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Aglycone specificity of *Escherichia coli* α-xylosidase investigated by transxylosylation

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The specificity of the aglycone-binding site of *Escherichia coli* α-xylosidase (YicI), which belongs to glycoside hydrolase family 31, was characterized by examining the enzyme's transxylosylation-catalyzing property. Acceptor specificity and regioselectivity were investigated using various sugars as acceptor substrates and α-xylosyl fluoride as the donor substrate. Comparison of the rate of formation of the glycosyl–enzyme intermediate and the transfer product yield using various acceptor substrates showed that glucose is the best complementary acceptor at the aglycone-binding site. YicI preferred aldopyranosyl sugars with an equatorial 4-OH as the acceptor substrate, such as glucose, mannose, and allose, resulting in transfer products. This observation suggests that 4-OH in the acceptor sugar ring made an essential contribution to transxylosylation catalysis. Fructose was also acceptable in the aglycone-binding site, producing two regioisomer transfer products. The percentage yields of transxylosylation products from glucose, mannose, fructose, and allose were 57, 44, 27, and 21%, respectively. The disaccharide transfer products formed by YicI, α-D-Xyl*p*-(1 → 6)-D-Man*p*, α-D-Xyl*p*-(1 → 6)-D-Fru*f*, and α-D-Xyl*p*-(1 → 3)-D-Fru*p*, are novel oligosaccharides that have not been reported previously. In the transxylosylation to cello-oligosaccharides, YicI transferred a xylosyl moiety exclusively to a nonreducing terminal glucose residue by α-1,6-xylosidic linkages. Of the transxylosylation products, α-D-Xyl*p*-(1 → 6)-D-Man*p* and α-D-Xyl*p*-(1 → 6)-D-Fru*f* inhibited intestinal α-glucosidases.

The enzymatic cleavage of carbohydrates and glycoconjugates is important in numerous biological processes. Glycoside hydrolases catalyzing these reactions have distinct substrate specificity, which can be determined by their specificity for both glycones and aglycones. For enzymes showing an exo-type action pattern, glycone specificity can be determined quite easily using various aryl glycoside substrates, such as nitrophenyl glycosides. In contrast, it is more difficult to determine aglycone specificity, because it is laborious and costly to synthesize several substrates having different aglycone moieties.

Many retaining glycoside hydrolases can catalyze both hydrolysis and transglycosylation through a double-displacement mechanism involving a covalent glycosyl–enzyme intermediate [1–3]. When glycosyl transfer of the glycosyl–enzyme intermediate occurs with an acceptor other than water, transglycosylation is catalyzed rather than hydrolysis. It is apparent that the conditions for aglycones at the glycosylation and

Abbreviations
ACN, acetonitrile; HMBC, heteronuclear multiple bond correlation; IPase, isoprimeverose-producing oligoxyloglucan hydrolase; OXG-RCBH, oligoxyloglucan reducing end-specific cellobiohydrolase; α-XF, α-xylosyl fluoride; XGO, xyloglucan-oligosaccharide; XTG, 4-nitrophenyl 6-thio-6-*S*-α-D-xylopyranosyl-β-D-glucopyranoside.

deglycosylation steps are identical, because both glycosylation and deglycosylation are considered to form the same transition-state structure and the same interaction takes place between the aglycone and its binding site. Thus, the nature of the transglycosylation products provides information on the interaction at the aglycone-binding site in both hydrolysis and transglycosylation. Transglycosylation is useful as a probe for studying the specificity of hydrolytic activity, thus avoiding the synthesis of many different oligosaccharide substrates.

Previously, we reported that the functionally unknown protein YicI of *Escherichia coli* was an α-xylosidase (EC 3.2.1.-) that recognizes an α-xylosyl residue at the nonreducing end and cleaves its linkage [4]; however, the aglycone specificity of this enzyme has remained obscure. In this study, we characterized the aglycone-binding site of YicI by determining acceptor specificity and regioselectivity during transxylosylation. Acceptor specificity and regioselectivity were investigated using α-xylosyl fluoride (α-XF) as the donor substrate, not only because α-XF has a good leaving group, but also because YicI shows the highest k_{cat}/K_m value toward α-XF in hydrolysis [4]. By using a donor substrate with a good leaving group, transglycosylation is enhanced by the accumulation of glycosyl–enzyme intermediates [5], and most transglycosylation products are not hydrolyzed until the donor substrate with the highest k_{cat}/K_m value is consumed completely [6].

Recently, the crystal structures of YicI in the free state and in complex with 4-nitrophenyl 6-thio-6-*S*-α-D-xylopyranosyl-β-D-glucopyranoside (XTG) have been solved and made available [7,8]. The crystal comprises six molecules of YicI per asymmetric unit, and these are packed as a noncrystallographic symmetry hexamer with 32-point group symmetry. The hexamer is composed of two piled-up planes of a trimeric equilateral triangle. The catalytic domain of each monomer is a triose-phosphate isomerase barrel flanked by two β-sandwich structures. The glycone-binding site is formed by the C-terminal ends of the barrel β strands and their connecting loops, but the aglycone-binding site is formed by loops from its own monomer and counterpart monomers. Using the YicI–XTG complex and biochemical data on acceptor specificity and regioselectivity during transxylosylation, we discuss important amino acid residues and hydroxy groups of the aglycone.

In addition, in order to explore the usefulness of the disaccharide transxylosylation products, they were tested as inhibitors of intestinal α-glucosidases. Inhibitors of intestinal α-glucosidases have a therapeutic use in diabetes, which causes higher blood sugar concentrations than in the normal state, because they delay the digestion of ingested carbohydrates. Acarbose and miglitol have been the most thoroughly investigated of these inhibitors, and are used medically as potent inhibitors [9–11]. Small intestinal brush-border sucrase–isomaltase (EC 3.2.1.48/10) and maltase–glucoamylase (EC 3.2.1.20/3) complexes are synthesized as single polypeptide chains that are eventually cleaved by protease to form heterodimers [12]; they complementarily serve as the final step in the digestion of carbohydrates in the small intestine [13–16]. Sucrose and isomaltose are recognized by the sucrase and isomaltase active sites, respectively, in the sucrase–isomaltase heterodimer [16]. Maltose is recognized as a hydrolytic substrate to all the active sites in sucrase–isomaltase and maltase–glucoamylase heterodimers [17]. These disaccharide substrates are structurally similar to α-xylosides. The only difference between xylosyl and glucosyl moieties at the nonreducing end is a hydroxy methylene group at the C-6 position, which implies that transxylosylation products may function as inhibitors of intestinal α-glucosidases.

Results

Acceptable substrate at the aglycone-binding site

To determine the acceptor specificity for subsite +1 of YicI (for nomenclature of sugar-binding subsites, see Davies *et al.* [18]), various monosaccharides were used as acceptor molecules in transxylosylation. The D-forms of seven monosaccharides, six aldohexoses (glucose, mannose, galactose, allose, gulose, and talose) and one ketohexose (fructose) were used as the acceptor molecules. As shown by TLC (Fig. 1A), YicI was able to transfer a xylosyl moiety to glucose, mannose, allose, and fructose, but not to galactose, gulose, or talose. YicI produced one transfer product from each acceptor substrate except for fructose, from which two isomers were produced based on TLC after the HPLC purification step (Fig. 1B). The formation of two isomers is attributed to the tautomerization of fructose [19] (discussed in detail below). Based on a comparison of acceptor substrate structures, galactose, gulose, and talose were found to have a common structural configuration for the hydroxy group at the C-4 position, and it is therefore thought that YicI could not transfer a xylosyl moiety to an aldopyranosyl sugar with an axial 4-OH.

Maltose, maltotriose, cellobiose, and cellotriose were also tested as acceptor substrates. HPLC (Fig. 1C) of the reaction mixture displayed one newly generated peak over 80 min of reaction time, indicating that YicI

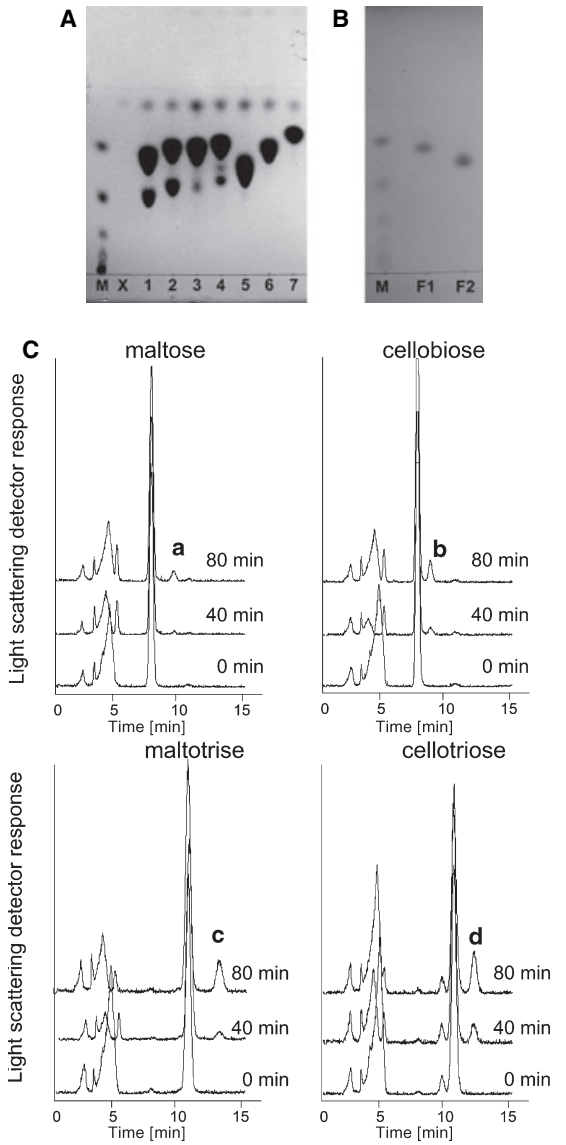

Fig. 1. Confirmation of tranxylosylation to various mono- and oligosaccharides by YicI. (A) TLC analysis of transxylosylation using each hexose monosaccharide as an acceptor. Lane M, malto-oligosaccharide standard; lane X, xylose; lanes 1–7: YicI (54 nM) was incubated with 10 mM of α-XF and 25 mM of each acceptor substrate (lane 1, glucose; lane 2, mannose; lane 3, allose; lane 4, fructose; lane 5, galactose; lane 6, gulose; lane 7, talose) in 0.1 M Hepes/NaOH (pH 7.0) at 37 °C for 50 min. (B) TLC analysis of two purified transfer products from fructose acceptor. Lane M, malto-oligosaccharide standard; lane F1, transfer product 1; lane F2, transfer product 2. (C) HPLC analysis of transxylosylation using oligosaccharides as acceptor substrates. Incubation for 0, 40, and 80 min at 37 °C with 54 nM YicI in 0.1 M Hepes/NaOH (pH 7.0), 10 mM of α-XF, and 25 mM of each acceptor substrate (maltose, cellobiose, maltotriose, and cellotriose in clockwise order). a, b, c, and d indicate newly generated peaks during the reaction.

Table 1. Initial rate of fluoride ion liberation in the presence of acceptor substrates. Initial velocity was estimated by amount of released fluoride ion from 2 to 10 min.

Acceptor substrate	F^- (s^{-1})	Relative velocity (%)
Glucose	75.0 ± 13.0	100
Mannose	72.2 ± 7.2	96
Allose	20.9 ± 1.67	28
Fructose	29.1 ± 1.7	39
Maltose	11.6 ± 0.8	15
Cellobiose	15.4 ± 0.8	21
Maltotriose	24.8 ± 1.1	33
Cellotriose	41.7 ± 5.0	56

produced one transxylosylation product from each acceptor substrate. This suggests that the aglycone-binding site of YicI is sufficiently large to accommodate an oligosaccharide.

Acceptor priority in transxylosylation

To estimate acceptor priority during transxylosylation, we measured the initial rate of fluoride-ion liberation for each acceptor. The rate of formation of the glycosyl–enzyme intermediate, i.e. the rate of fluoride-ion liberation, is governed by the breakdown of the intermediate by nucleophilic attack by water (hydrolysis) or acceptor molecules (transglycosylation) because α-XF, with a good leaving group, sustains the concentration of the intermediate at a near constant or steady-state level. In the reaction mixture, the effective concentration of water is constant, so the better the accommodation of the acceptor substrate in the aglycone site, the faster it accelerates fluoride-ion liberation. The initial rate of fluoride liberation in the presence of each acceptor substrate followed the order glucose > mannose > cellotriose > fructose > maltotriose > allose > cellobiose > maltose (Table 1) when 54 nM YicI, 10 mM α-XF, and 25 mM of each acceptor substrate were incubated at 37 °C.

To reconfirm and support the assumption that the rate of fluoride-ion liberation reflects the transxylosylation rate, we compared the transxylosylation product yield for each acceptor. Based on the time course of fluoride-ion liberation over 100 min under the same reaction conditions as used above, we fixed the reaction time for comparing transxylosylation yields at 80 min, when α-XF remained in all reaction mixtures containing each acceptor substrate (data not shown). The transxylosylation product yield for each acceptor substrate was in the order glucose, mannose, cellotriose, fructose, maltotriose, allose, cellobiose, and maltose (Table 2), consistent with the order seen for the initial rate of fluoride-ion liberation.

Table 2. Tranxylosylation yield of each acceptor. Each yield was estimated by decrease in acceptor substrate concentration for 80 min.

Acceptor substrates	Amount of transfer product (mM)	Relative yield (%)
Glucose	5.7	100
Mannose	4.4	77
Allose	2.1	37
Fructose	2.7	47
Maltose	1.2	21
Cellobiose	1.7	30
Maltotriose	2.6	46
Cellotriose	3.1	54

Structural analyses of transfer products from glucose, mannose, and fructose

The regioselectivity of YicI in transxylosylation was investigated by structural analysis of the transxylosylation products from glucose, mannose, and fructose. In MS analyses of all products, the signals corresponded to calculated molecular masses for sodium and potassium adducts of the xylose-transferred acceptor substrate (FD-MS: *m/z* 335 for $C_{11}H_{20}O_{10} + Na^+$, *m/z* 351 for $C_{11}H_{20}O_{10} + K^+$). For linkage analysis, chemical shifts of transfer products were assigned based on 2D NMR spectra (see data in Experimental procedures). The coupling constant of H-1′ in the xylosyl moiety ($J_{1',2'}$) was 3.7 and 3.4 Hz in all spectra for the transxylosylation disaccharide products, indicating an α-anomeric configuration. A correlation between C-1′ of the xylosyl moiety and H-6 of the reducing-end moiety was observed in the heteronuclear multiple bond correlation (HMBC) spectrum of each transfer product from glucose and mannose, confirming α-(1 → 6) linkage formation. Concerning the two different transfer products from fructose, one showed a correlation between C-1′ of the xylosyl moiety and H-6 of the fructosyl moiety, and the other showed a correlation between C-1′ of the xylosyl moiety and H-3 of the fructosyl moiety in the HMBC spectrum. This indicated that YicI formed 1,6 and 1,3 regioisomers toward fructose. The presence of a correlation between C-2 and H-6 of the fructose ring in the HMBC spectrum of the 1,3 regioisomer revealed that its fructosyl moiety was a pyranose conformer. Consequently, the structure of each transfer product was defined as α-D-Xyl*p*-(1 → 6)-D-Glc*p*, α-D-Xyl*p*-(1 → 6)-D-Man*p*, α-D-Xyl*p*-(1 → 6)-D-Fru*f*, and α–D-Xyl*p*-(1 → 3)-D-Fru*p*. Of note, α-D-xylopyranosyl-(1 → 6)-D-mannopyranose, α-D-xylopyranosyl-(1 → 6)-D-fructofuranose, and α-D-xylopyranosyl-(1 → 3)-D-fructopyranose are novel sugars.

Structural analyses of transfer products from cello-oligosaccharides

To conveniently purify the transfer products from cellobiose and cellotriose, unreacted acceptor substrates were hydrolyzed using *Aspergillus niger* β-glucosidase (EC 3.2.1.21) after it was confirmed that the transfer products were nonhydrolyzable substrates of that enzyme on the basis of TLC (data not shown). In MS analyses of transxylosylation products using cellobiose and cellotriose as acceptor substrates, the signals corresponded to the calculated molecular masses for sodium adducts of the xylose-transferred acceptor substrates (TOF-MS: *m/z* 498 for $C_{17}H_{30}O_{15} + Na^+$, *m/z* 659 for $C_{23}H_{40}O_{20} + Na^+$). Structural analysis was performed by HPLC coupled with enzymatic digestion by two oligoxyloglucan-specific enzymes, isoprimeverose-producing oligoxyloglucan hydrolase (IPase; EC 3.2.1.120) and oligoxyloglucan reducing end-specific cellobiohydrolase (OXG–RCBH; EC 3.2.1.150). Under the presumption that YicI would retain the same regioselectivity as was seen in the transxylosylation to glucose, i.e. an α-1,6-linkage, the transxylosylation products from cellobiose and cellotriose were compared with various xyloglucan-oligosaccharides (XGOs) that consisted of xylose α-1,6-linked to glucose units forming the β-(1,4)-glucan backbone.

IPase is highly specific for XGOs and splits off successive isoprimeverose residues from the nonreducing end of the backbone of the oligosaccharide [20,21]. Another XGO-specific enzyme, OXG-RCBH recognizes the structure of the reducing end of the oligoxyloglucan and releases the two glucosyl main-chain residues, such as GG (β-D-Glc*p*-(1 → 4)-D-Glc*p*), XG (α-D-Xyl*p*-(1 → 6)-β-D-Glc*p*-(1 → 4)-D-Glc*p*), and LG (β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)-β-D-Glc*p*-(1 → 4)-D-Glc*p*) [22] (here and hereafter, the structures of the units of xyloglucan are represented using the nomenclature of Fry *et al.* [23]). These two enzymes have been used to analyze the structure of xyloglucan [24,25] and have allowed us to identify the structures of the transxylosylation products from cellobiose and cellotriose. Each IPase- and OXG-RCBH-treated transxylosylation product was subjected to HPLC, and its composition was identified (Fig. 2). By treatment with IPase, which acts not on GX but on XG, the transxylosylation product from cellobiose was hydrolyzed into isoprimeverose (X) and glucose, and that from cellotriose was hydrolyzed into X and cellobiose (GG). Furthermore, OXG-RCBH treatment of the transxylosylation product from cellotriose also gave peaks corresponding to X and GG. The results showed the defined structures for the transxylosylation

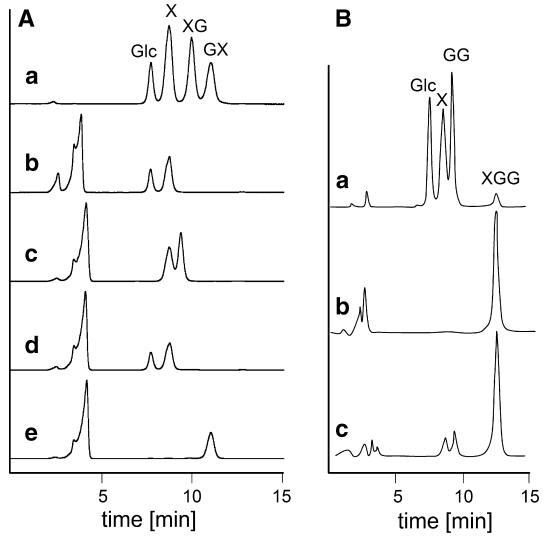

Fig. 2. HPLC analysis of IPase- and OXG-RCBH-treated transfer products. (A) Overlapped chromatograms of markers (a), IPase-treated transfer product from cellobiose (b), IPase-treated transfer product from cellotriose (c), IPase-treated stansdard XG (d), and IPase-treated standard GX. (B) Overlapped chromatograms of markers (a), transfer product from cellotriose (b), and OXG-RCBH-treated transfer product from cellotriose (c).

products from cellobiose and cellotriose to be α-D-Xyl*p*-(1 → 6)-β-D-Glc*p*-(1 → 4)-D-Glc*p* and α-D-Xyl*p*-(1 → 6)-β-D-Glc*p*-(1 → 4)-β-D-Glc*p*-(1 → 4)-D-Glc*p*, respectively. YicI thus transferred the α-xylosyl moiety to the nonreducing end of cellooligosaccharides by forming an α-1,6-linkage.

According to these results, YicI was thought to have no hydrolytic activity toward the branched xylosyl linkage in XGOs, because the structures of the tranxylosylation products reflected those of the hydrolytic substrates. To verify this, each of GX, GXG, XG, and XGG was treated with YicI, and they were analyzed by HPLC (data not shown). As expected, YicI was not able to hydrolyze GX or GXG, and it cleaved the α-xylosyl linkage of XG and XGG, revealing that hydrolyzable and synthesizable structures by YicI are equal.

Inhibition study of transxylosylation products

For the inhibition test, we used intestinal α-glucosidases from rat intestine extract without purification. TLC results showed that rat intestine extract did not show hydrolysis activity on each transxylosylation product (data not shown). Preliminary inhibition tests were performed at a fixed concentration of 2 mM sucrose, isomaltose, and maltose as the substrates in the presence of 10 mM of each transxylosylation product.

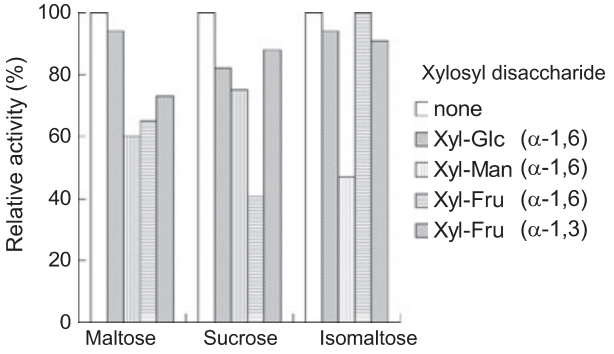

Fig. 3. Histograms of relative α-glucosidase activity in the presence of each disaccharide transxylosylation product when the activity without transxylosylation product (none) is taken as 100%. Ten millimoles of each disaccharide transxylosylation product and 2 mM of each substrate were incubated with rat intestine extract solution for 10 min at 37 °C in 40 mM sodium acetate buffer (pH 6.0).

Relative α-glucosidase activity, taking activity in the absence of the transxylosylation product as 100% for each substrate, is shown in Fig. 3. Of the transxylosylation products, α-D-Xyl*p*-(1 → 6)-D-Man*p* more strongly inhibited the hydrolysis of maltose and isomaltose by intestinal α-glucosidases than did the others, and α-D-Xyl*p*-(1 → 6)-D-Fru*f* most strongly inhibited the hydrolysis of sucrose.

To determine each concentration giving 50% inhibition (IC_{50}) of α-D-Xyl*p*-(1 → 6)-D-Man*p* and α-D-Xyl*p*-(1 → 6)-D-Fru*f*, we assayed maltase, sucrase, and isomaltase activities at various concentrations of the inhibitors in the presence of 2 mM of each substrate (Table 3). The IC_{50} values toward maltose and isomaltose hydrolysis by α-D-Xyl*p*-(1 → 6)-D-Man*p* were 18.1 and 8.62 mM, respectively. For the sucrose substrate, α-D-Xyl*p*-(1 → 6)-D-Fru*f* showed IC_{50} at 6.47 mM.

Discussion

YicI showed aglycone specificity, recognizing aldopyranose having an equatorial 4-OH at subsite +1, along with 1,6 regioselectivity and a preference for glucose over other monosaccharides. As a result, YicI transferred an α-xylosyl moiety to a specific hydroxy group in the acceptor sugar showing either 1,6 or 1,3 regioselectivity. Even though YicI gave two isomers from fructose, we should not consider them as two transxylosylation products from one reaction, because two regioisomers resulted from two different pyranosyl and furanosyl tautomers of fructose. Therefore, YicI produced only one transxylosylation product for each substrate molecular form. This suggests that subsite +1 of YicI allows only one binding mode toward one molecule, leading to a highly regioselective transferring

Table 3. Inhibitory effect of α-D-Xyl*p*-(1 → 6)-D-Man*p* and α-D-Xyl*p*-(1 → 6)-D-Fru*f* on rat intestinal α-glucosidase.

Substrate	Maltose	Sucrose	Isomaltose
Inhibitor	α-D-Xyl*p*-(1 → 6)-D-Man*p*	α-D-Xyl*p*-(1 → 6)-D-Fru*f*	α-D-Xyl*p*-(1 → 6)-D-Man*p*
IC_{50} (mM)	18.1 ± 1.5	6.47 ± 2.08	8.62 ± 0.87

reaction. Recently, the 3D structure of the YicI–XTG complex was determined [8]. XTG is a thiosugar, that is an analogue of *p*-nitrophenyl β-isoprimeveroside. Thus, the complex can show existing interactions at the active site, such as hydrogen bonding, between the enzyme and substrate having a xylosyl α-1,6 linkage in the ground state. In the structure, the 4-OH of the glucose moiety, accommodated at subsite +1, is fixed by hydrogen bonds to Arg466^{molF} (Arg466 in molecule F) and Asp185^{molF} from its own monomer (mol F), and 3-OH and 2-OH of that make hydrogen bonds to Asp185^{molF} from its own monomer and Asp49^{molA} from the threefold-related monomer, respectively. Furthermore, Trp8^{molE} from the twofold-related monomer is juxtaposed to the glucose ring, so its indole ring is involved in a typical hydrophobic stacking interaction with the nonpolar face of the glucose moiety (Fig. 4A). These hydrogen bonds and the stacking interaction seem to optimize subsite +1 for accommodating glucose at the orientation forming a 1,6-linkage to glycone. This is consistent with our data showing that YicI exhibited the highest yield for glucose and 1,6-regioselectivity toward glucose, mannose, fructofuranose, cellobiose, and cellotriose in transxylosylation.

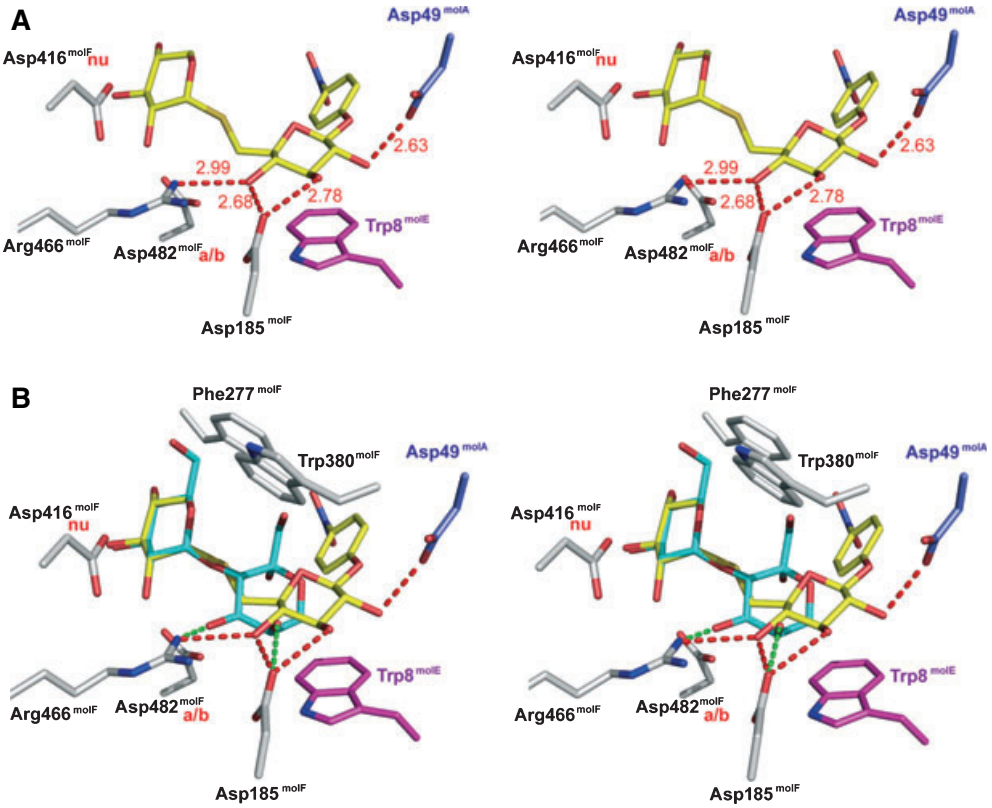

Fig. 4. Stereoviews of the YicI active site. The figures were made using PYMOL v. 0.99. (A) Structure of the YicI–XTG complex [8]. Four residues (Arg466, Asp185, Trp8, and Asp49) form subsite +1 for XTG. Asp416 and Asp482 are catalytic nucleophile and acid/base residues, respectively. Red dashes and numbers represent hydrogen bonds and their lengths. (B) Modeled structure of YicI with turanose (α-D-Glc*p*-(1 → 3)-Fru*p*). The structure was modeled by superimposing turanose with XTG in the active site of YicI. XTG is presented as yellow sticks, and turanose is presented as cyan sticks. Green dashes represent expected hydrogen bonds between the fuructopyranose moiety and amino acid residues of YicI, and red dashes represent existing hydrogen bonds between the glucose moiety of XTG and amino acid residues of YicI. Turanose structure was obtained from the Protein Data Bank (accession number, 1 N3Q).

In particular, hydrogen bonds from Arg466 and Asp185–4-OH of the glucose moiety seem to be more important because an aldopyranosyl sugar without an equatorial 4-OH could not function as the acceptor. Presumably, YicI cannot provide the aldopyranose having an axial 4-OH with sufficient energy and the appropriate orientation to attack the glycosyl–enzyme intermediate, even if the acceptor substrate happens to bind in subsite +1. Allose, a C3-epimer of glucose, was the weaker acceptor, whereas the transxylosylation yield of mannose, a C2-epimer of glucose, was comparable with that of glucose. Therefore, the contributions in transxylosylation catalysis of hydrogen bonds to hydroxyls in the glucose ring at subsite +1 would be in the order of 4-OH > 3-OH > 2-OH, even though hydrogen bonding distance between 2-OH and $Asp185^{molF}$ is shorter than that between 3-OH and $Asp49^{molA}$ in the YicI–XTG complex.

Considering the structure of α-D-Xyl*p*-(1 → 6)-D-Fru*f*, the transxylosylation product from fructofuranose, the 4-OH of the fructofuranose would interact with Arg446 and Asp185 at subsite +1. In the case of fructopyranose bound in subsite +1, however, it was unclear which of hydroxyls in the fructopyranose interacted with Arg446 and Asp185 to result in α-D-Xyl*p*-(1 → 3)-D-Fru*p*. Therefore, we modeled the structure of YicI complexed with turanose (α-D-Glc*p*-(1 → 3)-D-Fru*p*) using the xylose ring of XTG placed in subsite −1 as the basis for superimposition with the glucose ring of turanose (Fig. 4B). Turanose consists of a β-fructopyrnose in $^2C_5$ conformation, which is the same conformation as fructopyranose in aqueous solution. In the obtained turanose overlay-modeled structure of YicI, the regions of 4-OH and 5-OH in the fructose ring are oriented to $Arg446^{molF}$ and $Asp185^{molF}$. In the transxylosylation to fructopyranose 4-OH and 5-OH of fructopyranose seem to form hydrogen bonds with Arg446 and Asp185, resulting in αD-Xyl*p*-(1 → 3)-D-Fru*p*. The fructose ring and $Trp8^{molE}$ from a twofold-related monomer do not seem to be close enough to make the hydrophobic stacking interaction, but 1-CH_2OH of the fructose ring is closely positioned to $Phe277^{molF}$ and $Trp380^{molF}$ of subsite +1, where they form a hydrophobic wall. In the fructopyranose, 1-CH_2OH is a relatively hydrophobic part because of methylene, so presumably, Phe277, Trp380, and 1-CH_2 are involved in a hydrophobic interaction.

To elucidate the aglycone site beyond subsite +1, the acceptor priority in transxylosylation was compared between the oligosaccharides and glucose (Tables 1 and 2). Transxylosylation to disaccharide was much lower than that to glucose, whereas the trisaccharide acceptor was better than the disaccharide acceptor. These results imply that YicI has at least three plus subsites, including poor subsite +2 and good subsite +3. The rate of fluoride-ion liberation and the yield comparison between malto- and cello-oligosaccharide acceptors showed that YicI preferred cello-oligosaccharide to malto-oligosaccharide as an acceptor. This suggests that YicI has a suitable active site for XGOs.

Finally, we elucidated that the disaccharide transxylosylation products have the potential to be used as inhibitors of intestinal α-glucosidases. The two transxylosylation products, α-D-Xyl*p*-(1 → 6)-D-Man*p* and α-D-Xyl*p*-(1 → 6)-D-Fru*f*, are not strong inhibitors of intestinal α-glucosidases. Thus, they can avoid the side effects that occur in persons to whom strong inhibitors are administered. Based on their antidiabetic effect along with no calories, the two novel sugars could be used as substitute for sucrose or artificial sweeteners with further detailed safety and taste tests in the future. Some sugars partly [26]. L-Arabinose selectively inhibits sucrase. By contrast, α-D-Xyl*p*-(1 → 6)-D-Fru*f* suppresses sucrase suppress α-glucosidase activity, and one of them, L-arabinose, is known to have an intestinal α-glucosidase inhibitory effect and maltase, and α-D-Xyl*p*-(1 → 6)-D-Man*p* inhibits isomaltase and maltase. Accordingly, the novel sugars α−D-Xyl*p*-(1 → 6)-D-Fru*f* and α-D-Xyl*p*-(1 → 6)-D-Man*p* are broader inhibitors than is L-arabinose.

Experimental procedures

Materials

α-XF was synthesized according to a published method [27]. Allose was purchased from Sigma (St. Louis, MO, USA); talose and gulose were purchased from Wako Pure Chemical Inc. (Osaka, Japan). Solvents were of analytical grade and were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). All other chemicals were of analytical grade.

Purification of YicI

The *yicI*–pTrc99A plasmid was used for the production of His6-tagged YicI (hereafter referred to as YicI) [4]. *E. coli* MV1184 was used as the host strain for YicI expression. The transformed cells were grown in 200 mL of Luria–Bertani medium containing ampicillin (50 μg·mL^{-1}) at 37 °C. After A_{600} reached 0.5, isopropyl thio-β-D-galactoside was added at a final concentration of 0.1 mM. After additional incubation at 37 °C for 16 h, cells were harvested by centrifugation and resuspended in 10 mL of 50 mM Tris/HCl

buffer (pH 8.0) containing 0.3 M NaCl and 5 mM imidazole. The cell suspension was incubated at 4 °C for 30 min after the addition of lysozyme from egg white (Nacalai Tesque, Inc., Kyoto, Japan) at a final concentration of 0.2 mg·mL^{-1}. After disruption of the cells by sonication, the soluble fraction was obtained by centrifugation at 5800 ***g*** for 20 min. YicI was isolated from the soluble fraction using Ni-chelating Sepharose Fast Flow (Amersham Biosciences AB, Cardiff, UK) according to the manufacturer's protocol. The protein from the elution fraction was dialyzed against 50 mM Mops buffer (pH 7.0) containing 0.3 M NaCl. All purification steps were performed at 4 °C. The concentration of purified YicI was calculated from the amino acid contents of protein hydrolysate (6 M HCl, 24 h, 110 °C).

Confirmation of transferred products by TLC and HPLC

YicI (54 nM) was incubated with 10 mM α-XF and 25 mM of each acceptor substrate in 0.1 M Hepes/NaOH buffer (pH 7.0) for 50 min at 37 °C, followed by heating for 10 min at 100 °C. The reaction mixtures were desalted by an ion-exchange resin (Amberlite MB-3, Rhom & Haas Co., Philadelphia, PA) and then concentrated 10 times. To detect transfer products, TLC was performed using 0.25-mm layers of silica gel 60F_{254} (Merck, Darmstadt, Germany). The reaction mixture containing monosaccharide as the acceptor substrate was developed three times using a solvent system of acetonitrile (ACN)/water (85 : 15, v/v), whereas the reaction mixture containing di- or trisaccharide as the acceptor substrate was developed three times using a solvent system of nitromethane/1-propanol/water (4 : 10 : 3, v/v/v). The sugar and sugar derivatives in the reaction mixture were visualized by spraying α-naphthol/sulfuric acid solution (α-naphthol/sulfuric acid/methanol, 0.03 : 15 : 85, w/v/v) followed by heating at 110 °C for 5 min. Transfer products from maltose, cellobiose, maltotriose, and cellotriose were also reconfirmed using a Jasco HPLC system (Jasco, Tokyo, Japan) equipped with model 200 ELSD (Softa, Thornton, CO) with a TSK-GEL Amide-80 column (4.6 × 250 mm; Tosoh, Tokyo, Japan) and a mobile phase (ACN/water, 70 : 30, v/v) with a flow rate of 1 mL·min^{-1} at 70 °C.

Fluoride-ion assay for estimation of acceptor priority

The concentration of fluoride ion liberated from α-XF was measured using a fluoride-specific dye, Alfusone (Wako Pure Chemical Inc.) [28]. α-XF (10 mM) and 25 mM of each acceptor substrate in 0.1 M Hepes/NaOH buffer (pH 7.0) were incubated with 54 nM of YicI at 37 °C. To measure the initial rate, each of 2 and 10 min incubated reaction mixtures was mixed in 0.5% (w/v) Alfusone and 40% (v/v) acetone. To determine the time course of fluoride liberation, each of 30, 50, 70, 90, and 100 min incubated reaction mixtures was mixed in 0.5% (w/v) Alfusone and 40% (v/v) acetone. After the mixtures were incubated for 90 min, absorbance at 620 nm was measured spectrophotometrically.

Quantification of transfer products for the yield comparison

Peak areas from HPLC were used for quantitative calculation. α-XF (10 mM) and 25 mM of each acceptor substrate in 0.1 M Hepes/NaOH buffer (pH 7.0) were incubated with 54 nM of YicI for 80 min at 37 °C, followed by heating for 10 min at 100 °C. Before desalting with an ion-exchange resin (Rhom & Haas Co.) and filtration with a Steradisc 13 syringe filter unit (0.2 μm, Kurabo, Osaka, Japan), internal standard sugar was added to the reaction mixture. Lactose, maltotriose, and maltose were used as internal standards for reaction mixtures containing mono-, di-, and trisaccharide acceptor substrates, respectively. HPLC was performed on a Jasco HPLC system equipped with a refractive index detector (Hitachi 655A-30, Tokyo, Japan) and a TSK-GEL Amide-80 column (4.6 × 250 mm). Integration was performed using Borwin chromatography software (JMBS Developments, Grenoble, France). Transfer product quantification was calculated based on a decrease in acceptor substrate peak area when compared with control samples. Calibration of the peak area was performed based on internal standard sugar.

Structural analysis of transfer products from glucose, mannose, and fructose

YicI (54 nM) was incubated with 10 mM α-XF and 25 mM of each acceptor substrate in a final volume of 15 mL of 0.1 M Hepes/NaOH buffer (pH 7.0) for 100 min at 37 °C, followed by heating for 10 min at 100 °C. Transfer products were separated from the reaction mixtures by HPLC with a Shodex RSpak DC-613 normal phase column (6.0 × 300 mm; Showa Denko Co., Tokyo, Japan) under a constant flow (0.9 mL·min^{-1}) of mobile phase (CAN/water of 85 : 15, v/v) at 55 °C. The fraction of transfer product was collected, and its purity confirmed by TLC analysis. Each concentration of the transxylosylation products, α-D-Xyl*p*-(1 → 6)-D-Glc*p*, α-D-Xyl*p*-(1 → 6)-Man*p*, α-D-Xyl*p*-(1 → 6)-Fru*f*, and α-D-Xylp-(1 → 3)-Fru*p*, was measured by the phenol/sulfuric acid sugar assay method [29], and sugar mixtures of Xyl/Glc, Xyl/Man, and Xyl/Fru in a 1 : 1 molar ratio were used as the standards for the quantification. Field desorption mass spectra (FD-MS) of purified transfer products were recorded using a JEOL-SX102A spectrometer (Jeol Ltd, Tokyo, Japan). NMR analysis was performed as follows: 5 mg of each purified transfer product was exchanged three times

with D_2O (Aldrich Chemical Company, Inc., Milwaukee, WI), dissolved in 0.2 mL of D_2O, and transferred into a 5 mm NMR microtube. ^1H, ^{13}C, COSY, HMBC, and HMQC NMR spectra of the samples were recorded using a Bruker AMX-500 spectrometer (Bruker, Rheinstetten, Germany). All ^1H and ^{13}C NMR spectra were measured at 500 and 125 MHz, respectively, using sodium 3-(trimethylsylyl)propionate-2,2,3,3,-d_4 as an external standard.

α-D-Xyl*p*-(1 → 6)-D-Glc*p*

^1H NMR: δ 5.25 (1H, d, $J_{1,2} = 3.7$, H-1α), 4.92 (1H, d, $J_{1',2'} = 3.7$, H-1′), 4.68 (1H, d, $J_{1,2} = 3.7$, H-1β), 3.95 (1H, H-6a), 3.72 (1H, H-6b), 3.7 (1H, H-5′a), 3.68 (1H, H-2α), 3.63 (2H, H-5α and H-3′), 3.6 (1H, H-4′), 3.58 (2H, H-5β and H-5′), 3.52 (1H, H-2′), 3.51 (1H, H-4), 3.49 (1H, dd, $J_{2,3} = J_{3,4} = 9.1$, H-3β), 3.26 (1H, dd, $J_{1,2} = J_{2,3} = 8.5$, H-2β); ^{13}C NMR: δ 100.9 (C-1′), 98.9 (C-1β), 95 (C-1α), 78.8 (C-3β), 77.1 (C-5β), 76.9 (C-2β), 76 (C-3′), 75.8 (C-2α), 74.3 (C-2′), 72.9 (C-5α), 72.4 (C-4), 72.2 (C-4′), 68.6 (C-6), 64 (C-5).

α-D-Xyl*p*-(1 → 6)-D-Man*p*

^1H NMR: δ 5.18 (1H, H-1α), 4.92 (1H, d, $J_{1',2'} = 3.4$, H-1′), 4.91 (1H, H-1β), 4.2 (1H, H-6a), 3.95 (1H, H-5), 3.94 (1H, H-2), 3.84 (1H, H-3), 3.72 (1H, H-4), 3.7 (1H, H-5′a), 3.68 (1H, H-6b), 3.64 (1H, H-3′), 3.6 (1H, H-4′), 3.57 (1H, H-5′b), 3.54 (1H, H-2′); ^{13}C NMR: δ 100.9 (C-1′), 97.11 (C-1α), 96.8 (C-1β), 76 (C-3′), 74.3 (C-2′), 73.6 (C-5), 73.5 (C-2), 73.3 (C-3), 72.2 (C-4′), 68.7 (C-6), 62.2 (C-4).

α-D-Xyl*p*-(1 → 6)-D-Fru*f*

^1H NMR: δ 4.95 (1H, d, $J_{1',2'} = 3.7$, H-1′), 4.22 (1H, dd, $J_{3,4} = J_{4,5} = 8.1$, H-4), 4.13 (1H, d, $J_{3,4} = 8.1$, H-3), 3.98 (1H, H-5), 3.91 (1H, H-6a), 3.74 (1H, H-5′a), 3.71 (1H, H-3′), 3.7 (1H, H-6b), 3.69 (1H, H-5′b), 3.65 (1H, H-4′), 3.64 (1H, H-1), 3.56 (1H, dd, $J_{1',2'} = 3.7$, $J_{2',3'} = 10$, H-2′); ^{13}C NMR: δ 104.5 (C-2), 100.9 (C-1′), 81.8 (C-5), 78.1 (C-3), 77.3 (C-4), 76 (C-3′), 74.3 (C-2′), 72.2 (C-4′), 70.6 (C-6), 65.5 (C-1), 64.1 (C-5′).

α-D-Xyl*p*-(1 → 3)-D-Fru*p*

^1H NMR: δ 4.94 (1H, d, $J_{1',2'} = 3.7$, H-1′), 4.08 (1H, H-1a), 4.02 (1H, H-5), 3.92 (2H, H-4 and H-6a), 3.86 (1H, H-6b), 3.73 (1H, H-5′a), 3.64 (1H, H-3′), 3.59 (1H, H-4′), 3.57 (1H, H-2′), 3.55 (1H, H-5′b), 3.45 (1H, H-3); ^{13}C NMR: δ 101.5 (C-1′), 100.6 (C-2), 75.9 (C-3′), 74.3 (C-2′), 72.4 (C-4), 72.2 (C-4′), 72 (C-3), 71.9 (C-5), 70.7 (C-6), 66.4 (C-1), 64.2 (C-5′).

Preparation of transfer products from cellobiose and cellotriose

YicI (54 nM) was incubated with 10 mM α-XF and 25 mM of each acceptor substrate in a final volume of 30 mL of 0.1 M Hepes/NaOH buffer (pH 7.0) for 100 min at 37 °C, followed by heating for 10 min at 100 °C. For separation of the transfer products from reaction mixtures, hydrolysis of unreacted acceptor substrates was performed with Novozyme 188 (Novozyme, Bagsvaerd, Denmark) purified by anion-exchange chromatography using DEAE-Toyopearl 650 M (Tosoh, Tokyo, Japan). The purified Novozyme 188 was incubated with the transxylosylation mixtures in 20 mM sodium acetate buffer (pH 4.5) at 37 °C. After development (two ascents in a solvent system of nitromethane/1-propanol/H_2O, 4 : 10 : 3, v/v/v) of the concentrated acceptor substrate-removed mixtures on a TLC plate, the transfer products were recovered by scraping the silica gel adsorbent from the plate in the region of the transfer products and extracting the separated material from the silica gel using water. Purity was confirmed by TLC. The molecular mass of the transfer products was analyzed by MALDI TOF-MS, performed with a Voyager mass spectrometer (Perseptive Biosystems, Framingham, MA). 2,5-Dihydroxybenzoic acid dissolved in 50% ACN was used as the matrix. XXXG (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) served as an external calibration standard.

Preparation of standard XGOs (X, XG, GX, and XGG)

Isoprimeverose (X) was obtained by treating tamarind seed polysaccharide (Tokyo Kasei Kogyo, Tokyo, Japan) with Driselase (Sigma) according to a method described previously [4]. To obtain XG, XXXG was treated with OXG-RCBH, and the resultant XX and XG were separated using a TSK-GEL Amide-80 column (7.8 × 300 mm). GX was prepared by digesting XX with *Bacillus* α-xylosidase (Seikagaku Co., Tokyo, Japan). XGG was prepared by transglycosylation reactions using *Trichoderma viride* cellulase (Wako Pure Chemical Inc.) [30,31]. XXXG was incubated with *Trichoderma* cellulase and 20% glucose, generating the transfer product XXXGG. To remove the two X segments from the nonreducing end of XXXGG, *Bacillus* α-xylosidase and almond β-glucosidase (Sigma) were treated on it.

IPase and OXG-RCBH treatment of transxylosylation products

Each transfer product (5 mg·mL^{-1}) from cellobiose and cellotriose was incubated with *Oerskovia* sp. Y1 IPase [21] (50 μg·mL^{-1}) in 50 mM sodium acetate buffer (pH 4.5) at 50 °C for 30 min. The transfer product from cellotriose

(5 mg·mL^{-1}) was incubated with *Geotrichum* sp. M128 OXG-RCBH [22] (50 μg·mL^{-1}) in 50 mM sodium acetate buffer (pH 4.5) at 45 °C for 30 min. A TSK-GEL Amide-80 column (4.6 × 250 mm) was used for HPLC analysis with a mobile phase (CAN/water of 65 : 15, v/v) at a constant flow (0.8 mL·min^{-1}) at 25 °C.

YicI hydrolytic activity tests with various XGOs

Hydrolysis tests for various standard XGOs of GX, GXG, XGG, and XG were performed. The reaction mixtures containing 20 mM sodium phosphate (pH 7.0), 2.98 μM YicI, and each 0.2% XGO substrate were incubated for 30 min at 37 °C and then analyzed on the TSK-GEL Amide-80 column (4.6 × 250 mm) to confirm the xylose liberation.

Intestinal α-glucosidase preparation

Intestinal α-glucosidase was prepared from rat intestine acetone powder (Sigma) as follows. The rat intestine powder was added to 0.9% NaCl to a concentration of 100 mg·mL^{-1}. This rat intestine mixture was sonicated for 1 min three times. The supernatant obtained by centrifugation at 9100 ***g*** for 10 min was dialyzed against 20 mM sodium acetate buffer (pH 6.0).

Inhibition assay of sucrase, maltase, and isomaltase

Each substrate solution contained sucrose, maltose, and isomaltose for measuring sucrase, maltase, and isomaltase activities, respectively. The substrate solution (10 μL, 10 mM) and sodium acetate buffer (10 μL, 0.1 M, pH 6.0) were mixed with each transxylosylation product solution (20 μL, various concentrations: 3.13–50 mM). The reaction was initiated by the addition of 10 μL of intestinal α-glucosidase solution diluted suitably with 20 mM sodium acetate buffer (pH 6.0) to the mixture containing each substrate and each transxylosylation product. The reaction mixture was incubated for 10 min at 37 °C, and the reaction was stopped by adding 100 μL of 2 M Tris/HCl buffer (pH 7.0). The hydrolytic activity of the α-glucosidases was determined by the glucose released from each substrate. The concentration of liberated glucose was measured by the Tris/glucose oxidase/peroxidase color method with a Glucose C-II Test (Wako Pure Chemical Inc.) [32]. The IC_{50} value was determined from the curve of percentage inhibition versus inhibitor concentration by extrapolation.

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