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Structural elements responsible for the glucosidic linkage-selectivity of a glycoside hydrolase family 13 exo-glucosidase☆

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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 1300-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.

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

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

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of amino acid residues should determine their specificity for the glucosidic linkage. The amino acid residue next to the catalytic nucleophile is considered to be 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

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.