Structural Elements Responsible for the Glucosidic Linkage-selectivity of a Glycoside Hydrolase Family 13 Exo-glucosidase

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Running title: Structural element of substrate specificity

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Abstract

Glycoside hydrolase family 13 contains exo-glucosidases specific for α-(1→4)- and α-(1→6)-linkages including α-glucosidase, oligo-1,6-glucosidase, and dextran glucosidase. The α-(1→6)-linkage selectivity of *Streptococcus mutans* dextran glucosidase was altered to α-(1→4)-linkage selectivity through site-directed mutations at Val195, Lys275, and Glu371. V195A showed 1,300-fold higher k_{cat}/K_m for maltose than wild-type, but its k_{cat}/K_m for isomaltose remained 2-fold higher than for maltose. K275A and E371A combined with V195A mutation only decreased isomaltase activity.

V195A/K275A, V195A/E371A, and V195A/K275A/E371A showed 27-, 26-, and 73-fold higher k_{cat}/K_m for maltose than for isomaltose, respectively. Consequently, the three residues are structural elements for recognition of the α-(1→6)-glucosidic linkage.

Keywords: dextran glucosidase; α-glucosidase; oligo-1,6-glucosidase; substrate specificity; glycoside hydrolase family 13

Abbreviations

DG, dextran glucosidase; GH, glycoside hydrolase; GH13_31, GH family 13 subfamily 31; KE, K275A/E371A; O16G, oligo-1,6-glucosidase; pNPG, *p*-nitrophenyl α-D-glucoside; SAM1606 α-glucosidase, α-glucosidase from *Bacillus* sp. SAM1606; SmDG, *Streptococcus mutans* DG; VE, V195A/E371A; VK, V195A/K275A;VKE, V195A/K275A/E371A

1. Introduction

Glycoside hydrolases (GHs), catalyzing the hydrolysis of a glycosidic linkage, are important enzymes for carbohydrate metabolism in a wide variety of cells including archaea, bacteria, fungi, plants, and mammals. Numerous GHs, acting on various carbohydrates such as starch and cellulose, have been found thus far. Based on their amino-acid sequences, GHs are classified into 133 GH families [1]. GH family 13 is the largest family, and contains various GHs and glycosyltransferases that act on α-glucans such as starch, sucrose, and related sugars. α-Amylases (EC 3.2.1.1), cyclodextrin glucanotransferases (EC 2.4.1.19), branching enzymes (2.4.1.18), and α-glucosidases (EC 3.2.1.20) are typical members of this family. GH family 13 enzymes show low similarity within their amino acid sequences, and are further divided into 40 subfamilies [2]. They share three common domains, A, B, and C: domain A, the catalytic domain folded in a $(\beta/\alpha)_8$-barrel; domain B, a long loop connecting β-strand 3 and α-helix 3 of domain A; domain C, a domain following domain A and made up of β-strands [3]. Four short conserved regions (regions I–IV) include essential amino acid residues for catalysis. Regions I, II, III, and IV are located at the C-termini of the third, fourth, fifth, and seventh β-strands of domain A, respectively, and are involved in the formation of the catalytic site. The catalytic amino acid residues, catalytic nucleophile and general acid/base catalyst, are included in the conserved regions II and III, respectively.

GH family 13 contains several exo-glucosidases: α-glucosidase, oligo-1,6-glucosidase (EC 3.2.1.10, O16G), and dextran glucosidase (EC 3.2.1.70, DG). These enzymes have distinct specificity for the scissile glucosidic linkage: α-glucosidase has high hydrolytic activity to the α-(1→4)-linkage at the non-reducing end of substrates, whereas O16G and DG are specific to the α-(1→6)-linkage. Most of these enzymes show high amino acid sequence similarity, and are classified into GH family 13 subfamily 31 (GH13_31) [3]. Thus, within this subgroup of exo-glucosidases, a small number of amino acid residues should determine their specificity for the glucosidic linkage. The amino acid residue next to the catalytic nucleophile is considered the primary determinant for the enzyme's specificity [4, 5]. α-(1→4)-Specific glucosidases have Ala or Thr at this position, whereas Val is

conserved in the α-(1→6)-specific glucosidases (Table 1). Mutant enzymes of α-(1→6)-specific glucosidases, in which the conserved Val and its neighboring amino acid residues were mutated, hydrolyzed the α-(1→4)-glucosidic linkage, but the mutants retained hydrolytic activity toward the α-(1→6)-linkage in all the cases [4, 5]. This suggests that other important amino acid residues (i.e., structural elements) involved in the recognition of α-(1→6)-linkage are present.

DG from *Streptococcus mutans* (SmDG) is a typical α-(1→6)-linkage specific exo-glucosidase. Both SmDG and O16G prefer short isomaltooligosaccharides, isomaltose [α-D-glucopyranosyl-(1→6)-α-D-glucopyranose] or isomaltotriose [α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranose], but SmDG has higher activity toward long-chain substrates than O16G [6]. SmDG catalyzes transglucosylation at high substrate concentrations to generate an α-(1→6)-glucosidic linkage. Transglucosylation was enhanced by the replacement of the catalytic nucleophile aspartyl residue with cysteine sulfinate [7]. SmDG is composed of three domains commonly found in GH family 13 enzymes [8]. The β→α Loop 8 of domain A contains three α-helices (Aα8′, Aα8″, and Aα8‴), and contributes to the formation of the pocket-shaped substrate binding site. One calcium ion, which is tightly coordinated by the amino acid residues on the β→α loop 1 of domain A (Asp21, Asn23, Asp25, Ile27, and Asp29), is predicted to enhance the thermostability of SmDG [9]. The short β→α loop 4 of domain A and Trp238 located at the C-terminal of β→α loop 5 are important determinants for the high preference for long-chain substrate [6]. The structure of an inactive SmDG mutant (general acid/base mutant, E236Q) in complex with isomaltotriose occupying the −1 to +2 subsites revealed that Lys275 and Glu371 form hydrogen bonding interactions with the 2OH and 3OH groups of a glucosyl residue in the +1 subsite [8]. Both the amino acid residues are almost completely conserved in the α-(1→6)-linkage specific exo-glucosidases, whereas these amino acid residues are not present in α-(1→4)-specific enzymes (Table 1). Hence we predict that Lys275 and Glu371 are important for hydrolytic activity toward α-(1→6)-linked substrates together with Val195 next to the catalytic nucleophile. This study describes the conversion of selectivity of glucosidic linkage in SmDG from

α-(1→6)-linkage to α-(1→4)-linkage through site-directed mutations at Val195, Lys275, and Glu371 in an effort to understand the structural element which contributes to the α-(1→6)-linkage specificity.

2. Materials and methods

2.1 Preparation of mutant SmDGs

Site-directed mutagenesis was introduced by the megaprimer PCR method [10], in which the expression plasmid for wild-type SmDG [6] was used as a template. Recombinant enzyme was produced in *Escherichia coli* BL21 (DE3)-CodonPlus™ RIL (Stratagene; La Jolla, CA) on a 1 L scale, and purified to homogeneity by Ni-chelating column chromatography as described previously [7]. The concentration of the mutant enzymes prepared was determined by amino acid analysis.

2.2 Enzyme assay

In a standard enzyme assay, the reaction velocity for the release of *p*-nitrophenol from 2 mM *p*-nitrophenyl α-D-glucoside (pNPG, Nacalai Tesque, Kyoto, Japan) was measured as described previously [6]. The optimum pH was determined from the enzyme activity at various pH levels. To vary the reaction pH, 40 mM Britton Robinson buffer (pH 3.5-11) was used as the reaction buffer. The selectivity of glucosidic linkage was investigated based on the rate of hydrolysis of a series of glucobioses at 1 mM. A reaction mixture (50 μL), containing an appropriate concentration of enzyme, 1 mM substrate, 40 mM sodium acetate buffer, and 0.2 mg/mL bovine serum albumin, was incubated at 37°C for 10 min. The pH of the reaction buffer was 6.0, but was 5.6 for the Val195 variants (pH 6.0 for only V195I and V195L), K275A, V195A/K275A (VK), and V195A/E371A (VE). Isomaltose (Tokyo Chemical Industry, Tokyo, Japan), maltose [α-D-glucopyranosyl-(1→4)-α-D-glucopyranose, Nacalai Tesque], kojibiose [α-D-glucopyranosyl-(1→2)-α-D-glucopyranose, Wako Pure Chemical Industries, Osaka, Japan], nigerose [α-D-glucopyranosyl-(1→3)-α-D-glucopyranose, Wako Pure Chemical Industries], and trehalose (α-D-glucopyranosyl α-D-glucopyranoside, Nacalai Tesque) were used as the substrates. The enzyme reaction was terminated by the addition of 100

μL of 2 M Tris-HCl buffer (pH 7.0), and liberated D-glucose was measured with a Glucose CII Test (Wako Pure Chemical Industries). Steady state kinetic parameters for the hydrolysis of the indicated substrates were determined by fitting the reaction velocities at 1–20 mM to the Michaelis–Menten equation using Kaleidagraph ver. 3.6.1 (Synergy Software, Reading, PA).

3. Results and discussion

3.1 Selectivity of glucosidic linkage in SmDG Val195 variants.

Val195 of SmDG, located next to the catalytic nucleophile, was replaced by Ala, Asp, Gly, His, Ile, Leu, Met, Phe, Ser, Thr, and Trp. From 1 L of culture broth of the *E. coli* transformant, 33–126 mg of purified mutant enzymes were obtained. All SmDG variants had lower specific activity (0.0019–26% of wild-type) compared with the wild-type (171 U/mg) [6]. Specific activities of the mutant enzymes to 2 mM pNPG were as follows: V195A, 40.0 U/mg; V195D, 17.9 U/mg; V195G, 1.12 U/mg; V195H, 0.00326 U/mg; V195I, 12.4 U/mg; V195L, 0.596 U/mg; V195M, 43.7 U/mg; V195F, 0.281 U/mg; V195S, 6.40 U/mg; V195T, 26.8 U/mg; and V195W, 0.0143 U/mg. As the specific activities of V195H and V195W were very low, these mutants were not investigated further. The optimum pH was 5.6 for V195A/G/F/M/T, whereas those of the V195I/L mutants were 6.0, the same as wild-type. V195S/D showed an optimum pH at 5.1, although their stabilities were not sufficient at pH 5.1 to determine accurate initial velocities. Kinetic analyses of the V195A/D/G/F/M/S/T mutants were carried out at pH 5.6

The selectivity of glucosidic linkage in the Val195 SmDG variants was investigated based on the hydrolytic velocities toward 1 mM substrate and the associated kinetic parameters. No mutant enzyme hydrolyzed trehalose at all as the wild-type. All the mutant enzymes except for V195S showed the highest activity toward isomaltose among the tested disaccharides. The K_m values of the Val195 SmDG variants for isomaltose were 1.6–8.8-fold higher than that of wild-type, and the k_{cat}/K_m values of these mutants were 3.9–73,000-fold lower than that of wild type (Table 2). In particular, V195L/M/F, harboring a bulky amino acid residue at the Val position, showed a high K_m

value for isomaltose. The large introduced side-chain presumably caused enough steric hindrance to prevent the substrate from binding. V195A showed higher maltase activity than wild-type as shown in the equivalent mutant enzyme of *G. thermoglucosidasius* O16G [5]. In addition to V195A, V195D/G/S also showed higher maltase activity than wild-type. V195A/D/G/S had 2.1–3.9-fold lower K_m and 5.8–620-fold higher k_{cat} than wild-type. The k_{cat}/K_m of V195A for maltose was the highest among the mutant enzymes, and was 1,300-fold higher than that of wild-type. Some α-(1→4)-specific glucosidases such as yeast maltase have Thr at the corresponding position of Val195 in SmDG, and the mutant enzyme of yeast isomaltase, V216T, has maltase activity [4]. However, the substitution of Val195 with Thr did not yield an enzyme with any maltase activity. In the case of SmDG, Val195 was predicted to cause steric hindrance to the binding of an α-(1→4)-linked substrate [8], and elimination of the steric hindrance by the substitution of Val195 with a small amino acid residue was thought to increase maltase activity. V195D/S/T showed much higher preference for nigerose than wild-type. In particular, the k_{cat}/K_m of V195S for nigerose was comparable with that for isomaltose. V195S also showed a 2-fold higher k_{cat}/K_m for kojibiose than wild-type. As V195S and V195T had high hydrolytic activity towards nigerose, an OH group on the side chain of the amino acid residue at the Val195 position might be necessary for recognition of the α-(1→3)-linkage.

3.2 Selectivity of glucosidic linkage in SmDG multiple-mutants of Val195, Lys275, and Glu371

Lys275 and Glu371, located on the long β→α loop 6 and the second α-helix on β→α loop 8 (Aα8″), respectively, are predicted to be involved in the recognition of the α-(1→6) glucosidic linkage as described above (Fig. 1). Single mutants, K275A and E371A; double-mutants, K275A/E371A (KE), VK, and VE; and a triple-mutant enzyme, V195A/K275A/E371A (VKE) were prepared. From 1 L of the culture broth of the *E. coli* transformant, 23–207 mg of purified enzyme was obtained. The specific activities of the mutant enzymes were as follows: K275A, 24.3 U/mg; E371A, 54.9 U/mg; KE, 164 U/mg; VK, 6.07 U/mg; VE, 5.74 U/mg; and VKE, 5.45 U/mg. The optimum pH

levels of E371A, KE, and VKE were 6.0, the same as wild-type. K275A, VK, and VE had a lower optimum pH than wild-type: the optimum pH of K275A and VK was 5.6; and that of VE was 5.0 (VE was unstable at pH 5.0). Kinetic analysis of K275A, VK, and VE was carried out at pH 5.6. Based on the kinetic parameters, the maltase and isomaltase activities of these mutant enzymes were compared with wild-type and V195A (Table 3). Both the single mutant enzymes, K275A and E371A, showed lower hydrolytic activities toward isomaltose and maltose than wild-type. In fact, their isomaltase activity was more severely decreased than their maltase activity: the k_{cat}/K_m values of K275A and E371A for isomaltose were 75–320-fold lower than those of wild-type, whereas those for maltose were only 2.3–10.9-fold lower than those of wild-type. This indicates that Lys275 and Glu371 are important for the enzyme's high specificity for the α-(1→6)-glucosidic linkage.

In the double mutants of VK and VE, the maltase activity was enhanced by the V195A mutation, and isomaltase activity was suppressed by the substitution of Lys275 or Glu371 (Table 3). The maltase activities of these double mutants were much higher than K275A and E371A, and similar to that of V195A. The k_{cat}/K_m values of VK and VE for maltose were 33% and 110% of that of V195A, respectively. However, the isomaltase activities of these double mutants were close to K275A and E371A, and much lower than V195A: the k_{cat}/K_m value of VK was 49% of that for K275A, and that of VE was 41% of that of E371A. The combination of the V195A mutation with the K275A or E371A mutation resulted in a large enhancement of selectivity for the α-(1→4)-linkage. The VK and VE mutant enzymes showed 27- and 26-fold higher k_{cat}/K_m values for maltose than for isomaltose, whereas V195A had a 2-fold lower k_{cat}/K_m for maltose than for isomaltose. As the double-mutation containing K275A and/or E371A did not significantly enhance maltase activity, both Lys275 and Glu371 can be assumed to only minimally contribute to the recognition of the α-(1→4)-linkage. Hydrogen bonding interactions of Lys275 and Glu371 with the substrate, observed in the complex of SmDG and isomaltotriose [8], are probably important only for the hydrolysis of the α-(1→6)-linkage. In the triple mutant enzyme, VKE, the regioselectivity for the α-(1→4)-linkage was further enhanced. VKE showed a 73-fold

higher k_{cat}/K_m for maltose than for isomaltose. Compared with wild type, the k_{cat}/K_m values of VKE for isomaltose and maltose were 500-fold lower and 1,600-fold higher, respectively.

As shown in Table 1, α-glucosidase from *Bacillus* sp. SAM1606 (SAM1606 α-glucosidase) has structural elements of α-(1→6)-linkage specific glucosidases (Val, Lys, and Glu at positions 195, 275, and 371 of SmDG, respectively), but shows considerably high maltase activity with a k_{cat}/K_m for maltose, which is only 7.1-fold lower than that for isomaltose [11]. This enzyme has Gly273 in the position two residues after the general acid/base catalyst, whereas most GH family 13 exo-glucosidases have a more bulky amino acid residue such as Asn, Gln, Pro, Tyr, or Trp at the corresponding position (Table 1). The substitution of Gly273 of SAM1606 α-glucosidase with a bulky amino acid residue decreased maltase activity more than isomaltase activity, confirming that Gly273 is important for maltase activity [12, 13]. Consistent with this result, in yeast isomaltase (devoid of maltase activity), Gln279, corresponding to Gly273 of SAM1606 α-glucosidase, is predicted to sterically hinder the binding of maltose. Replacement of Gln to Ala at position 279 gives a small maltase activity [14]. Therefore, the high maltase activity of *Bacillus* sp. SAM1606 α-glucosidase may be explained by the absence of steric hindrance two residues after the acid/base catalyst.

In this study, we proposed three amino acid residues in SmDG, Val195, Lys275, and Glu371, were essential for hydrolytic activity toward the α-(1→6)-linkage at the non-reducing end of substrates. The V195A mutation did not significantly reduce isomaltase activity, but enhanced hydrolytic activity to α-(1→4)-glucosidic linkage. On the other hand, the substitutions of the other two amino acid residues, resulting in the large loss of isomaltase activity, did not give maltase activity. Therefore V195A mutation is the most significant mutation to affect recognition of scissile α-(1→4)-linkage among three mutations. Val195 causes steric hindrance to the binding of an α-(1→4)-linked substrate [8], and is an impeditive amino acid residue for the reaction with maltose. The Val corresponding to Val195 of SmDG is easily identified because it is located in the conserved region II (next to the catalytic nucleophile). The

Trp residue next to the Lys (Lys275 in SmDG) is conserved in the α-(1→6)-linkage specific glucosidases (Table 1). In SmDG, Trp276 forms an aromatic stacking interaction with Tyr375, which is contained in the Aα8″ helix together with Glu371 (Fig. 1). The substitution of Trp276 with Ala resulted in large loss of hydrolytic activities towards both isomaltase and maltose (Table 3). The k_{cat}/K_m of W276A for isomaltose was 3,500-fold higher than that for maltose, and the selectivity of glucosidic linkage in wild-type SmDG was retained in this mutant enzyme. Thus Trp276 does not directly contribute to the selectivity of glucosidic linkage. The Asp residue is completely conserved at the position two residues before the Glu (Glu371 in SmDG), and a hydrophobic amino acid residue is found between the Asp and Glu. These sequence features should help to identify enzymes with regioselectivity for the α-(1→6)-linkage.

Acknowledgements

We are grateful to Tomohiro Hirose of the Instrumental Analysis Division, Equipment Management Center, Creative Research Institute, Hokkaido University for amino acid analysis.

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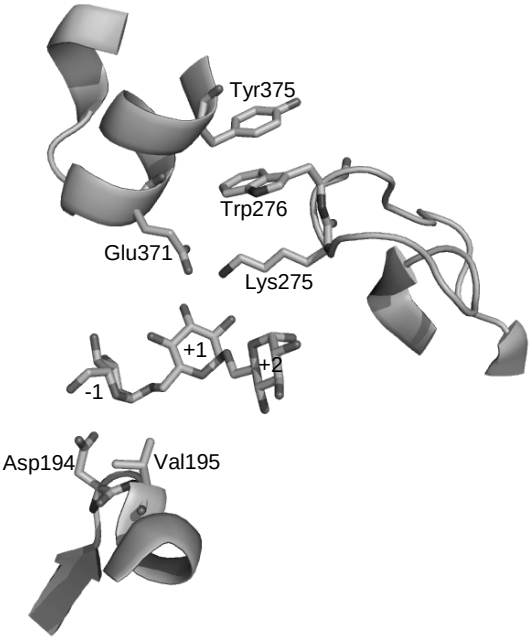
Figure caption

Fig. 1. Close-up view of the key amino acid residues of SmDG.

The active site region of SmDG (complex of E236Q and isomaltotriose; protein data bank code, 2ZID), including Val195, Lys275, and Glu371, is shown.


Tyr375
Trp276
Glu371
Lys275
+1
+2
-1
Asp194
Val195

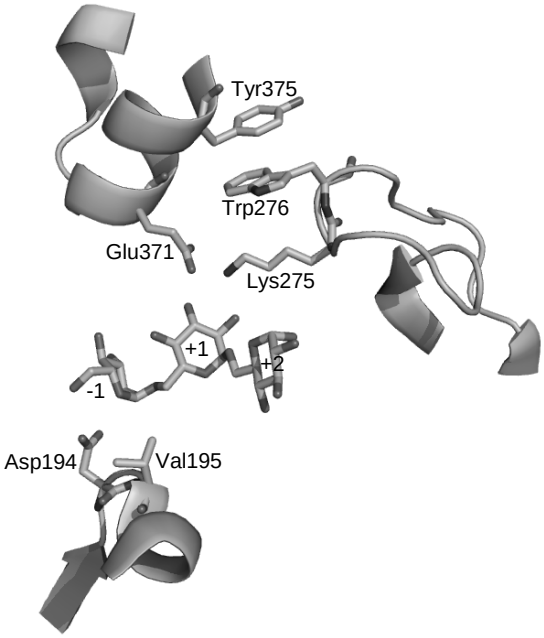

Tyr375
Trp276
Glu371
Lys275
+1
+2
-1
Asp194
Val195

Table 1. Multiple-sequence alignment of GH family 13 exo-glucosidases.

Enzyme	Origin	Sequence			
Dextran glucosidase	*Streptococcus mutans* (SmDG)	190 GFRMDVIDMI	233 TVGETWGAT	267 LQHKPE--APKWDYVKELNV	364 LNELDDIESLN-Y
	Lactobacillus acidophilus	194 GFRMDVIELI	237 TVGETWNAT	271 LDQQPG--KEKWD-LKPLDL	367 IDEVEDIESINMY
Oligo-1,6-glucosidase	*Bifidobacterium adolescentis*	217 GFRMDVITQI	287 NVGEAPGIT	321 IDQE----GSKWN-TVPFEV	416 LEQYRDLEALNGY
	Bifidobacterium breve (Agl1)	220 GFRMDVITLI	290 TVGEAPGIT	324 FDCD----GVKWK-PLPLDL	419 LDQYRDLESLNAY
	Bifidobacterium breve (Agl2)	219 GFRMDVITLI	289 TVGEAPGIT	323 VDQTP---ESKWD-DKPWTP	419 LDQYRDLESINAY
	Bacillus cereus	195 GFRMDVINFI	252 TVGEMPGVT	286 LDSGE---GGKWD-VKPCSL	380 LDEYRDIETLNMY
	Bacillus coagulans	195 GWRMDVIGSI	252 TVGEAIGSD	286 VDTKPGSPAGKWA-LKPFDL	382 LEEYDDIEIRNAY
	Bacillus subtilis	195 GWRMDVIGSI	252 TVGEANGSD	286 IDKEQNSPNGKWQ-IKPFDL	382 LEMYDDLEIKNAY
	Bacillus sp. F5	194 GWRMDVIGSI	251 TVGEAGGSD	285 IDTKQHSPNGKWQ-MKPFDP	381 LEMYDDLEIKNAY
	Geobacillus thermoglucosidasius	195 GFRMDVINMI	253 TVGETPGVT	287 LDSGP---GGKWD-IRPWSL	381 IEDYRDIETLNMY
	Saccharomyces cerevisiae (Ima1)	211 GFRMDVGSLY	274 TVGEMQHAS	308 VGTSP---LFRYN-LVPFEL	404 VEKYEDVEIRNNY
α-Glucosidase	*Saccharomyces cerevisiae* (Mal1S)	210 GFRMDTAGLY	273 TVGEVAHGS	305 VGTSP---FFRYN-IVPFTL	401 IEKYEDVDVKNNY
	Geobacillus stearothermophilus	195 GFRIDAISHI	253 TVGEANGVT	287 LWKRK---AD-----GSIDV	377 IRDYRDVAALRLY
	Geobacillus sp. HTA-462	195 GFRIDAISHI	253 TVGEANGVT	287 LWERR---AD-----GSIDV	377 IRDYRDVSALRLY
	Halomonas sp. H11	198 GFRLDTVNFY	268 TVGEIGDDN	302 MPHSAS----------	377 ---EADVPFERIQ
	Bacillus sp. SAM1606	210 GFRMDVINAI	268 TVGETGGVT	302 IDATD---GDKWR-PRPWRL	396 IDEYRDVEIHNLW
	Apis mellifera (HBGI)	226 GFRIDAVPHL	296 LLTEAYSSL	315 SNVPFN-FKFITDANSSSTP	403 IYKY-DV------
	Apis mellifera (HBGII)	219 GFRIDAINHM	289 ILTEAYTEF	308 STVPFN-FMFIADLNNQSTA	398 YQETVDPAGCNAG
	Apis mellifera (HBGIII)	219 GFRVDALPYI	283 MLIEAYTNL	302 ADFPFN-FAFIKNVSRDSNS	392 WEDTQDPQGCGAG

Amino acid residues of SmDG mutated in this study. The corresponding amino acid residues of the related enzymes are shaded.

Table 2. Kinetic parameters of Val195 SmDG mutant enzymes.

	Isomaltose			Maltose			Nigerose			Kojibiose		
	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 ($s^{-1}\cdot mM^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\cdot mM^{-}$
Wild type	418 ± 8	8.25 ± 0.29	50.7	0.198 ± 0.006	41.9 ± 1.4	0.00473	1.46 ± 0.11	56.2 ± 5.0	0.0260	6.03 ± 0.41	33.7 ± 3.4	0.179
V195A	268 ± 6	20.4 ± 0.7	13.1	122 ± 1	19.5 ± 0.4	6.26	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
V195D	2.68 ± 0.04	20.3 ± 0.5	0.132	1.14 ± 0.02	17.2 ± 0.6	0.0663	0.309 ± 0.013	17.7 ± 1.1	0.0175	N.D.	N.D.	N.D.
V195F	0.0425 ± 0.042	61.6 ± 8.9	0.000690	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
V195G	7.06 ± 0.21	16.4 ± 1.3	0.430	2.64 ± 0.06	10.8 ± 0.3	0.244	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
V195I	27.7 ± 1.3	20.3 ± 1.7	1.36	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
V195L	0.924 ± 0.018	46.2 ± 1.9	0.0200	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
V195M	39.5 ± 1.7	39.6 ± 2.8	0.997	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
V195S	71.8 ± 1.8	15.8 ± 0.9	4.54	17.0 ± 1.0	13.0 ± 0.4	1.31	79.6 ± 4.6	17.2 ± 1.4	4.63	5.59 ± 0.08	15.3 ± 0.5	0.365
V195T	69.6 ± 2.6	13.3 ± 0.7	5.23	N.D.	N.D.	N.D.	10.9 ±0.6	17.1 ± 1.5	0.637	N.D.	N.D.	N.D.

Each value represents the mean ± standard deviation of three independent experiments.

Table 3. Kinetic parameters of SmDG multiple-mutant enzymes.

	Isomaltose			Maltose		
	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	418 ± 8	8.25 ± 0.29	50.7	0.198 ± 0.006	41.9 ± 1.4	0.00473
V195A	268 ± 6	20.4 ± 0.7	13.1	122 ± 1	19.5 ± 0.4	6.26
K275A	7.10 ± 0.16	45.0 ± 1.0	0.158	0.00867 ± 0.00047	20.0 ± 3.6	0.000433
W276A	80.5 ± 3.7	54.0 ± 3.3	1.53	N.D.	N.D.	0.000440
E371A	14.7 ± 0.5	21.7 ± 1.5	0.677	0.0780 ± 0.004	37.3 ± 2.9	0.00209
K275A/E371A	40.7 ± 2.4	10.3 ± 0.6	3.94	N.D.	N.D.	0.0015
V195A/K275A	4.48 ± 0.24	57.9 ± 6	0.0773	99.7 ± 2.8	48.1 ± 2.4	2.07
V195A/E371A	15.3 ± 0.87	55.5 ± 4.5	0.275	175 ± 13	24.9 ± 2.7	7.04
V195A/K275A/E371A	5.70 ± 0.57	55.7 ± 9.1	0.102	136 ± 1	18.1 ± 0.4	7.48

Each value represents the mean ± standard deviation of three independent experiments. N.D., not determined due to too high K_m value.

·V195, K275, and E371 of GH13 dextran glucosidase are conserved in α-1,6-glucosidases.
·V195A mutant showed 1,300-fold higher k_{cat}/K_m for maltose than wild-type.
·Isomaltase activity was severely decreased by K275A and E371A mutations.
·V195A/K275A/E371A showed 73-fold higher k_{cat}/K_m for maltose than for isomaltase.