

Role of Dipeptide at Extra Sugar-Binding Space of *Thermus* Maltogenic Amylase in Transglycosylation Activity

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Abstract Two conserved amino acid residues in the extra sugar-binding space near the catalytic site of *Thermus* maltogenic amylase (ThMA) were analyzed for their role in the hydrolysis and transglycosylation activity of the enzyme. Site-directed mutagenesis was carried out by replacing N331 with a lysine (N331K), E332 with a histidine (E332H), or by replacing both residues at the same time (N331K/E332H). The measured K_m values indicated that affinities toward all substrates tested, including starch, pullulan, β -cyclomaltodextrin, and acarbose, were lower in all the mutants compared to that of wild-type ThMA, leading to reduced hydrolysis activity. In addition, the lower ratio of transglycosylation to hydrolysis in the mutants compared to that in the wild-type ThMA indicated that these mutants preferred hydrolysis to the transglycosylation reaction. These results demonstrated that the conserved dipeptide at 331 and 332 of ThMA is directly involved in the formation and accumulation of transfer products by accommodating acceptor sugar molecules.

Key words: *Thermus* maltogenic amylase (ThMA), site-directed mutagenesis, transglycosylation, acarbose

Various types of oligosaccharides have been synthesized by enzymatic transfer reactions involving glucan sucrases [1, 15, 17], cyclomaltodextrin glucanotransferase [CGTase; 14], and maltogenic amylases [MAases; 2, 5, 6, 9]. MAases have attracted special attention due to their capability of forming α -1,6-linked transglycosylation products from starch. MAases are members of α -amylase family 13 and possess properties distinct from those of other amylolytic

enzymes. The enzymes hydrolyze various substrates having α -(1,4)- and/or α -(1,6)-glycosidic linkages, such as soluble starch, pullulan, and cyclomaltodextrin (CD), to yield maltose as the major product. In addition, they have strong transglycosylation activity in the presence of various acceptor molecules, thereby accumulating branched oligosaccharides [8, 12, 13]. Furthermore, MAases hydrolyze acarbose, a potent amylase inhibitor, to glucose and acarviosine-glucose (pseudotrisaccharide; PTS), simultaneously transferring PTS to various acceptor molecules to form α -(1,3)-, α -(1,4)-, and α -(1,6)-glycosidic linkages [2, 6, 11].

Kim *et al.* [4] reported that maltogenic amylase of *Thermus* sp. (ThMA) was a homo-dimer in solution, and that the *N*-terminal domain of one subunit partly covered the top of the $(\beta/\alpha)_8$ -barrel of the other. As a result, the interface of the dimer formed a narrow and deep groove at the top of the barrel in contrast to the wide and shallow active site clefts in α -amylases. An extra space at the deep bottom of the active site cleft can accommodate disaccharides such as maltose. Kim *et al.* [4] proposed that this extra sugar-binding area might be important for the transglycosylation activity of ThMA. Sugar molecules occupying the site can serve as acceptors competing with water molecules in attacking the enzyme-substrate intermediate. Recent X-ray crystallography analysis of ThMA-dimer indicated that the N331 and E332 residues were located in a pocket, the so-called "extra sugar-binding space" [4]. Comparison of the primary structures of ThMA and other closely related enzymes suggested that these residues are involved in transferring activities (Fig. 1). The transglycosylation activity of MAase from *Bacillus stearothermophilus* (BSMA) was reduced to 50% of the wild-type activity, when E332 was replaced with a histidine [2]. Kim *et al.* [7] replaced the E332 of ThMA with a histidine

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and observed a drastic decrease in transfer products. The result strongly suggested that glutamic acid at 332 is located at a subsite of ThMA that binds to a part of the substrate.

Based on these previous findings, in the present study, the role of the conserved dipeptide, N331 and E332, of ThMA in the transglycosylation activity of the enzyme was investigated. This report provides evidence that N331 and E332 of MAases are involved in transglycosylations, forming α -(1,3)-, α -(1,4)-, and α -(1,6)-linkages. Based on the present results together with previous information on the tertiary structure of ThMA [4], a mechanism for initiating transglycosylation reactions in MAases and related enzymes is proposed.

MATERIALS AND METHODS

Purification of ThMA

Escherichia coli MC1061 [F, *araD139*, *recA13*, Δ (*araABC-leu*)7696, *galU*, *galK*, Δ *lacX74*, *rpsL*, *thi*, *hsdR2*, *mcrB*] was used as a host for DNA manipulation and transformation. *E. coli* transformants were grown in Luria-Bertani (LB) medium (1% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl) containing ampicillin (100 μ g/ml) at 37°C. An expression vector, p6xHis119, was used for the overproduction of wild-type and mutant enzymes [6]. Six-His-tagged ThMA and its mutants were efficiently purified by a Ni-NTA column (1 \times 4 cm, Ni-NTA superflowTM, QIAGEN, Germany) as described previously [7]. Purity of the enzyme was confirmed by SDS-PAGE analysis.

Site-Directed Mutagenesis

Amino acid substitutions of Asn331, Glu332, or both amino acid residues were constructed separately by cassette mutagenesis of the ThMA gene [16]. Primers ThN331KN (5'-CTAGATGTGGCGAAAGAAATCGATCATCAATTTGCG CG-3') and ThN331KC (5'-AATTCGCGCCAAAATTGATGATCGATTCTTTTCGCCACAT-3') were used for the construction of N331K; ThNE-KHN (5'-CTAGATGTGGCGAAACATATCGA TCATCAATTTTGGCGCG-3') and ThNE-KHC (5'-AATTCGCGCCAAAATTGATGATCGATAT CTTTCGCCACAT-3') for N331K-E332H. Construction of E332H was as described previously [7]. All mutations were confirmed by dideoxy-chain termination sequencing using an ABI377 PRISM DNA sequencer (Perkin-Elmer, Wellesley, U.S.A.).

Enzyme Reaction on Various Substrates

The enzyme activity was assayed at 60°C for 10 min in 50 mM sodium acetate buffer (pH 6.0) by the dinitrosalicylate (DNS) method [10] for the hydrolyzing activity toward β -CD, soluble starch, and pullulan. The glucose oxidase/peroxidase method [3] was used for acarbose hydrolyzing activity. One unit of hydrolyzing activity toward β -CD

(CU), starch (SU), or pullulan (PU) was defined as the amount of enzyme producing 1 μ mol equivalent of maltose in 1 min. One unit of acarbose hydrolyzing activity (AU) was defined as the amount of enzyme producing 1 μ mol of glucose in 1 min. Acarbose was kindly donated by Bayer Korea (Seoul, Korea). Methyl α -D-glucopyranoside (α -MG), maltotriose, and pullulan were purchased from Sigma Chemicals (St. Louis, U.S.A.).

Kinetics of Acarbose Hydrolysis

Hydrolysis of acarbose was carried out in 50 mM sodium acetate buffer (pH 6.0) at 60°C for 10 min, and the reaction was stopped by boiling for 5 min. Glucose released from acarbose was measured using the glucose oxidase-peroxidase method [3]. The initial velocities were obtained directly from the time course plots of the reaction. The k_{cat} and K_m were calculated from the Lineweaver-Burk plot.

Analysis of Transglycosylation Products

For transglycosylation reactions, wild-type and mutant ThMAs (1 CU/mg acarbose) were added to the reaction buffer (50 mM sodium acetate buffer, pH 6.0) containing 77.4 mM (5%) acarbose (donor) and 514.9 mM (10%) α -MG (acceptor), and the reaction was carried out at 60°C for 24 h. The transfer products were analyzed by high-performance anion-exchange chromatography (HPAEC) using a CarboPac PA1 column (0.4 \times 25 cm, Dionex, Sunnyvale, U.S.A.) and an electrochemical detector (ED40, Dionex, Sunnyvale, U.S.A.) as described previously [7].

Reaction Rate Constants

In order to understand the reaction mode of wild-type and mutant ThMAs precisely, formation and degradation rates of each transfer product were determined. The entire reaction was divided into two stages including formation of the transfer products from acarbose (transglycosylation reactions) and degradation of each transfer product (hydrolysis reactions). To determine the transglycosylation and hydrolysis reaction rates of each transfer product, ThMA and its mutants were added to the reaction buffer containing the K_m concentration of acarbose and 514.9 mM α -MG, and the reaction mixtures were incubated at 60°C. Each aliquot of reaction mixture taken at various time points was analyzed by HPAEC after being boiled for 5 min. Reaction rate constants for the hydrolysis and transglycosylation reactions were determined by the equations for a first-order reaction.

RESULTS AND DISCUSSION

Construction of Mutants

In order to test the role of the dipeptide in the extra sugar-binding space, the N331 and E332 residues were mutagenized

Enzyme		Conserved regions							
		I	II	III	IV				
MAase	<i>Thermus</i> strain (ThMA)	242	DAVFNH	324	GWRLDVANE	357	E I W H	419	LLGSHD
	<i>B. licheniformis</i> (BLMA)	245	DAVFNH	324	GWRLDVANE	357	E I W H	419	LLGSHD
	<i>B. stearothermophilus</i> (BSMA)	242	DAVFNH	324	GWRLDVANE	357	E I W H	419	LLGSHD
Cyclodextrinase TVaII	Alkalophilic <i>Bacillus</i>	238	DAVFNH	321	GWRLDVANE	354	E I W H	416	LLGSHD
	<i>Thermoactinomyces vulgaris</i>	239	DAVFNH	321	GWRLDVANE	354	E I W H	416	LLGSHD
Neopullulanase α -Amylase	<i>B. stearothermophilus</i>	242	DAVFNH	324	GWRLDVANE	357	E I W H	419	LLGSHD
	<i>Aspergillus oryzae</i>	117	DVVANH	202	G I R I D T V K H	230	E V L D	292	F V E N H D
	<i>B. stearothermophilus</i>	101	DVVFDD	230	G F R L D G L K H	264	E Y W S	326	F V D N H D
CGTase	Barley	101	D I V I N H	127	D G R L D W G P H	218	E V W D	299	F V D N H D
	<i>B. macerans</i>	135	D F A P N H	225	G I R F D A V K H	258	E W F L	324	F I D N H D
	<i>B. stearothermophilus</i>	131	D F A P N H	221	G I R M D A V K H	253	E W F L	319	F I D N H D
	<i>Klebsiella pneumoniae</i>	130	D Y A D N H	219	A I R I D A I K H	257	E W F G	328	F M D N H D

Fig. 1. Comparison of amino acid residues in the conserved regions of maltogenic amylases and related enzymes. The asparagine and glutamate residues conserved among MAases and the equivalent residue in other amylolytic enzymes are indicated by black highlight. Closed circles (●) represent the residues constituting the catalytic site.

individually or together. N331 and E332 were substituted with a lysine and a histidine, respectively, based on their sequence alignment with other related enzymes. The residues were conserved as a lysine and a histidine in α -amylases and CGTases (Fig. 1). The complementary oligonucleotide primers containing specific mutations were annealed and inserted into the cassette sequence of p6xHTMX between the *Xba*I and *Eco*RI sites (Fig. 2). Transformants carrying each construction were screened by agar plate assay using the iodine test. As the colonies appeared on a 1% starch agar plate, 0.6% soft agar containing D-cycloserine

(1.2 mg/ml) was layered over the plate. The transformants that formed smaller clear zones around the colonies, when iodine solution (I_2 , 0.203 g; KI, 5.2 g, H_2O to 100 ml) was applied, were selected as putative mutants, and the mutations (N331K, E332H, and N331K/E332H) were confirmed by sequencing using an ABI377 PRISM DNA sequencer (Perkin-Elmer, Wellesley, U.S.A.). Wild-type and mutant ThMAs with a histidine (6xHis)-tag were successfully expressed in *E. coli*, and each enzyme was purified by Ni-NTA affinity chromatography.

Catalytic Properties of the Mutant Enzymes

The hydrolysis activities of the wild-type and mutant enzymes on soluble starch, pullulan, β -CD, and acarbose were determined. All of the mutant enzymes showed considerably less hydrolyzing activity toward soluble starch, pullulan, and β -CD than that of the wild-type enzyme (Table 1). For soluble starch, pullulan, and β -CD, the E332H mutation caused more severe impairment of hydrolyzing activities (0.5–2.1% of the wild-type activities) than the N331K mutation did (10.4–17.3% of wild-type activities). The combined effect of both mutations (0.2–0.4% of wild-type activities) was even greater in impairing the hydrolysis activity. The N331K mutation did not affect the acarbose hydrolyzing activity at all. On the other hand, the E332H mutation impaired the activity quite significantly (22.1% of the wild-type activity), and the double mutation did so even more severely (3.0% of the wild-type activity). The kinetic parameters of acarbose hydrolysis, K_m and k_{cat} , are summarized in Table 2. The mutant enzymes showed a lower affinity to acarbose molecules than the wild-type. The K_m value of N331K and E332H was 3.6 and 5 times higher, respectively, than that of the wild-type. The double mutation did not seem to affect the substrate affinity,

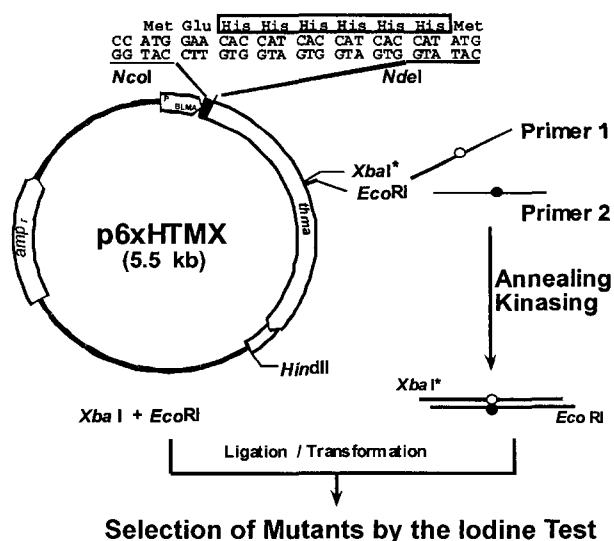


Fig. 2. Construction of the mutant enzymes by cassette mutagenesis using the p6xHTMX vector.

The annealed primers containing appropriate mutation(s) were inserted into the *Xba*I and *Eco*RI sites in the middle of the ThMA gene, which was fused to 6x-histidine residues in frame at the amino terminus.

Table 1. Specific hydrolysis activities of wild-type and mutant ThMAs on various substrates.

ThMA	Specific activity (Units/mg)			
	β -CD ^a	Soluble starch ^a	Pullulan ^a	Acarbose ^b
Wild-type	105.60 (100.0%)	4.60 (100.0%)	4.89 (100.0%)	22.3 (100.0%)
N331K	18.04 (17.08%)	0.79 (17.3%)	0.51 (10.42%)	23.7 (106.2%)
E332H	2.26 (2.1%)	0.053 (1.16%)	0.027 (0.55%)	4.94 (22.1%)
N331K/E332H	0.28 (0.26%)	0.018 (0.41%)	0.013 (0.27%)	0.72 (3.0%)

^aUnits for hydrolysis of β -CD, soluble starch, and pullulan were measured by the DNS method.

^bAcarbose hydrolyzing units were measured by the glucose oxidase-peroxidase method.

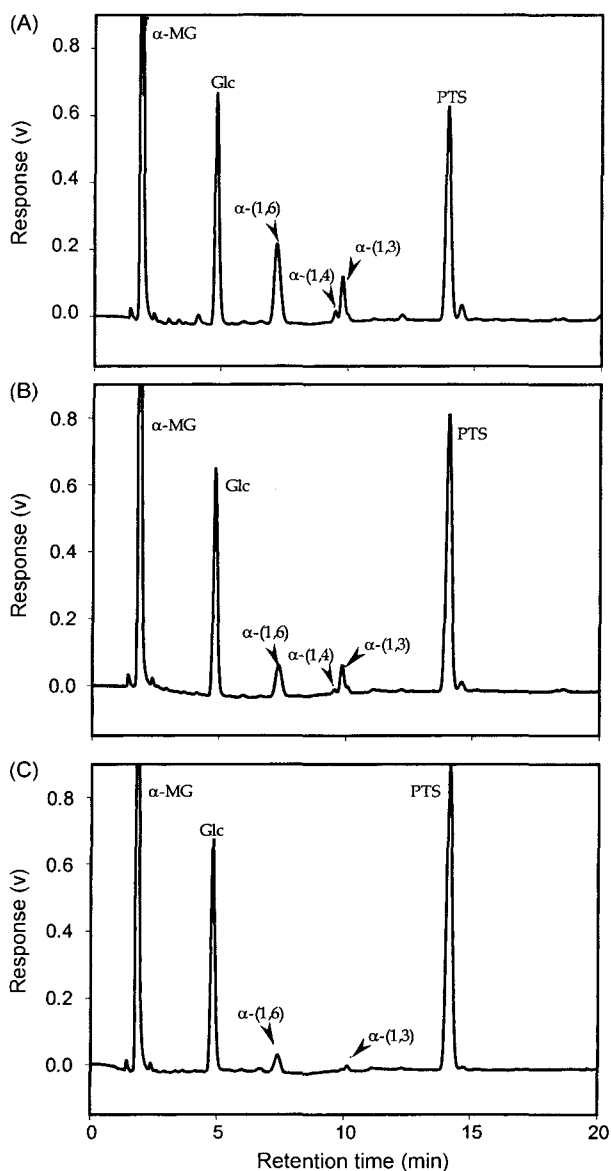


Fig. 3. HPAEC analysis of transfer products from 5% acarbose and 10% methyl- α -D-glucopyranoside by ThMA WT (A), N331K (B), and E332H (C).

α -MG represents methyl- α -D-glucopyranoside; Glc, glucose; α -(1,6), PTS-1,6- α -D-glucopyranoside; α -(1,4), PTS-1,4- α -D-glucopyranoside; α -(1,3), PTS-1,3- α -D-glucopyranoside; and PTS, pseudotrisaccharide (acarviosine-glucose).

having a K_m value similar to that of E332H. The overall k_{cat}/K_m values of mutants were greatly reduced. According to these results, both N331 and E332 seemed to be involved more tightly in accommodating bulky substrates such as pullulan, starch, and β -CD than in accommodating a small substrate like acarbose for hydrolysis. E332 was likely to play a more critical role in substrate binding and hydrolysis than N331.

Kinetics of Transglycosylation Reaction Carried Out by the Mutant Enzymes

Acarbose and α -MG were used as a donor and an acceptor, respectively, to investigate the transglycosylation reactions carried out by the wild-type and mutant enzymes. HPAEC analysis of a 24 h reaction mixture indicated that wild-type ThMA produced α -(1,6)-, α -(1,3)-, and α -(1,4)-linked transfer products (Fig. 3A), while all mutants produced less transfer products than the wild-type (Figs. 3B and 3C). HPAEC analysis revealed that the decrease of α -(1,6)-linked transfer product of the N331K mutant (Fig. 3B) was more remarkable, compared to the other products. In E332H, not only α -(1,6)-linked transfer product but also α -(1,3)- and α -(1,4)-linked transfer products were decreased significantly (Fig. 3C). Almost the same patterns were observed with the double mutant (data not shown). These results suggested that N331 was most directly involved in the α -(1,6)-transglycosylation activity of ThMA, but E332 played more important roles in forming α -(1,3)-, α -(1,4)-, and α -(1,6)-glycosidic linkages via transglycosylation.

Time course analysis of acarbose hydrolysis and the transfer products formation by the mutant and wild-type enzymes are represented in Fig. 4. For the transglycosylation reaction, acarbose concentration was 0.12 mM for the wild-type, 0.4 mM for N331K, 0.64 mM for E332H, and 0.69 mM for the double mutant, based on their K_m values. In the case of the wild-type, the α -(1,4)-linked transglycosylation product was predominant at the beginning of the reaction. However, the resulting product was rapidly hydrolyzed for the rest of the reaction. On the other hand, the amount of α -(1,3)- and α -(1,6)-linked transglycosylation products increased during the first 8 h of the reaction, and maintained at a steady rate during the rest of the reaction. Rate constants for the formation and degradation of each

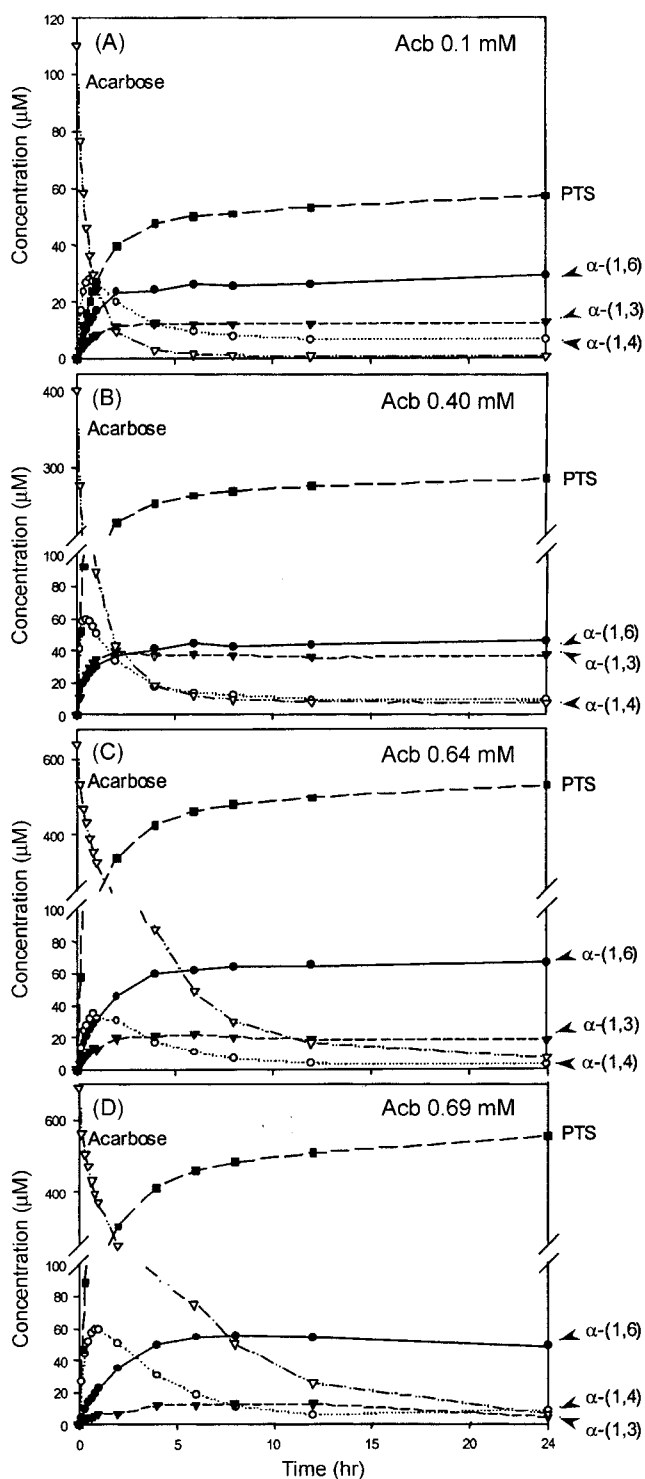


Fig. 4. Composition of reaction products as a function of reaction time.

ThMA and its mutants were reacted with acarbose at the appropriate K_m concentration with 10% α -MG (w/v) at 60°C for 24 h. Panel A represents the reaction carried out by the wild-type; B, the N331K mutant; C, the E332H mutant; and D, the N331K/E332H double mutant. Graphs represent the hydrolysis of acarbose (∇) and the formation of α -(1,6)-linked products (\circ), α -(1,4)-linked products (\bullet), α -(1,3)-linked products (\blacktriangledown), and PTS (\blacksquare).

Table 2. Kinetic parameters of acarbose hydrolysis by WT and mutant ThMAs^a.

Enzyme	K_m [mM]	k_{cat} [sec^{-1}]	k_{cat}/K_m [$\text{mM}^{-1}\text{sec}^{-1}$]
Wild-type	0.11 ± 0.02	24.29 ± 1.06	219.44
N331K	0.40 ± 0.01	29.34 ± 0.41	73.70
E332H	0.63 ± 0.02	5.65 ± 0.12	8.93
N331K/E332H	0.68 ± 0.04	0.63 ± 0.02	0.92

^aAcarbose hydrolyzing activities were measured by the glucose oxidase-peroxidase method.

transglycosylation product were determined by assuming that the reactions followed first-order kinetics with substrate concentration at the K_m value of each (Table 2). The reaction rate constants were evaluated by the following equations:

$$\frac{d[\text{Acb}]}{dt} = -(k_1 + k_2 + k_3 + k_4)[\text{Acb}]$$

$$\frac{d[1, 4]}{dt} = k_2[\text{Acb}] - k_5[1, 4]$$

$$\frac{d[1, 3]}{dt} = k_3[\text{Acb}] - k_6[1, 3]$$

$$\frac{d[1, 6]}{dt} = k_4[\text{Acb}] - k_7[1, 6]$$

The kinetic values are shown in Table 3. The rate constants for α -(1,6)-, α -(1,4)-, and α -(1,3)-linked transglycosylation product formation by N331K were reduced to 14.9%, 18.0%, and 31.3%, respectively, of those of wild-type ThMA. For the E332H mutant, they were reduced to 1.8%, 0.9%, and 1.3%, respectively, of the wild-type. For the double mutant, N331K/E332H, they were reduced to less than 0.5% of the wild-type constants. The ratio of hydrolysis to transglycosylation ($[T]/[H]$) represents the total accumulation of each transfer product by the wild-type and mutant enzymes. As expected from a higher $[T]/[H]$, more α -(1,6)-linked product was accumulated in the reaction mixture than α -(1,4)- or α -(1,3)-linked products. These results indicated that accumulation of transfer products depended on the relative reaction rate between the transglycosylation and the hydrolysis. The N331K mutant had much lower $[T]/[H]$ ratios for α -(1,6)- and α -(1,4)-linked transfer product than those of the wild-type, but the ratio for α -(1,3)-linked product was almost the same as that of wild-type ThMA. The mutants, E332H and N331K/E332H, had lower $[T]/[H]$ ratios for all transfer products, with the largest ratio for α -(1,6)-linked products. It could be deduced from these results that the Glu332 residue was involved in the transglycosylation reaction for the formation of not only α -(1,6)-, but also α -(1,3)- and α -(1,4)-glycosidic linkages between donor and acceptor molecules, while N331 seemed to play an important role in the transglycosylation of α -(1,6)- and α -(1,4)-glycosidic linkage formation, but not the α -(1,3)-glycosidic linkage formation.

Table 3. Reaction rate constants of acarbose hydrolysis and transglycosylation by WT and mutants.

ThMA	Reaction rate constants ($10^{-3} \text{ sec}^{-1} \text{ mol}^{-1}$) ^a							[T]/[H] ratio		
	Transglycosylation [T]				Hydrolysis [H]			α -(1,4)	α -(1,3)	α -(1,6)
	k_2	k_3	k_4	k_1	k_5	k_6	k_7	k_2/k_5	k_3/k_6	k_4/k_7
Wild-type	8.115	1.134	2.028	2.924	9.547	0.656	0.298	0.850	1.727	6.800
N331K	1.464	0.355	0.302	1.664	3.569	0.177	0.124	0.410	2.000	2.429
E332H	0.075	0.015	0.036	0.245	0.690	0.078	0.034	0.109	0.198	1.069
N331K/E332H	0.026	0.001	0.004	0.043	0.160	0.014	0.006	0.164	0.102	0.740

^aReaction rate constants were expressed as $k_i/[E]$.

Role of N331 and E332 in the Transglycosylation Reaction

ThMA forms a homo-dimer via the extra N-terminal domain unique to MAase and cyclomaltodextrinase. The space formed by adjacent N-terminal domains of each homo-dimer subunit could provide room for an acceptor molecule in such a way as to compete with a water molecule. From the docking model, acceptor molecules can compete with the water molecule to act on the PTS-enzyme intermediate complex. N331 and E332 at the extra sugar-binding space might be involved in binding small oligosaccharide acceptors. Upon binding the acceptor molecule (α -MG in this study), the conserved dipeptide could position the acceptor molecule in an appropriate orientation, allowing the formation of an α -(1,6)-, an α -(1,3)-, or an α -(1,4)-linkage between α -MG and PTS. Based on the results obtained from a mutagenesis study of ThMA at N331 and E332, this study concluded that the conserved E332 as well as N331 were involved in aligning the acceptor molecule to compete with water

molecules in the nucleophilic attack on the glycosyl-enzyme intermediate, thereby promoting transglycosylation (Fig. 5). This conclusion was supported by the reduced [T]/[H] values of the mutants N331K, E332H, and N331K/E332H.

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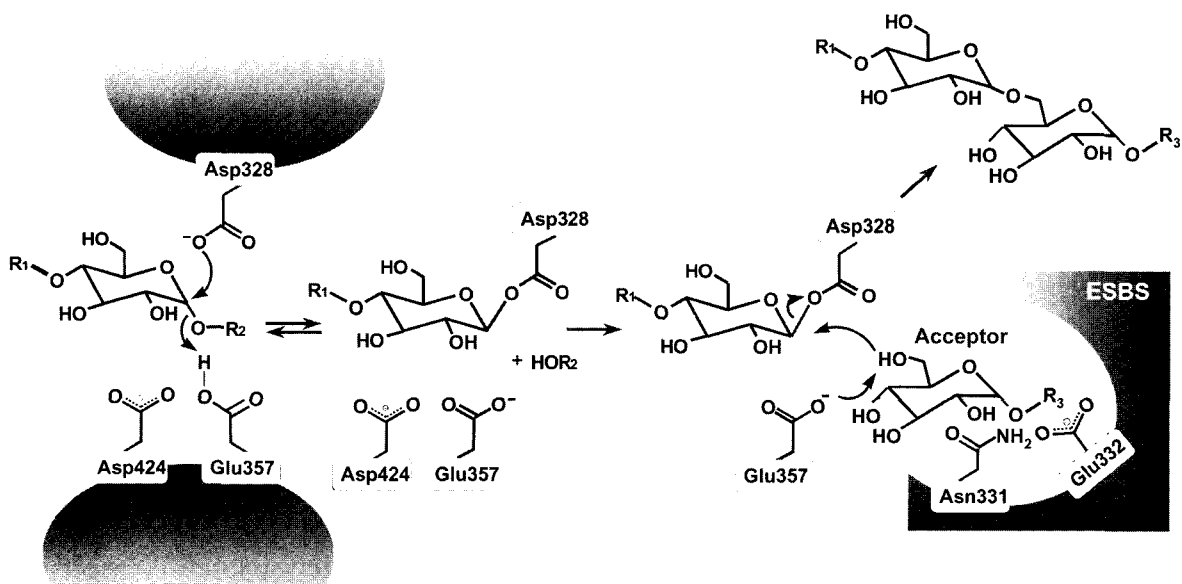


Fig. 5. Scheme of the transglycosylation reaction mechanism of ThMA.

R_1 represents a sugar moiety of a donor (substrate) molecule; R_2 , a sugar moiety leaving group of the donor (substrate); R_3 , a sugar moiety of acceptor molecule or -H; ESBS, extra sugar-binding space. D328, E357, and D424 are the amino acid residues constituting the catalytic site of ThMA.

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