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Characterization of the (Engineered) Branching Sucrase GtfZ-CD2 from *Apilactobacillus kunkeei* for Efficient Glucosylation of Benzenediol Compounds

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**ABSTRACT** Branching sucrases, a subfamily of Glycoside Hydrolase family (GH70), display transglycosidase activity using sucrose as donor substrate to catalyze glucosylation reaction in the presence of suitable acceptor substrates. In this study, the (α1→3) branching sucrase GtfZ-CD2 from *Apilactobacillus kunkeei* DSM 12361 was demonstrated to glucosylate benzenediol compounds (i.e., catechol, resorcinol, and hydroquinone) to form monoglucoside and diglucoside products. The production and yield of catechol glucosylated products were significantly higher than that of resorcinol and hydroquinone, revealing a preference for adjacent aromatic hydroxyl groups in glucosylation. Amino residues around acceptor substrate binding subsite +1 were targeted for semirational mutagenesis, yielding GtfZ-CD2 variants with improved resorcinol and hydroquinone glucosylation. Mutant L1560Y with improved hydroquinone mono-glucosylated product synthesis allowed enzymatic conversion of hydroquinone into α-arbutin. This study thus revealed the high potential of GH70 branching sucrases for glucosylating noncarbohydrate molecules.

**IMPORTANCE** Glycosylation represents one of the most important ways to expand the diversity of natural products and improve their physico-chemical properties. Aromatic polyphe-rol compounds widely found in plants are reported to exhibit various remarkable biological activities; however, they generally suffer from low solubility and stability, which can be improved by glucosylation. Our present study on the glucosylation of benzenediol compounds by GH70 branching sucrase GtfZ-CD2 and its semirational engineering to improve the glucosylation efficiency provides insight into the mechanism of acceptor substrates binding and its glucosylation selectivity. The results demonstrate the potential of using branching sucrase as an effective enzymatic glucosylation tool.

**KEYWORDS** GH70, branching sucrase, glucosylation, benzenediol, α-arbutin

Glycosylation represents one of the most important tailoring reactions in nature for modification of small organic molecules (1, 2), resulting in the generation of high structural diversity of natural products. In addition, glycosylation may significantly improve the physicochemical properties of small organic molecules (3, 4), which generally suffer from low solubility and stability in industrial applications. The synthesis of glycosidic bonds, especially with respect to the position specificity and stereoselectivity, has always been a challenge. Chemical glycosylation reactions are often very tedious, requiring multiple activation and protection steps, and can cause environmental pollution due to the use...
of toxic chemicals. Enzymatic glycosylation, however, has the advantages of high stereoselectivity and catalytic efficiency under mild conditions (4), at least potentially resulting in high yields. Among the various enzymes capable of mediating glycosylation, glycoside hydrodolase 70 family (GH70) glucansucrases, that are widely found in lactic acid bacteria, are efficient transglucosidases, catalyzing the cleavage of the glycosidic bond in sucrose and the transfer of the glucosyl unit to an acceptor substrate using the α-retaining double displacement mechanism (5). Structural analysis of GH70 glucansucrases revealed that their polypeptide chains follow a U shape-path to form domains A, B, C, IV and V (6, 7). Depending on the acceptor substrates, GH70 glucansucrases catalyze three types of reactions, namely, polymerization, acceptor reaction, and hydrolysis. In the polymerization reaction, a growing α-glucan chain is used as acceptor substrate, resulting in the synthesis of structurally diverse α-glucans with one or more glycosidic linkage types [(α1→2), (α1→3), (α1→4), or (α1→6)] (5). In the hydrolysis reaction, water is used as acceptor substrate, leading to the hydrolysis of sucrose into glucose and fructose. In the acceptor reaction, the glucosyl unit of sucrose is transferred to carbohydrate or noncarbohydrate molecules containing a hydroxyl group, producing oligosaccharides or glucosides (5, 8). More importantly, for glycosylation reaction, GH70 glucansucrase do not require expensive sugar nucleotides, but use the inexpensive and easily available sucrose as donor substrate (2). Therefore, GH70 family glucansucrases are considered attractive biocatalysts for α-D-glycosylation and have been extensively evaluated for glycosylating various hydroxyl group containing organic molecules, including simple phenolic compounds (such as catechol, resorcinol, and hydroquinone) (9–12), phenolic acid compounds (such as caffeic acid, ferulic acid, chlorogenic acid, and gallic acid) (13–15), complex polycyclic flavonoids (16–19), and linear primary alcohol compounds (9, 20, 21).

An inherent problem in using GH70 glucansucrases to catalyze glucoside synthesis lies in the fact that α-glucan polysaccharides and oligosaccharides are also synthesized, which would compete with noncarbohydrate organic molecules as acceptor and also complicate the downstream purification. Indeed, it was previously shown that inhibiting α-glucan synthesis of glucansucrases is beneficial for improving the glucosylation of noncarbohydrate organic molecules (9). Moreover, GH70 glucansucrase enzymes frequently generate polyglucoside products of small organic molecules, in accordance with their functionality to catalyze polysaccharide synthesis. It has been reported that glucansucrase GtfA from Limosilactobacillus reuteri 121 glucosylated catechol with the formation of more than 10 glucosylated products containing up to 5 glucosyl units at a single hydroxyl group (C1). Similarly, Gtf180 from L. reuteri 180 also efficiently glucosylated catechol with the formation of glucosylated products with various glucosyl moieties at the C1 hydroxyl group, including products containing more than 3 glucosyl units. The production of glucoside products with multiple glucosyl units is not conducive to downstream purification and the biological activity of glucosylated products (22).

An opportunity to avoid the unwanted α-glucan and polyglucoside product synthesis may be provided by the branching sucrase, belonging to a newly established subfamily of GH70 (23). Unlike canonical GH70 glucansucrases, branching sucrases mainly hydrolyze sucrose into glucose and fructose, but do not catalyze the synthesis of α-glucan polysaccharides and oligosaccharides when only sucrose is used as the substrate. With sucrose as donor substrate and dextran as acceptor substrate, branching sucrases catalyze the synthesis of (α1→2) or (α1→3) branches with a single glucosyl unit onto the dextran backbone (24–26), resulting in the formation of polysaccharides and oligosaccharides with a highly branched comb-like structure (27). In this regard, branching sucrases naturally do not catalyze the synthesis of α-glucans but retain transglycosidase activity in the presence of acceptor substrates, which makes these enzymes attractive as catalysts for glucosylating small organic molecules (23). Currently, only 6 branching sucrases have been biochemically characterized, including (α1→2) branching sucrase DSRE-CD2 (28), BRS-A (25), BRS-D (25) and (α1→3) branching sucrase BRS-B (24), BRS-C (24) and GfZ-CD2 (26). Recently, DSRE-CD2 was demonstrated to efficiently glucosylate quercetin with a 60% conversion yield (29), which was significantly higher than that of Leuconostoc mesenteroides NRRL B-1299 dextransucrase (23%) and L. mesenteroides NRRL B-23192 dextransucrase (4%) (16). In addition, DSRE-CD2 was also capable of glucosylating
naringenin, morin, apigenin and chrysin, albeit with a lower efficiency (29). Apart from this, the glucosylation capacity of these branching sucrases has not been extensively evaluated.

Benzenediol compounds are simple aromatic compounds with two hydroxyl groups that display a variety of biological effects (30, 31), such as anti-inflammatory, anti-neoplastic, and antimicrobial activities. Depending on the relative position of the two hydroxyl groups, benzenediol compounds can be divided into catechol, resorcinol, and hydroquinone. However, the application of these compounds is impeded by their slight toxicity and low stability, which can be largely improved by glycosylation. Of note, the monoglucosylation product of hydroquinone, α-arbutin, is a commonly used whitening agent in the cosmetic industry (32). Benzenediol compounds also represent important structural building blocks of natural polyphenol compounds such as flavonoids. Glucosylation of benzenediol compounds is thus considered relevant for industrial applications and may also provide important insights into the glucosylation of other polyphenol compounds. We previously cloned the GtfZ-CD2 encoding gene from *Apilactobacillus kunkeei* DSM 12361 and detailed biochemical analyses revealed that GtfZ-CD2 catalyzes the formation of $(\text{Cat-G1}, \text{Cat-G2})$ branching linkages onto dextran via the Ping Pong Bi Bi mechanism (26). In the present study, we investigated the glucosylation of benzenediol compounds (i.e., catechol, resorcinol, and hydroquinone) using GtfZ-CD2 from *A. kunkeei* DSM 12361 and engineered the enzyme by semirational mutagenesis to improve its glucosylation efficiency for resorcinol and hydroquinone.

**RESULTS**

*Glucosylation of benzenediol compounds by GtfZ-CD2.* In order to evaluate the glucosylation capacity of GtfZ-CD2, and possible effects of the relative position of hydroxyl group on the efficiency of glucosylation, we chose catechol, resorcinol and hydroquinone as acceptor substrates for glucosylation reactions. HPLC analysis showed that GtfZ-CD2 was capable of glucosylating catechol, resorcinol, and hydroquinone, generating two glucosylated products in each case (Fig. 1).

Glucosylated products were purified by fractionation on a Bio-gel P2 column for structure elucidation. Mass spectrometry analysis revealed that the two glucosylated products of catechol, resorcinol, and hydroquinone represent their monoglucoside $(\text{IM-H}^{-}, 271.08$) and diglucoside products $(\text{IM-H}^{-}, 433.13$), respectively (Fig. S1). 1H NMR analysis showed that the monoglucoside product of catechol, resorcinol, and hydroquinone are $\alpha$-D-Glcp-1,1-catechol (Cat-G1), $\alpha$-D-Glcp-1,1-resorcinol (Res-G1), $\alpha$-D-Glcp-1,1-hydroquinone (Hq-G1, i.e., α-arbutin). Their $^1$H NMR spectra (Fig. 2) are identical to those previously reported by Devlamynck et al. (9). The catechol diglucoside product (Cat-G2) was identified as $\alpha$-D-Glcp-α-(1→3)-Glcp-1,1-catechol, and its $^1$H NMR spectrum (Fig. 2) matched that previously reported by Devlamynck et al. (9). The NMR spectra of Hq-G2 showed two $\alpha$-anomeric signals at $\delta$ 5.538 (A1) and 5.431 (B1), fitting a di-glucosylated structure. From 2D $^1$H-$^1$H TOCSY and $^{13}$C-$^1$H HMQC spectra all $^1$H chemical shifts were assigned (Fig. S2, Table S1); residue B showed an H-4 signal at $\delta$ 3.49 ppm, which is a structural reporter signal for a terminal residue (33). All $^{13}$C chemical shifts for residue B, assigned from the 2D $^{13}$C-$^1$H HMQC spectrum, fit a terminal glucose residue. Residue A showed an H-3:C-3 combination of $\delta$ 4.04/81.1 ppm, indicative of a 3-substituted residue (33). The 3-substitution of residue A is further reflected in the downfield shift of H-2. Residue B H-5 is also shifted downfield to $\delta$ 4.05, which was previously observed for $\alpha$-D-Glcp-(1→3)-RESOR (33), fitting the structure $\alpha$-D-Glcp-α-(1→3)-Glcp-1,1-Hydroquinone. Similar structural reporters were observed for Res-G2 with residue A H-3:C-3 at $\delta$ 4.04/81.5, fitting a 3-substituted residue (Fig. S3, Table S1). Residue B showed a terminal-residue pattern with a downfield shifted H-5 fitting a $\alpha$-D-Glcp-(1→3)-residue. These data fit the structure of $\alpha$-D-Glcp-α-(1→3)-Glcp-1,1-Resorcinol. Quantification of glucosylated products showed that the yields for catechol, resorcinol and hydroquinone were 59.3%, 19.7% and 5.8%, respectively, when 150 mM sucrose and 150 mM the corresponding acceptor substrates were used.

***Improving the synthesis of glucosylation products by optimizing the acceptor substrate concentration.*** Compared to catechol, the yields of resorcinol and hydroquinone glucosylation by GtfZ-CD2 were relatively low. Increasing the concentration of sucrose was reported to be beneficial for the stability of glucansucrase and to promote
The transglycosylation reaction (10), thereby increasing the yield of glucosylated products. Therefore, we chose to use 1 M sucrose as donor substrate for subsequent experiments. Also, the concentration of the acceptor substrate is a key factor that affects the production and yield of glucosylated products. When the concentration of catechol was increased from 100 to 200 mM, the yield of glucosylated products increased from 60% to 88% (Fig. 3). However, the yield of glucosylated product decreased when the catechol concentration exceeded 200 mM, and only 0.2% was obtained with 400 mM catechol, indicating an inhibitory effect on the activity of GtfZ-CD2 at a high concentration of catechol. The total production of glucosylated catechol products was highest at 200 mM catechol, reaching 175.8 mM. For resorcinol and hydroquinone, the yield of glucosylated products increased at lower acceptor substrate concentrations, probably reflecting the increased donor/acceptor substrate ratio. At 25 mM resorcinol and hydroquinone, the yield of corresponding glucosylated products reached 57.9% and 30.9%, respectively (Fig. 3). Although the yields were higher at low acceptor substrate concentrations, the total amounts of glucosylated products were relatively low. The total amounts of glucosylated products obtained for both resorcinol and hydroquinone were highest at 200 mM, reaching 72.7 mM and 30.9 mM, respectively, with yields of 36.4% and 15.5%, respectively.

**Molecular docking of catechol, resorcinol, and hydroquinone.** As shown above, GtfZ-CD2 has considerable higher catalytic efficiency with catechol than with resorcinol and hydroquinone. This resulted in highest yields of catechol glucosylated products. The relative position of the two hydroxyl groups on the aromatic ring are thus believed to significantly affect GtfZ-CD2-mediated glucosylation of these benzenediol compounds. Aiming to improve the glucosylation of resorcinol and hydroquinone, catechol, resorcinol, and hydroquinone were docked in the GtfZ-CD2 modeled structure.
FIG 2 ¹H NMR spectra and structures of benzenediol (catechol, resorcinol, and hydroquinone) glucosylated products, i.e., Cat-G1, Cat-G2, Res-G1, Res-G2, Hq-G1, Hq-G2.
constructed previously (26). The modeled structure of GtfZ-CD2 showed that the poly-peptide chain adopts a U-shaped path to form domains A, B, C, IV, and V, which is similar to reported GH70 glucansucrase crystal structures (6). Notably, GtfZ-CD2 has a wide acceptor substrate binding site, which is less favorable for the binding of the relatively small benzenediol compounds. Restricting the docking space to subsite 1 resulted in productive binding modes where an acceptor hydroxyl group is favorably positioned to attack the C1 atom of the covalent-glucosyl intermediate (Fig. 4). Notably, the spatial position of this hydroxyl group is shared between all three acceptor ligands (Fig. S4) and nearly coincides (within 1.4 Å) with that of the C6-hydroxyl of the superposed structure of the Gtf180-ΔN-maltose complex (PDB: 3KLL) (8). This attacking hydroxyl group is at hydrogen-bond distance with the acid/base residue E1672 of GtfZ-CD2, an interaction that is also observed in the Gtf180-ΔN-maltose complex between the C6-OH of the glucosyl moiety in subsite 1 and the corresponding acid/base residue E1063. Additionally, this hydroxyl group may engage in hydrogen bond interactions with the transition state stabilizing residue D1744, as well as with the 2-OH group of the covalently linked glucosyl moiety. The productive binding modes of all three benzenediol compounds thus, via transglycosylation, lead to monoglucoside synthesis. The docking results for catechol show that this compound may have two productive poses, where the second (non-attacking) hydroxyl can be stabilized through hydrogen-bonded interactions with either E1672 (left panel in Fig. 4A) or with the O of the covalently linked glucosyl moiety (right panel of Fig. 4A). For resorcinol and hydroquinone, it was noticed that in productive poses, the second (non-attacking) hydroxyl group is surrounded by several hydrophobic amino acid side chains (Fig. 4B and C). This is obviously not favorable for productive binding of these acceptor molecules. Therefore, based on the molecular docking analysis, 6 hydrophobic amino acid residues close to the second hydroxyl group at the acceptor substrate binding site, namely, L1559, L1560, L1590 (all in domain B) as well as A1635, F1638 and F2065 (all in domain A), were selected for semirational mutagenesis and the resulting GtfZ-CD2 mutants were screened for improved glucosylation efficiency.

**Screening and characterization of mutant enzymes.** For each of the 6 selected amino acid residues, 11 to 16 mutant enzymes were constructed. In total, 80 mutant enzymes were obtained from semirational mutagenesis (Table S2). Our initial screening based on TLC analysis revealed that although a few mutant enzymes (F1638Y, F2065W and F2065V) were still active and retained the ability to glucosylate benzenediol compounds, most enzymes with mutations targeted at residues L1590, A1635, F1638 and F2065 were inactivated. In contrast, L1559 and L1560 were found to be more tolerant to mutations and only L1559Q were completely inactive. Based on the initial screening, 11 mutant enzymes were selected for purification and their hydrolytic activities and glucosylation abilities were further quantitatively evaluated. All the selected mutant enzymes showed comparable or even higher enzyme activities with 200 mM sucrose as the substrates (Table S3). Regarding glucosylation, the yield of glucosylated catechol products synthesized by these mutant enzymes was not significantly affected (Fig. 5A).
For resorcinol glucosylation, the yield of glucosylated products produced by L1559E and L1559D increased from 31% (wild type) to about 40% (Fig. 5B). For hydroquinone glucosylation, L1559K, L1560C and L1560Y mutations remarkably improved the yield of glucosylated products, with L1560Y giving the highest yield (25.0%) (Fig. 5C). Notably, the L1560Y mutant showed an increased monoglucoside Hq-G1 yield (21.5%) but a reduced synthesis of diglucoside Hq-G2 (3.5%), compared to 9.7% and 6.2% for wild-type GtfZ-CD2, respectively. In order to confirm the decreased ability of the L1560Y mutant to elongate the glucosyl unit of Hq-G1, we incubated GtfZ-CD2 and L1560Y with 200 mM Hq-G1 (acceptor substrate) and 1 M sucrose (donor substrate). Compared to wild-type GtfZ-CD2, the L1560Y mutant synthesized diglucoside products with a significantly lower yield (15.4% versus 38.1%) when 200 mM Hq-G1 was used as acceptor substrate.

Kinetic analysis of GtfZ-CD2 and L1560Y with catechol, resorcinol, and hydroquinone as acceptor substrates. GtfZ-CD2 and L1560Y followed Michaelis-Menten kinetics in the transglucosylation reactions with catechol, resorcinol, and hydroquinone as acceptor substrates. The $K_m$ values of GtfZ-CD2 for catechol, resorcinol, and hydroquinone were determined to be 37.76 ± 6.74 mM, 43.48 ± 4.56 mM and 61.44 ± 10.55 mM, respectively. Compared to GtfZ-CD2, L1560Y showed a comparable $K_m$ value for resorcinol,
a slightly lower $K_m$ for hydroquinone but a significantly lower (70%) $K_m$ for catechol (Table 1). The $k_{cat}$ values of GtfZ-CD2 for catechol, resorcinol, and hydroquinone were determined to be $13.40 \pm 0.58 \text{ s}^{-1}$, $0.55 \pm 0.02 \text{ s}^{-1}$, and $0.50 \pm 0.03 \text{ s}^{-1}$, respectively. L1560Y displayed more than 2 times higher $k_{cat}$ values for catechol and hydroquinone and a 65% higher $k_{cat}$ value for resorcinol than GtfZ-CD2 (Table 1). Taken together, the catalytic efficiency ($k_{cat}/K_m$) of L1560Y for catechol, resorcinol, and hydroquinone was more than 10 times, 60% and 3 times higher than that of GtfZ-CD2, respectively (Table 1).

**Optimization of the synthesis of α-arbutin by L1560Y.** The monoglucoside product of hydroquinone, α-arbutin, is one of the most common whitening agents on the market, with important applications in the cosmetic industry. The GtfZ-CD2 L1560Y mutant enzyme was found to produce α-arbutin as its predominant product (Fig. 5).

We further optimized the reaction conditions for α-arbutin synthesis by L1560Y.

**TABLE 1** Kinetic analysis of the GtfZ-CD2 and L1560Y mutant enzyme with catechol, resorcinol, and hydroquinone as acceptor substrates

<table>
<thead>
<tr>
<th>Acceptor substrates</th>
<th>Kinetic parameters</th>
<th>GtfZ-CD2</th>
<th>L1560Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol</td>
<td>$K_m$ (mM)</td>
<td>$37.76 \pm 4.76$</td>
<td>$11.13 \pm 0.84$</td>
</tr>
<tr>
<td></td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$13.40 \pm 0.58$</td>
<td>$41.58 \pm 0.77$</td>
</tr>
<tr>
<td></td>
<td>$k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$)</td>
<td>$0.35$</td>
<td>$3.74$</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>$K_m$ (mM)</td>
<td>$43.48 \pm 4.56$</td>
<td>$43.28 \pm 5.806$</td>
</tr>
<tr>
<td></td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$0.55 \pm 0.02$</td>
<td>$0.91 \pm 0.04$</td>
</tr>
<tr>
<td></td>
<td>$k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$)</td>
<td>$0.013$</td>
<td>$0.021$</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>$K_m$ (mM)</td>
<td>$61.44 \pm 10.55$</td>
<td>$48.92 \pm 5.34$</td>
</tr>
<tr>
<td></td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$0.50 \pm 0.03$</td>
<td>$1.29 \pm 0.05$</td>
</tr>
<tr>
<td></td>
<td>$k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$)</td>
<td>$0.008$</td>
<td>$0.026$</td>
</tr>
</tbody>
</table>
mutant in terms of enzyme concentration, hydroquinone concentration and the ratio of sucrose/hydroquinone (Fig. S5). The production of $\alpha$-arbutin first increased with increasing enzyme loading, reaching a maximum at 5 U/mL and subsequently dropped at higher enzyme loadings. The optimum hydroquinone concentration was determined to be 200 mM. Production of $\alpha$-arbutin also increased with the increase of the sucrose/hydroquinone ratio. The optimum condition for $\alpha$-arbutin synthesis by L1560Y was thus determined to be 5 U/mL enzyme, 200 mM hydroquinone and 1.4 M sucrose. The production of $\alpha$-arbutin reached 55.8 mM with a yield of 27.9% under these conditions.

**DISCUSSION**

GH70 branching sucrases lose the ability to catalyze the synthesis of $\alpha$-glucan polysaccharides but retain the transglycosidase activity to efficiently catalyze the transfer of glucosyl unit onto acceptor substrates (23). However, they have not been extensively evaluated for glucosylation of non-carbohydrate organic molecules. In the present study, we explored the potential of GH70 branching sucrase GtfZ-CD2 from *A. kunkeei* DSM 12361 in glucosylation of benzenediol compounds. Unlike canonical GH70 glucansucrases that have been reported to catalyze the formation of more than 10 glucosylated products containing up to 5 glucosyl units at a single hydroxyl group of catechol (C1) (5, 10), only monoglucoside and diglucoside products was found in the GtfZ-CD2-mediated glucosylation of benzenediol compounds. In addition, GtfZ-CD2 introduced ($\alpha$1→3) linkages in all benzenediol diglucoside products, displaying the same linkage specificity as observed for glucosylation of dextran (26).

Glucosylating organic molecules with a limited number of glucosyl units by branching sucrases like GtfZ-CD2 is desirable considering that polyglucosylation of organic molecules, including benzenediol compounds, may have negative effects on their biological activity (22), especially antioxidant activity. Uncontrolled polyglucosylation to various degrees may also cause difficulties in downstream purification of the target product. In this respect, branching sucrases, including GtfZ-CD2 hold a great potential as a catalyst to achieve the efficient glucosylation of benzenediol or polyphenol compounds.

Quantification of glucosylated products revealed that GtfZ-CD2 has a much higher glucosylation efficiency with catechol as an acceptor substrate than with resorcinol and hydroquinone, suggesting that the relative position of hydroxyl groups on the benzene ring has a significant impact on the glucosylation. There has been discrepancy regarding the effect of the hydroxyl group position on glucosylation by glucansucrases. Whereas glucansucrase GtfD from *Streptococcus mutans* GS-5 has been shown to glucosylate catechol, 4-methylcatechol and 3-methoxy catechol with a high efficiency (over 50%) (12), but not resorcinol, hydroquinone, and monohydroxyl aromatic compounds (12), a dextran sucrase from *L. mesenteroides* B-1299CB BF563 was otherwise shown to be capable of glucosylating hydroquinone (34) as well as the monohydroxyl aromatic compound phenol (35). Although the precise mechanism for achieving this position selectivity is not clear yet, our results are consistent with previous reports that compounds with two adjacent hydroxyl groups on the aromatic ring are preferred for glucosylation by glucansucrase enzymes (9, 12). The observed much higher catalytic efficiency of GtfZ-CD2 toward catechol may result from the additional hydrogen bond interactions between the C2 hydroxyl group of catechol and GtfZ-CD2 residues at acceptor binding subsite +1. In contrast with catechol, the non-glucosylated C3 hydroxyl group of resorcinol and C4 hydroxyl group of hydroquinone are surrounded by hydrophobic residues, which may disfavor the catalysis. Similar glucosylation preference has also been reported for the glucansucrase Gtf180 from *L. reuteri* 180 (9). However, the $K_m$ value of Gtf180 (103.3 mM) for catechol was significantly higher than that of GtfZ-CD2 (37.76 mM) (9). Correspondingly, the yields for resorcinol and hydroquinone glucosylation by GtfZ-CD2 (36.4% and 15.5%, respectively) are significantly higher than those reported for Gtf180 (17% and 1%, respectively) (9). This comparison demonstrates the potential of GtfZ-CD2 as an efficient biocatalyst for glucosylation of benzenediol molecules.

Compared to polysaccharide and oligosaccharide synthesis, GH70 glucansucrase enzymes are usually less efficient in catalyzing the glucosylation of non-carbohydrate molecules. Enzyme engineering is thus often required to improve their glucosylation.
efficiency. In this respect, mutants of glucansucrase Gtf180 (Gtf180-\(\triangle\)N L981A, Gtf180-\(\triangle\)N L938F, and Gtf180-\(\triangle\)N N1029M) have been shown to display a greatly reduced \(\alpha\)-glucan synthesis but a significantly improved glucosylation efficiency toward small organic molecules (9). Importantly, their glucosylation product distribution was altered with an enhanced synthesis of monoglucoside compounds (9). Of note, mutating a leucine residue (L242, equivalent to L981 of Gtf180) of a dextran sucrase from \(L.\) reuteri TMW 1.106 and a asparagine residue (N555, corresponding to N1029 of Gtf180) of dextran sucrase from \(L.\) mesenteroides has been also reported to significantly enhance the glucosylation efficiency of flavonoid compounds and caffeic acid phenethyl ester, respectively (17, 36). Our molecular docking and mutagenesis analyses demonstrated different effects of hydrophobic amino acid residues (L1559, L1560, L1590, A1635, F1638 and F2065) surrounding the benzenediol binding site of GtfZ-CD2 on the glucosylation activity. The fact that most L1590, A1635, F1638 and F2065 mutant enzymes completely lost their activity implicates that these residues have critical interactions with the donor substrate sucrose. The importance of residues corresponding to L1590 and F1638 (L981 and N1029 of Gtf180, respectively) to the activity and polysaccharide synthesis of glucansucrase Gtf180 has been shown in our previous study (37). In contrast to the above residues, GtfZ-CD2 L1559 and L1560 are more tolerant to mutation and may thus indirectly participate in sucrose binding and cleavage, probably due to their farther location from the active site. A similar phenomenon has been observed for L940 in Gtf180 (corresponding to L1560 of GtfZ-CD2) that has been reported to be important for linkage specificity determination, but the activities of the Gtf180 L940 mutants were not significantly affected (38). The improved glucosylation of resorcinol by L1559E and L1559D may be a result of their establishing additional hydrogen bonds with the non-glucosylated hydroxyl group of resorcinol. Of note, the L1560Y not only displayed a higher catalytic efficiency toward hydroquinone, catechol, and resorcinol, but also changed the product distribution, resulting in predominantly monoglucoside products. These may be explained by a reduction in the size of the acceptor subsite and/or additional hydrogen bond interactions formed between the substrate and the tyrosine side chain in the mutant.

To improve the yield and production of glucosylated products, reaction conditions have to be optimized with key parameters, including the amount of enzyme, donor, and acceptor substrate concentrations and the ratio of donor/acceptor substrate. On one hand, the trans-glucosylation activity of glucansucrases has been shown to increase with the increase of sucrose concentrations (10). On the other hand, it has been reported that the glucosylated products can be used as donor substrate for glucoside and oligosaccharide synthesis when sucrose is depleted (11), thereby reducing the yield of glucosylated products. In the present study, we found that the glucosylation yields were higher at low acceptor substrate concentrations while high concentrations of acceptors decreased the production of glucosylated products. The observed drop in the production is probably due to the inhibition of GtfZ-CD2 activity, a phenomenon that has reported for glucansucrase Gtf180 (9) and GtfD from \(S.\) mutans GS-5 (12). Regardless of this, our optimized production of \(\alpha\)-arbutin (55.8 mM) is a significant improvement over the reported use of dextran sucrase from \(L.\) mesenteroides B-1299CB BF563 as catalyst, wherein only 2 mM \(\alpha\)-arbutin was synthesized under optimum conditions (34). The present production is comparable to the highest enzymatic production of \(\alpha\)-arbutin (59 mM) reported in the literature, which was achieved by a sucrose phosphorylase from \(L.\) mesenteroides (39) or an \(\alpha\)-amylase form \(Bacillus\) sultis X-23 (40).

**Conclusion.** Branching sucrase GtfZ-CD2 is capable of efficiently glucosylating dihydroxylaromatic compounds catechol, resorcinol, and hydroquinone to form monoglucoside and diglucoside products. The highest glucosylation efficiency was found for catechol, which has adjacent aromatic hydroxyl groups. Structural analysis of glucosylated products demonstrated that GtfZ-CD2 catalyzed the synthesis of \((\alpha1\rightarrow3)\) linkages, the same specificity as for branching linkage synthesis onto dextran. The observed product profiles are consistent with molecular docking results, revealing favorable productive binding modes for catechol, but less so for resorcinol and hydroquinone. Semirational mutagenesis was further performed with selected residues at the acceptor substrate binding site, generating mutant enzymes.
with improved resorcinol and hydroquinone glucosylation. The optimized synthesis of α-arbutin by L1560Y mutant enzyme reached 55.8 mM with a yield of 27.9%. Using benzenediols as model compounds, our present study provides insight into their binding as acceptor substrates and demonstrates the capacity of the branching sucrase GtfZ-CD2 for the glucosylation of non-carbohydrate molecules.

MATERIALS AND METHODS

GtfZ-CD2 expression and purification. GtfZ-CD2 was produced and purified as previously described (26). Briefly, the O/N pre-culture of E. coli BL21 star (DE3) harboring p15b-gtfZ-CD2 was inoculated into fresh LB media with 100 μg/mL ampicillin. The expression of gtfZ-CD2 was induced by adding 0.1 mM IPTG when the culture reached OD600 of 0.4–0.6. The culture was continuously incubated at 16°C with shaking at 160 rpm for 26 h. The cells were collected by centrifugation (8000 g, 10 min) and disrupted by B-Per (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. GtfZ-CD2 was purified by Ni-NTA affinity chromatography. The protein concentration was determined with a Nanodrop Q5000 spectrophotometer by measuring the absorbance at 280 nm. The molar extinction coefficient of GtfZ-CD2 was calculated by ProPam (41) as 159870 M$^{-1}$ cm$^{-1}$ and used for protein concentration determination.

Hydrolytic activity assay with sucrose as the substrate. The hydrolytic activity of branching sucrase was determined by measuring the released reducing sugar with 200 mM sucrose as the substrate, which was quantified using the DNS method (42) and glucose as standard. Sucrose hydrolysis results in the release of glucose and fructose, therefore one unit of enzyme activity (U) was defined as the release of 2 μmol of reducing sugar per minute. The enzyme assays were performed in 50 mM sodium acetate buffer pH 5.5, 1 mM CaCl$_2$ at 30°C using 200 mM sucrose as the substrate. Samples of 120 μL incubation mixture at 10 min were inactivated by adding 120 μL DNS reagent and boiled for 10 min. The reducing sugar was quantified by measuring the absorbance at OD540, with a Tecxan Infinite M200 Pro plate reader.

High Performance Liquid Chromatography (HPLC) analysis of glucosylation products. Glucosylation products were obtained by incubating GtfZ-CD2 with sucrose and benzenediol compounds (i.e., catechol, resorcinol, and hydroquinone) in 50 mM sodium acetate buffer pH 5.3, 1 mM CaCl$_2$. Reaction mixtures (10 μL) were injected into a HPLC system equipped with a refractive index detector and analyzed on Rezex ROA-Organic Acid H ($^+$) column (Phenomenex, Torrance, CA, USA) using 0.005 N sulfuric acid as mobile phase with a flow rate of 0.6 mL/min. The temperature of the column was set to 50°C.

Purification of glucosylated products. Enzymatic reactions (10 mL) were performed by incubating 1 M sucrose and 200 mM benzenediol compounds (i.e., catechol, resorcinol and hydroquinone) with 0.5 U/mL GtfZ-CD2 in 50 mM sodium acetate buffer 1 mM CaCl$_2$ for 120 h. The glucosylated products were partitioned 3 times with an equal volume of 2-butanol. The combined 2-butanol phase was concentrated to dryness by rotary evaporation at 45°C and the dry residues were dissolved in 2 mL deionized water. The extracted glucosylation products were fractionated on a Bio-Gel P-2 column (50 × 2.5 cm), eluted with 10 mM NH$_4$HCO$_3$ at a flow rate of 4 mL/h. Fractions containing pure glucosylated products were collected and lyophilized for further structural analysis.

Structural analysis of glucosylation products. The purified glucosylated products were injected into a LC-MS system (LTQ-Orbitrap velos pro ETD) for mass spectrometry analysis using negative mode. One-dimensional 1H NMR, 2D 1H-H and 2D 13C-1H correlation spectra were recorded on a Bruker 600 MHz Spectrometer (Core Facilities For Life and Environmental Sciences, Shandong University) at a probe temperature of 300 K. Prior to NMR analysis, samples of purified glucosylated products were prepared by dissolving them in D$_2$O (Cambridge Isotope Laboratories, Inc., MA, USA) followed by freeze-drying. After repeating this process for two times, samples were dissolved in 0.6 mL D$_2$O. Chemical shifts (δ) were expressed in ppm by reference to internal acetone (δ 1H 2.225, δ 13C 31.07). MestReNova (Mestrelabs Research SL, Santiago de Compostela, Spain) was used to process all the recorded spectra.

Acceptor substrate concentration optimization. Different concentrations of benzenediol compounds (100 to 400 mM catechol, 25 to 400 mM resorcinol and 25 to 400 mM hydroquinone) were incubated with 1 M sucrose and 0.5 U/mL GtfZ-CD2 in 50 mM sodium acetate buffer pH 5.5, 1 mM CaCl$_2$ at 30°C for 120 h. The reactions were stopped by incubating at 95°C for 10 min. The glucosylated products were quantified by HPLC using respective purified standards.

Molecular docking of catechol, resorcinol, and hydroquinone in GtfZ-CD2 modeled structure. A homology model of GtfZ-CD2, which was generated with the Phyre2 server (43) in intensive mode using residues 1364 to 2621 of the GtfZ sequence in the previous study (26), was used for molecular docking. The model covered 87% of the input sequence with >90% confidence. Construction of the model for the covalent glucosyl intermediate at the GtfZ-CD2 D1634 residue was guided by the model of the covalent glucosyl intermediate of glucansucrase Gtf180-1N (6). Molecular docking studies of GtfZ-CD2 were performed with catechol, resorcinol and hydroquinone using AutoDock Vina (44). The docking space was confined to the acceptor substrate binding subsite +1 as guided by the location of the covalent glucosyl intermediate and the size of the docked substrates. The homology model and docking results were visualized using PyMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC).
China. All the mutants constructed are summarized in Table S2. The isolated mutant plasmids were transformed into E. coli BL21 star (DE3) and the mutant enzymes were produced as described for the wild-type enzyme but in a small scale (50 mL). The induced cells were collected by centrifugation (8000 g, 10 min) and the wet weights were determined. Cells were lysed with B-per reagent (4 mL/g wet weight). The supernatants obtained were used as crude enzymes, which were incubated with 150 mM catechol, resorcinol, or hydroquinone and 150 mM sucrose in 50 mM sodium acetate buffer, pH 5.5, 1 mM CaCl$_2$ at 30°C for 120 h. Synthesis of glucosylated products was evaluated by TLC analysis and compared with that of wild-type enzyme by evaluating the intensity of transglucosylation product spots. Reaction mixtures (1 μL) were spotted on TLC sheets (Merck Silica Gel 60 F254, 20 × 20 cm), which were developed with 2-butanol/acetic acid/water = 2:1:1. A mixture of glucose and malto-oligosaccharides (DP2-7) was used as a standard. The TLC plates were stained with 10% (vol/vol) sulfuric acid solution containing 2 g/L orcinol for carbohydrate detection.

**Characterization of selected mutants.** Based on the initial screening results, 11 mutant enzymes were selected for further characterization. The enzymes were purified and were incubated (0.5 U/mL) with 1 M sucrose and 200 mM catechol, resorcinol or hydroquinone in 50 mM sodium acetate buffer pH 5.5, 1 mM CaCl$_2$ at 30°C for 120 h. The synthesis of glucosylated products was quantified by HPLC using respective standards.

**Kinetic analysis of GtfZ-CD2 and L1560Y for the acceptor substrates catechol, resorcinol, and hydroquinone.** Kinetic parameters of GtfZ-CD2 and L1560Y for the acceptor substrates catechol, resorcinol and hydroquinone were determined using different concentrations of catechol, resorcinol, and hydroquinone (ranging from 1 to 200 mM). The concentration of the donor substrate sucrose was kept constant at 1 M. All the reactions were performed in 50 mM sodium acetate buffer pH 5.5, 1 mM CaCl$_2$ at 30°C. With catechol as acceptor substrate, reactions were stopped after 20 min by incubating at 95°C for 10 min. In case of resorcinol and hydroquinone, reactions were run for 4 h before being terminated. Glucosylated products were quantified by HPLC using respective standards. One unit of benzenediol glucosylation activity was defined as the formation of 1 μmol of the corresponding glucosylated product per minute.

**Optimization of α-arbutin production by L1560Y mutant enzyme.** The L1560Y mutant enzyme was selected for α-arbutin synthesis based on its improved monoglucoside synthesis with hydroquinone as acceptor substrate. The production of α-arbutin was optimized by varying the enzyme concentration, hydroquinone concentration and the ratio of sucrose/hydroquinone. All reactions were performed in 50 mM sodium acetate buffer pH 5.5, 1 mM CaCl$_2$ at 30°C for 72 h. Different concentrations of mutant enzyme L1560Y (0.5 to 15 U/mL) were incubated with 1 M sucrose and 200 mM hydroquinone. For hydroquinone concentration optimization, 10 to 300 mM hydroquinone was incubated with 5 U/mL GtfZ-CD2 and 1 M sucrose. Regarding the optimization of the sucrose/hydroquinone ratio, reactions were performed with 200 mM hydroquinone and various concentration of sucrose to achieve different ratios of sucrose/hydroquinone (from 1:1 to 7:1). The synthesis of α-arbutin was quantified by HPLC with the standard.

**Data availability.** All relevant data are included in the main body or the Supplementary file of this article.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.**

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