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4-*O*-Methyl Modifications of Glucuronic Acids in Xylans Are Indispensable for Substrate Discrimination by GH67 α-Glucuronidase from *Bacillus halodurans* C-125

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Abstract: A GH67 α-glucuronidase gene derived from *Bacillus halodurans* C-125 was expressed in *E. coli* to obtain a recombinant enzyme (*Bh*GlcA67). Using the purified enzyme, the enzymatic properties and substrate specificities of the enzyme were investigated. *Bh*GlcA67 showed maximum activity at pH 5.4 and 45 °C. When *Bh*GlcA67 was incubated with birchwood, oat spelts, and cotton seed xylan, the enzyme did not release any glucuronic acid or 4-*O*-methyl-glucuronic acid from these substrates. *Bh*GlcA67 acted only on 4-*O*-methyl-α-D-glucuronopyranosyl-(1→2)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranose (MeGlcA^{3}Xyl$_3$), which has a glucuronic acid side chain with a 4-*O*-methyl group located at its non-reducing end, but did not on β-D-xylopyranosyl-(1→4)-[4-*O*-methyl-α-D-glucuronopyranosyl-(l→2)]-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranose (MeGlcA^{3}Xyl$_4$) and α-D-glucuronopyranosyl-(l→2)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranose (GlcA^{3}Xyl$_3$). The environment for recognizing the 4-*O*-methyl group of glucuronic acid was observed in all the crystal structures of reported GH67 glucuronidases, and the amino acids for discriminating the 4-*O*-methyl group of glucuronic acid were widely conserved in the primary sequences of the GH67 family, suggesting that the 4-*O*-methyl group is critical for the activities of the GH67 family.

Key words: α-glucuronidase, glycoside hydrolase family 67, *Bacillus halodurans*, 4-*O*-methyl glucuronoxylan, glucuronoxylan, xylan

INTRODUCTION

The plant cell wall is mainly composed of cellulose, hemicellulose, and lignin, and it contains about one third of each component.[1)] Xylan is the major component of hemicellulose, which is the second most abundant lignocellulosic biomass resource on earth next to cellulose.[2)] Generally, xylans consist of a backbone of β-1,4-linked xylopyranose residues, which is substituted for α-1,3-linked L-arabinofuranose and α-1,2-linked 4-*O*-methyl glucuronic acid (MeGlcA) and/or glucuronic acid (GlcA) side chains.[3)] Enzymes such as β-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), and α-glucuronidase (EC 3.2.1.139) are necessary to hydrolyze xylan completely.[4)]

The importance of using biomass is increasing due to considerations of environmental problems such as global warming. Plant cell walls are the most abundant biomass on earth and are created through the fixation of carbon dioxide by photosynthesis. Therefore, this biomass is carbon neutral and does not increase the carbon dioxide in the atmosphere when it burns. In recent years, the concept of bioeconomy has been advocated globally, and the scale of industry using biotechnology is increasing year by year (OECD, 2009, http://www.oecd.org/futures/bioeconomy/2030). Because the industry aims to use only cellulose, diluted sulfuric acid pretreatment and hydrothermal treatment

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Abbreviations: CAZy, Carbohydrate-Active enZymes; GH, glycoside hydrolase; GH67, glycoside hydrolase family 67; *Bh*GlcA67, GH67 α-glucuronidase from *Bacillus halodurans* C-125; TLC, thin-layer chromatography; SDS-PAGE, sodium dodecyl sulfate poly-acrylamide gel electrophoresis; MeGlcA, 4-*O*-methyl-D-glucuronic acid; GlcA, D-glucuronic acid; MeGlcA^{3}Xyl$_3$, 4-*O*-methyl-α-D-glucuronopyranosyl-(l→2)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranose; MeGlcA^{3}Xyl$_4$, β-D-xylopyranosyl-(1→4)-[4-*O*-methyl-α-D-glucuronopyranosyl-(l→2)]-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranose; GlcA^{3}Xyl$_3$, α-D-glucuronopyranosyl-(l→2)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranose; X$_1$, xylose; X$_2$, xylobiose; X$_3$, xylotriose; X$_4$, xylotetraose; X$_5$, xylopentaose.

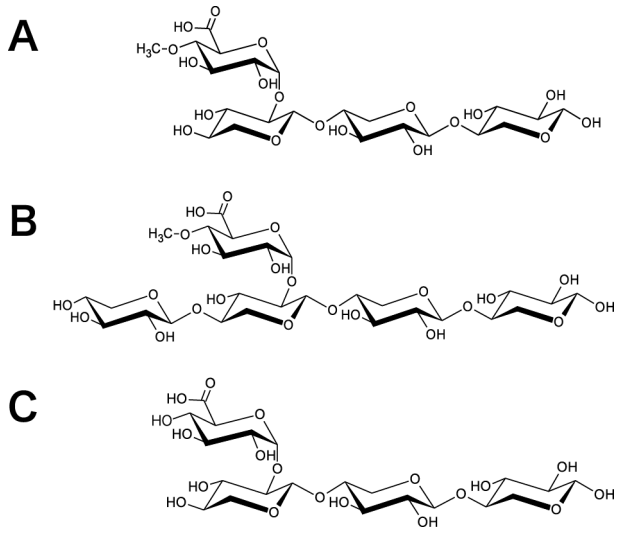

Fig. 1. Structures of the purified oligosaccharides.
A: MeGlcA^{3}Xyl$_3$, a hydrolysate of birchwood xylan catalyzed by SoXyn10A, is the non-reducing end xylose residue of xylotriose and accommodates an α-1,2-linked 4-*O*-methyl-glucuronic acid. B: MeGlcA^{3}Xyl$_4$, a hydrolysate of birchwood xylan catalyzed by SoXyn11A, is the second xylose residue from the non-reducing end of xylotetraose and accommodates an α-1,2-linked 4-*O*-methyl-glucuronic acid. C: GlcA^{3}Xyl$_3$, a hydrolysate of cotton seed xylan catalyzed by SoXyn10A, is the xylose residue at the non-reducing end of xylotriose and accommodates an α-1,2-linked glucuronic acid.

that remove hemicelluloses from plant cell walls are usually employed as pretreatments for the enzymatic saccharification in the biomass utilization process.[5] This means that more than half of the resource is wasted. Technological development for utilizing hemicellulose, which accounts for about one-third of biomass, is important from the viewpoint of bio-economy.

Due to its chemical complexity and because it contains a large amount of pentoses unsuitable for fermentation, efficient utilization of hemicellulose is not highly developed. Because diluted sulfuric acid pretreatment and hydrothermal treatment are not specific for the structure of hemicelluloses, a mixture of several kinds of sugars together with fermentation inhibitors produced by these severe hydrolysis conditions make it difficult to utilize the resulting hemicellulose hydrolysate. In contrast, the enzymes that highly discriminate among hemicellulose structures may be developed to target the production of specific sugar products from hemicellulose and would be expected to be useful tools to use hemicelluloses. These enzymes have great potential to develop the process of biomass utilization because it is possible to regulate the structure and size of products by the substrate specificity of the enzymes. It has been demonstrated that the degradation of hemicelluloses is restricted by the branches of hemicelluloses. Therefore, it is important to prepare the enzymes that are characterized to be specific for the branched structure of hemicelluloses to develop hemicellulose utilization technology using enzymes as a tool.

In this study, we focused on an accessary enzyme, α-glucuronidase, which acts on the linkage of α-glucuronic acid substitution in xylans. Carbohydrate active enzymes are classified in the Carbohydrate Active enZymes (CAZy) database (http://www.cazy.org/) based on their amino acid sequence.[6] α-Glucuronidases are classified into GH67 and GH115. It has been reported that GH67 enzymes cannot act on polysaccharides, and they specifically act on oligosaccharides having MeGlcA and/or GlcA side chains at the non-reducing end.[7] In contrast, GH115 enzymes can cleave MeGlcA and/or branches of both polysaccharides and oligosaccharides.[8]

Although many papers have reported that "α-glucuronidases act on GlcA and/or MeGlcA of xylans", they did not test the activity for GlcA branches in oligosaccharides and there are no reports on the GH67 enzymes activity for non-methylated GlcA branches in the substrates. The oligosaccharides having GlcA branches were not commercially available even the natural substrate contains a large amount of GlcA side chains not modified with a methyl group.[9][10]

In this study, in order to make GH67 enzymes a useful tool of xylan study, oligosaccharides having GlcA side chains modified with or without a methyl group were prepared, and the substrate specificities of a GH67 α-glucuronidase (*Bh*GlcA67) derived from *Bacillus halodurans* C-125 for these substrates were investigated.

MATERIALS AND METHODS

Protein expression and purification. The gene encoding a putative GH67 α-glucuronidase (BH1061; GenBank accession number BAB04780) was amplified from the *B. halodurans* C-125 genomic DNA by polymerase chain reaction using the following primers: forward, 5′-CATATGAATCGAGGAGAAACTGGTTATGAAAC ATG-3′; and reverse, 5′-GCGGCCGCTATCGGATAAATGGTT-3′. The amplified DNA was cloned into a pET30a vector (Novagen, Madison, USA) at NdeI and NotI restriction enzyme sites (underlined). The resulting pET30-*bhglca67* recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) (Merck KGaA, Darmstadt, Germany). The transformants were grown in Luria-Bertani medium[11] at 37 °C with shaking at 200 rpm. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.1 mM when the OD_{600} reached 0.2. After the addition of IPTG, the cultures were grown at 25 °C for 22 h. The cells were collected by centrifugation (6,500 × G for 15 min, 4 °C) and resuspended in 50 mM sodium phosphate buffer (pH 7.0) followed by sonication. Insoluble materials were removed by centrifugation (14,000 × G for 30 min, 4 °C). The *Bh*GlcA67 protein was purified by immobilized metal-affinity chromatography (HisTrap HP, GE Healthcare, Chicago, USA) by fusion of the protein to an in-frame C-terminal 6 × histidine tag and was subsequently dialyzed against distilled H_2O. The elution of the enzyme was monitored by SDS-PAGE.[12] The final preparation obtained was used as the purified enzyme.

Substrates. 4-*O*-Methyl-glucuronoxylan from beechwood and birchwood, and 4-*O*-methyl-glucurono-arabinoxylan from oat spelts were obtained from Merck KGaA. Glucuronoxylan from cotton seeds was prepared by the combination of chromatographies of active carbon, anion exchange and gel filtration as described previously.[13] 4-*O*-Methyl-α-

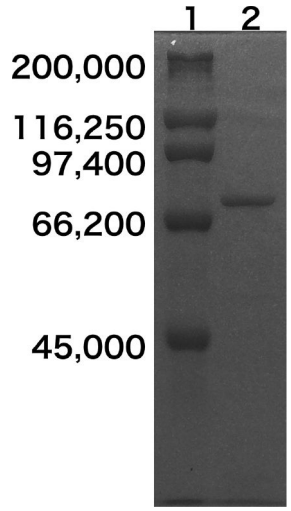

Fig. 2. SDS-PAGE of purified *Bh*GlcA67.
Lane 1, molecular weight standards (1 μg in each band); lane 2, purified *Bh*GlcA67 (1 μg. Calculated as 1 mg/mL when the absorbance at 280 nm was 1).

D-glucuronopyranosyl-(l→2)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranose (MeGlcA^{3}Xyl$_3$) and α-D-glucuronopyranosyl-(l→2)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranose (GlcA^{3}Xyl$_3$) was prepared from hydrolysates of birchwood xylan and cotton seed xylan by GH10 xylanase from *Streptomyces olivaceoviridis,* E-86[14] respectively, as described previously.[15] β-D-Xylopyranosyl-(1→4)-[4-*O*-methyl-α-D-glucuronopyranosyl-(l→2)]-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranose (MeGlcA^{3}Xyl$_4$) were purified from hydrolysates of birchwood xylan by GH11 xylanase from *Streptomyces olivaceoviridis* E-86[16], as described previously[15] (Fig. 1).

Enzyme assay and substrate specificity. *Bh*GlcA67 activity was determined by measuring the liberated GlcA and/or MeGlcA by the method of Milner–Avigad[17] using the acidic oligosaccharides fraction as the substrate. The acidic oligosaccharides fraction was prepared by anion exchange chromatography of GH10 xylanase hydrolysate of birchwood xylan as described previously.[15] The enzyme assay mixture contained 200 μL of McIlvaine buffer (0.2 M Na_2HPO_4 and 0.1 M citric acid) at a pH of 6.0, 250 μL of 10 mg/mL of acidic oligosaccharides fraction, and 50 μL of the enzyme preparation. The reactions were performed at 40 °C for 15 min. At regular time intervals, 50-μL aliquots of the reaction mixture were collected, and 150 μL of copper reagent was added to the reaction mixture. The reaction was terminated by heating at 100 °C for 10 min. Arsenomolybdate reagent (100 μL) was added to the solution and mixed until the precipitate dissolved. The solution was diluted by adding 200 μL of water. A blank solution was prepared by using water in the reaction mixture. The amount of uronic acids was determined by measuring the increase in absorbance at 600 nm, and the assays were performed in duplicate.

The effects of pH on the activity and stability of *Bh*GlcA67 were examined in a series of McIlvaine buffers ranging from pH 2.2 to 8.0 and in Atkins–Pantin buffer (0.2 M boric acid/0.2 M KCl/0.2 M Na_2CO_3) ranging from pH 7.0 to 10.0. The activities were assayed under the conditions described for the standard method. In order to determine the effect of pH on enzyme stability, the enzymes were preincubated at various pH values in the absence of substrate at 30 °C for 30 min, and the residual activity was then assayed using the standard method. The effects of temperature on the activity of α-glucuronidase were determined from 30 to 60 °C. With the exception of temperature, the assay conditions were the same as described for the standard method. For the temperature stability measurements, the enzymes were pre-incubated at 30, 40, and 50 °C at pH 5.5 for 0, 1, 3, 6, 12, and 24 h, and the residual activity was determined using the standard method. These assays were performed in triplicate.

To determine the substrate specificities, xylans from birchwood, beechwood, oat spelts, and cotton seeds were used as substrates. A solution was prepared containing 5 μL of enzyme solution (1 mg/mL), 20 μL McIlvaine buffer (pH 5.5), 5 μL of 10 mg/mL bovine serum albumin solution, and 25 μL 10 mg/mL substrates. After incubation at 30 °C for 24 h, the reaction was stopped by boiling for 20 min. Released GlcA and/or MeGlcA was determined by the Milner–Avigad method.[17] For oligosaccharides, the enzyme (1 mg/mL) was incubated with 5 mg/mL substrate at 30 °C for 0, 0.16, 0.33, 0.5, 0.66, 1, 2, 4, and 24 h. The reaction was stopped by boiling for 20 min, and then the reaction mixtures were subjected to thin layer chromatography (TLC) and the Milner–Avigad method.[17] TLC was done by ascending on a silica gel TLC plate with a solvent system of *n*-butanol-acetic acid-water (2:1:1, v/v/v). The sugars on the plate were detected by heating at 140 °C for a few minutes in a hot dry oven after spraying with a 6.5 mM solution *N*-(1-naphtyl) ethylenediamine dihydrochloride in methanol containing 3 % (v/v) sulfuric acid.[18]

RESULTS

Expression, purification, and properties of BhGlcA67.

The gene *bhglca67* from *B. halodurans* C-125 is 2,046 bp and encodes 682 amino acids. *Bh*GlcA67 consists of only a catalytic domain belonging to GH67 without any secretory signal sequence. The purified recombinant *Bh*GlcA67 showed a single band on SDS-PAGE with an estimated molecular weight of 78,500 (Fig. 2). The value was almost the same as the molecular weight calculated from the amino acid sequence of *Bh*GlcA67 (78,251).

The enzymatic properties of recombinant *Bh*GlcA67 were examined (Fig. 3). The enzyme achieved maximal activity at 45 °C and at pH 5.5 (Figs. 3A and 3B). It retained more than 60 % activity in the pH range from pH 5.0 to 8.0 (Fig. 2C) after treatment at 30 °C for 30 min. It also retained more than 60 % of its activity after the incubation at pH 6.0, 40 °C for 3 h, and at pH 6.0, 30 °C for 12 h (Fig. 3D).

Substrate specificity of BhGlcA67.

*Bh*GlcA67 did not show any activity for the xylans from birchwood, beechwood, oat spelts, or cotton seeds (data not shown). Next, we investigated the substrate specificity of

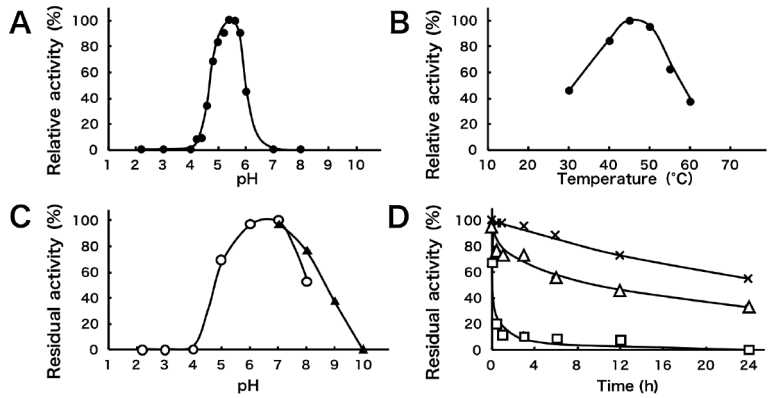

Fig. 3. Enzymatic properties of *Bh*GlcA67.

A: the effect of pH on enzyme activity. B: the effect of temperature on enzyme activity. C: the effect of pH on enzyme stability. D: the effect of temperature on enzyme stability. Open circles, McIlvaine buffer; closed triangles, Atkins–Pantin buffer; crosses, 30 °C; open triangles, 40 °C; open squares, 50 °C.

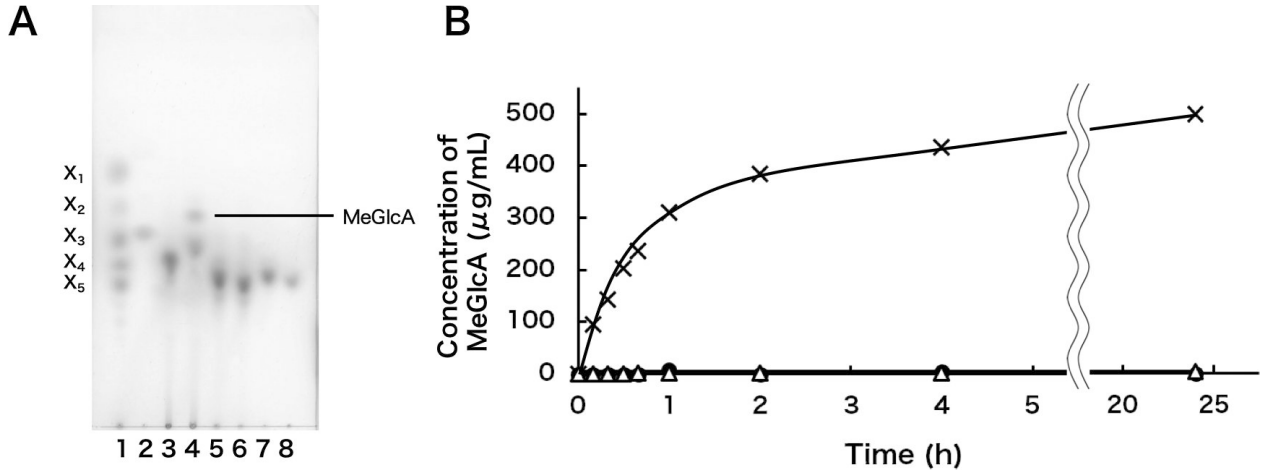

Fig. 4. Substrate specificities of *Bh*GlcA67 for glucuronoxylooligosaccharides.

A: thin-layer chromatography of the hydrolysis products. Lane 1, xylose standard of xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4) and xylopentaose (X_5), lane 2, D-glucuronic acid (GlcA); lane 3, MeGlcA^{3}Xyl$_3$, lane 4, hydrolysis products of MeGlcA^{3}Xyl$_3$ by *Bh*GlcA67; lane 5, MeGlcA^{3}Xyl$_4$, lane 6, hydrolysis products of MeGlcA^{3}Xyl$_4$ by *Bh*GlcA67; lane 7, GlcA^{3}Xyl$_3$; lane 8, hydrolysis products of GlcA^{3}Xyl$_3$ by *Bh*GlcA67; B: time course of hydrolysis of glucuronoxylooligosaccharides by *Bh*GlcA67. Crosses, MeGlcA^{3}Xyl$_3$; black circles, MeGlcA^{3}Xyl$_4$; open triangles, GlcA^{3}Xyl$_3$.

*Bh*GlcA67 against the three types of oligosaccharides, the structures of which are shown in Fig. 1. Figure 4A shows the TLC of the hydrolysis products of MeGlcA^{3}Xyl$_3$, MeGlcA^{3}Xyl$_4$, and GlcA^{3}Xyl$_3$ by *Bh*GlcA67. The enzyme acted only on MeGlcA^{3}Xyl$_3$ but not on MeGlcA^{3}Xyl$_4$ and GlcA^{3}Xyl$_3$. Because TLC is not a sensitive method, the released GlcA and/or MeGlcA was analyzed by the Milner–Avigad method (Fig. 4B). The specific activity of *Bh*GlcA67 for MeGlcA^{3}Xyl$_3$ was 42 nmol/min/mg and the amounts of GlcA or MeGlcA released from each oligosaccharide after 24 h were 497.5 μg/mL, 0 μg/mL, and 3.8 μg/mL from MeGlcA^{3}Xyl$_3$, MeGlcA^{3}Xyl$_4$, and GlcA^{3}Xyl$_3$, respectively. Thus, only small amounts of GlcA could be detected from GlcA^{3}Xyl$_3$, and the reaction rate was much slower than that from MeGlcA^{3}Xyl$_3$, which has a methylated MeGlcA side chain (Fig. 4B).

Because it is interesting that such a small modification of the sugars that possess a methyl group or not affected the enzyme activity so significantly, the crystal structure of *Geobacillus stearothermophilus* T-6[19] with 98 % similarity to *Bh*GlcA67 was used to observe the environment of the sub-

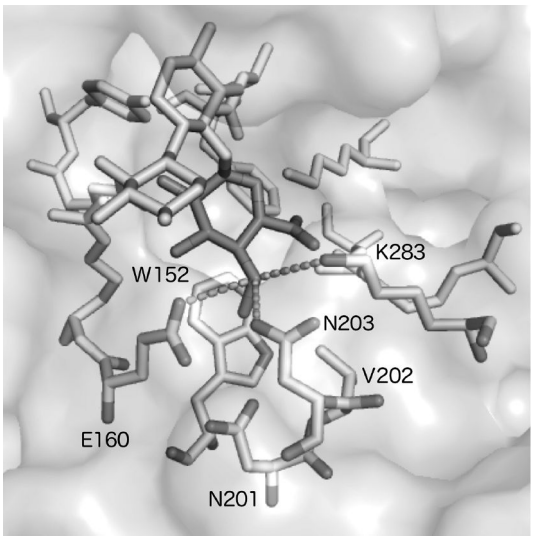

Fig. 5. The catalytic pocket of *Bh*GlcA67.

The homology model structure of *Bh*GlcA67 with a substrate was modeled on the basis of the crystal structure of α-glucuronidase from *Geobacillus* stearothermophilus T-6[20] (PDB: 1K9F) using the software Modeller (https://salilab.org/modeller/) .[25]

strate binding pocket (Fig. 5). The amino acids such as Trp-152, Glu-160, Asn-201, Val-202, Asn-203, and Lys-283 in model structure of *Bh*GlcA67 exist so as to surround the methyl group to interact with the 4-*O*-methyl group of MeGlcA, suggesting the enzyme positively recognizes the methyl group of the substrates.

DISCUSSION

The oligosaccharides MeGlcA^{3}Xyl$_3$ and MeGlcA^{3}Xyl$_4$ used in this study were hydrolysis products of GH10 and GH11 xylanases, respectively. The substrate specificity for which the enzymes acted on MeGlcA^{3}Xyl$_3$ and not on MeGlcA^{3}Xyl$_4$ has been reported in the previous papers and is understood as a common property of GH67.[7)20)] When *Bh*GlcA67 was incubated with GlcA^{3}Xyl$_3$, a very small amount of GlcA (3.8 μg/mL) was detected after 24 h (Fig. 4B). This amount may be increased if the amount of enzyme used for the hydrolysis and the reaction time are increased. Hydrolysis of the linkage of nonmethylated GlcA is significantly slower than that of methylated MeGlcA.

To date, the crystal structures of two GH67 enzymes of *G. stearothermophilus* and *Cellbibrio japonicus* have been reported.[20)21)] The structure for recognizing the 4-*O*-methyl group of MeGlcA in the substrate binding pocket such as the structure shown in Fig. 5 was completely conserved in these three enzymes: even *C. japonicus* GlcA67A showed comparatively low similarity (less than 60 %) with *Bh*GlcA67. Nagy *et al*. investigated the affinity of *C. japonicus* GlcA67A and its catalytic pocket mutant enzymes for GlcA and MeGlcA.[22)] The K_is of wild type *C. japonicus* GlcA67A for GlcA and MeGlcA were 3.84 mM and 0.54 mM, respectively. This suggests that the enzyme recognizes the 4-*O*-methyl group of MeGlcA and thereby shows a higher affinity for MeGlcA than GlcA, as expected from the crystal structure. The Val-210 in *C. japonicus* GlcA67A has a detrimental effect on substrate binding. The k_{cat}/K_m of V210A and V210S mutants of *C. japonicus* GlcA67A for 4-nitrophenyl 2-*O*-(4-*O*-methyl-α-D-glucuronopyranosyl)-β-D-xylopyranoside were 2.7 and 23.3 times decreased, respectively. When Val-210 was replaced by Asn (V210N), the activity of the enzyme for the substrate significantly decreased, and the relative k_{cat}/K_m for the substrate was 9.4 × 10^{-2} fold with the wild type enzyme, suggesting that bind-

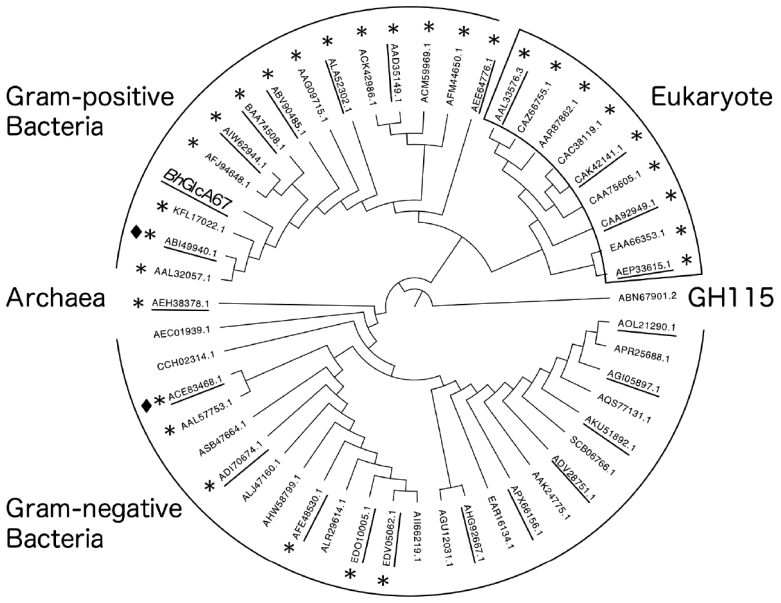

Fig. 6. The phylogenetic relationships of α-glucuronidases belonging to GH67.

The analysis was performed using the ClustalW program (http://www.genome.jp/tools-bin/clustalw). The sequences used in Fig. 7 were underlined. Asterisk and black diamond indicate functionally characterized and crystalized, respectively. α-Glucuronidase from ABN67901.2 (*Scheffersomyces stipitis* CBS 6054), AEC01939.1 (*Sphaerochaeta coccoides* DSM 17374), ADI70674.1 (*Prevotella bryantii* B14), AEH38378.1 (*Haloopiger xanaduensis* SH-6), EAA66353.1 (*Aspergillus nidulans* FGSC A4), AEP33615.1 (*Chrysosporium lucknowense*), CAA92949.1 (*Trichoderma reesei*), AAR87862.1 (*Aureobasidium pullulans*), AAL33576.3 (*Rasamsonia emersonii*), CAZ66755.1 (*Penicillium aurantiogriseum*), CAA75605.1 (*Aspergillus tubingensis*), CAC38119.1 (*Aspergillus niger*), CAK42141.1 (*Aspergillus niger*), AEE64776.1 (*Ruminococcus albus* 8), AFM44650.1 (*Caldanaerobius polysaccharolyticus*), ACM59969.1 (*Caldicellulosiruptor bescii* DSM 6725), ACK42986.1 (*Dictyoglomus turgidum* DSM 6724), AAD35149.1 (*Thermotoga maritima* MSB8), ALA52302.1 (*Bacillus clausii*), AAG09715.1 (*Geobacillus stearothermophilus*), BAB04780.1 (*Bacillus halodurans* C-125), KFL17022.1 (*Geobacillus stearothermophilus*), AAL32057.1 (*Geobacillus stearothermophilus*), ABI49940.1 (*Geobacillus stearothermophilus*), ABV90485.1 (*Paenibacillus* sp. JDR-2), AFJ94648.1 (uncultured bacterium), BAA74508.1 (*Aeromonas caviae*), AIW62944.1 (*Paenibacillus curdlanolyticus*), CCH02314.1 (*Fibrella aestuarina* BUZ 2), ASB47664.1 (*Alkalitalea saponilacus*), ALR29614.1 (*Chryseobacterium* sp. IHB B 17019), AHW58799.1 (*Draconibacterium orientale*), AFE48530.1 (uncultured rumen bacterium), ALJ47160.1 (*Bacteroides ovatus*), AII66219.1 (*Bacteroides dorei*), EDV05062.1 (*Bacteroides intestinalis* DSM 17393), EDO10005.1 (*Bacteroides ovatus* ATCC 8483), EAR16134.1 (*Robiginitalea biformata* HTCC2501), AAL57753.1 (*Cellvibrio mixtus*), ACE83468.1 (*Cellvibrio japonicus* Ueda107), AHG92667.1 (*Gemmatirosa kalamazoonesis*), AGU12031.1 (uncultured organism), APX66156.1 (*Sphingomonas* sp. LK11), AAK24775.1 (*Caulobacter crescentus* CB15), ADV28751.1 (*Pseudoxanthomonas suwonensis* 11-1), SCB06766.1 (*Xanthomonas translucens* pv. *translucens* DSM 18974), AKU51892.1 (*Xanthomonas arboricola* pv. *juglandis*), AQS77131.1 (*Xanthomonas perforans* 91-118), AGI05897.1 (*Xanthomonas citri* subsp. *citri* Aw12879), AOL21290.1 (*Xanthomonas citri* subsp. *malvacearum*), APR25688.1 (*Xanthomonas citri* subsp. *citri*).

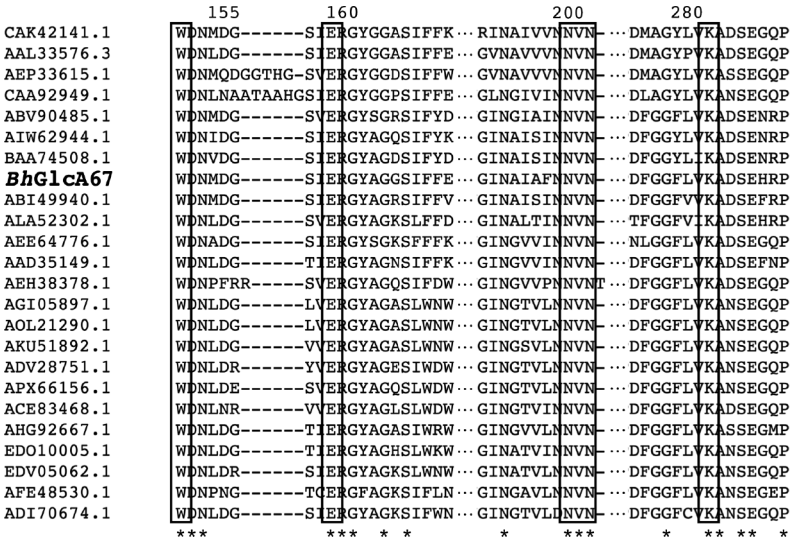

Fig. 7. Amino acid residues important for the accommodation of the 4-*O*-methyl group of MeGlcA.
Conserved amino acids important for the accommodation of the 4-*O*-methyl group of MeGlcA are boxed. The aligned sequences were selected uniformly from each phylogenetic tree group shown in Fig. 6.

ing with the 4-*O*-methyl group of MeGlcA is very important for the enzyme activity. However, they did not test the activity of *C. japonicus* GlcA67A for the substrates that have a nonmethylated GlcA side chain, so it is unclear whether the *C. japonicus* GlcA67A shows a similar activity with *Bh*GlcA67 for $GlcA^3Xyl_3$.

Septiningrum *et al*. investigated the activity of a GH67 enzyme from *Paenibacillus curdlanolyticus* B-6 toward the oligosaccharides having hexenuronic acid side chains, which convert from MeGlcA by alkali-catalyzed formation. The k_{cat}/K_m of the enzyme for the oligosaccharides having hexenuronic acid side chains decreased by 470 times than that for $MeGlcA^3Xyl_3$, also suggesting that accommodation of the 4-*O*-methyl group of MeGlcA is important for the enzyme activity.[23)]

These facts suggest that the existence of methyl groups greatly influences the xylan decomposition mechanism of GH67. In order to investigate whether these features are common to GH67 enzymes, the amino acid sequences of the 24 characterized members belonging to GH67 were compared. The alignment of the 24 sequences indicates that 6 amino acids, Trp-152, Glu-160, Asn-201, Val-202, Asn-203, and Lys-283, were completely conserved. Furthermore, we compared 477 sequences belonging to GH67, which were the sequences after removal of apparently incomplete sequences. The four important amino acids for recognizing the 4-*O*-methyl group of MeGlcA were also completely conserved. This strongly suggests that the GH67 enzyme has a structure for recognizing the 4-*O*-methyl group of MeGlcA and can hydrolyze the oligosaccharides having a methylated MeGlcA side chain (Figs. 6 and 7). The 4-*O*-methylation of MeGlcA in xylans may have some biologically important function in the plant cell wall.

Before α-glucuronidases were classified in the CAZy database, it was reported that a α-glucuronidase from *Aspergillus niger* 5–16 acted much more on $GlcA^3Xyl_3$ than on $MeGlcA^3Xyl_3$.[24)] Because the amino acid sequence of the α-glucuronidase from *A. niger* 5–16 is not reported, we could not know to which GH family this enzyme belongs. However, we speculate that this enzyme belongs to GH115 because the molecular weight of this enzyme estimated by SDS-PAGE is 150,000, which is too large for the general molecular weight of GH67. In addition, we also studied GH115 α-glucuronidase and confirmed that GH115 enzyme clearly releases GlcA under the same conditions as this study (data not shown). Thus we believe that GH115 enzymes are less specific than GH67. But the hypothesis will be a challenge for the future because no GH115 structure has been solved with MeGlcA and/or GlcA.

In conclusion, we demonstrated that modification of the 4-*O*-methyl group in the substrate significantly affected the activity of *Bh*GlcA67 because the enzyme acted only on $MeGlcA^3Xyl_3$ but not $GlcA^3Xyl_3$. Observation of the structure of the catalytic site of GH67 enzymes and amino acid sequence alignment of GH67 members strongly suggested that GH67 enzymes accommodate the 4-*O*-methyl group of MeGlcA in the substrates. Even if it is unknown whether or not the other enzymes act on $GlcA^3Xyl_3$, *Bh*GlcA67 showed tight substrate specificity for $MeGlcA^3Xyl_3$. We believe that *Bh*GlcA67 will be a useful tool for xylan study because the exact specificity of the enzyme will be available to analyze and modify the structure of glucuronoxylooligosaccharides.

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