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Modulation of Hydrolysis and Transglycosylation Activity of *Thermus* Maltogenic Amylase by Combinatorial Saturation Mutagenesis

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The roles of conserved amino acid residues (Val329-Ala330-Asn331-Glu332), constituting an extra sugar-binding space (ESBS) of *Thermus* maltogenic amylase (ThMA), were investigated by combinatorial saturation mutagenesis. Various ThMA mutants were firstly screened on the basis of starch hydrolyzing activity and their enzymatic properties were characterized in detail. Most of the ThMA variants showed remarkable decreases in their hydrolyzing activity, but their specificity against various substrates could be altered by mutagenesis. Unexpectedly, mutant H-16 (Gly-Leu-Val-Tyr) showed almost identical hydrolyzing and transglycosylation activities to wild type, whereas K-33 (Ser-Gly-Asp-Glu) showed an extremely low transglycosylation activity. Interestingly, K-33 produced glucose, maltose, and acarviosine from acarbose, whereas ThMA hydrolyzed acarbose to only glucose and acarviosine-glucose. These results propose that the substrate specificity, hydrolysis pattern, and transglycosylation activity of ThMA can be modulated by combinatorial mutations near the ESBS.

Keywords: *Thermus* maltogenic amylase, combinatorial saturation mutagenesis (CSM), substrate specificity, acarbose hydrolysis, transglycosylation activity

Maltogenic amylase (MAase; EC 3.2.1.133), cyclomaltodextrinase (CDase; EC 3.2.1.54), and neopullulanase (NPase; EC 3.2.1.135) belong to the glycoside hydrolase (GH) family 13 [9, 10], which are distinguishable from typical α-amylases by their versatile hydrolysis and transglycosylation activities against various carbohydrate substrates [5, 31]. These enzymes can hydrolyze α-(1,4)-glycosidic linkages of cyclodextrins (CDs) and starch to mainly maltose, and pullulan to panose. In addition, the hydrolyzed sugar moiety can be transferred to various carbohydrate acceptors by mainly forming an α-(1,6)-glycosidic linkage [6, 30, 31, 34].

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Thermus maltogenic amylase (ThMA) is a typical MAase, which hydrolyzes CDs much faster than it does starch or pullulan [19]. Furthermore, it can hydrolyze acarbose, a potent amylase inhibitor, to glucose and acarviosine-glucose, simultaneously transferring acarviosine-glucose to various acceptor molecules to form α-(1,3)-, α-(1,4)-, or α-(1,6)-glycosidic linkages. Kim *et al.* [15] reported that ThMA possesses the unique N-terminal domain and exists as a homodimer in solution. Dimeric MAase-family enzymes were widely known [3, 4, 11, 14, 15, 28], but an interesting dodecameric structure of CDase from an alkalophilic *Bacillus* strain was recently reported [23]. Dimer formation of ThMA can transform an wide and shallow active site cleft in a monomer to a pair of narrow and deep substrate-binding sites in a dimer, making the access of β-CD or maltooligosaccharides preferable to a bulky substrate, starch or pullulan [15, 31]. At the bottom of the active site cleft, an extra sugar-binding space (ESBS) was found in the dimer structure of ThMA, which makes the enzyme possess the versatile transglycosylation activity [15]. The amino acid residues at the interface and the N-domain of the dimeric enzyme are likely to play a critical role in the formation of oligomeric structures [20, 22].

Owing to their catalytic versatility including transglycosylation activity, MAases and related enzymes have been investigated for the enzymatic development of functional carbohydrate materials. Cha *et al.* [2] and Kuriki *et al.* [21] showed that the hydrophobicity of the residues near the active site is critical to the transglycosylation reaction of these enzymes. Sequence alignment of α-amylases and related enzymes revealed a common $(\beta/\alpha)_8$-barrel as the catalytic core domain with four highly conserved regions [12, 13, 25]. Among them, the second conserved region is clearly distinguished from that of other amylolytic enzymes. Kim *et al.* [17] and Baek *et al.* [1] reported that both Asn331 and Glu332 residues of ThMA play important roles in transglycosylation activity. Nevertheless, there have been no further investigations about the amino acid residues at the second conserved region near the ESBS.

In the present work, intensive combinatorial mutations were introduced into well-conserved motif residues forming

the ESBS *via* saturation mutagenesis technique. Mutant enzymes were screened and their enzymatic properties were characterized on the basis of multi-substrate specificities and transglycosylation activity. This report shows the possibility to modulate the hydrolysis and transglycosylation activities of ThMA by combinatorial mutations of the amino acid residues near the ESBS.

Materials and Methods

Bacterial Strains and Growth Conditions

Escherichia coli MC1061 was used as a host for the cloning and expression of target genes and the resulting transformants were grown in Luria-Bertani (LB; 1% bactotryptone, 0.5% yeast extract, 1% NaCl) medium containing ampicillin (100 μg/ml) at 37°C.

Enzymes and Reagents

Taq DNA polymerase, modifying enzymes, and restriction endonucleases were purchased from Roche Applied Science (Mannheim, Germany) or Takara Biomedical Inc. (Otsu, Japan). *AccuPrep* plasmid extraction kit, PCR purification kit, and PCR primers were provided by Bioneer (Daejeon, Korea). Other chemicals and reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.), Merck (Darmstadt, Germany), or Duchefa Biochemie (Haarlem, The Netherlands).

Combinatorial Saturation Mutagenesis

Random substitutions of amino acid residues from Val329 to Glu332 of ThMA were performed by a combinatorial saturation mutagenesis (CSM) technique. The degenerate mutagenic primer TMX-DP2N (5'-TGGCGTCTAGANNNNNNNNNNNNATCGA-TCAATTTTGGCGC-3') and M13F universal sequencing primer (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') were used for the amplification of part of the ThMA gene by using a Px2 thermal cycler (Thermo-Hybaid, Middlesex, U.K.). The amplified DNA fragment digested with XbaI and HindIII was ligated into the p6xHTMX [17] and transformed into *E. coli* MC1061 by electroporation (GenePulser Xcell, Bio-Rad Co., Hercules, CA, U.S.A.). The mutants with desirable starch-hydrolyzing activity were screened on the LB agar plate containing 1% soluble starch. DNA sequencing was performed by the Genome Research Facility in Seoul National University (Seoul, Korea) using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

Expression and Purification of ThMA variants

Recombinant *E. coli*, harboring p6xHTMX or its derivative, was cultivated in LB broth containing ampicillin (100 μg/ml) at 37°C for 12 h. The cells were harvested and disrupted by sonication (VCX750, Sonics & Materials, Inc., Newtown, CT, U.S.A.). Each ThMA variant with N-terminal six-histidines was simply purified by using nickel-nitrilotriacetic acid (Ni-NTA; Qiagen, Hilden, Germany) column chromatography.

Activity Assays for ThMA Variants

Purified enzyme was incubated with 0.5% of each substrate, including soluble starch, pullulan, β-CD, maltotriose, or acarbose, in 50 mM sodium-acetate buffer (pH 6.0) at 60°C for an appropriate time to determine its hydrolyzing activity. The 3,5-dinitrosalicylic acid (DNS) reducing sugar method [26] was used for the hydrolyzing activity assay against β-CD, soluble starch, or pullulan. The glucose oxidase-peroxidase method [7] was used for the hydrolysis of acarbose or maltotriose. One unit of hydrolyzing activity towards β-CD (CU), starch (SU), or pullulan (PU) is defined as the amount of enzyme producing 1 μmol equivalent of maltose for 1 min, and one unit of acarbose (AU) or maltotriose (MU) hydrolyzing activity is determined as the amount of enzyme producing 1 μmol equivalent of glucose for 1 min.

Analysis of Enzyme Reaction Products

Transglycosylation reactions with ThMA and its mutants were carried out. For the maltotriose reaction, enzyme (0.2 U/mg maltotriose) was added to 10% (w/v) of maltotriose in sodium acetate buffer (50 mM, pH 6.0). For acarbose reaction, enzyme (0.3 U/mg acarbose) was added to the same reaction buffer containing 5% acarbose (donor) and 10% α-methyl glucopyranoside (acceptor). After the reaction at 60°C for 24 h, transfer products were analyzed by thin layer chromatography (TLC) or high-performance anion-exchange chromatography (HPAEC). A CarboPac PA1 column (0.2×25 cm; Dionex Co., Sunnyvale, CA, U.S.A.) was used for HPAEC analysis (Bio-LC ICS-3000; Dionex). Buffer A (150 mM NaOH; Fisher Scientific, Rockford, IL, U.S.A.) and buffer B (600 mM sodium-acetate; Sigma-Aldrich) were used for elution along with a linear gradient. For TLC analysis, the resulting products were separated by the solvents of isopropanol/ethylacetate/water (3:1:1, v/v/v). The silica gel K5F TLC plate (Whatman, Düsseldorf, Germany) was visualized by dipping it into a solution containing 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol and then heating it for 10 min at 110°C.

Results and Discussion

Generation and Screening of ThMA Mutants

Among most amylolytic enzymes, four well-conserved regions including catalytic amino acid residues have been commonly reported [12, 25, 29]. Jespersen *et al.* [13] earlier classified MAase-family enzymes into glycoside hydrolase family 13 on the basis of their sequence similarity. As shown in Fig. 1, most amylolytic enzymes possess similar amino acid residues at the front part of the second conserved region, but the later part residues of the conserved region II (gray-shaded in Fig. 1) are relatively variable between typical amylases and MAase-type enzymes. Common fungal α-amylase (Thr-Val-Lys-His) and *Bacillus* cyclodextrin glucanotransferase (Ala-Val-Lys-His) possess typical hydrolysis and transglycosylation activities specific for α-(1,4)-glycosidic linkages, respectively [8, 27]. On the other hand, a novel enzyme (Cys-Gly-His-Asp) with dual activities of CDase and α-glucosidase was recently cloned and characterized from *Thermotoga maritima*, which showed quite different action patterns and substrate specificity compared with MAases [24]. Accordingly, four amino acid residues (Val329-Ala330-Asn331-Glu332) located around the ESBS of ThMA were chosen to be mutated in this study.

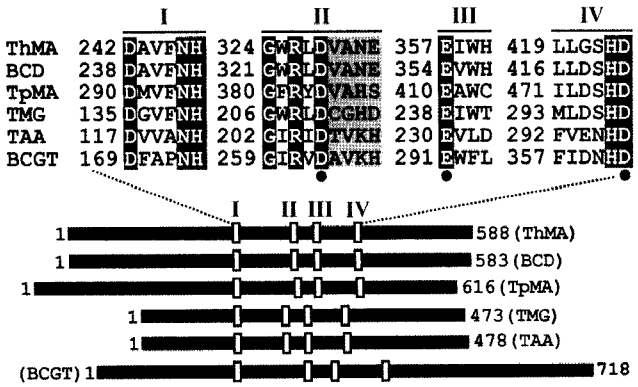

Fig. 1. Comparison of amino acid residues in the four conserved regions found in various amylolytic enzymes.
Four conserved regions (I-IV) are schematically shown on the basis of sequence alignment between ThMA and related enzymes. ThMA, *Thermus* MAase [19]; BCD, alkalophilic *Bacillus* CDase [18]; TpMA, *Thermoplasma volcanium* MAase [16]; TMG, *Thermotoga maritima* glucosidase [24]; TAA, *Aspergillus oryzae* α-amylase [33]; BCGT, *Bacillus circulans* cyclodextrin glucanotransferase [27]. Invariant residues are indicated as white letters on a black background, whereas the target residues in this work are shaded in dark gray. Putative catalytic residues are shown with closed circles.

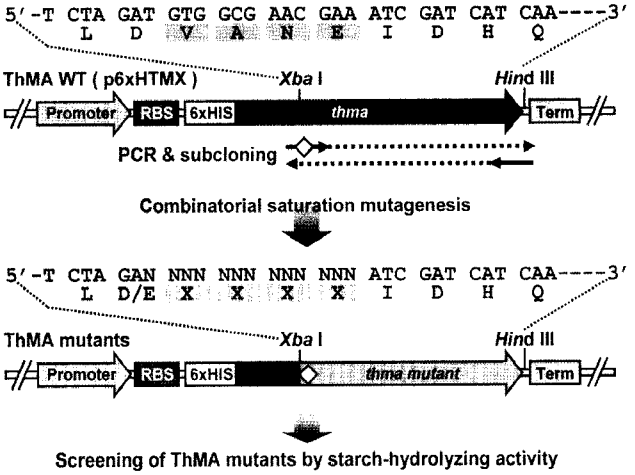

Fig. 2. Scheme for the generation of ThMA mutants by combinatorial saturation mutagenesis technique.
PCR-products containing various mutagenic sequences (shown as a diamond) were amplified and introduced between the XbaI and HindIII sites of p6xHTMX. The resulting variants were screened by using their starch-hydrolyzing activity.

Even though site-directed mutagenesis is routinely used for understanding the function of any specific residue, extremely low activity with unexpected conformational change can often restrict its availability. On the contrary, combinatorial saturation mutagenesis (CSM) can be applicable to generation of various mutants with considerable activity and stable structure followed by high throughput

Table 1. Specific hydrolysis activities of ThMA and its mutants on various substrates.

No.	ThMA	AA sequence	Specific activity (Units/mg)					Activity ratio		
			CU[a]	PU[a]	SU[a]	MU[b]	AU[b]	C/S	P/S	M/A
*	WT	-VANE-	55.66	3.33	2.60	38.24	11.02	21.41	1.28	3.47
A	H-16	-GLVY-	59.89	3.36	3.62	20.70	9.26	16.55	0.93	2.24
B	C-20	-VGGC-	2.02	0.27	0.49	0.62	2.20	4.12	0.55	0.28
C	F-80	-VSAE-	21.23	0.78	0.49	2.62	4.43	43.33	1.60	0.59
D	C-43	-VGPG-	2.19	0.11	0.44	1.38	3.11	4.98	0.25	0.44
E	G-22	-VGGS-	3.39	0.16	0.39	0.37	1.06	8.69	0.41	0.35
F	I-69	-VASG-	3.55	0.20	0.39	1.21	3.50	9.10	0.51	0.35
G	F-18	-VGCC-	2.63	0.22	0.34	0.02	0.03	7.74	0.65	0.67
H	I-70	-FTGW-	3.64	0.14	0.31	0.94	2.66	11.74	0.45	0.35
I	G-90	-VGVG-	1.36	0.03	0.26	0.37	0.93	5.23	0.12	0.40
J	G-91	-VGGG-	6.69	0.33	0.25	1.21	3.89	26.76	1.32	0.31
K	K-37	-VSGT-	1.47	0.08	0.23	0.34	1.20	6.39	0.35	0.28
L	G-13	-AGVA-	1.59	0.08	0.20	0.47	0.41	7.95	0.40	1.15
M	D-3	-CAHR-	0.27	0.17	0.18	0.01	0.02	1.50	0.94	0.50
N	A-39	-ACGV-	1.13	0.04	0.16	1.16	1.69	7.06	0.25	0.69
O	K-33	-SGDE-	4.41	0.17	0.13	2.57	1.79	33.92	1.31	1.44
P	B-4	-AALE-	1.76	0.12	0.12	0.11	0.03	14.67	0.10	3.67
Q	C-56	-IGWE-	1.87	0.08	0.11	0.17	0.28	17.00	0.73	0.61
R	A-18	-SCSP-	0.52	0.09	0.09	0.04	0.06	5.78	1.00	0.67
S	B-96	-VMGC-	0.32	0.05	0.06	0.02	0.05	5.33	0.83	0.40
T	E-50	-TCTV-	0.24	0.06	0.06	0.02	0.04	4.00	0.10	0.50
U	E-74	-SGGV-	0.55	0.05	0.06	0.06	0.11	9.17	0.83	0.55

[a]CU (cyclodextrin-hydrolyzing units), PU (pullulan-hydrolyzing units), and SU (starch-hydrolyzing units) were measured using the DNS reducing sugar assay method.
[b]MU (maltotriose-hydrolyzing units) and AU (acarbose-hydrolyzing units) were determined using the glucose oxidase-peroxidase assay method.

screening procedures [35, 36]. Therefore, the CSM technique was used here to investigate the factors modulating the hydrolysis and transglycosylation activities of ThMA.

As shown in Fig. 2, DNA fragments containing a combination of saturated mutations were PCR-amplified and inserted into the target region between the XbaI and HindIII sites of p6xHTMX. After transformation into *E. coli*, the ThMA variants were screened by the iodine test for their starch-hydrolyzing activity. In total 4,500 transformants were obtained and approximately 60% of them showed varied activity against starch. Twenty-one clones forming desirable clear zones were finally selected as variant candidates. The sequence analyses revealed that various combinatorial mutations were successfully introduced into the target region of ThMA by the CSM technique (shown in Table 1). ThMA wild type and mutants, fused with N-terminal six-histidines, were well-expressed in *E. coli* and simply purified to homogeneity by Ni-NTA column chromatography.

Substrate Specificity of ThMA Mutants

Hydrolysis activities of wild-type and mutant ThMAs were determined against starch, pullulan, β-CD, maltotriose, or acarbose as a substrate. As shown in Table 1, the hydrolysis activities of mutant enzymes decreased remarkably, but an exclusive mutant, H-16 (Gly-Leu-Val-Tyr), showed comparable activities with ThMA wild type. Interestingly, although four corresponding residues were completely substituted, the hydrolyzing activities of H-16 were very similar or slightly higher than those of the wild type. This result suggests that the CSM technique can be a potential alternative to site-directed mutagenesis to generate the fully active mutants.

In order to compare the changes in substrate specificity of ThMA variants, their hydrolysis activities were shown as the activity ratios between substrates (Table 1), and the variations of relative substrate specificity were comparatively and graphically summarized by designing the relative substrate specificity index (Fig. 3). For an example of the index calculation, the activity ratio for CD/starch of ThMA wild type was subtracted from that of each mutant and then the resulting value was divided by the ratio of wild type. Positive index value means the higher activity ratio of variant than that of ThMA wild type. According to the index values in Fig. 3, mutations in residues near the ESBS generally resulted in a significant decrease of relative specificity on β-CD or pullulan to starch substrate, except for a few ThMA variants such as F-80. Specifically, their relative preferences on maltotriose or acarbose were completely reduced through all the mutants. On the contrary, the mutants F-80 (Val-Ser-Ala-Glu) and K-33 (Ser-Gly-Asp-Glu) showed much higher CD/starch indexes than that of wild type (Val-Ala-Asn-Glu), which suggested that the mutations around the ESBS may cause any subtle

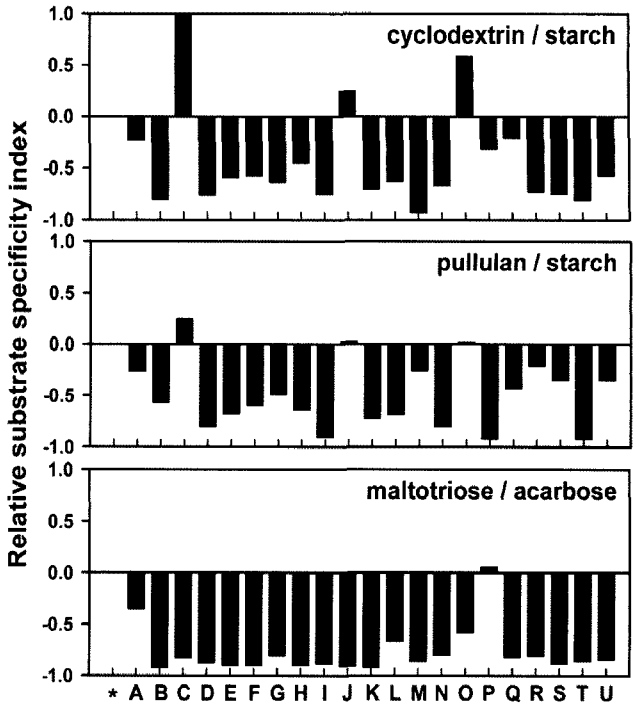

Fig. 3. Changes in relative substrate specificity of ThMA and its mutants.
Considering the multi-substrate specificity of ThMA, the variations in relative hydrolysis activity against various substrates were compared by using the relative substrate specificity index. ThMA mutants are numbered by alphabetical orders identical to those in Table 1.

changes in substrate binding to modulate substrate preferences towards a specific direction.

Hondoh *et al.* [11] reported that the valine residue at 329 of neopullulanase plays an important role in substrate-binding *via* hydrophobic interaction with various substrates. The corresponding valine residue was conserved in 10 out of 21 ThMA mutants, which suggested that the valine residue plays a significant role in starch-hydrolyzing activity. In addition, ThMA mutants with relatively high activity possess abundant glycine residues in the mutated region, which may have resulted from the structural stabilization by adding small and flexible glycine residues to the ESBS.

Acarbose Hydrolysis of ThMA Mutant K-33

Hydrolysis patterns of ThMA mutants on each substrate were carefully examined by TLC and HPAEC analyses. Despite the changes in substrate specificities of mutant enzymes, there were little differences in hydrolysis patterns between the wild type and mutants. However, ThMA variant K-33 showed significantly different action patterns on acarbose substrate. Acarbose is the well-known substrate analog used as an α-glucosidase inhibitor for the treatment of diabetes. Most of amylolytic enzymes cannot hydrolyze acarbose, but ThMA hydrolyzes it to mainly glucose and acarviosine-glucose. This means that the binding mode of

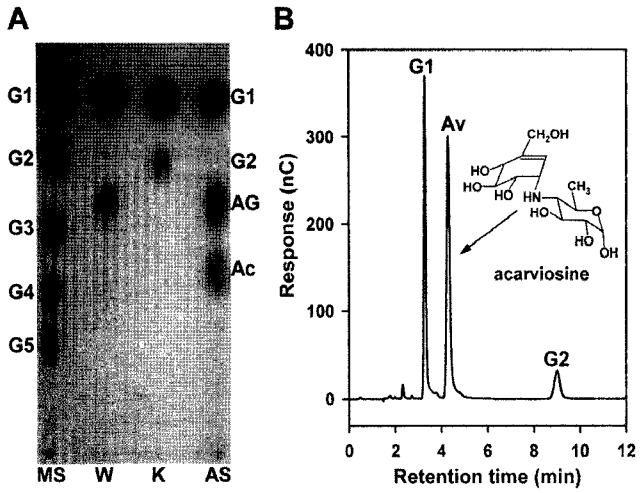

Fig. 4. Differences in acarbose hydrolysis patterns between ThMA and mutant K-33.

A. TLC analysis of the acarbose hydrolysate by ThMA (W) and K-33 (K): MS, maltooligosaccharides standard from glucose (G1) to maltopentaose (G5); AS, acarbose derivatives standard containing glucose; AG, acarviosine-glucose; Ac, acarbose. **B.** HPAEC analysis of the acarbose hydrolysate by mutant K-33: G2, maltose; Av, acarviosine.

acarbose with ThMA can be quite different from that with any typical amylases.

Interestingly, mutant K-33 produced glucose, maltose, and acarviosine from acarbose molecules, whereas ThMA produced only glucose and acarviosine-glucose (Fig. 4). The resulting acarviosine molecule could not be visualized by the common detection method used in TLC [24], but the corresponding peak was clearly identified by HPAEC analysis. Even though the excess amount of ThMA may make very little amount of acarviosine *via* further hydrolysis, it can predominantly cleave acarbose to glucose and acarviosine-glucose. However, K-33 produced a significant amount of glucose, maltose, and acarviosine as final products, which indicated that its preferences to attack the glycosidic linkages in acarbose molecule were definitely shifted. Moreover, mutant K-33 is likely to possess a much higher hydrolyzing activity on acarviosine-glucose than that of wild type. All the possible action modes of ThMA variant K-33 against acarbose are schematically proposed and compared with wild type in Fig. 5.

As previously mentioned, it was reported that the valine329 residue of neopullulanase plays an important role in substrate binding *via* hydrophobic interaction [11]. In addition, Park *et al.* [32] substituted the Ala290 residue at the interface of the ThMA dimer in the vicinity of the substrate-binding site with an isoleucine, which caused structural changes due to its bulky side chain and revealed that ThMA mutant Ala290Ile hydrolyzes maltotetraose to produce mainly maltose. In a similar way, the mutations of three residues (from Val-Ala-Asn to Ser-Gly-Asp) in the ESBS can modulate its subsite affinity for acarbose, which may cause the shift in the acarbose-binding with mutant K-33. This result implied that the substitution of amino acid residues around the ESBS can modulate the substrate binding and hydrolyzing patterns of ThMA.

Transglycosylation Activity of ThMA Mutants

From the transglycosylation with maltotriose, ThMA synthesized various transfer products including isomaltose,

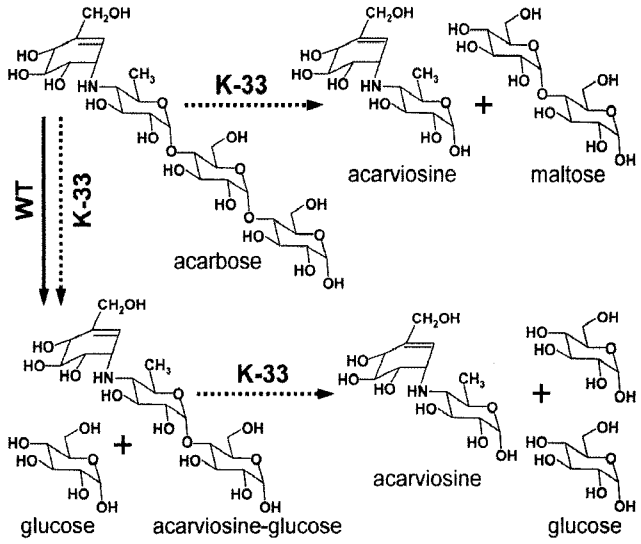

Fig. 5. Proposed mechanisms in acarbose hydrolysis by ThMA and its mutant K-33.

Whereas ThMA wild type can hydrolyze acarbose to glucose and acarviosine-glucose, mutant K-33 can alternatively produce maltose or acarviosine, as well as glucose and acarviosine-glucose. Probable directions of acarbose hydrolysis with ThMA and its mutant K-33 are shown by solid and dashed arrows, respectively.

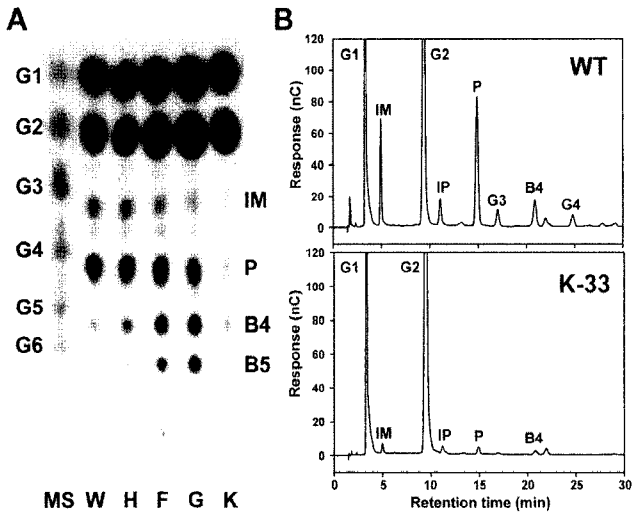

Fig. 6. Analyses of maltotriose transglycosylation products by ThMA and its mutants.

A. TLC analysis of the transfer products from maltotriose by ThMA wild type (W), H-16 (H), F-80 (F), G-91 (G), and K-33 (K): MS, maltooligosaccharides standard (G1-G5); IM, isomaltose; IP, isopanose; P, panose; B4, branched tetraose; B5, branched pentaose. **B.** HPAEC analysis of the transfer products from maltotriose with ThMA and mutant K-33.

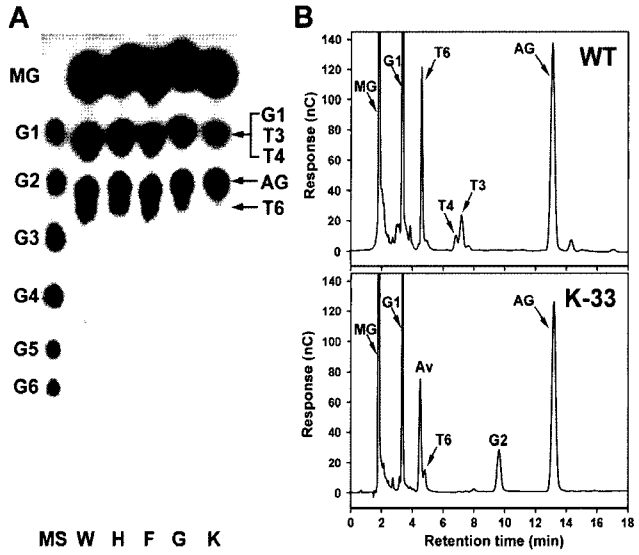

Fig. 7. Analyses of acarbose transglycosylation products with an α-methyl glucoside acceptor by ThMA and its mutants.
A. TLC analysis of the transfer products from acarbose by ThMA wild type (W), H-16 (H), F-80 (F), G-91 (G), and K-33 (K): MS, maltooligosaccharides standard (G1-G5); AG, acarviosine-glucose; T3, α-(1,3)-transfer product; T4, α-(1,4)-transfer product; T6, α-(1,6)-transfer product. **B.** HPAEC analysis of the transfer products from acarbose with ThMA and mutant K-33.

isopanose, panose, and branched oligosaccharides (Fig. 6). ThMA variant H-16 showed similar transglycosylation patterns to wild type, whereas both F-80 and G-91 produced a high amount of branched tetraose and pentaose. However, mutant K-33 generated mainly hydrolysis products, with very little amount of transfer products. In the acarbose transglycosylation with K-33, a dramatic decrease of transfer products was observed (Fig. 7). Moreover, ThMA mutant K-33 produced a significant amount of acarviosine and maltose (Fig. 7B), which probably coincided with its unique hydrolysis patterns on acarbose in Fig. 5. These results indicated that the mutations of amino acid residues near the ESBS can deeply affect the transglycosylation activity of ThMA.

To date, the importance of Asn331 and Glu332 at the ESBS in transglycosylation of ThMA has been experimentally confirmed by site-directed mutagenesis [1, 17]. There has been no doubt that both amino acids are key residues for the transglycosylation activity of ThMA. Nevertheless, the results found in this work gave rise to questions for the additional factors affecting the transglycosylation of ThMA. Even though the H-16 mutant has no essential Glu332 residue, combinatorial mutations near key residues may cause the conformational adaptation for the comparable transglycosylation activity. In the opposite way, the K-33 variant possessing Glu332 may lose its transglycosylation ability by a subtle conformational change in the ESBS. Accordingly, Glu332 can be one of the key residues for the transglycosylation of ThMA, but other residues around Glu332 may also play roles in structural stabilization and modulation. All the variants obtained here proposed that additional structural factors should be considered for modulating the specificity or the activity of ThMA.

In the present study, the substrate specificity, hydrolysis, and transglycosylation activities of ThMA could be modulated by the combinatorial substitutions of key residues and their neighbors in the ESBS. Various efforts to find the additional key residues for the versatile activity of ThMA and related enzymes are in progress, which can give us an opportunity to develop novel enzymes engineered for the production of specific functional carbohydrate materials.

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