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Biological synthesis of isorhamnetin 3-*O*-glucoside using engineered glucosyltransferase

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ABSTRACT

The gene for one of the glycosyltransferases from *Populus deltoids*, PGT-3, was cloned and was expressed as a glutathione S-transferase fusion protein in *Escherichia coli*. Various flavonoids were used as potential substrates of the purified recombinant PGT-3. Flavones having two adjacent hydroxyl groups were served as substrate. The regioselectivity of PGT-3 depends on the hydroxyl groups of the substrate. Flavones having two adjacent hydroxyl groups in the B ring were glucosylated at the 4′-hydroxyl group. However, PGT-3 transferred a glucose group to the 3-hydroxyl group of isorhamnetin. Molecular modeling and docking and site-directed mutagenesis were carried out to engineer a PGT-3 having a specificity for isorhamnetin but not for quercetin. Glu82Leu turned out to display this activity. Using the Glu82Leu mutant and a quercetin 3′-*O*-methyltransferase, isorhamnetin 3-*O*-glucoside was synthesized.

1. Introduction

Use of glycosyltransferases to increase solubility or to alter biological activity of small molecules is one of challenging areas but is of great interest in the food and pharmaceutical industries [1–3]. Microorganisms and plants have been valuable sources of glycosyltransferases (http://www.cazy.org/fam/GT1.html). As sugar donors, glycosyltransferases from plants utilize nucleotide diphosphate sugars, usually uridine diphosphate (UDP)-sugars. Thus, these glycosyltransferases are known as UDP-dependent glycosyltransferases (UGTs) [4].

Flavonoids are phytochemicals which have various biological roles in animals and plants [5,6]. As nutraceuticals, their low solubility is problematic. In addition, the physical and biological properties of flavonoids are changed depending on the position of glycosylation and on the sugar [7]. Regioselective glycosylation using UGTs may be a valuable tool to increase solubility and to produce flavonoids with desired biological properties [4].

Plants produce various small compounds, most of which are glycosylated. Thus, UGTs responsible for glycosylation of small chemicals are diverse [8]. For example, *Arabidopsis thaliana* and *Oryza sativa* contain more than 100 UGTs [9,10]. Flavonoid UGTs have also been characterized from various plants. The most common glycosylation position in flavonoid is the 3-hydroxyl group. Among 14 quercetin O-monoglucosyltransferases from *A. thaliana*, 11 are 3-hydroxyl group specific and 3 are 7-hydroxyl group specific [11]. Flavonoid 3-*O*-glucoyltransferase transfers a glucose into 7-hydroxyl group when 3-hydroxyl group is not available [12]. Furthermore, flavonoid 3′-*O*-glucosyltransferases and 4′-*O*-glucosyltransferases have been characterized in various plants [13,14].

Flavonoids displayed various biological activities [15]. Their biological activities were tested using extracts from herbs or other traditional medicinal plants. Thus, limited supplement of flavonoid is a bottleneck of exploring their various biological activities. Chemical synthesis of flavonoids has been carried out. However, problems with regioselectivity and multiple step reactions need to be addressed. Instead of extractions or chemical synthesis, bioconversion or transformation will be more convenient.

Flavonoid UGTs from various plants have been characterized. In some cases, their structures were determined using X-ray crystallography [16,17]. However, the molecular basis of regioselectivity of flavonoid UGTs remains elusive. To expand the source for flavonoid bioconversion, we studied a new flavonoid O-glycosyltransferase, which has a novel regioselectivity. We found that PGT-3 from *Populus deltoids* could utilize both quercetin and isorhamnetin but that it showed different regioselectivity toward quercetin and isorhamnetin. PGT-3 was engineered to use only isorhamnetin (3′-*O*-methylquercetin) but not quercetin. The mutant PGT-3, which exclusively uses isorhamnetin but not quercetin, along with ROMT9 (quercetin 3′-*O*-methyltransferase) [18], was used to biosynthesize isorhamnetin 3-*O*-glucoside which has been known to be used for the treatment of diabetic complications [19].

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2. Materials and methods

2.1. Cloning and characterization of PGT-3

PGT-3 was cloned using reverse-transcription and polymerase chain reaction. Total RNA was isolated from leaves of *P. deltoids* Marsh using a plant total RNA isolation kit (Qiagen, Hilden, Germany). Reverse-transcription was carried out as described in Kim et al. [20]. The primers, CAGCCATGGAGGAGGCCATA and GACGATACATCGACCCAATTCA (underlined initiation codon and stop codon) for PCR were designed based on sequences from The Institute of Genome Research (TC60608). The PCR product was subcloned into pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced.

Escherichia coli expression vector pGEX-5X-3 was used to express PGT-3. Induction and purification of the recombinant PGT-3 were carried out as described in Ko et al. [10]. Site-directed mutagenesis was carried out using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, USA). The list of primers is in Table 1. UGT assay was done as described in Jeon et al. [21].

2.2. Enzyme reaction and analysis of reaction product

The reaction mixture of glucosyltransferase contained 4 μg of the purified recombinant protein, 1 mM UDP-glucose and 60 μM flavonoid in 200 μl of 50 mM Na_2HPO_4 buffer (pH 7.5). The reaction mixture was incubated at 37 °C for 30 min. The reaction was stopped by boiling for 2 min and the mixture was centrifuged for 15 min at 13,000 rpm. The supernatant was analyzed by high performance liquid chromatography (HPLC). A Varian HPLC equipped with a photodiode array (PDA) detector and a Varian C18 reverse-phase column (Varian, 4.60 mm × 250 mm, 3.5-μm particle size) was used.

Analysis of the reaction product using nuclear resonance spectroscopy (NMR) was performed as described in Kim et al. [18].

2.3. Homology model of PGT-3

The protein sequence of PGT-3 was aligned against that of the *Medicago truncatula* UGT71G1 structure (PDB: 2acv) [16]. The PGT-3 homology model was then built from this alignment using Prime (Schrödinger, Inc.), and then subjected to multiple iterative rounds of energy minimization and side chain optimization to relieve any steric strain present in the model. Due to the significant sequence identity (35%) with the template, the overall three-dimensional structures as well as the active site conformation of PGT-3 were in good accordance with UGT71G1 [16]. UDP-glucose from the crystal structure of UGT71G1 (PDB: 2acw) was directly merged into the superimposed homology model of PGT-3 to provide the enzyme-cofactor complex. The stability of the modeled PGT-3–UDP-glucose complex was confirmed by performing a molecular mechanics energy minimization and molecular dynamics simulation by using the molecular graphics and simulation program MacroModel, version 7.0 (Schrödinger, Inc.).

Table 1
Primers for site-directed mutagenesis of PGT-3.

Mutation position	Primer (5′ → 3′)
Glu82Leu-F	AACCACCCATCATctAGAACTCACTTTTG
Glu82Leu-R	CAAAAGTGAGTTCTagATGATGGGTGGTT
Asp45Val-F	TCTGTGCCCTATGtTTCTGGCTCCACT
Asp45Val-R	AGTGGAGCCAGAAaCATAGGGCACAGA

Mutagenized positions are presented as lower-case letters.

2.4. Ligand preparation and docking study

Quercetin, luteolin, and isorhamnetin were sketched using the sketch module in the SYBYL package (version 7.2). Conformational searches were performed by grid search which calculates energies by systematically changing the dihedral angles of each ligand using the standard TRIPOS force field. The lowest energy structures were selected as the conformer for the FlexX studies. Finally, the ligand was fully optimized using the standard TRIPOS force field with Gästeiger–Hückel charges until the energy gradient converged to below 0.05 kcal/mol. The homology model of PGT-3 complexed with UDP-glucose was constructed as described above. For the docking study, the FlexX module was used, which gives the best poses at the binding site by an incremental algorithm with flexible conformations. The binding site for calculations was defined with default parameters as all atoms of the homology model of PGT-3 within 15 Å of the active site. The formal charge of each compound was assigned during the calculations by FlexX, and all FlexX parameters were set to standard conditions. Sampling was done with 100 poses and, after scoring by the original FlexX scoring function, 30 poses were saved in mol2 files for further analysis. All stored poses were rescored using the Cscore™ module of SYBYL 7.2, which is comprised of five different scoring functions including Dock, Chem, FlexX, PMF, and Gold. In order to eliminate conformations with impossible torsion energy values, we introduced torsion energy constraints by setting up the maximal torsion energy term to the default value (20 kJ/mol).

2.5. Biological synthesis of isorhamnetin 3-O-glucoside

To produce isorhamnetin 3-*O*-glucoside, two *E. coli* BL21 (DE3) transformants containing either PGT-3 Glu82Leu or ROMT9 were used. ROMT9 encodes a flavonoid 3′-*O*-methyltransferase from rice and was subcloned into pET15b [18]. To express both PGT-3 Glu82Leu mutant and ROMT9, the transformants were grown for seed culture in 2 ml LB medium containing 50 μg/ml ampicillin, respectively. The seed culture was inoculated into 50 ml fresh LB medium containing 50 μg/ml ampicillin. The culture grew until absorbance at 600 nm reached 0.6. At this time, IPTG was added to a final concentration of 0.1 mM, and the transformant was grown for an additional 20 h at 18 °C. Cells were harvested by centrifugation and resuspended in fresh LB/ampicillin medium. Equal amount of cells (OD_{600} = 2) were mixed. The substrate, quercetin was added to a final concentrations of 100 μM. The mixture was incubated at 25 °C with careful shaking for 48 h and the reaction mixtures were harvested periodically. The resulting samples were extracted twice with an equal volume of ethyl acetate and dried in vacuo. The dried sample was dissolved in 70 μl DMSO and analyzed using HPLC.

3. Results and discussion

3.1. Functional characterization of PGT-3

PGT-3 was originally cloned based on the sequence in TIGR. The PCR product was subcloned and sequenced. Both strands of several clones were sequenced. However, the nucleotide sequence of PGT-3 did not match exactly with that in TIGR. Fourteen amino acids were changed from those in TIGR to those in the cloned PGT-3 (A6V, S48P, A54T, T72I, H81L, S123F, R145I, P174L, N189Y, G249C, L262P, V320L, K439N and K468E). Thus, the new nucleotide sequence of PGT-3 was deposited in GenBank with accession number of GU390542.

The open reading frame of PGT-3 was subcloned into the *E. coli* expression vector, pGEX-5X-3 and expressed. The recombinant PGT-3 was expressed as a soluble protein and purified. Naringenin,

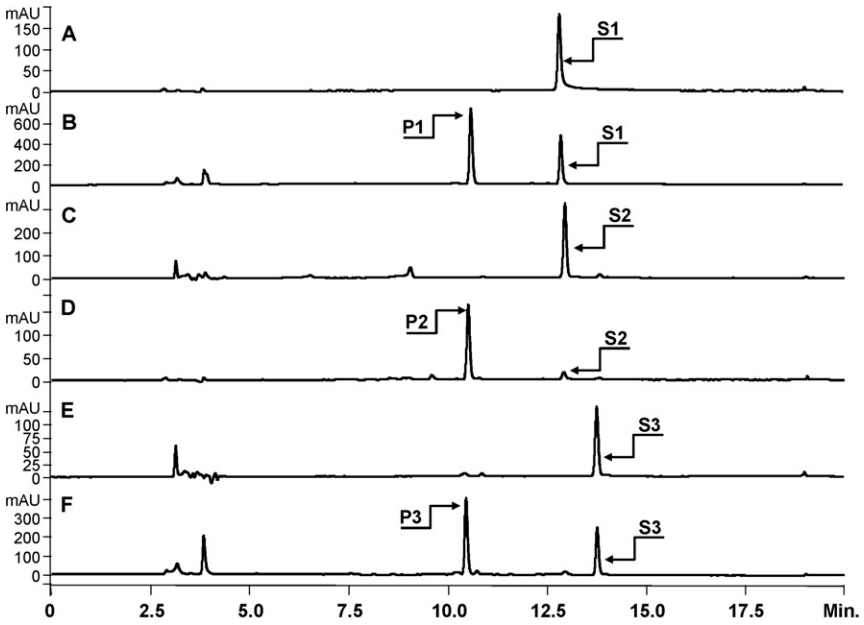


Fig. 1. HPLC analysis of reaction products. (A) Standard luteolin; (B) reaction of luteolin with PGT-3; (C) standard quercetin; (D) reaction of quercetin with PGT-3; (E) standard isorhamnetin; (F) reaction of isorhamnetin with PGT-3.

apigenin, kaempferol, luteolin, and quercetin were examined as potential substrates using purified recombinant PGT-3. HPLC analysis of reaction from naringein, apigenin, and kaempferol did not show any product. However, the product of the reactions from luteolin (Fig. 1B) and quercetin (Fig. 1D) revealed a new peak which had different retention time from substrate itself. In addition, molecular mass of the reaction product increased 162 Da, indicating that one glucose molecule is attached to the hydroxyl group of luteolin or quercetin (data not shown). Structures of luteolin and quercetin reaction product were determined by using ^{1}H NMR to be luteolin 4′-*O*-glucoside and quercetin 4′-*O*-glucoside, respectively (Table 2) by comparison of the published data [22]. Apigenin, a flavone also containing the 4′-hydroxyl group, was not a substrate of PGT-3, indicating that PGT-3 needs both the 3′- and 4′-hydroxyl groups for the reaction. Eriodictyol, a flavanone, which contains both 3′- and 4′-hydroxyl groups, did not give any reaction product. Taken together, these results indicated that a double bond between carbon 2 and 3 as well as two adjacent hydroxyl groups (see below) is required in a substrate of PGT-3.

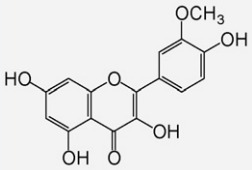
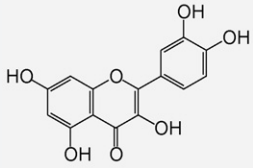
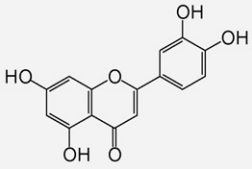
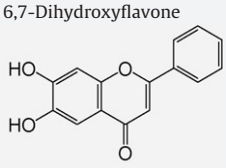
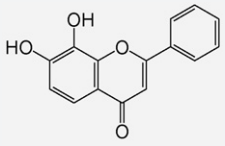
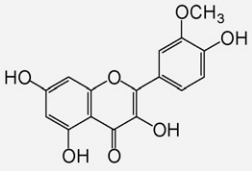
To determine if flavones containing two adjacent hydroxyl groups in the A ring could serve as a substrate of PGT-3, we tested 6,7-dihydroxyflavone and 7,8-dihydroxyflavone as substrates. Both flavones were served as a substrate. Kinetic parameters of five flavones were calculated and 6,7-dihydroxyflavone was the best substrate for PGT-3 (Table 3). Molecular docking of 6,7- and 7,8-dihydroxyflavones showed that these two flavones were fit better into substrate binding site of PGT-3 than luteolin, quercetin or isorhamnetin. It is because both flavones do not contain hydroxyl group in the B ring, which results in the optimal size for the substrate binding site of PGT-3 (Fig. 2D). In addition, the glucosylation position of 6,7-dihydroxyflavone and 7,8-dihydroxyflavone was likely to be at the 7-hydroxyl group based on the molecular docking. UGT90A7 from *Hieracium pilosella* also produced flavonoid 4′-*O*-glucoside with coproduction of 7-*O*-glucoside. However, it also converted apigenin and naringenin into the corresponding 7-*O*-glucoside instead of 4′-*O*-glucoside [14]. In addition, UGT73A4 also displayed a preference for 4′-hydroxyl group of flavonoids in addition to the 7-hydroxyl group [23]. PGT-3 is believed to be a first flavonoid *O*-glucosyltransferase that requires two adjacent hydroxyl groups in flavones. Some of *O*-methyltransferases from plants and microbes were found to need two adjacent hydroxyl groups for their activity [24–26].

Flavonoid O-methyltransferase from poplar (POMT-7: TIGR accession number: TC29789) showed altered regioselectivity when an O-methylated substrate was used as a substrate. Altered regioselectivity of O-methyltransferase from poplar was shown previously [27]. Isorhamnetin was used as a substrate for PGT-3. Analysis of the reaction product using HPLC (Fig. 1F) showed a new peak which had a same retention with an authentic isorhamnetin 3-*O*-

Table 2
Structural determination of luteolin, quercetin, and isorhamnetin reaction products using NMR.

Position	Luteolin	
	Luteolin-4′-*O*-β-glucopyranoside ^{1}H/ppm (Hz)	Metabolite ^{1}H/ppm (Hz)
3	6.83 (s)	6.82 (s)
6	6.19 (d;2.1)	6.20 (d;2.0)
8	6.49 (d;2.1)	6.50 (d;2.0)
2′	7.50 (d;2.1)	7.50 (d;2.1)
5′	7.23 (d;8.5)	7.24 (d;8.5)
6′	7.52 (dd;2.1,8.5)	7.52 (dd;2.2,8.4)
Position	Quercetin (spiraeoside)	
	Quercetin-4′-*O*-β-glucopyranoside ^{1}H/ppm (Hz)	Metabolite ^{1}H/ppm (Hz)
6	6.22 (d;2.0)	6.20 (d;2.0)
8	6.46(d;2.0)	6.45(d;2.0)
2′	7.71(d;2.2)	7.71(d;2.2)
5′	7.26(d;8.7)	7.24(d;8.8)
6′	7.63 (dd;2.2,8.7)	7.62 (dd;2.2,8.7)
Position	Isorhamnetin	
	Isorhamnetin-3-*O*-β-glucopyranoside ^{1}H/ppm (Hz)	Metabolite ^{1}H/ppm (Hz)
6	6.22 (d;2.0)	6.20 (d;2.0)
8	6.48 (d;2.0)	6.44 (d;2.0)
2′	7.96 (d;2.0)	7.94 (d;2.0)
3′	3.85 (OCH_3)	3.84 (OCH_3)
5′	6.99 (d;8.0)	6.91 (d;8.4)
6′	7.50 (dd;2.0, 8.0)	7.50 (dd;2.0, 8.4)

Table 3
Kinetic parameters of wild type PGT-3.

Enzyme	Substrate	K_m (μM)	V_{max} (nk_{cat}/mg)	V_{max}/K_m	K_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
PGT-3	Isorhamnetin 	39 ± 4	1823 ± 196	47	0.15	3.7×10^{-3}
	Quercetin 	46 ± 6	1252 ± 45	27	0.10	2.2×10^{-3}
	Luteolin 	74 ± 5	1483 ± 92	20	0.12	1.6×10^{-3}
	6,7-Dihydroxyflavone 	60 ± 3	12261 ± 46	204	0.98	16.3×10^{-3}
	7,8-Dihydroxyflavone 	16 ± 2	1612 ± 73	100	0.13	7.9×10^{-3}
PGT-3 E82L	Isorhamnetin 	78 ± 6	5118 ± 52	66	0.42	5.4×10^{-3}

Enzyme assays were carried out using 3–30 μg of the purified PUGT-3, 10–150 μM of each substrate, and 1 mM UDP-glucose in 50 mM Na_2HPO_4 buffer (pH 7.5). Reaction mixtures were incubated at 37 °C for 30 min.

glucoside. The structure of the reaction product was determined to be isorhamnetin 3-*O*-glucoside using NMR (Table 2) [28]. PGT-3 altered its regioselectivity when 3′-*O*-methyl substrate was utilized.

3.2. Alternation of PGT-3 based on molecular docking

The three-dimensional structure of PGT-3 was predicted via protein structure prediction program Prime implemented in Maestro interface using the X-ray structure of the grape UDP-glucose: flavonoid glycosyltransferase, VvGt1 complexed with kaempferol (PDB ID: 2C1Z). In order to determine whether flavonoids such as quercetin, luteolin, isorhamnetin, 6,7-dihydroxyflavone, or 7,8-dihydroxyflavone could fit into the substrate binding site of the modeled structure of PGT-3, docking study was carried out using Glide program (extra precision mode, XP). For each flavonoid, the docking pose with the lowest Glide XP score was extracted from the docked complex. Molecular docking of quercetin, luteolin or isorhamnetin into the modeled structure of PGT-3 was carried out in order to determine whether quercetin, luteolin or isorhamnetin could fit into the substrate binding site. Both quercetin and luteolin were docked in a position to glucosylate the 4′-hydroxyl group (Fig. 2A and B). In this configuration, Asp45 forms a hydrogen bond with the 5-hydroxyl group of quercetin or luteolin, and His15 serves as a base for the catalytic reaction. In addition, Glu82 appeared to form a hydrogen bond with the 3′-hydroxyl group of either quercetin or luteolin through water. The validation of this model was evaluated by site-directed mutagenesis of Asp45 and Glu82. Glu82 was mutated into Leu. The resulting mutants were purified using the same method as the wild type (Fig. 3). The configuration of isorhamnetin into the substrate binding pocket of PGT-3 was somewhat different from that of either quercetin or luteolin (Fig. 2C). Asp45 forms a hydrogen bond with the 5-hydroxyl group. Instead of forming a hydrogen bond between

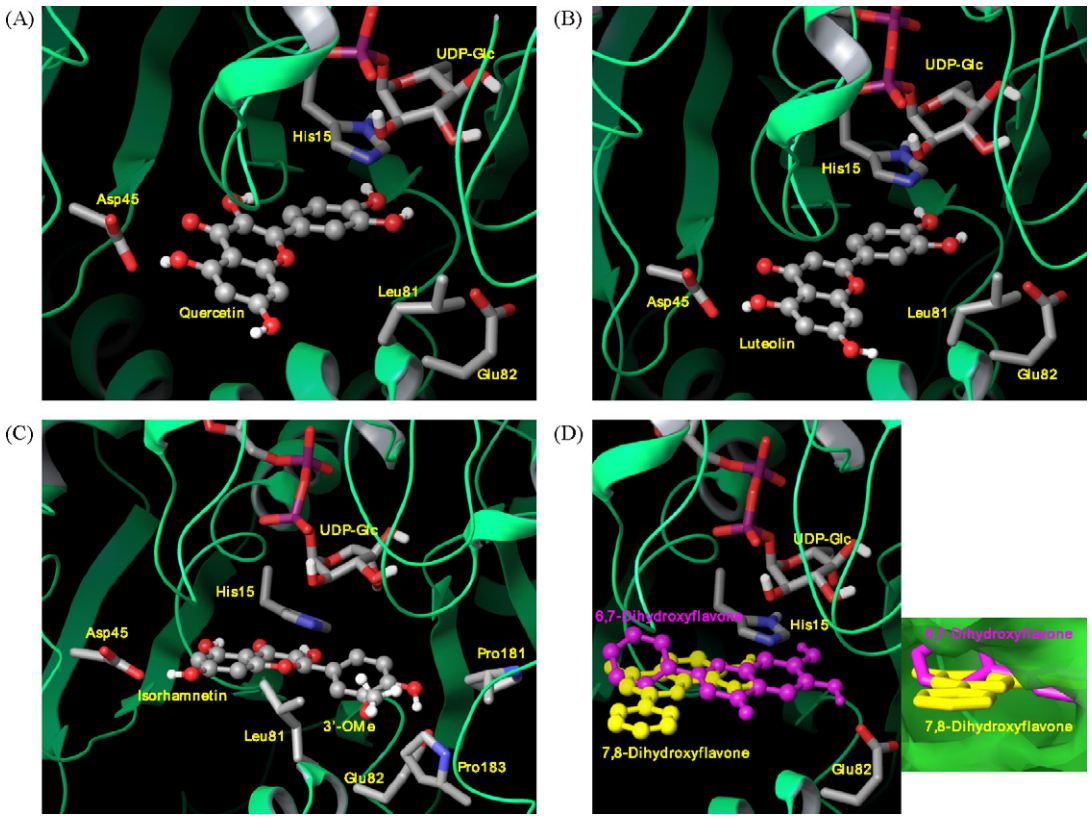


Fig. 2. Molecular docking of quercetin (A), luteolin (B), isorhamnetin (C), 7,8-dihydroxyflavone and 6,7-dihydroxyflavone (D) into PGT-3.

Glu82 and the 3′-hydroxyl group (as found in luteolin or quercetin), a hydrogen bond is formed between Glu82 and the 4′-hydroxyl group of isorhamnetin. In addition, the hydrophobic interaction between the 3′-methoxy group of isorhamnetin and a hydrophobic pocket formed by Leu81, Pro181, and Pro183, is also important for the position of isorhamnetin in the substrate binding pocket. Based on the modeled structure of PGT-3, these three interactions seem to place isorhamnetin into a position for 3-*O*-glucosylation.

The reaction of quercetin or luteolin with Glu82Leu did not yield a product (data not shown). This indicated that the mutation of Glu82 into Leu totally abolished the reactivity toward quercetin or luteolin and that hydrogen bond formation between Glu82 and the 3′-hydroxyl group of quercetin or luteolin was critical for the reactivity of PGT-3. On the other hand, reactivity of Glu82Leu toward isorhamnetin increased approximately 68.5% compared to the wild type (Table 3). These results suggested that the substrate specificity of PGT-3 was changed by a point mutation (Fig. 4A). Also, the result of the reaction of isorhamnetin with Glu82Leu indicates that the hydrophobic interaction between the 3′-methoxy group of isorhamnetin and the hydrophobic pocket is more important than a hydrogen bond formed by Glu82 and the 4′-hydroxyl group of isorhamnetin.

An Asp45Val mutant was also created by site-directed mutagenesis. Asp45Val converted only 20% of quercetin into its corresponding glucoside compared to the wild type. However, this mutation reduced activity toward isorhamnetin by 20%, though. This indicated that the hydrogen bond between Asp45 and the 5-hydroxyl group of the substrate contributed to the reactivity of PGT-3.

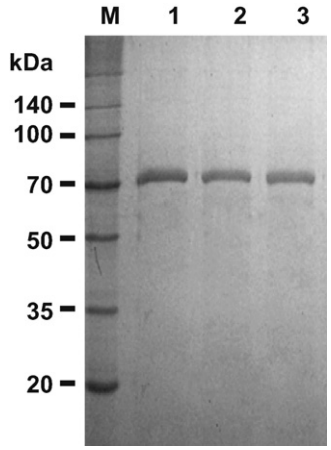


Fig. 3. SDS-PAGE analysis of the purified recombinant wild type and mutant PGT-3. 1, wild type; 2, Glu82Leu; 3, Asp45Val.

3.3. *Biosynthesis of isorhamnetin 3-O-glucoside from quercetin using E. coli transformant harboring two genes*

Isorhamnetin 3-*O*-glucoside has an effect on diabetic complications by inhibiting aldose reductase (AR) [29]. AR catalyzes the conversion of glucose into sorbitol using NADPH and is considered as a target for the control of diabetic complications [19] and cataract formation in the lens of the eye [30]. The major source of this compound is from *Salicornia herbacea* [29] or *Brassica campestris* [13]. We reasoned that isorhamnetin 3-*O*-glucoside was able to be synthesized from quercetin using two sequential enzymatic reactions; 3′-*O*-methylation followed by 3-*O*-glucosylation (Fig. 4B). We previously showed that *O*-methyltransferase from rice (ROMT9) converted quercetin to isorhamnetin [18], which was eventually converted into isorhamnetin 3-*O*-glucoside by PGT-

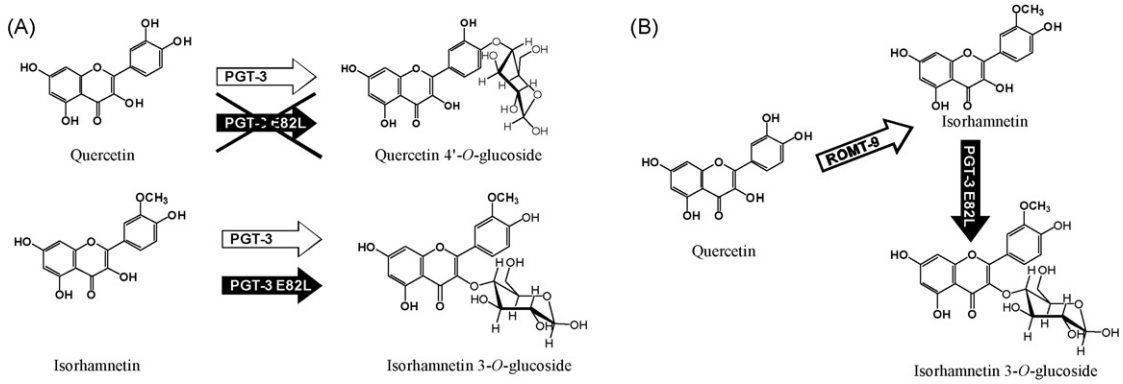


Fig. 4. (A) Glucosylation reaction of PGT-3 or PGT-3 Glu82Leu mutant with quercetin and isorhamnetin and (B) production of isorhamnetin 3-*O*-glucoside from quercetin with ROMT9 and PGT-3 Glu82Leu.

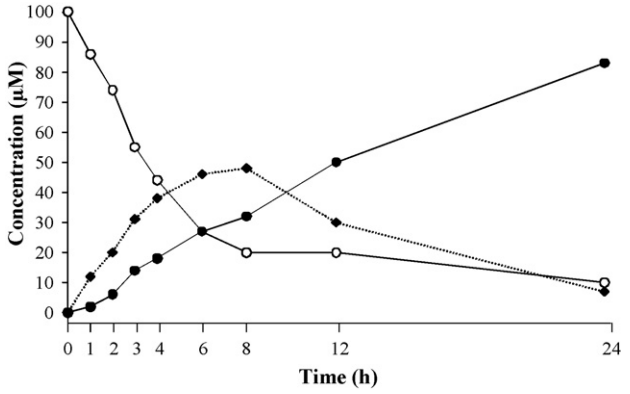


Fig. 5. Production of isorhamnetin 3-*O*-glucoside from quercetin using two *E. coli* transformants. (–○–) Quercetin; (–♦–) isorhamnetin; (–●–) isorhamnetin 3-*O*-glucoside.

3. However, wild type PGT-3 can catalyze the reaction of both isorhamnetin and quercetin, making two glucosylated flavonoids, isorhamnetin 3-*O*-glucoside and quercetin 4′-*O*-glucoside. Thus, the PGT-3 mutant, Glu82Leu was used because it showed specificity only for isorhamnetin. In addition, this mutant was more efficient toward isorhamnetin than quercetin. Using the Glu82Leu mutant along with ROMT9, the conversion of isorhamnetin 3-*O*-glucoside from quercetin was carried out. Two *E. coli* transformants, one with ROMT9 and the other with PGT-3 Glu82Leu mutant, were induced separately. The same amounts of both *E. coli* transformants were mixed. And then, 100 μM of quercetin was added to the culture medium and the conversion of quercetin was monitored. Isorhamnetin was appeared more quickly and continued to increase for 8 h. The concentration of quercetin decreased for 24 h to a final concentration of 10 μM. Production of isorhamnetin 3-*O*-glucoside was observed after only 1 h incubation and its concentration was increased to 83 μM (Fig. 5). Using this approach, we successfully synthesized isorhamnetin 3-*O*-glucoside from quercetin. The synthesis of flavonoid backbones from caffeic acid using several genes in flavonoid biosynthetic pathway has been accomplished [31]. Construction of an entire pathway in a heterologous system like *E. coli* is valuable, but the final yield of the flavonoid is not high enough for the efficient mass production for biosynthesis of other flavonoids with biological activities. In addition, the *E. coli* strain harboring several genes suffered from metabolic load. Thus, the addition of any other flavonoid modification genes could increase the metabolic load. Alternative approaches to produce a particular flavonoid would be bioconversion of simple and inexpensive flavonoids to valuable flavonoid. Usually, *E. coli* transformants containing one or two genes are sufficient for the production of valuable flavonoids.

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