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Molecular Determinants of Substrate Recognition in Thermostable α-glucosidases Belonging to Glycoside Hydrolase Family 13

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Bacillus stearothermophilus **α-1,4-glucosidase (BS) is highly specific for α-1, 4-glucosidic bonds of maltose, maltooligosaccharides and α-glucans. *Bacillus thermoglucosdasius* oligo-1,6-glucosidase (BT) can specifically hydrolyse α-1,6 bonds of isomaltose, isomaltooligosaccharides and α-limit dextrin. The two enzymes have high homology in primary structure and belong to glycoside hydrolase family 13, which contain four conservative regions (I, II, III and IV). The two enzymes are suggested to be very close in structure, even though there are strict differences in their substrate specificities. Molecular determinants of substrate recognition in these two enzymes were analysed by site-directed mutagenesis. Twenty BT-based mutants and three BS-based mutants were constructed and characterized. Double substitutions in BT of Val200→Ala in region II and Pro258→Asn in region III caused an appearance of maltase activity compared with BS, and a large reduction of isomaltase activity. The values of k_0/K_m ($s^{-1} \cdot mM^{-1}$) of the BT-mutant for maltose and isomaltose were 69.0 and 15.4, respectively. We conclude that the Val/Ala200 and Pro/Asn258 residues in the α-glucosidases may be largely responsible for substrate recognition, although the regions I and IV also exert a slight influence. Additionally, BT V200A and V200A/P258N possessed high hydrolase activity towards sucrose.**

Key words: α-glucosidase, glycoside hydrolase family 13, substrate specificity.

Abbreviations: BS, *Bacillus stearothermophilus* α-1,4-glucosidase; BT, *Bacillus thermoglucosidasius* oligo-1,6-glucosiade; GH, glycoside hydrolase family; LA, LB medium supplemented with 100 μg/ml ampicillin; *p*NPG, *p*-nitro-phenyl-α-D-glucopyranoside; PAGE, polyacrylamide gel electrophoresis; SAM, *Bacillus* sp. SAM1606 α-glucosidase.

Carbohydrates are very diverse and important biomolecules for all living organisms, and their hydrolysing enzymes play central roles in many biological processes. In particular starch, one of the most common storage sugars, and the starch-digesting enzymes such as amylase have been noticed and researched (*1*, *2*). The classification of glycoside hydrolases depends on amino acid sequence similarities [(*3*–*6*) http://afmb.cnrs-mrs.fr/CAZY]. Based on computer analysis, many α-glucosidases belong to glycoside hydrolase family 13 (GH13, α-amylase family), which contains α-amylase (EC 3.2.1.1), pullulanase (EC 3.2.1.41), cyclomaltodextrin glucanotransferase (EC 2.4.1.19), cyclomaltodextrinase (EC 3.2.1.54), trehalose-6-phosphate hydrolase (EC 3.2.1.93), oligo-α-1,6-glucosidase (EC 3.2.1.10), α-1,4-glucosidase (EC 3.2.1.20), neopullulanase (EC 3.2.1.135) and so on. The GH13 enzymes have four high conserved regions (I, II, III, IV) in primary structure and $(\beta/\alpha)_8$ barrel folding structures, and generally have broad substrate specificities. The common structure consists of three domains: a catalytic domain folded in a $(\beta/\alpha)_8$ barrel in the N-terminus, an additional domain extending out of the barrel, and a β-sheet-rich domain in the C-terminus (*1*, *2*, *7*).

Enzymes from thermophilic microorganisms developed unique structure-function properties of thermostability and optimal activity at higher temperatures, and can be used in several industrial processes. The molecular determinants of protein thermostability have been focused on, and some models have been proposed. We previously found a strong correlation between the increase in the number of proline residues and the rise in the thermostability of bacillary oligo-1,6-glucosidases. Based on this correlation, we proposed the proline rule, a general principle for increasing thermostability (*8*, *9*). This proposal was verified by protein engineering and three-dimensional structure analysis (*7*). In addition, protein-engineering studies of substrates specificities have also been initiated, although without much success (*2*). Recently, some research groups reported on the relationship between three-dimensional structure and substrate specificity. Verdoucq *et al*. (*10*) recently reported the structure determinants of substrate specificity in GH1 β-glucosidases. Neopullulanase from *B. stearotheromophilus* is a member of GH13, and hydrolyses both α-1,4 and α-1,6 linkages (*11*). Three-dimensional structure analysis of the neopullunase

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showed that the hydrolysis of both α-1,4 and α-1,6 linkages must take place at the same catalytic site and that this duality resulted from characteristic features of the subsite structures of the enzyme (*12*). The relationship of structure and substrate specificity of an α-glucosidase in GH13 was described by Nakayama and his collaborators (*13*, *14*). The enzyme used by the group was α-glucosidase from *Bacillus* sp. SAM1606, which had the broad substrate specificity (*15*, *16*). By protein engineering, some mutant enzymes were constructed, assayed and discussed in the context of substrate specificity. Although the information is seen to be of great value and use for understanding the relationship between structure and substrate recognition of the GH13 enzymes, the molecular determinants of very strict specificities to α-1,4 and α-1,6-bonds remain obscure in some respects.

Bacillus stearothermophilus (*Geobacillus stearothermophilus*) ATCC12016 α-1,4-glucosidase (BS) is highly specific for α-1,4-glucosidic bonds of maltose, maltooligosaccharides and α-glucans (*17–19*). *Bacillus thermoglucosdasius* (*Geobacillus thermoglucosdasius*) KP1006 oligo-1,6-glucosidase (BT) can hydrolyse α-1,6 bonds of isomaltose, isomaltooligosaccharides and α-limit dextrin, but not act on α-1,4 bonds (*20*). The two enzymes belong to GH13 (α-amylase family), and have high homology (56% identity, 73% similarity) in amino acid sequence. Thus, these two enzymes are highly interesting as model systems for understanding substrate specificities. Both enzymes, BS and BT, appear to have the same, or nearly the same, catalytic domain, in the form of a $(\beta/\alpha)_8$ barrel whose folding structure consists of eight parallel β-strands surrounded by eight α-helices. Thus, we are interested in determining the reason for the highly specific difference in their substrate specificity. The wild-type BT can hydrolyse isomaltose, which consists of two glucoses and contains an α-1,6 bond, but not maltose, which consists of two glucoses and contains an α-1,4 bond. Double substitutions in BT of Val200 → Ala and Pro258 → Asn caused the appearance of maltose-degrading activity compared with BS, and a large reduction in the enzyme specificity constant (k_0/K_m) for isomaltose. The values of k_0/K_m ($s^{-1} \cdot mM^{-1}$) for maltose, isomaltose and sucrose of BT V200A were 8.26, 10.8 and 21.8, respectively. The mutant enzyme preferred sucrose to isomaltose and maltose. The present research is expected to add a new aspect to our understanding of the relationship between structure and activity in GH13.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions and DNA Manipulations—All DNA manipulations were performed essentially as described by Sambrook *et al*. (*21*). *Escherichia coli* JM109 was used for overexpression and preparation of subclones for DNA sequencing. pUC vectors were used as routine cloning vectors. *Escherichia coli* transformants were cultured at 37°C in LB medium supplemented with 100 μg/ml ampicillin (LA). Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA, USA), Toyobo (Osaka, Japan) and Takara (Kyoto, Japan) and were used as recommended by the respective suppliers. Polymerase chain reaction (PCR) was performed with LA-*Taq* DNA polymerase (Takrara) for 30 cycles according to the manufacturer's directions.

Site-directed Mutagenesis and Construction of Plasmids—Site-directed mutagenesis was done using a site-directed mutagenesis kit (Takara) and by PCR amplification using two primers for mutagenesis. Primers used in this study are listed in Table 1, and the universal primers were also used as recommended by the manufacturer. The resultant DNA fragments, which contained the desired mutations as confirmed by sequence analyses described subsequently, were recloned, using approximate restriction enzymes and DNA ligase. For constructing BT-based mutants, DNA fragments of *Spe*I-*Sal*I (296 bp), *Sal*I-*Xho*I (370 bp), *Mlu*I-*Xho*I (285 bp) and *Xho*I-*Bam*HI (423 bp) were used for recloning in regions I, II, III and IV, respectively. For constructing BS-based mutants, the *Sal*I-DNA fragment (418 bp) was used for recloning in regions II and III.

DNA fragments were sequenced on both strands by the dideoxy chain termination method of Sanger (*22*), using a DYEnamic™ ET terminator sequencing kit (Perkin Elmer, Applied Biosystems Division, USA). Oligonucleotide primers used in this study were RT-SEQ1 (5′-ATTTGGATTCAGGGCCTGGCGG-3′), RT-SEQ2 (5′-CGGGTCGTCTTCCAAAATTAAGTC-3′), M13RV (5′-CAGGAAACAGCTATGAC-3′) and M13M4 (5′-GTTTTCCCAGTCAACGAC-3′).

Analytical Methods—Hydrolase activity was determined by measuring the initial rate of the hydrolysis of *p*-nitro-phenyl-α-D-glucopyranoside (*p*NPG), maltose, isomaltose and sucrose as described before (*7*, *17–20*, *23*). Briefly, the activity was determined at 60°C in a reaction system consisting of 33 mM potassium phosphate buffer (pH 6.8). *p*-Nitrophenol released from *p*NPG by the action of enzymes was photometrically determined at 400 nm. Glucose was measured by Trinder's method using glucose-oxidase (*24*). Protein was assayed by the method of Lowry *et al*. (*25*) with bovine serum albumin as the standard. SDS- and native-polyacrylamide gel electrophoresis (PAGE) were performed by the method of Laemmli (*26*).

Purification of the Recombinant Thermostable Glucosidases—Escherichia coli JM109 bearing each expression vector was grown aerobically for 10 h at 37°C in LA medium and then harvested, washed and suspended in 50 mM phosphate buffer/5 mM EDTA (pH7.0, Buffer A). The cells were sonicated in Buffer A. The sonicate was centrifuged. The supernatant was then treated for 10 min at 55°C and centrifuged again. The enzyme in the supernatant was treated by 3 M KSCN to break the non-specific association between the desired enzyme and the other proteins, and then dialysed to Buffer A. The enzyme solution was purified by three-column chromatography, DEAE-cellulose SH, Butyl-Toyopearl 650S and Sephadex G-100. The purity was judged by both SDS–PAGE and gel filtration.

Double Immunodiffusion—Rabbit antiserum against the wild-type enzyme (5 μl), was applied to the centre well (3.3 mm diameter) on a 1% agarose gel that included 25 mM borate-HCl/0.1%NaN_3 (pH 8.0). The purified wild-type or mutant enzymes were added to each of the

Table 1. **Primers and plasmids used for this study.** Mutation sites for restriction site and site-directed mutagenesis are shown by italic and underline. Unless specific primers are described for constructing each plasmid, other primers were used together.

Plasmids	Mutants	Primers for mutations	Source
pBT6-2	Wild-type BT		Watanabe *et al*. (*27*)
pBT3467	Wild-type BT BT34 SalI	5′-GCGGAAACC*GTCGAC*GCCTT-3′	A derivative from pBT6-2
	BT67 XhoI	5′-GCCCTTTCC*CTCGAG*TTCTTTT-3′	
pBT1 I	V101I	5′-G GAT TTA GTC ATC AAC CAT ACA TCG GA-3′	A derivative from pBT3467
pBT2 A__	V200A	5′-CGC ATG GAT GCC ATT AAT ATG ATT TCC-3′	A derivative from pBT3467
pBT2 _S_	N202S	5′-GAT GTC ATT TCT ATG ATT TCC AAA GTG-3′	A derivative from pBT3467
pBT2 __H	M203H	5′-AAT CAC ATT TCC AAA GTG CCG GAA TTG CCG-3′	A derivative from pBT3467
pBT2 AS_	V200A, N202S	5′-GAT GCC ATT TCT ATG ATT TCC AAA GTG-3′	A derivative from pBT3467
pBT2 A_H	V200A, M203H	5′-GAT GCC ATT AAT CAC ATT TCC AAA GTG-3′	A derivative from pBT3467
pBT2 _SH	N202S, M203H	5′-GTC ATT TCT CAC ATT TCC AAA GTG CCG GAA TTG CCG GA-3′	A derivative from pBT3467
pBT2 ASH	V200A, N202S, M203H	5′-GGA AAT GTG ACT AAT GGC ATC CAT GCG-3′	A derivative from pBT3467
pBT3 A_	T257A	5′-TT GGA GAA GCG CCG GGT GTC ACA CCA-3′	A derivative from pBT3467
pBT3 _N	P258N	5′-GGA GAA ACG AAC GGT GTC ACA CCA AAA-3′	A derivative from pBT3467
pBT3 AN	T257A, P258N	5′-GTT GGA GAA GCG AAC GGT GTC ACA CCA-3′	A derivative from pBT3467
pBT4 F	Y325F	5′-CTT TTC TTA AAT AAC CAT GAC CAG CC-3′	A derivative from pBT3467
pBT4 L	Q331L	5′-AC CTG CCG CGC GCT GTT-3′	A derivative from pBT3467
pBT4 FL	Y325F, Q331L		A derivative from pBT4 F
pBT2ASH 3AN	V200A, N202S, M203H, T257A, P258N		A derivative from pBT2 ASH and pBT3 AN
pBT2A 3N	T257A, P258N		A derivative from pBT2 A and pBT3N
pBT1I 2A 3N	V101I, T257A, P258N		A derivative from pBT1 I and pBT2A 3N
pBT1I 4 FL	V101I, Y325F, Q331L		A derivative from pBT1 I and pBT4 FL
pBT1I2A3N4FL	V101I, T257A, P258N, Y325F, Q331L		A derivative from pBT4 FL and pBT1I2A3N
pBT1I2ASH3N4FL	V101I,V200A, N202S, M203H,P258N, Y325F, Q331L		A derivative from pBT2 ASH and pBT1I2A3N4FL
pBST(XP)	Wild-type BS		Takii et al. (18, 19)
pBS”(RP)	Wild-type BS		A derivative from pBST(XP)
pBS3467	Wild-type BS (I193V)		A derivative from pBS”(RP)
pBS2 V	A200V	5′-C GAC GTG ATT TCC CAC ATT AAG-3′	A derivative from pBS3467
pBS3 P	N258P	5′-GTC GGC GAG GCG CCG GGA GTA ACG GTT G-3′ Forward	A derivative from pBS3467
		5′-C AAC CGT TAC TCC CGG CGC CTC GCC GAC-3′ Reverse	

other wells. After diffusion for 24 h at 25°C, the plate was washed by soaking it in 0.85% NaCl/0.1% NaN_3 at 4°C overnight.

RESULTS

Assessment of BT- and BS-based Mutants in Comparison with Wild-type Enzymes—The enzymes in GH13 contain four conserved regions in their primary structures and have a catalytic domain in the form of a $(\beta/\alpha)_8$ barrel in their tertiary structures, and the elements of the secondary structures of the proteins have the nomenclature Nβ1 to Nβ8 for β-strands and Nα1 to Nα8 for α-helices of the N-terminal and catalytic domain (*7*). As shown in Fig. 1, five α-glucosidases were compared. SAM has broad substrate specificity, and catalyses the hydrolysis of α-1,4- and α-1,6-bonds (*15*, *16*). The other enzymes are very specific for α-1,4- or α-1,6-bonds (*7*, *17–20*, *27*). We focused on two thermostable BS and BT that have high homology

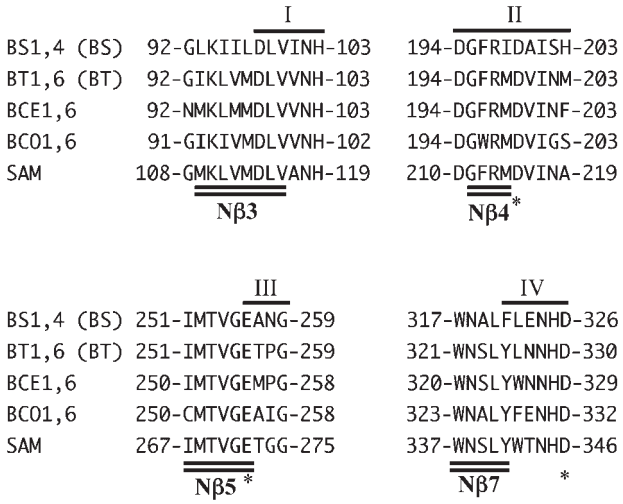

Fig. 1. **Alignment of the amino acid sequences around conserved regions I, II, III and IV of five glucosidases of GH13.** The enzymes used for alignment are: BS1,4, *B. stearothermophilus* α-1,4-glucosidase (D84648); BT1,6, *B. thermoglucosidasius* oligo-1,6-glucosidase (D104879); BCE1,6, *B. cereus* oligo-1,6-glucosidase (X53507); BCO1,6, *B. coagulans* oligo-1,6-glucosidase (D78342); SAM, *Bacillus* sp. SAM1606 α-glucosidase (X76947). Accession numbers of DDBJ/Genbank/EMBL are shown in parentheses. Four regions conserved in GH13 (α-amylase family) are indicated by underline. Catalytic residues are indicated by asterisk. Secondary structures β-strands are shown by duplicate lines.

(56% identity, 73% similarity) in amino acid sequence. Target residues were judged by the alignment of α-glycosidases from *Bacillus* species and the structure predictions. Eight amino acid residues in BT were substituted with the corresponding ones in BS (Table 2). Twenty mutant enzymes of BT in total were constructed and their enzyme activities were at 55°C investigated.

The structures of all mutant proteins were immunologically confirmed. On double immunodiffusion, the rabbit serum against the wild-type BT identically produced single and spur-free precipitation-lines against the wild-type and mutant proteins (data not shown). These results showed that each site-directed mutagenesis allowed the eight interesting residues to be respectively substituted with other ones without significantly affecting the structure, surface integrity and the foldings of mutated enzymes with respect to those of wild-type.

Analysis of substrate specificities using 20 crude extracts showed that amino acid residues in region II were strongly involved in substrate recognition (Table 2), while the substitution of residues in region III had a small effect. The highest value obtained for the ratio of maltase to isomaltase activities ($1620/202 = 8.0$) was achieved by the eight-residue substitution V101I/V200A/N202S/M203H/T257A/P258N/Y325F/Q331L, indicating that all of the eight interesting residues might play an important role in substrate specificity. However, its *p*NPG hydrolase activity remarkably decreased. The *p*NPG hydrolysis activity of the enzymes represents α-glucosidase activity. Thus, a large loss of this activity might indicate a decrease of catalytic efficiency and/or thermostability of a mutant. Three substitutions, BT V200A, BT P258N and BT V200A/P258N were used for further analysis, because these three mutants, showed little change in their *p*NPG hydrolase activities as compared with wild-type BT. Judging from the results thus far the substitution s of V200A and P258N appeared to be the most effective in alternation of substrate specificity.

As described earlier, Val200 and Pro258 of BT and Ala200 and Asn257 of BT-mutants, might be important in recognizing isomaltose and maltose, respectively. Next, two BS-mutants, BS A200V and N258P were investigated. These BS mutants did not show hydrolysis activity towards isomaltose, contrary to our expectation (Table 2). A triply BS-mutant A200V/S202N/H203M was constructed, expressed and purified. The values of k_0/K_m for maltose and isomaltose were no more than $0.0001\,s^{-1}\cdot Mm^{-1}$ together, while k_0/K_m for maltose of wild-type BS was 157 (unpublished data).

Effect of Temperature on the Hydrolase Activity of Three Mutants, BT V200A, BT P258N and BT V200A/P258N—The effect of temperature, using *p*NPG as a substrate, was investigated. Optimal temperatures for BT V200A, BT P258N and BT V200A/P258N were 73°C, 70°C and 68°C, respectively, while for WT it was 72°C (Table 3). There was little difference on the thermostability expressed as temperature (T_m) for 50% inactivation and apparent activation energy calculated using Arrenius plots of the enzymes (Table 3). These results showed that these mutation(s) might have little effect on the physical properties of the mutant enzymes.

Substrate Specificities of Three Mutants, BT V200A, BT P258N and BT V200A/P258N—Double substitutions in BT of Val200 → Ala in region II and Pro258 → Asn in region III caused an appearance of maltase activity compared with BS, and a large reduction in isomaltase activity (Tables 2 and 4). The value of K_m for maltose and isomaltose of the mutant, BT V200A/P258N were 5.62 and 8.83, respectively, indicating that it had more affinity to maltose rather than isomaltose. Also, the values of k_0 and k_0/K_m showed that the enzyme could hydrolyse maltose better than isomaltose.

Single substitution of Val200 → Ala in region II caused an alteration of substrate specificity, such that the enzyme could hydrolyse maltose and isomaltose more or less equally well (Table 4). However, the values of K_m, k_0 and k_0/K_m of BT V200A for isomaltose were slightly more than the ones for maltose. Interestingly, BT P258N possessed maltase activity (Tables 2 and 4), indicating that region III as well as region II might be involved in substrate recognition.

In addition, we investigated the hydrolase activity of these enzymes for the sucrose linked α-1 position of glucose and β-2 of fructose. Using crude enzymes, specific activities (mU/mg protein) of wild-type BT, V200A, P258N and V200A/P258N were 85.6, 2830, 60.2 and 3130, respectively, indicating that the V200A mutation was highly effective for the recognition of sucrose. The values of k_0/K_m ($s^{-1}\cdot mM^{-1}$) for maltose, isomaltose and sucrose of BT V200A were 8.26, 10.8 and 21.8, respectively. The mutant enzyme preferred sucrose to isomaltose and maltose.

Table 2. **Hydrolase activities of various enzymes.** Each transformant was cultured in a test tube containing 5 ml of LB medium (0.5% yeast extract, 1% peptone, 1% NaCl, pH 7.2) supplemented with100 mg/ml of ampicillin at 37°C, with shaking for 16 h. Cells were harvested by centrifugation (2000*g* at 4°C for 10 min) and washed at least twice with cold saline (0.85% NaCl). Each pellet was suspended in buffer A (50 mM potassium phosphate buffer, 5 mM EDTA, pH 7.0) and sonicated. The lysate was centrifuged. The supernatant was used for enzyme assay at 55°C.

Enzymes		Region I	Region II	Region III	Region IV	Hydrolase activity (mU/mg protein)		
						pNPG	Maltose	Isomaltose
Wild-types	BS	98-DLVINH-103	194-DGFRIDAISH-203	256-EANG-259	321-FLENHDL-327	2230	6890	4.8
	BT	98-DLVVNH-103	194-DGFRMDVINM-203	256-ETPG-259	325-YLNNHDQ-331	12600	0.8	4670
Mutant of BT	V101I	98-DLVINH-103				19400	1.7	4490
	V200A		FRIDAISH-203			6790	1180	1970
	N202S		194-DGFRIDAISH-203			6920	1.0	6320
	M203H		194-DGFRIDAISH-203			6940	1.3	8400
	V200A/N202S		194-DGFRIDAISH-203			4840	972	2330
	V200A/M203H		194-DGFRIDAISH-203			6300	2790	5420
	N202S/M203H		194-DGFRIDAISH-203			2960	3.0	5380
	V200A/N202S/M203H		194-DGFRIDAISH-203			5580	1780	2000
	T257A			256-EANG-259		35700	1.6	7810
	P258N			256-EANG-259		19300	21.4	5500
	T257A/P258N			256-EANG-259		11600	22.1	4190
	Y325F				321-FLENHDL-327	14300	0.8	3640
	Q331L				321-FLENHDL-327	6360	0.7	1380
	Y325F/Q331L				321-FLENHDL-327	7010	1.1	1610
	V200A/N202S/M203H/ T257A/P258N		194-DGFRIDAISH-203	256-EANG-259		11600	9050	2170
	A200A/P258N		194-DGFRIDAISH-203	256-EANG-259		14500	7100	3200
	V101I/V200A/P258N	98-DLVINH-103	194-DGFRIDAISH-203	256-EANG-259		16700	9900	3640
	V101I/Y325F/Q331L	98-DLVINH-103			321-FLENHDL-327	14600	0.8	1820
	V101I/V200A/P258N/ Y325F/Q331L	98-DLVINH-103		256-EANG-259	321-FLENHDL-327	9710	2540	612
	V101I/V200A/N202S/ M203H/T257A/ P258N/Y325F/Q331L	98-DLVINH-103	194-DGFRIDAISH-203	256-EANG-259	321-FLENHDL-327	1200	1620	202
Mutants of BS	A200V		194-DGFRMDVINM-203			71	1.7	0.1
	N258P			256-ETPG-259		4580	3280	14.4

DISCUSSION

This study showed that eight amino acid residues are particularly important in determining the substrate specificity of the thermostable α-glucosidases. The ratio of maltase to isomaltase activities of the mutant in which all eight amino acids, Val101, Val200, Asn202, Met203, Thr257, Pro258, Tyr325 and Gln331 of BT were modified to the corresponding BS-type amino acids by site-directed mutagenesis was 8.0 (=1620/202), the highest value of all of our BT-mutants (Table 2). These results showed that all of these residues might be involved in substrate specificity. Analysis of tertiary structures predicted using *Bacillus cereus* oligo-1,6-glucosidase (*7*, *27*) showed that these eight amino acid residues were located on the same side of the catalytic triad without exception (data not shown). They seem to be in an aglycone site.

Of the eight amino acids, Val200 showed the most effect on substrate recognition, with Pro258 showing the next largest effect (Tables 2 and 4). The Val200 residue is located next to Asp199, a catalytic nucleophile in region II (Fig. 1). In yeast, a similar analysis was done using isomaltase and maltase, which are specific to isomaltose and maltose, respectively (*28*). The Val216 residue located next to the catalytic Asp in region II was modified. Specific activities of theVal216 mutant (V216T) were 16.5 (maltose) and 16.5 (isomaltose), indicating that Val216 is an important residue that enables the enzyme to effectively discriminate between maltose and isomaltose. Our results do not contradict this previous data. Additionally, it was reported that doubly- and triply-mutated α-glucosidase enzymes in yeast (V216T/G217A, V216T/S218G, V216T/G217A/S218G) exhibited changes in the hydrolysis ratio of maltose:isomaltose as 5 : 1, 3 : 1 and 10: 1, respectively (*28*). All three enzymes preferred maltose to isomaltose. These observations demonstrate that three residues in region II play a key role in distinguishing between maltose (α-1,4 bond) and isomaltose (α-1,6 bond). However, our data showed that seven mutated enzymes, which were modified only in region II, still preferred isomaltose to maltose (Table 2). The difference in results between these studies illustrates the challenge in obtaining a comprehensive understanding of the molecular mechanism behind the substrate specificity of α-glucosidase.

α-Glucosidase from *Bacillus* sp. SAM1606 (SAM) has a very similar structure to BS and BT, but it has broad substrate specificity (*15*, *16*). Site-directed mutagenesis analysis showed that Gly273 (or perhaps Gly274) of SAM plays an important role in substrate specificity (*14*). The Gly273 residue of SAM corresponds to Pro258 of BT (Fig. 1). Maltase activity of four SAM mutants, G273I, G273M, G273V and G273S did not increase, though sucrase activity of G273V was about twice as high as that of wild-type SAM (*14*). As shown in Tables 2 and 4, P258N of BT has apparent maltase activity, while wild-type BT does not. Therefore, the second residue behind the catalytic Glu in region III of α-glucosidase seems to be important for substrate specificity. The relative catalytic efficiency (sucrase/isomaltase) of SAM G273V, and BT V200A were 69% and 202%, respectively (14; Table 4), suggesting that region II might play a more important role than region III in recognizing sucrose. Further analysis will provide an answer the question.

Some BS-based mutants also were constructed and investigated as described previously. Large losses of hydrolase activity were observed, suggesting that they might be sensitive to high temperature. It is very difficult to mutate the target enzyme without effects on stability and structural changes near a catalytic site of a mutated enzyme. From the standpoint of structural biology, the structure of the enzymes and substrates should be described and discussed. However, it is beyond the scope of this article to illustrate the detailed

Table 3. **Effect of temperature on the activities and stabilities of the wild-type and mutant enzymes.** Purified preparations were used for enzyme assay at different temperatures. Each enzyme was heated for 10 min at different temperatures and assayed for the residual activity at 60°C. Thermostability is expressed as temperature (T_m) for 50% inactivation.

Wild-types and BT-variants	Optimal temperature (°C)	Activation energy (kJ/mol)	T_m (°C)
BS	70	71.2 (13–40°C)	68
		50.2 (40–70°C)	
BT	72	117 (30–50°C)	73
		23.0 (65–75°C)	
BT V200A	73	86.3 (30–70°C)	68
		16.7 (70–75°C)	
BT P258N	70	86.0 (35–65°C)	67
		11.3 (65–73°C)	
BT V200A/P258N	68	96.8 (35–63°C)	67
		40.0 (60–70°C)	

Table 4. **Kinetic parameters of the wild-type and mutant enzymes for the hydrolysis of various substrates.** Purified preparations were used for enzyme assay at 60°C. N.D.; not determined; (*1), the values are quoted from reference (*17*); (*2), the values were quoted from reference (*23*).

	Substrates											
	*p*NPG			Maltose			Isomaltose			Sucrose		
Wild-types and BT-variants	K_m (mM)	k_0 (s^{-1})	k_0/K_m (s^{-1}/mM)	K_m (mM)	k_0 (s^{-1})	k_0/K_m (s^{-1}/mM)	K_m (mM)	k_0 (s^{-1})	k_0/K_m (s^{-1}/mM)	K_m (mM)	k_0 (s^{-1})	k_0/K_m (s^{-1}/mM)
BS (*1)	0.63	123	195	5.6	877	157	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
BT (*2)	0.24	233	971	N.D.	N.D.	N.D.	3.3	74.2	22.5	N.D.	N.D.	N.D.
BT V200A	0.33	275	841	11.3	93.3	8.26	10.8	116	10.8	8.63	188	21.8
BT P258N	0.19	365	1950	12.7	2.33	0.183	3.57	136	38.1	22.9	7.89	0.345
BT V200A/P258N	0.49	347	704	5.62	388	69.0	8.83	136	15.4	16.2	280	17.3

structures, because we could not obtain the crystals of enzyme–substrate complexes and it was very difficult to predict the structure of sugars around the catalytic site. Iterated PSI-BLAST database searches demonstrated that a distinct kinship of GH13 with GHs 27, 31, 36 and 66, which catalyse diverse reactions, exists (*29*). Our work here provides important information necessary for comprehending substrate specificities and recognitions of GH 13 enzymes, as well as those of related families.

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