Altering UDP-Glucose Donor Substrate Specificity of Bacillus licheniformis Glycosyltransferase towards TDP-Glucose

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The specificity of a Bacillus licheniformis uridine diphosphate (UDP) glycosyltransferase, YjiC, was increased towards thymidine diphosphate (TDP)-sugar by site-directed mutagenesis. The Arg-282 of YjiC was identified and investigated by substituting with Trp. Conversion rate and kinetic parameters were compared between YjiC and its variants with several acceptor substrates such as 7-hydroxyflavone (7-HF), 4',7-dihydroxyisoflavone, 7,8-dihydroxyflavone and curcumin. Molecular docking of TDP-glucose and 7-HF with YjiC model showed \(\pi\)-alkyl interaction with Arg-282 and His-14, and \(\pi\)-\(\pi\) interaction with His\textsuperscript{14} and thymine ring. YjiC (H14A) variant lost its glucosylation activity with TDP-glucose validating significance of His-14 in binding of TDP-sugars.

Keywords: Glycosyltransferase, protein engineering, enzyme kinetics, mutation, TDP-glucose substrate specificity

Natural products (NPs), decorated with sugar residues constitute a library of compounds with a variety of biological activities. Currently used clinical compounds, such as antibiotics and anticancer agents are found to be decorated by diverse kinds of sugars. For example, anticancer (doxorubicin, calicheamicin, AT2433), antibiotics (vancomycin, erythromycin, megamycin, kanamycin, streptomyycin), antifungal (nystatin, amphotericin B, saponins), and antiparasitic (avermectins) drugs contain highly modified sugars in their structures. These sugars usually participate in the interaction between the drug and the cellular target [1]. Notably, these sugars are biosynthesized in the activated form (NDP-sugar) so that they can be used by glycosyltransferases (GTs). The phosphonucleotidyl moiety has dual purposes: serving as a recognition element for enzymes and functioning as a good leaving group during the glycosylation reaction [2]. TDP-activated sugars are the most structurally diverse class of nucleotide sugars found in nature and the preferred sugar donors in the biosynthesis of most of the bacterial glycosylated natural products [3].

Recently, a Bacillus licheniformis UDP-glycosyltransferase (UGT)-YjiC has been extensively applied for biosynthesis of various small molecule glycosides such as flavonoids [4–7], xanthone [8, 9] and macrolides [10] with non-regiospecificity. In addition, YjiC showed remarkable flexibility toward five NDP-glucoses and different sugar moieties in in vitro reaction [5]. UDP-glucose is the favored substrate for YjiC. To employ YjiC for biotechnological production of microbial sugar-conjugated plant metabolites, it needs to be specific towards TDP-sugars as these are abundantly present in microbial cytosol.

In this study, we generated mutated variants of YjiC to explore the TDP-sugar specificity of the enzyme. By homology modeling and amino acid sequence alignment, two amino acids, His-14 and Arg-282, were identified as
potential candidate amino acids for TDP-sugar specificities. We generated H14A and R282W mutated variants of YjiC and studied their role to get insight on TDP-glucose binding and glucosylation activity. The YjiC and its variants were expressed to soluble fraction, purified by His-tag affinity resin and quantified using Bradford assay [11–14].

Prior to enzymatic assay, the in silico analysis of YjiC was performed by docking 7-hydroxyflavone (7-HF) and TDP-glucose and comparing the amino acid sequence with previously known UGTs. The BLAST search in Discovery Studio 2018 was used for searching homologous structure of YjiC based on its amino acid sequence. Two structures, OleD (UGT) (PDB number: 2IYF) and CalG4 (TDP-glycosyltransferase; TGT) (PDB code: 3IA7) with the highest identity (40%) were chosen as the templates for building homology models of YjiC using Discovery Studio 2018 (DS 2018; BIOVIA, USA). The coordinates for UDP were obtained from 2IYF and positioned as in the template. The predicted YjiC model displayed the GT-B fold typical of several GT families including GT-1, consisting of two Rossmann-like β/α/β domains that are not tightly associated. Catalytic diad His-14 and Asp-110 of YjiC were well conserved based on comparison of the modeled YjiC structure and structure of OleD. The crystal structure of several UGTs showed highly conserved residues of Trp and Val (Ala) for uridine moiety base recognition of UDP-sugars. In the crystal structure of OleD bound UDP, the uridine stacks against the aromatic side chain of Trp-289 and forms hydrogen bonds with Val-290. Similar interactions are observed in the other GT-1 enzymes that are not highly...

Fig. 1. Amino acid sequence alignment of various glycosyltransferase from Bacillus licheniformis, Arabidopsis thaliana, Micromonospora echinospora, Medicagro truncatula, Streptomyces lividans, Streptomyces antibioticus to check conserved region of W282. The alignment was performed using Clustal X. The aligned amino acid sequences are YjiC (WP_003182014.1) from Bacillus licheniformis, UGT71G1 (2ACW_B), UGT74F2 (OAP07463.1), and UGT85A1 (OAP13723.1) from Arabidopsis thaliana, The Olel (AAC12648.1), OleD (ABA42199.2) from Streptomyces antibioticus, MGT (ABA28305.2) from Streptomyces lividans and CalG2(3RSC_A), CalG4 (3IA7_A) from Micromonospora echinospora. Conserved W282 residues are marked by a black diamond below the alignment.
related to OleD, for example, in UGT71G1, the equivalent residues to OleD are Trp-289 (Trp-339) and Val-290 (Ala-340), whereas in VvGT1, the corresponding amino acids are Trp-332 and Ala-333 [15–17]. Similar to the UGTs, some TGTs such as CalG2 and CalG4, have conserved Trp-Val, Trp-Ile, respectively, in the same position. These amino acids could play the same function like UGTs (pi-pi interaction). Interestingly, in YjiC model, two equivalent residues to these amino acids were Arg-282 basic amino acid and Val-283 (Fig. 1). Therefore, the Arg-282 of YjiC was selected for substitution by the aromatic amino acid Trp.

The donor and acceptor binding pattern can influence the catalytic activity of GT [18–22]. Firstly, UDP-glucose binds to the donor binding site of GT in catalytic manner followed by acceptor substrate binding to the active site in proper orientation. In YjiC, the interactions with TDP-glucose include pi-alkyl interaction between Arg-282 with thymine ring, and His-14 with methyl group of thymine ring (Fig. 2A). In addition His-14 forms pi-pi interactions with thymine ring of TDP-glucose (Fig. 2A). Furthermore, the amino acids Thr-234, Thr-263, Gln-281, Arg-282, Met-301 and Asn-302 showed hydrogen bonding network with TDP-glucose (Fig. 2A). Also, R282W substitution led to breaking of pi-alkyl interactions and the hydrogen bonds with thymine ring (Figs. 2A and 2B). However, the same pi-alkyl and pi-pi interactions are shown in His-14 (Figs. 2A and 2B). Thus, His-14 and Arg-282 residues are very important for proper binding of TDP-glucose in YjiC. R282W changed the binding orientation of TDP-glucose due to breaking the pi-alkyl interaction and hydrogen bonds (Fig. 2C). As a result, R282W distance was decreased from 3.39 Å to 2.97 Å in between donor α carbon of TDP-glucose and oxygen of 7-hydroxyl group of 7-HF (Figs. 2B and 2D). The distance between the nitrogen of His-16 and the proton of 7-hydroxyl group of 7-HF was decreased from 3.36 Å to 2.52 Å (Figs. 2B and 2D). Direct displacement SN2-like mechanism is important for inverting GTs. So, reduced distance influences the conversion rate of 7-HF while using TDP-glucose donor in the reaction, while R282W is anticipated to exhibit more favorable glycosylation reaction using TDP-glucose than YjiC. Moreover, pi-alkyl interactions can be seen between Ala-13 with A ring of 7-HF, Ala-13, Ala-14, and Ala-15 in both YjiC and R282W.

![Fig. 2. Molecular docking of TDP-glucose and 7-HF with YjiC model.](image-url)

(A) TDP-glucose docking in the NDP-sugar binding site of YjiC. (B) TDP-glucose and 7-HF docking in the donor and acceptor substrates binding pocket of YjiC. (C) TDP-glucose docking in the NDP-sugar binding site of R282W. (D) TDP-glucose and 7-HF docking in the substrates binding pocket of R282W. All atoms are colored to standard coloring. Amino acid residues are represented by the stick model, whereas bound donor and acceptor substrate atoms are shown by the ball-and-stick model. Bond lengths are displayed in Å. Hydrogen atoms were removed in amino acid residues to improve clarity, and images were made by Discovery Studio 2018.
Leu60, Met-71, Ala-80, Pro-81, Ile-145 with B ring and Leu-60 with C ring, respectively (Fig. S1). Identical interactions were also observed in R282W mutant (Fig. S1). These results clearly indicate that only TDP-glucose binding will be influenced but not the binding of 7-HF in R282W variant in comparison to YjiC.

H14A and R282W were generated, cloned, and expressed in *Escherichia coli* BL21 (DE3) along with pET302/NT-His vector. Analysis by SDS/PAGE demonstrated that isolated H14A and R282W both exhibited protein size in the range of 46 kDa, the same molecular weight as wild-type YjiC (Fig. S2). Purified YjiC and R282W were assayed with 7-HF as acceptor and various NDP-glucoses (U/T/C/A/GDP-glucose) as donors as described in supporting methods. In case of wild type, UDP-glucose resulted in the highest conversion (79.9%) of 7-HF to its glucoside formation among other NDP-sugars (Fig. 3A). The conversion of 7-HF to its glucoside was 33.6, 19.4, 22.1 and 15% when TDP, CDP, ADP and GDP-glucose were used in the reaction mixture, respectively. Only 5.5%, 0.4%, 13.8%, and 1% conversion rate was increased in case of UDP-glucose, CDP-glucose, ADP-glucose and CDP-glucose while comparing between wild type and R282W (Fig. 3A and Table S1A). But in case of TDP-glucose, there was 34.4% increase in the conversion rate (Fig. 3A and Table S1A). Thus, we were interested in identification of the governing factor that can influence on the increased conversion rate of R282W using TDP-glucose.

We also performed kinetic studies of R282W mutant. Reaction condition was optimized for enzyme kinetic studies. The highest conversion of 7-HF to glycosylated products was observed at pH 9 (Fig. S3A) and optimal MgCl₂ concentration of 10 mM (Fig. S3B). These results suggested that His-14 has more effect on the conversion of TDP-glucose in YjiC. In the H14A mutant, there was loss of two *pi-π* and *π*-alkyl interactions with thymine ring (Fig. 2A). So, TDP-glucose was not properly bound to the active site resulting in a loss of enzyme activity.

Enzyme reactions were also performed with various other acceptor molecules (4′,7-dihydroxy isoflavone, 7.8-dihydroxyflavone, curcumin) to confirm the reaction pattern of R282W (Fig. 4 and Tables S1C–S1E). Enzyme reaction was performed based on previous reports [23–25]. In case of UDP-glucose, the conversion rate of all glucosylated products was similar (1~4% different) compared to YjiC and R282W (Fig. 4 and Tables S1C–S1E). In contrast there was enhanced conversion rate (4~39%) with TDP-glucose (Fig. 4 and Tables S1C–S1E). While TDP-glucose was used for enzyme reaction, R282W mutant and YjiC exhibited 47%, 28%, and 46% increment on total conversion of 4′,7-dihydroxy isoflavone, 7.8-dihydroxyflavone.

![Fig. 3.](image) (A) The conversion of 7-HF to glucoside by R282W (in gray) with various NDP-glucoses. (B) The conversion of 7-HF to glucoside by H14A (in white) and wt-YjiC (in black) with U(T)DP-glucose.
and curcumin, respectively. Again, this result clearly demonstrates the increased affinity of R282W mutant with TDP-glucose.

We further characterized the glucosylated reaction products of 7-HF, 4',7-dihydroxy isoflavone, 7,8-dihydroxyflavone and curcumin with U(T)DP-glucose by HPLC-PDA (Fig. S5 and Table S2) and QTOF-HR ESI/MS (Figs. S6-S9 and Table S3). The HR ESI/MS analysis confirmed the product to be 7-HF 7-\(\beta\)-D-glucoside (H1, \([M+H]^{+}\) m/z ~401.1231) as 7-HF contains a single hydroxyl group at the C-7 position. The mass analysis identified a 4',7-dihydroxy isoflavone (I1) (\(t_R = 13.1\) min, \([M + H]^{+}\) m/z ~255.0640), 4-\(O\)-glucoside derivative of 4',7-dihydroxy isoflavone (I2, \(t_R = 10.6\) min, \([M + H]^{+}\) m/z ~417.1179), and 7-\(O\)-glucoside derivative of 4',7-dihydroxy isoflavone (I3, \(t_R = 10.2\) min, \([M + H]^{+}\) m/z ~417.1169). Similarly, with 7,8-dihydroxyflavone, a 7-\(O\)-glucoside derivative (F1, \(t_R = 11.5\) min, \([M + H]^{+}\) m/z ~417.1181), 8-\(O\)-glucoside derivative (F2, \(t_R = 11.2\) min, \([M + H]^{+}\) m/z ~417.1172) were characterized based on previous reports [24]. With curcumin, a curcumin 4'-\(O\)-\(\beta\)-glucoside (C1, \(t_R = 13.4\) min, \([M + H]^{+}\) m/z ~531.1848), a curcumin 4',4'' di-\(O\)-\(\beta\)-glucoside (C2, \(t_R = 11.1\) min, \([M + H]^{+}\) m/z ~693.2390) were detected in the reaction mixture.

In conclusion, we tuned donor substrate specificity of YjiC from preferred UDP-glucose to TDP-glucose by site-directed mutagenesis. This study provides evidence for better understanding of YjiC catalytic activity and donor substrate specificity. Two residues (Arg 282 and His14) identified by \textit{in silico} study and characterized by mutagenesis studies present evidence for promiscuous activity of YjiC with diverse donor substrates. The study also provides information for further engineering GT by saturated mutagenesis for target NDP-sugar specificity in the near future.

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**Conflict of Interest**

The authors have no financial interests to declare.

**Reference**


