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Article

Glycosylation of Anthraquinone by UGT-1: Biosynthesis of compound with potential biological activity

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Abstract: Anthraquinones are secondary metabolites of plants and are widely distributed in various species. They are known to exhibit a wide range of biological activities, including anticancer, anti-inflammation, antioxidant, antibacterial, anti-damage, and anti-osteoporosis. Anthraquinones are generally lipophilic, which results in relatively low solubility and bioavailability. These limitations can be addressed through enzymatic reactions such as glycosylation and hydroxylation. In this study, we attempted the glycosylation of two anthraquinone compounds, anthraflavic acid and xanthopurpurin, using microbial UDP-glycosyltransferase UGT-1. UGT-1 could catalyze the O-glucosylation of anthraquinones to form glucoside-type products, which were confirmed by HPLC and high-resolution LC-MS analyses. This study demonstrates that glycosyltransferase UGT-1 could be a good candidate for producing modified anthraquinones with poogical activtential biolities

Keywords: Glycosyltransferase, Anthraquinone, Biosynthesis

1. Introduction

Natural compounds have been of significant interest in the fields of pharmacology, food science, and the cosmetics industry due to their diverse biological properties [1, 2]. In particular, anthraquinone compounds are excellent research subjects due to their various biological properties such as antibacterial, antioxidant, antitumor, and antihypertensive effects. Anthraquinone is a multifunctional polyphenol compound widely distributed in the plant kingdom. It is a compound derived from anthracene with two ketone groups attached at positions 9 and 10 [3, 4]. These compounds are structurally complex and have limited physicochemical properties such as hydrophilicity and lipophilicity, often resulting in reduced efficiency in absorption, distribution, metabolism, and excretion processes within the body. Various biosynthetic processes are being studied to improve bioavailability and stability [5, 6]. In particular, glycosylation, which involves adding sugars to compounds, is known to be an effective method for increasing the solubility of compounds and ensuring stability against enzymatic or chemical degradation within the body [7]. Consistently, it has been reported that the presence of a sugar moiety in anthraquinone glycosides, such as aloe-emodin-8-glucoside, increases aqueous solubility compared with their aglycones, thereby enhancing their applicability in biological systems [8].

Glycosyltransferases (GTs) catalyze glycosylation reactions on a wide range of substrates, such as secondary metabolites, nucleic acids, polysaccharides, and protein lipids [9]. Glycosylation is the most common modification in plant secondary metabolites, enhancing the biological activity, stability, and solubility of receptor molecules. It also has significant physiological effects, such as regulating the balance of plant hormones and contributing to plant detoxification and defense responses. These reactions generally occur through UDP-glucosyltransferases, which catalyze glycosylation reactions using UDP-glucose as a donor [10].

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In particular, UGT-1 has been reported to exhibit high catalytic efficiency in the glycosylation of steroid compounds, specifically catalyzing O-glycosylation at hydroxyl group. For example, Yu et al. demonstrated that glycosylation of testosterone and nandrolone by UGT-1 resulted in reduced cytotoxicity and enhanced protective effects. In addition, it has been reported that cortisone and prednisone are glycosylated by UGT-1 [11, 12].

This study focuses on exploring the glycosylation potential of anthraquinone derivatives using UGT-1. Through this, we aim to present the applicability of enzymatic modification using UGT-1.

2. Materials and Methods

2.1. Materials

Xanthopurpurin used in this study was purchased from ChemFaces (Wuhan ChemFaces Biochemical Co., Ltd., Wuhan, China), and anthraflavic acid was obtained from TCI (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). UDP-glucose was purchased from GeneChem, South Korea. All other high-grade reagents were obtained from commercial sources.

2.2. Enzymatic in-vitro assay of anthraquinones

The heterologous expression and purification of glucosyltransferase UGT-1 were carried out according to previously published papers [11]. In the case of in vitro analysis, the reaction mixture consisted of a final concentration of 10 μg/ml UGT-1, 100 mM Tris-HCl buffer (pH 8.5), 10 mM $MgCl_2 \cdot 6H_2O$, 2 mM UDP-glucose, and 0.4 mM substrate. The reaction was carried out at 35℃ for 2 h with shaking. We added twice the amount of methanol relative to the reaction volume to stop the reaction and then centrifuged it. Later, the supernatant was collected and analyzed using high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS). All enzymatic assays were performed in triplicate (three independent experiments), and the reported results represent mean values.

2.3. Analysis of glycosylated anthraquinones

Products were analyzed with a Nexera UHPLC system (Shimadzu, Japan) using a GIS C18 column (Shim-pack, 4.6 × 250 mm, particle size 5 μm HSS). The mobile phase consisted of solvent A (HPLC grade water + 0.05% trifluoroacetic acid (TFA)) and solvent B (HPLC grade 100% acetonitrile). The flow rate was set at 1 ml/min for a 35 min program. The oven temperature was maintained at 30°C. Acetonitrile (ACN) concentrations were 10% (0 – 10 min), 10 to 20% (25 min), 100 % (28 min), 70% (30 min), and then 10% (30 - 35 min). The injection volume was 20 μL. Detection of both substrate and product was performed by UV absorbance at 280 nm.

After completing the in-vitro experiment, the supernatant collected by adding HPLC-grade methanol was used for mass spectrometry analysis. High-resolution quadrupole time-of-flight electrospray ionization mass spectrometry (HQ-QTOF ESI/MS) was performed in positive ion mode, and the analysis was conducted using Waters Crop. (Milford, MA, USA) ACQUITY UPLC and SYNAPT GS-2 equipment.

2.4. In-silico analysis

The *in-silico* protein structure of UGT-1 was performed through the AlphaFold server (https://alphafold-server.com/) [13]. The homology model was validated for structural accuracy using ERRAT [14], Verify3D [15], and PROCHECK [16]. AutoDock Vina [17] was used to conduct molecular docking studies and predict the binding affinity between anthraflavic acid and xanthofulvin, and ProteinPlus (https://proteins.plus/)[18] was utilized to thoroughly examine the interactions between the enzyme residues and the substrate. We selected the predominant ligand conformation and analyzed the binding mode with UGT. The visualization of the results was performed using PyMoL [19].

3. Results and Discussion

3.1. In-vitro biotransformation of anthraquinones

We performed the in vitro biotransformation of anthraquinone. The HPLC results showed two peaks in the biotransformation analysis of anthrapurpurin and xanthopurpurin, respectively. The peaks of the products were

observed at shorter retention times compared to the substrate peaks, indicating the presence of glucosylated products (Figure 1).

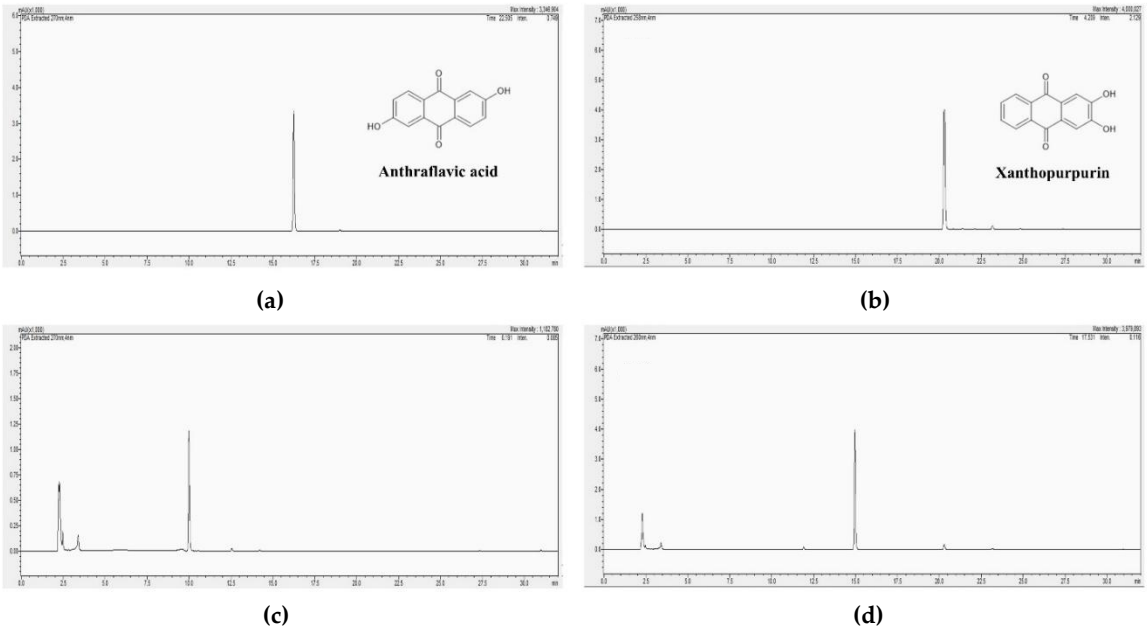

Figure 1. HPLC chromatogram of glycosylated anthraquinones: (a) Anthraflavic acid standard; (b) Xanthopurpurin standard; (c) Reaction product of anthraflavic acid with UGT-1; (d) Reaction product of xanthopurpurin with UGT-1.

The conversion rates of anthraflavic acid and xanthopurpurin were both over 95%. The reaction products were further analyzed by LC-QTOF-ESI/MS in positive ion mode for accurate confirmation. The mass spectrum of anthraflavic acid showed a precursor ion at m/z 241.049 $[M + H]^+$, and its reaction nd di-glucoside derivatives. Similarly, xanthopurpurin displayed a precursor ion at m/z 241.050 $[M + H]^+$, and the products exhibited peaks at m/z 403.102 $[M + H]^+$ and 565.154 $[M + H]^+$, corresponding to the mono- and di-glucose conjugaproducts showed additional peaks at m/z 403.102 $[M + H]^+$ and 565.156 $[M + H]^+$, which were similar to the mono- ates (Figure 2).

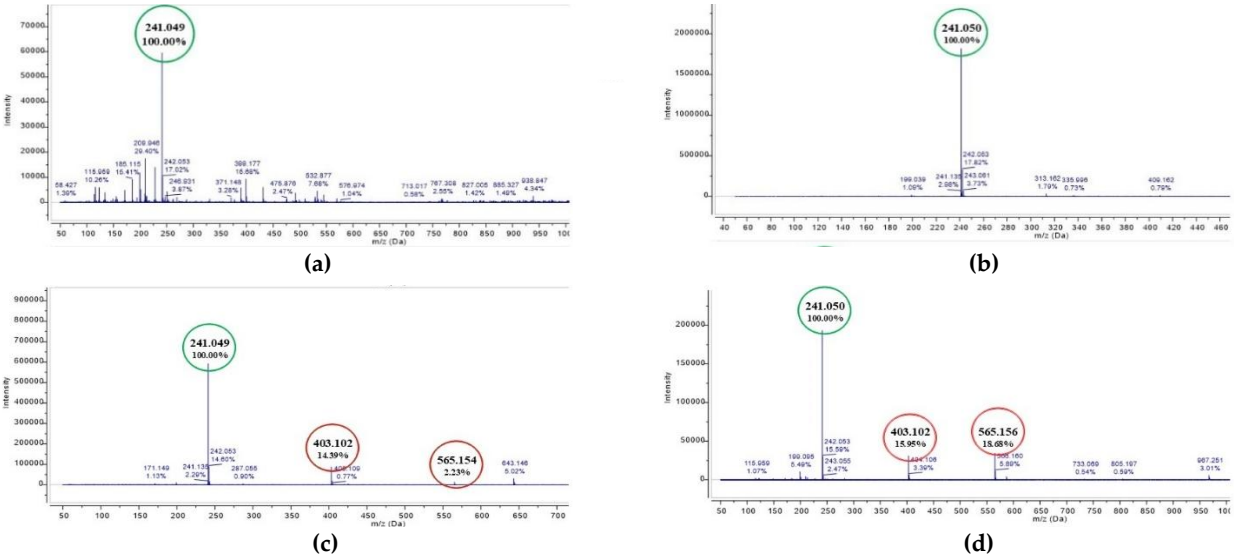

Figure 2. LC-MS spectrum of glycosylated anthraquinones: (a) Anthraflavic acid standard; (b) reaction product of anthraflavic acid with UGT-1; (c) xanthopurpurin standard; (d) reaction product of xanthopurpurin with UGT-1. The mass of the substrate was indicated using a green circle, and the mass of the reactant was indicated using a red circle.

3.2. Homology Modeling of UGT-1

The homology model of UGT-1 was obtained using the AlphaFold Server. If the predicted template modeling (pTM) of a homology model is above 0.5, it means that the predicted structure is similar to the actual structure, and the higher the value, the more similar it is to the actual structure [20, 21]. Since the pTM of the UGT-1 homolgy model is 0.93, it means that the structure predicted by the AlphaFold Server is similar to the actual structure. To verify the quality of the homology model of UGT-1, we used the online programs ERRAT, VERIFY3D, and ERRAT. The ERRAT analysis results showed an overall quality index of 98.11, indicating that the homology model of UGT-1 is a highly reliable structure. In the VERIFY3D analysis, 87.02% of the residues in the model recorded values above 0.1 in the 3D/1D profile score, indicating that most of the residues are in environmentally suitable positions within the structure. As a result of evaluating the stereochemical quality of the protein structure using PROCHECK, the Ramachandran plot analysis showed that 95.4% of the residues were located in the core region, and the remaining 4.6% were found in the additionally allowed region. When comprehensively reviewing these results, the homology model of UGT-1 obtained using the AlphaFold Server is considered to be of reliable structural quality.

The modeled 3D structure of UGT-1 showed a structure composed of two separate Rossmann-fold domains (β/α/β) connected by a linker region, which is a form typically seen in GT-B folds. The active site was located between the two Rossmann-fold domains [22]. The active site was located in the inter-domain cleft formed by two flexibly linked Rossmann fold domains. The substrate binding site was present in the N-terminal domain, which had a flexible and structurally plastic loop region. In contrast, the donor substrate binding site was located in the more conserved and structurally rigid C-terminal domain [23].

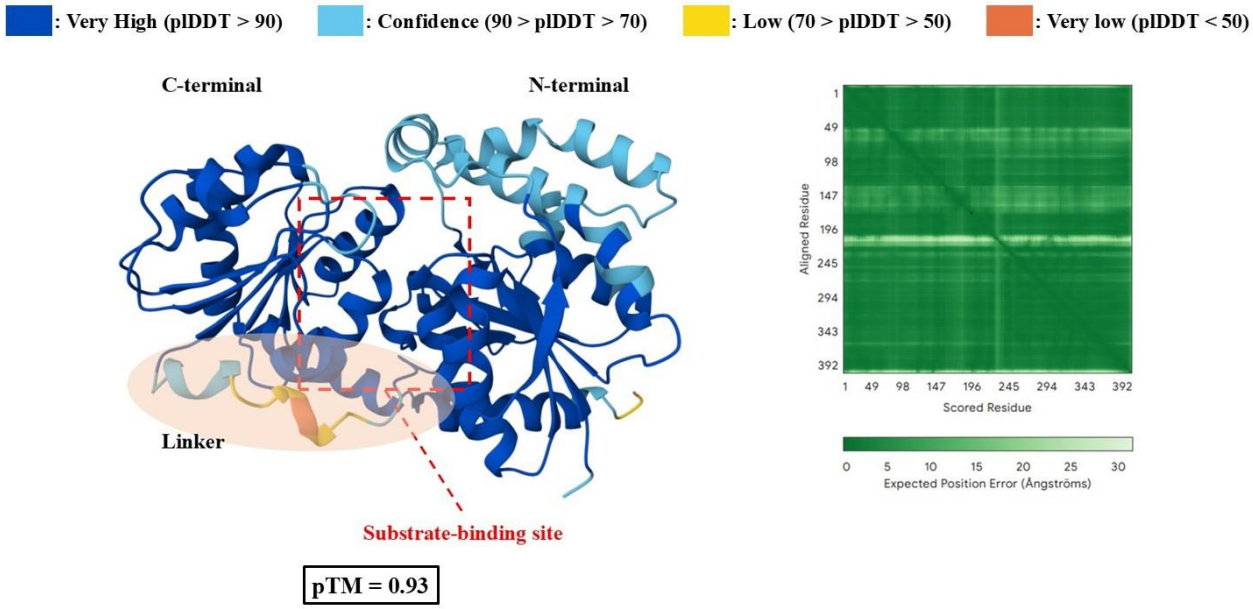

Figure 3. The homology model of UGT-1 obtained from the AlphaFold Server. The N-terminal domain and C-terminal domain are labeled, and the predicted linker region is highlighted in orange. The putative substrate binding site is marked with a red dashed box. The model is colored according to the plDDT confidence score, where values closer to 100 indicate higher per-residue confidence. plDDT, predicted local Distance Difference Test; and pTM, predicted template modeling.

3.3. Molecular docking

Molecular docking analyses were performed to anticipate the potential binding configurations of sugar donors and receptor molecules at the active site. The analysis of the docked complex of UGT-1 with the ligand revealed a predilection for a comparable orientation within the active site. The docking locations of the sugar source UDP-glucose, anthraflavic acid, and xanthopurpurin were identified in a conformation conducive to the glucosyl transfer process, signifying a thermodynamically advantageous orientation. The projected lowest binding energies for anthraflavic acid and xanthopurpurin are -7.6 kcal/mol and -7.8 kcal/mol, respectively. The docked complex of UGT-1 and UDP-glucose revealed the presence of UGT-glucose in the C-domain. The docking

analysis of anthraflavic acid and UGT-1 revealed interactions with His294, Asn298, and Glu318 residues of UGT-1. The docking results of xanthopurpurin and UGT-1 indicated interactions with His294, Asn298, and His16. Given these observations, Asn298 and His294 are anticipated to be pivotal in the reaction with UGT-1 (Figure 4). Furthermore, numerous investigations have demonstrated that histidine and aspartic acid function as catalytic bases, and this has been identified as a highly conserved residue. UGT-1 possesses histidine and aspartic acid at analogous locations relative to other UGTs, serving as catalytic bases [12]. The results are also evident in our UGT-1 and xanthopurpurin complex. Nonetheless, the structure of UGT-1 remains incompletely characterized. Importantly, docking analyses did not identify any residues within 3 Å of UDP-glucose or the substrate, indicating an absence of direct coordination. Therefore, the docking results should be viewed primarily as qualitative predictors of potential binding poses rather than as quantitative structural evidence. These limitations highlight the need to validate the binding interactions with a higher resolution structure in the future.

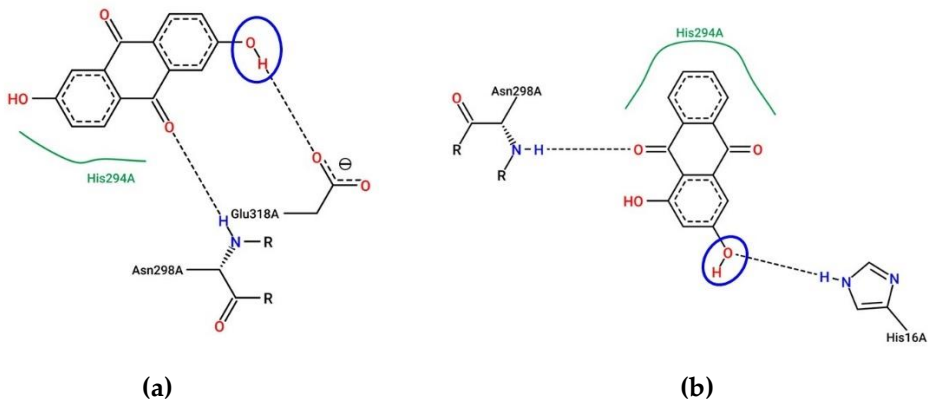

Figure 4. Analysis of the interaction between UGT-1 and substrates based on ProteinPlus: (a) UGT-1 and anthraflavic acid complex; (b) UGT-1 and xanthopurpurin complex. Blue circles indicate the predicted glycosylation sites.

4. Conclusions

In this study, we successfully performed the enzymatic glycosylation of anthraquinone compounds using UDP-glycosyltransferase UGT-1. Through in vitro experiments, anthraflavic acid and xanthopurpurin were each efficiently converted into mono- and di-glucoside derivatives, achieving conversion rates of over 95%. These results were confirmed by HPLC and LC-MS analyses, demonstrating that glycosylation is an effective approach for enhancing the solubility and bioavailability of anthraquinone derivatives.

Additionally, through homology modeling of UGT-1, we identified important structural features, including the conserved Rossmann-fold domain and the active site structure. Molecular docking analysis predicted that the His294 and Asp298 residues play crucial roles in substrate binding and catalytic activity. These results not only expand the functional understanding of UGT-1 but also highlight the potential application of UGT-1 in the enzymatic modification of bioactive compounds.

However, although the docking results indicated thermodynamically favorable interactions, additional validation through experimental methods is necessary due to the structural limitations of the predicted UGT-1 model. Future research should focus on precisely improving the UGT-1 structure of glycosylated anthraquinone to enhance docking accuracy.

This study suggests the potential of UGT-1 as a versatile biocatalyst for the development of new bioactive compounds and demonstrates its applicability in the pharmaceutical and biotechnology fields.

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Conflicts of Interest: The authors declare no conflict of interest.

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