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Citation

Febs letters, 589(7), 865-869

https://doi.org/10.1016/j.febslet.2015.02.023

Issue Date

2015-03-25

Doc URL

http://hdl.handle.net/2115/60921

Type

article (author version)

File Information

69722 Kimura-A.pdf

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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 kcat/Km for maltose than wild-type, but its kcat/Km 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 $\alpha$-(1→6)-glucosidic linkage.

Keywords: dextran glucosidase; $\alpha$-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 $\alpha$-D-glucoside; SAM1606 $\alpha$-glucosidase, $\alpha$-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 (β/α)_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 megaprimrer 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 $\alpha$-(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
$\alpha$-(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
$\alpha$-(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 $\beta$→$\alpha$ loop 6 and the second $\alpha$-helix on $\beta$→$\alpha$
loop 8 (Aa8”), respectively, are predicted to be involved in the recognition of the
$\alpha$-(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 $\alpha-(1\rightarrow6)$-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 $\alpha-(1\rightarrow4)$-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 $\alpha-(1\rightarrow4)$-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 $\alpha-(1\rightarrow6)$-linkage. In the triple mutant enzyme, VKE, the regioselectivity for the $\alpha-(1\rightarrow4)$-linkage was further enhanced. VKE showed a 73-fold
higher $k_{\text{cat}}/K_m$ for maltose than for isomaltose. Compared with wild type, the $k_{\text{cat}}/K_m$
values of VKE for isomaltose and maltose were 500-fold lower and 1,600-fold higher, respectively.

As shown in Table 1, $\alpha$-glucosidase from *Bacillus* sp. SAM1606 (SAM1606
$\alpha$-glucosidase) has structural elements of $\alpha$-(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 $k_{\text{cat}}/K_m$ 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 $\alpha$-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 $\alpha$-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 $\alpha$-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 $\alpha$-(1→6)-linkage at the non-reducing end of substrates. The V195A mutation did not significantly reduce isomaltase activity, but enhanced hydrolytic activity to $\alpha$-(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 $\alpha$-(1→4)-linkage among three mutations. Val195 causes steric hindrance to the binding of an $\alpha$-(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.

References


the broad substrate specificity of \( \alpha \)-glucosidase from *Bacillus* sp. SAM1606. *J. Biochem.* 134, 543-550.


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.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Origin</th>
<th>Sequence</th>
<th>Amino acid residues of SmDG mutated in this study. The corresponding amino acid residues of the related enzymes are shaded.</th>
</tr>
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<tbody>
<tr>
<td>Dextran glucosidase</td>
<td><em>Streptococcus mutans</em> (SmDG)</td>
<td>190 GFRMDVIDMI 233 TVGGETWGAT 267 LQHKPE---APKMDYVKELNV 364 LNELDDIESLNLN-Y</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td></td>
<td>194 GFRMDWIELI 237 TVGGETWNAT 271 LDQQPG---KEKWD--LKPLDL 367 IDEVEDEIESINMY</td>
<td></td>
</tr>
<tr>
<td>Oligo-1,6-glucosidase</td>
<td><em>Bifidobacterium adolescentis</em></td>
<td>217 GFRMDVIQIQ 287 NVGEAFPGIT 321 IDQE----GSKWN--TVFPFEV 416 LEQYRDEALNGY</td>
<td></td>
</tr>
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<td><em>Bifidobacterium breve</em></td>
<td>(Ag1)</td>
<td>220 GFRMDWITLI 290 TVGEAPGIGT 324 FDCD----GVKWK--PLPLDL 419 LDQYRDLESLNAY</td>
<td></td>
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<tr>
<td><em>Bifidobacterium breve</em></td>
<td>(Ag12)</td>
<td>219 GFRMDWITLI 289 TVGEAPGIGT 323 VDQTP----ESKWD--KPKWDL 419 LDQYRDLESINAY</td>
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<tr>
<td><em>Bacillus cereus</em></td>
<td></td>
<td>195 GFRMDVINFI 252 TVGEMPGVT 286 LDSGE---GKWD--VKPCSL 380 LDEYRDIELTNMY</td>
<td></td>
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<tr>
<td><em>Bacillus coagulans</em></td>
<td></td>
<td>195 GWRMDWIGSI 252 TVGEAIGSD 286 VDTKPGSAGKMA--LKPFDL 382 LEEYDDIEIRNAY</td>
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<tr>
<td><em>Bacillus subtilis</em></td>
<td></td>
<td>195 GWRMDWIGSI 252 TVGEANGSD 286 VDOKPGSAGKMA--LKPFDL 382 LEEYDDIEIRNAY</td>
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<tr>
<td><em>Bacillus sp. F5</em></td>
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<td>194 GWRMDWIGSI 251 TVGEAGGSD 285 IDTQHSPAGKMA--MKPFDP 381 LEMYDDEIKNAY</td>
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<td><em>Geobacillus thermoglucosidasius</em></td>
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<td>195 GFRMDVIMNI 253 TVGETPFGVT 287 LDSPG---GKWD--IRFWSL 381 LIDYRDEETLNM</td>
<td></td>
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<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>(Ima1)</td>
<td>211 GFRMDVGLSY 274 TVGEMQHAS 308 VGTS---LFRYN--LVFPEL 404 VEKYEDEVIRNNY</td>
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<td>α-Glucosidase</td>
<td><em>Saccharomyces cerevisiae</em></td>
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<td><em>Geobacillus stearotherophilus</em></td>
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<td><em>Geobacillus sp. HTA-462</em></td>
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<td><em>Bacillus sp. SAM1606</em></td>
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<td><em>Apis mellifera</em> (HBGI)</td>
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Table 2. Kinetic parameters of Val195 SmDG mutant enzymes.

<table>
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<th>Nigerose</th>
<th>Kojibiose</th>
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<tr>
<td></td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$K_m$ (mM)</td>
<td>$k_{cat}/K_m$ (s$^{-1}$·mM$^{-1}$)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>Wild type</td>
<td>418 ± 8</td>
<td>8.25 ± 0.29</td>
<td>50.7</td>
<td>0.198 ± 0.006</td>
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<tr>
<td>V195A</td>
<td>268 ± 6</td>
<td>20.4 ± 0.7</td>
<td>13.1</td>
<td>122 ± 1</td>
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<tr>
<td>V195D</td>
<td>2.68 ± 0.04</td>
<td>20.3 ± 0.5</td>
<td>0.132</td>
<td>1.14 ± 0.02</td>
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<tr>
<td>V195F</td>
<td>0.0425 ± 0.042</td>
<td>61.6 ± 8.9</td>
<td>0.000690</td>
<td>N.D.</td>
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<td>V195I</td>
<td>7.06 ± 0.21</td>
<td>16.4 ± 1.3</td>
<td>0.430</td>
<td>2.64 ± 0.06</td>
</tr>
<tr>
<td>V195L</td>
<td>27.7 ± 1.3</td>
<td>20.3 ± 1.7</td>
<td>1.36</td>
<td>N.D.</td>
</tr>
<tr>
<td>V195M</td>
<td>0.924 ± 0.018</td>
<td>46.2 ± 1.9</td>
<td>0.0200</td>
<td>N.D.</td>
</tr>
<tr>
<td>V195S</td>
<td>39.5 ± 1.7</td>
<td>39.6 ± 2.8</td>
<td>0.997</td>
<td>N.D.</td>
</tr>
<tr>
<td>V195T</td>
<td>71.8 ± 1.8</td>
<td>15.8 ± 0.9</td>
<td>4.54</td>
<td>17.0 ± 1.0</td>
</tr>
<tr>
<td>V195T</td>
<td>69.6 ± 2.6</td>
<td>13.3 ± 0.7</td>
<td>5.23</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Each value represents the mean ± standard deviation of three independent experiments.
Table 3. Kinetic parameters of SmDG multiple-mutant enzymes.

<table>
<thead>
<tr>
<th></th>
<th>Isomaltose</th>
<th></th>
<th>Maltose</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$</td>
<td>$K_m$</td>
<td>$k_{cat}/K_m$</td>
<td>$k_{cat}$</td>
</tr>
<tr>
<td></td>
<td>(s$^{-1}$)</td>
<td>(mM)</td>
<td>(s$^{-1}$·mM$^{-1}$)</td>
<td>(s$^{-1}$)</td>
</tr>
<tr>
<td>Wild type</td>
<td>418 ± 8</td>
<td>8.25 ± 0.29</td>
<td>50.7</td>
<td>0.198 ± 0.006</td>
</tr>
<tr>
<td>V195A</td>
<td>268 ± 6</td>
<td>20.4 ± 0.7</td>
<td>13.1</td>
<td>122 ± 1</td>
</tr>
<tr>
<td>K275A</td>
<td>7.10 ± 0.16</td>
<td>45.0 ± 1.0</td>
<td>0.158</td>
<td>0.00867 ± 0.00047</td>
</tr>
<tr>
<td>W276A</td>
<td>80.5 ± 3.7</td>
<td>54.0 ± 3.3</td>
<td>1.53</td>
<td>N.D.</td>
</tr>
<tr>
<td>E371A</td>
<td>14.7 ± 0.5</td>
<td>21.7 ± 1.5</td>
<td>0.677</td>
<td>0.0780 ± 0.004</td>
</tr>
<tr>
<td>K275A/E371A</td>
<td>40.7 ± 2.4</td>
<td>10.3 ± 0.6</td>
<td>3.94</td>
<td>N.D.</td>
</tr>
<tr>
<td>V195A/K275A</td>
<td>4.48 ± 0.24</td>
<td>57.9 ± 6</td>
<td>0.0773</td>
<td>99.7 ± 2.8</td>
</tr>
<tr>
<td>V195A/E371A</td>
<td>15.3 ± 0.87</td>
<td>55.5 ± 4.5</td>
<td>0.275</td>
<td>175 ± 13</td>
</tr>
<tr>
<td>V195A/K275A/E371A</td>
<td>5.70 ± 0.57</td>
<td>55.7 ± 9.1</td>
<td>0.102</td>
<td>136 ± 1</td>
</tr>
</tbody>
</table>

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.