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Form 3

Abstract of Thesis

Name （Rakesh Maharjan）	
Title	Structural and functional study of uridine diphosphate glycosyltransferases from *Phytolacca americana* (*Phytolacca americana*由来ウリジン二リン酸グリコシルトランスフェラーゼの構造と機能に関する研究)

Polyphenols are natural products with several health benefits and serve as an important lead compound for drug discovery. These compounds have gained significant interest in pharmaceutical, cosmetics, and food industries since they are effective against cancer, diabetes, inflammation, cardiovascular diseases, neurodegenerative diseases, UV radiation protection, aging, and obesity. However, the poor water solubility and stability of polyphenols, which results in low bioavailability, are limiting their exploitation. Glycosylation is one of the important methods to improve the solubility and stability of polyphenols. In plants, uridine diphosphate glycosyltransferases (UGTs) catalyze glycosylation of small molecules. Glycosylation is important for the cellular localization, storage, and metabolism of such compounds. Thus, understanding the structure and glycosylation mechanism of UGTs will be useful for improving the solubility and stability of compounds with pharmaceutical interest.

Here, two glycosyltransferases, *Pa*GT2 and *Pa*GT3, from a plant *Phytolacca americana*, "*Yoshuyamagobou*" in Japanese, were studied. Among the three glycosyltransferases (*Pa*GT1/2/3) identified in *P. americana*, *Pa*GT2 had the least and *Pa*GT3 had the highest glycosylation activities with different phenolic compounds. Understanding the reasons for this discrepancy will add information to the substrate recognition and glycosylation mechanisms of UGTs, which will be useful for developing efficient enzymes with higher substrate specificity. For this purpose, the crystal structures of *Pa*GT2 and *Pa*GT3 with and without substrates were determined. Molecular docking, site-directed mutagenesis, and glycosylation assays were also performed. The information was used to modify *Pa*GT2 for resveratrol glycosylation, which was negligibly glycosylated by wildtype *Pa*GT2.

In chapter 1, the crystal structures of *Pa*GT2 with and without substrates were determined. *Pa*GT2 has a very low activity with resveratrol and pterostilbene that are present in the crystal structures. The crystal structures show that the OH groups on these substrates are not close enough to the conserved catalytic base (His18) for effective interactions and catalysis. As *Pa*GT2 does not glycosylate the substrates in the crystal structures, molecular docking with good substrates piceatannol and kaempferol is performed in the structure of *Pa*GT2. Besides the conserved catalytic pair (His18-Asp119), both the crystal structures and molecular docking suggests His81 as an additional catalytic residue in *Pa*GT2. The catalytic roles of His18 and His81 are studied by site-directed mutagenesis and glycosylation assays. The structural and functional studies identified two separate catalytic residues in a single UGT for the first time.

In chapter 2, *Pa*GT2 was modified to enable resveratrol glycosylation. Resveratrol is one of the most studied stilbenoid polyphenols for its beneficial bioactivities. In the crystal structure, the OH groups on resveratrol are far from both catalytic bases. It is assumed that changing the binding position of resveratrol could enable its glycosylation by *Pa*GT2. A residue in the active-site that could affect the binding of resveratrol is identified as Cys142. Cys142 is mutated to glycine, alanine, phenylalanine, and glutamine. The mutant enzymes transform resveratrol into resveratrol glucosides. The

crystal structure of the Cys142Phe mutant shows the substitution of Cys142 could alter the position of resveratrol in the active-site than in the wildtype enzyme. Besides, the crystal structure of the Cys142Ala mutant is determined with UDP, which reveals that His81 could inhibit glycosylation, despite being a complementary catalytic residue. This is also verified by the measurement of resveratrol glycosylation activity using the His18Ala/Cys142Ala and the His81Ala/Cys142Ala double mutants. The His81Ala/Cys142Ala double mutant has higher activity and regioselectivity for resveratrol glycosylation than both the Cys142Ala and the His18Ala/Cys142Ala double mutants.

In chapter 3, the crystal structures of *Pa*GT3 with and without substrates were discussed. Although *Pa*GT3 glycosylates a range of compounds, the glycoside products of smaller compounds have low regioselectivity. The crystal structures showed *Pa*GT3 has a large, hydrophobic, and flexible acceptor binding pocket. In the kaempferol complexed structure, the substrate positions in two different orientations in two molecules of *Pa*GT3 present in the asymmetric unit. Besides kaempferol, the acceptor-binding pocket also contains some ethylene glycol molecules. This suggests the large acceptor-binding pocket in *Pa*GT3 allows smaller polyphenols to bind in different orientations, which results in low product regioselectivity. The PaGT3 structure with capsaicin shows that the acceptor binding pocket in *Pa*GT3 easily accommodates long-chain phenolic compounds. The elongated acceptor binding pocket in *Pa*GT3 is an important feature that allows it to accept and glycosylate long-chain phenolic compounds such as capsaicin and retinol. Besides, crown ether was used as an additive for the crystallization of *Pa*GT3. Although crown ethers have been used in protein crystallization, the structures of *Pa*GT3 reveals a new role of these compounds in protein crystallization. In the structures of *Pa*GT3, crown ether-metal complex acts as molecular glue for the crystal growth.

The structural and functional studies of *Pa*GT2 identified two separate catalytic residues in the enzyme wherein one of the catalytic residue (His18) is conserved among UGTs and the other (His81) is not. The study finds His18 is the primary residue for catalysis. Although His81 in *Pa*GT2 catalyzes glycosylation, it also partly inhibits the enzyme. The crystal structure of the Cys142Phe mutant shows that the mutation of Cys142 changes the binding position of resveratrol in the active-site which allows the Cys142 mutant enzymes to glycosylate the compound. Mutation of both His81 and Cys142 significantly improved the enzyme's glycosylation activity towards resveratrol. The crystal structure of *Pa*GT3 shows that the acceptors in the enzyme's active-site are stabilized mainly through hydrophobic interactions. The stabilization of substrates through hydrophobic interactions does not require their specific orientation. Thus, the product regioselectivity is lower in *Pa*GT3 although it has a higher glycosylation activity compared to *Pa*GT2. The larger hydrophobic acceptor binding pocket in *Pa*GT3 also allows it to recognize long-chain phenolic compounds such as capsaicin and retinol, which are not glycosylated by *Pa*GT2. The findings in this study provide information for the customization of UGTs with high substrate specificity and product regioselectivity for glycosylation of compounds of pharmaceutical, nutraceutical, and cosmetics interests.

様式 7

論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

ポリフェノールは、いくつかの健康効果を持つ天然物であり、創薬のための重要なリード化合物としての役割を果たす。誘導体の中にはがん、糖尿病、炎症、心血管疾患、神経変性疾患、紫外線防御、老化、肥満などに効果があることから、製薬、化粧品、食品等の業界では大きな関心を集めている。しかし、ポリフェノールの水への溶解性と安定性が悪いため、その利用が制限されており、ポリフェノールの水溶性と安定性の向上が重要な課題の一つとなっている。植物では、ウリジン二リン酸グリコシルトランスフェラーゼ（UGT）がポリフェノール類のグリコシル化を触媒し、細胞内での局在化、貯蔵、代謝に重要な役割を果たしており、UGTの構造や糖転移機構を理解することは、医薬品として注目されているポリフェノール類の溶解性や安定性の向上による工業的な応用のためにも重要となる。

そこで申請者は、ヨシヤマゴボウ（*P. americana*）という植物に由来する 2 つの糖転移酵素 PaGT2 と PaGT3 についてX線結晶解析や分子動力学計算の手法を駆使した触媒機構の解明や、遺伝子工学的手法による酵素反応の選択性や反応性の向上に関する研究に着手した。その結果、以下の成果を得た。

１つ目は、基質としては活性の低いレスベラトロールとプテロスチルベン分子の存在下／非存在下のPaGT2のX線結晶構造を決定し、活性の高いピセアタノールやカエンフェロール分子とのドッキング計算にも成功した。これらの構造情報を基に、基質のグリコシル化を受けるOH基が、触媒残基と考えられてきた保存性の高いHis18から遠くに位置し、His81が付加的な触媒残基として働いていることが示唆された。そこでHis18とHis81に部位特異的変異を加え、変異体の生化学的実験を行うことによって、1つのUGTに2つの触媒残基が存在することを初めて明らかとした。

２つ目は、医薬品等への応用化でも価値の高いレスベラトロールの位置選択的グリコシル化反応を可能にするためのPaGT2の遺伝子改変を行い、これに成功した。レスベラトロールは、その生理学的価値が高いために最も研究されているスチルベノイドポリフェノールの一つであるが、水溶性が低く、グリコシル化の位置によっても生理活性が大きく異なっている。申請者は、PaGT2とレスベラトロールの複合体の構造モデルを基にCys142の変異体を検討し、グリシン、アラニン、フェニルアラニン、グルタミンに変えた変異体のグリコシル化反応の活性を調べると共に、基質との複合体のX線構造解析にも成功した。C142F変異体は基質の結合位置を変化させ、レスベラトロールの4位の水酸基を位置選択的にグリコシル化することが判った。一方、C142A変異体と基質との複合体のX線構造から相補的触媒残基であるHis81が基質の結合を阻害していることを明らかとし、さらに変異を加えた二重変異体のうち、H81A/C142Aの二重変異体が種々の応用化においても価値の高い3位の水酸基を位置選択的にグリコシル化し、かつ、高い酵素活性も有することが判明した。このように、X線構造を基に医薬品としても有用なことが実証されているポリフェノールを位置選択的に、かつ、効率よくグリコシル化する変異体の作製に成功した。

３つ目は、PaGT3のカプサイシンなどの基質の存在下／非存在下のX線構造解析に成功し、疎水性の基質結合ポケットがPaGT2に比べて大きく、反応の位置選択性が低い代わりに、幅広い基質に対してもグリコシル化できる構造要因について追究した。

以上、PaGT2やPaGT3の蛋白質工学的、構造化学的研究を通じ、ポリフェノール類のグリコシル化による医薬品、栄養補助食品、化粧品などへの応用が可能な、高い基質特異性と位置選択性を有するUGTを創出する変異体を作製したことから博士（薬科学）の学位論文に値するものと認める。