes M, oligosaccharide markers. The abbreviations are as in Fig. 3. Lane P, pullulan. Lanes 1–3, 4–6, and 7–9 are samples incubated with the wild-type, QY/KH, and QY/NE enzymes, respectively. Lanes 1, 4, 7 are samples at 1 h, lanes 2, 5, 8 are samples at 3 h, and lanes 3, 6, 9 are samples at 20 h.

trin glucanotransferase-maltotetraose complex (*5*), the Ala residue corresponds to Ala-357 of TVA I located near the maltotetraose.

The mutations altered the bond-cleavage pattern of TVA I not for maltooligosaccharides but for pullulan. It has been reported that the latter part of region II is included in the subsite structure of α-amylase family members. Matsui *et al.* modified this region of group I α-amylase and observed certain shifts of the bond-cleavage patterns for maltooligosaccharides (*26*). Therefore, the role of the latter part of region II seems to differ between pullulan-hydrolyzing and non-pullulan-hydrolyzing enzymes.

For maltotriose, pullulan and α-cyclodextrin, significant decreases in the K_m values of the mutated enzymes, especially of the Del11+AQY/VNE enzyme, were observed. These decreases were concomitant with greater decreases in the k_{cat} values (Table I). As these mutants were prepared intending to convert TVA I to a TVA II-type enzyme, one may think that the structures of the substrate binding site of TVA I mutants became similar to that of TVA II. However, as the K_m values for pullulan and maltotriose of the Del11+AQY/VNE enzyme were significantly smaller than those of TVA II, this seems unlikely. Another possibility is that, according to the subsite theory (*27*), if a mutation results in a large decrease in the affinity of a subsite which is far from the catalytic site, decreases in both the K_m and k_{cat} values should be observed because the reduction of the amount of the non-productive ES complex. In this case, the k_{cat}/K_m values should be unaltered. Our data indicated even the k_{cat}/K_m values were greatly decreased in the mutants. Thus, the decreases in the K_m values may be due to stabilization of a conformation which favors these substrates, but with scarce turn over.

There are several explanations as to why Del11+AQY/VNE TVA I behaves not like TVA II (or neopullulanase) but an amylopullulanase. The α-amylase family enzymes have been reported to have several semi-conserved regions in addition to the four highly conserved regions (*28, 29*). It seems that these regions are strongly conserved within the pullulan-hydrolyzing enzymes or within the cyclodextrin glucanotransferases, *etc.*, rather than between the different subfamilies, and we believe that these regions determine the properties of the α-amylase family. Also, a small separate structure which protrudes from the C-terminus of the third β-strand of the $(\beta/\alpha)_8$-barrel framework composes part of the catalytic cleft of α-amylase family enzymes, and Svensson's group designated this structure as domain B (*30*). They constructed a series of chimeric barley α-amylases, and reported that domain B contributed to several of the functional and stability properties of amylase (*31*). These findings suggest that several regions determine the distinct substrate specificities of TVA I and TVA II. We tried to construct several chimeric forms of

TVA I and TVA II but they could not be produced by *E. coli* (Matsumoto, H., Sakai, H., and Matsuzawa, H., unpublished data). We are now modifying other amino acid residues of TVA I.

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