Change of *Bacillus cereus* Flavonoid *O*-Triglucosyltransferase Into Flavonoid *O*-Monoglucosyltransferase by Error-Prone Polymerase Chain Reaction

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The attachment of sugar to flavonoids enhances their solubility. Glycosylation is performed primarily by uridine diphosphate-dependent glycosyltransferases (UGTs). The UGT from *Bacillus cereus*, BcGT-1, transferred three glucose molecules into kaempferol. The structural analysis of BcGT-1 showed that its substrate binding site is wider than that of plant flavonoid monoglucosyltransferases. In order to create monoglucosyltransferase from BcGT-1, the error-prone polymerase chain reaction (PCR) was performed. We analyzed 150 clones. Among them, two mutants generated only kaempferol *O*-monoglucoside, albeit with reduced reactivity. Unexpectedly, the two mutants harbored mutations in the amino acids located outside of the active sites. Based on the modeled structure of BcGT-1, it was proposed that the local change in the secondary structure of BcGT-1 caused the alteration of triglucosyltransferase into monoglucosyltransferase.

Keywords: *Bacillus cereus*, flavonoid, glycosyltransferase

Secondary metabolites from plants and microorganisms are modified by glycosylations. The attachment of sugars to small compounds not only enhances their solubility but also modifies their biological activity [2, 9]. The glycosyltransferases (GTs) include 91 distinct families. Among the various types of GTs, family 1 GTs utilize uridine diphosphate nucleotide (UDP)-sugar as a sugar donor, and are referred to as the UDP-dependent glycosyltransferases (UGTs) [5]. The target compounds of UGTs are diverse, and include plant hormones, secondary metabolites such as terpenoids, alkaloids, and phenylpropanoids, as well as antibiotics and a variety of xenobiotics [6, 11]. The N-terminal region of UGTs binds to sugar acceptors and its C-terminal binds to sugar donors. Thus, the N-terminal region tends to exhibit greater variance than the C-terminal region [4]. UGTs from various sources have previously been biochemically characterized, and the molecular structures of these UGTs have been thoroughly elucidated [2, 11, 15].

The glycosyltransferase from *Bacillus cereus* (BcGT-1) is a member of the UGTs. Even though the *in vivo* substrate of BcGT-1 remains unknown, it has been demonstrated that it can utilize flavonoids as a substrate [10]. Additionally, we determined that it was capable of transferring three sugar molecules into three different hydroxyl groups of kaempferol, thereby generating kaempferol-3,7,4′-triglucosides (Fig. 4A). In an effort to evaluate the structural bases of multiple glucosylation of BcGT-1, the molecular structure of BcGT-1 was constructed using a molecular modeling method [9]. Briefly, a homology modeling software PRIME incorporated into the Schrödinger modeling software suite was used to generate a structure of the BcGT-1 homologous to the crystallographic structure of the flavonoid 3′-*O*-glucosyltransferase (UGT71G1) from *Medicago truncatula* (PDB 2ACW) [16]. The optimal model was selected based on bond angle stereochemistry using PROCHECK. After refinement of the loop structures, the model was subjected to energy minimization and molecular dynamics simulations (MD) in order to obtain a stable, low-energy conformation. Energy minimization was performed using a conjugate gradient minimize (0.05 convergence criteria), the OPLS-AA force field, and GB/SA continuum water model. MD simulations were performed by pre-equilibration for 100 ps and simulation for 1 ns at 300K with a 1-fs time step and SHAKE applied to all bonds to hydrogen. The root mean square deviation (RMSD) between the main chain atoms of the models and the template was 1.5 Å, indicating a close homology. We overlapped the structure of the modeled BcGT-1 with that of the template UGT71G1 from
Medicago truncatula. The overall structures of both enzymes were similar, but the most apparent difference was that BcGT-1 harbored an open active site as compared with that of UGT71G1. The loop formed by amino acids 126–185 in BcGT-1 was separate from the other part of the enzyme, which made the active site readily accessible to the substrate (Fig. 1). Other glycosyltransferases from bacteria also harbor acceptor binding sites that are more open than in the UGTs from plants [1, 13]. Additionally, the substrate binding site of BcGT-1 was wider than that of UGT71G1, which enables the placement of a larger flavonoid \( O \)-glucoside for the second glucosylation. This appears to be one of the major reasons that BcGT-1 generated flavonoid \( O \)-triglucoside, whereas UGT71G1 generated flavonoid \( O \)-monoglucoside.

With the modeled structure, the docking of kaempferol or kaempferol 3-\( O \)-glucoside was carried out. We used the protein preparation utilities in Maestro to assign the charge state of ionizable residues, add hydrogens, and carry out energy minimization. The ligand kaempferol or kaempferol 3-\( O \)-glucoside was then docked into the homology models using GLIDE (http://www.schrodinger.com). The default setting of the extreme precision mode of GLIDE was employed for the docking, and up to ten poses were saved for analysis. All of the saved poses were similar, and therefore, the top scored pose was chosen for the binding mode analysis. Kaempferol fitted very well into the substrate binding site of BcGT-1 (Fig. 2A). His14 functions as a base for the catalysis of BcGT-1 and is conserved in all UGTs. The deprotonation from the 3-hydroxyl group of kaempferol is one of the driving forces behind the process of catalysis. Asp106 stabilizes protonated histidine and functions as a charge stabilizer. This kaempferol-His-Asp triad is generally considered to be a basic mechanism of glycosylation [3, 16]. However, recently, some plant UGTs have been shown to use a cysteine and arginine/asparagines/serine network rather than histidine and aspartic acid [14]. The TDP-epi-vancosaminyltransferase GtfA harbors asparagines rather than histidine, and can thus function as a general base for glycosylation [13].

Gln142 formed a hydrogen bond with the 4'-hydroxyl group of kaempferol and positioned kaempferol for 3-\( O \)-glucosylation. Kaempferol 3-\( O \)-glucoside was detected after a brief incubation of kaempferol and UDP-glucose with BcGT-1. Once kaempferol 3-\( O \)-glucoside was formed, it became a substrate for the second glucosylation reaction.

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**Fig. 1.** A, Structure of BcGT-1; B, structure of flavonoid 3-\( O \)-glucosyltransferase from Medicago truncatula (UGT71G1); C, the overlapping of the structure of BcGT-1 with that of UGT71G1. The dotted box indicates the loop from BcGT-1 that makes the substrate binding site more accessible.

**Fig. 2.** A, Binding of kaempferol in the substrate binding pocket of BcGT-1 [a kaempferol (KMP)-His14-Asp106 triad is formed for the glucosylation reaction] B, Docking of kaempferol 3-\( O \)-glucoside (3-\( O \)-Glc-KMP) into the substrate binding site of BcGT-1 (His14 also served as a base for catalysis).
Kaempferol 3-O-glucoside was subsequently docked into BeGT-1. Kaempferol 3-O-glucoside was placed in the proper position for 4'-O-glycosylation; the 4'-hydroxyl group was positioned close to His14 for deprotonation (Fig. 2B). The results of a molecular modeling study showed the amino acid residues required for substrate binding and catalysis.

We then conducted error-prone polymerase chain reactions of BeGT-1 to isolate and identify a mutant(s) that transferred one glucose into kaempferol. BeGT-1 was previously cloned into the E. coli expression vector pGEX [10]. Using this as a template, error-prone PCR was performed. Two sets of dNTPs were used; the first set contained the final concentration of 5 µM of dATP and dTTP, and 25 µM of dCTP and dGTP. The second set consisted of a final concentration of 25 µM of dATP and dTTP, and 5 µM of dCTP and dGTP. pGEX 5' and pGEX 3' primers were used. The resultant PCR products were then digested with EcoRI/NotI and subcloned into the corresponding site of pGEX. The resultant constructs were transformed into E. coli BL21. A total of 150 colonies created by error-prone PCR were screened. Each transformant was inoculated into 2 ml of LB containing 50 µg/ml of ampicillin and grown to OD600 = 0.6~0.7. IPTG was added at a final concentration of 0.1 mM and the mixture was grown for 4 h at 30°C to induce the protein. The cells were harvested and resuspended with 2 ml of phosphate buffer (pH 7.5) containing 1% glucose. Then, 100 µM of kaempferol was added to the culture and incubated overnight at 30°C with shaking. The supernatant was collected after centrifugation and analyzed via HPLC [10]. Two colonies turned out to generate only kaempferol 3-O-glucoside. The plasmids from each colony were isolated and sequenced. Mutant 1 evidenced a Pro replacing a Leu at position 343. Mutant 2 evidenced three alterations at Met161Thr, Glu193Gly, and Met361Thr. None of these mutations occurred near the substrate binding sites (Fig. 3). The mutant enzymes were expressed in E. coli as glutathione fusion protein and purified. Two mutants were purified via the same procedure used for wild-type BeGT-1. After 30 min of incubation with the same amount of enzyme, only the wild-type BeGT-1 generated kaempferol O-triglucoside (Fig. 4A), but the mutants produced only kaempferol O-monoglucoside (Fig. 4B and 4C). Longer incubation (5 h) of mutant enzymes with kaempferol did not generate any kaempferol O-triglucoside. In order to evaluate each point mutation in mutant 2, site-directed mutageneses of BeGT-1 were conducted, and their independent mutants (Met161Thr, Glu193Gly, or Met361Thr) were obtained. The kaempferol reaction product from Met161Thr, Glu193Gly, or Met361Thr was similar to that of the wild-type.

The structural base of kaempferol O-monoglucoside formation by these mutants remains to be elucidated. The modeled structure of BeGT-1 provided no definitive clues regarding the functions of each mutation on the structure of BeGT-1. From the location of the mutant, Leu343 was deemed likely to mediate the interaction between one helix and...
and an adjacent sheet through van der Waals interactions. The mutation of Leu343 to Pro appears to weaken these van der Waals interactions. Met161 and Met361 are also likely to mediate interactions among helixes or between the local helix and sheets, and mutations resulted again in alterations in the strength of van der Waals interactions (Fig. 5). However, it remains to be determined why a single mutation in Met161 or Met361 did not significantly alter the enzyme activity, whereas the triple mutant evidenced significantly altered reactivity. The sequential change effected by modulating the sites away from the catalytic site might induce the conformational change at the catalytic site. The binding of activator to a site distant from the substrate binding site exerted an apparent influence over the substrate binding site [7].

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References


