

Purification and characterization of UDP-glucose: anthocyanin 3',5'-*O*-glucosyltransferase from *Clitoria ternatea*

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Abstract A UDP-glucose: anthocyanin 3',5'-*O*-glucosyltransferase (UA3'5'GT) (EC 2.4.1.-) was purified from the petals of *Clitoria ternatea* L. (Phaseoleae), which accumulate polyacylated anthocyanins named ternatins. In the biosynthesis of ternatins, delphinidin 3-*O*-(6''-*O*-malonyl)- β -glucoside (**1**) is first converted to delphinidin 3-*O*-(6''-*O*-malonyl)- β -glucoside-3'-*O*- β -glucoside (**2**). Then **2** is converted to ternatin C5 (**3**), which is delphinidin 3-*O*-(6''-*O*-malonyl)- β -glucoside-3',5'-di-*O*- β -glucoside. UA3'5'GT is responsible for these two steps by transferring two glucosyl groups in a stepwise manner. Its substrate specificity revealed the regioselectivity to the

anthocyanin's 3'- or 5'-OH groups. Its kinetic properties showed comparable k_{cat} values for **1** and **2**, suggesting the subequality of these anthocyanins as substrates. However, the apparent K_{m} value for **1** (3.89×10^{-5} M), which is lower than that for **2** (1.38×10^{-4} M), renders the $k_{\text{cat}}/K_{\text{m}}$ value for **1** smaller, making **1** catalytically more efficient than **2**. Although the apparent K_{m} value for UDP-glucose (6.18×10^{-3} M) with saturated **2** is larger than that for UDP-glucose (1.49×10^{-3} M) with saturated **1**, the k_{cat} values are almost the same, suggesting the UDP-glucose binding inhibition by **2** as a product. UA3'5'GT turns the product **2** into a substrate possibly by reversing the B-ring of **2** along the C2-C1' single bond axis so that the 5'-OH group of **2** can point toward the catalytic center.

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Abbreviations

DTT	Dithiothreitol
<i>p</i> APMSF	<i>p</i> -Amidinophenylmethylsulfonylfluoride
TFA	Trifluoroacetic acid
UA3'GT	UDP-glucose: anthocyanin 3'- <i>O</i> -glucosyltransferase
UA3GT	UDP-glucose: anthocyanidin 3- <i>O</i> -glucosyltransferase
UA3'5'GT	UDP-glucose: anthocyanin 3',5'- <i>O</i> -glucosyltransferase
UA5'GT	UDP-glucose: anthocyanin 5'- <i>O</i> -glucosyltransferase

Introduction

Flower colors ranging from reddish to bluish are mainly due to the various structures of anthocyanins which accumulate in the flowers. They are the glycosides or acylated glycosides of anthocyanidin aglycones. Although anthocyanin structure is of primary importance, there are other factors which influence flower color by interacting with anthocyanin molecules including some flavone and flavonol glycosides, metal ions, and vacuolar pH (Brouillard and Dnagles 1994).

The blue flowers in many plants such as *Clitoria ternatea*, *Delphinium hybridum*, *Eustoma grandiflora*, *Gentiana triflora*, contain delphinidin as anthocyanidin aglycone which are generally glycosylated, and acylated by aromatic organic acids (Honda and Saito 2002). These modifications are important enabling the plant to produce blue flowers because the mauve flower line of *C. ternatea* accumulates delphinidin 3-*O*-(6''-*O*-malonyl)- β -glucoside (**1**) which lacks the modifications (Kazuma et al. 2003). The effect of acylation can be explained as the intramolecular stacking between delphinidin chromophore and aromatic-acyl residue(s), which produces a bathochromic shift in the visible light absorption wavelength of anthocyanins. Thus, polyacylation of anthocyanins with aromatic-acyl groups is one of the mechanisms to produce blue flowers. The acylation at anthocyanin's B-ring sugar residue (3'-*O*-glucosyl residue) has been suggested as the most appropriate site for the bluish color in the *G. triflora* anthocyanin, gentiodelphin (Yoshida et al. 2000).

Ternatins are a group of polyacylated anthocyanins found in the deep blue flower of *C. ternatea* (Terahara et al.

1998). They all have 3',5'-di-*O*-glucosylated structure in delphinidin's B-ring (Figs. 1, 2), and both glucosyl residues are alternately acylated and glucosylated in various repetition by *p*-coumaroyl and glucosyl residues. The importance of 3',5'-di-*O*-*p*-coumaroylglucosylated structure is that two *p*-coumaroyl residues are thought to sandwich delphinidin chromophore between them (Terahara et al. 1996). In addition, ternatins are significantly more stable than gentiodelphin, a 5,3'-di-*O*-acylglucosyldelphinidin, in a neutral solution (Saito et al. 1986).

Anthocyanin glycosyltransferases are important for the flower colors and the stability of the anthocyanins because they decide the positions where glycosyl residues are transferred. Among them, anthocyanin B-ring glycosyltransferases are of interest in relation to the blue flower color. Recently, anthocyanin 3'-*O*-glucosyltransferase was isolated (Fukuchi-Mizutani et al. 2003) from *G. triflora*. However, no anthocyanin 5'-*O*-glucosyltransferase activity has been reported. In this study, we purified anthocyanin 3',5'-*O*-glucosyltransferase, a bifunctional enzyme which has both anthocyanin 3'- and 5'-*O*-glucosyltransferase activities, for the first time from *C. ternatea* and characterized its properties in detail.

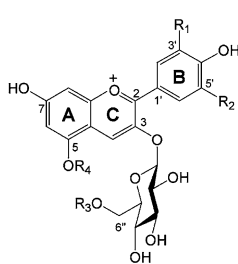
Materials and methods

General

For HPLC analysis of reaction mixtures, a Waters 600s HPLC system equipped with a 717 autosampler and a 996

Fig. 1 Chemical structures of anthocyanins and some spectroscopic and other chemical data for anthocyanins

	R ₁	R ₂	R ₃	R ₄
Delphinidin 3- <i>O</i> -(6''- <i>O</i> -malonyl)- β -glucoside (1)	OH	OH	malonyl	H
Delphinidin 3- <i>O</i> -(6''- <i>O</i> -malonyl)- β -glucoside-3'- <i>O</i> - β -glucoside (2)	<i>O</i> -glucosyl	OH	malonyl	H
Delphinidin 3- <i>O</i> -(6''- <i>O</i> -malonyl)- β -glucoside-3',5'-di- <i>O</i> - β -glucoside, ternatin C5 (3)	<i>O</i> -glucosyl	<i>O</i> -glucosyl	malonyl	H
Pelargonidin 3- <i>O</i> -(6''- <i>O</i> -malonyl)- β -glucoside (4)	H	H	malonyl	H
Cyanidin 3- <i>O</i> -(6''- <i>O</i> -malonyl)- β -glucoside (5)	OH	H	malonyl	H
Cyanidin 3- <i>O</i> -(6''- <i>O</i> -malonyl)- β -glucoside-3'- <i>O</i> - β -glucoside (6)	<i>O</i> -glucosyl	H	malonyl	H
Petunidin 3- <i>O</i> -(6''- <i>O</i> -malonyl)- β -glucoside (7)	OCH ₃	OH	malonyl	H
Petunidin 3- <i>O</i> -(6''- <i>O</i> -malonyl)- β -glucoside 5'- <i>O</i> - β -glucoside (8)	OCH ₃	<i>O</i> -glucosyl	malonyl	H
Delphinidin 3- <i>O</i> - β -glucoside (9)	OH	OH	H	H
Delphinidin 3,3',5'-tri- <i>O</i> - β -glucoside (10)	<i>O</i> -glucosyl	OH	H	H
Delphinidin 3,3',5'-tri- <i>O</i> - β -glucoside (11)	<i>O</i> -glucosyl	<i>O</i> -glucosyl	H	H
Delphinidin 3,5-di- <i>O</i> - β -glucoside (12)	OH	OH	H	glucosyl
Delphinidin 3,5-di- <i>O</i> -(6- <i>O</i> -acetyl)- β -glucoside (13)	OH	OH	acetyl	acetylglucosyl



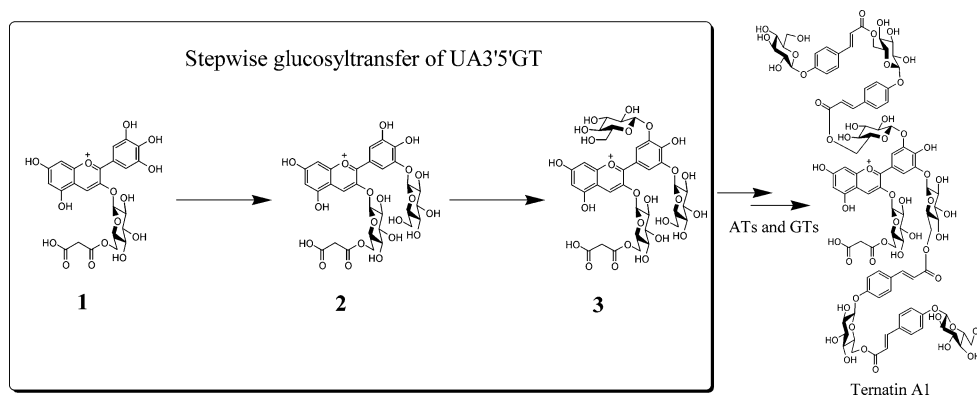
Spectroscopic and other chemical data for several compounds

Cyanidin 3-*O*-(6''-*O*-malonyl)- β -glucoside-3'-*O*- β -glucoside (**6**). UV (0.1% HCl-MeOH) λ_{\max} nm (log ϵ): 515 (4.4), 277 (4.2); AlCl₃/HCl: 519 and 276. The lack of a bathochromic shift on addition of AlCl₃ is the evidence of 3'-*O*-glucosylation (Markham 1982); HR-MALDI-TOF-MS, *m/z* 697.1598 [M+H]⁺, calc. for C₃₀H₃₃O₁₉, 697.1616; ESI-MS, *m/z* 697 [M+H]⁺; ESI-MS/MS from *m/z* 697, 653 [M-CO₂+H]⁺, 611 [M-malonyl+H]⁺, 535 [M-glucosyl+H]⁺, 449 [M-malonylglucosyl+H]⁺, 287 [cyanidin+H]⁺

Petunidin 3-*O*-(6''-*O*-malonyl)- β -glucoside 5'-*O*- β -glucoside (**8**). UV (0.1% HCl-MeOH) λ_{\max} nm (log ϵ): 530 (4.3), 279 (4.0); AlCl₃/HCl: 535, 361, and 276. The lack of a bathochromic shift on addition of AlCl₃ is the evidence of 5'-*O*-glucosylation (Markham 1982); HR-MALDI-TOF-MS, *m/z* 727.1699 [M+H]⁺, calc. for C₃₁H₃₅O₂₀, 727.1722; ESI-MS, *m/z* 727 [M+H]⁺; ESI-MS/MS from *m/z* 727, 683 [M-CO₂+H]⁺, 641 [M-malonyl+H]⁺, 565 [M-glucosyl+H]⁺, 479 [M-malonylglucosyl+H]⁺, 317 [petunidin+H]⁺

Delphinidin 3,5-di-*O*- β -glucoside (**12**). TLC on cellulose with aq. 10% acetic acid, R_f 0.34 for **12**, R_f 0.60 for **13**; ESI-MS, *m/z* 627 [M+H]⁺; MS/MS from *m/z* 627, 465 [M-glucosyl+H]⁺, 303 [delphinidin+H]⁺

Fig. 2 Early biosynthetic steps of ternatins. UA3'5'GT is a bi-functional glucosyltransferase which possesses both UA3'GT and UA5'GT activities. UA3'5'GT is responsible for the conversion of **1** to **3** via **2**, the key biosynthetic steps of ternatins production. Then, **3** is further converted to ternatin A1, the largest ternatin, by *p*-coumaroyltransferases (ATs) and glucosyltransferases (GTs)



photodiode array detector was used with a Develosil ODS-UG-5 column (1.5 i.d. × 250 mm; Nomura Chemical Co. Ltd., Seto, Japan). For DEAE Toyopearl and Mono-Q chromatography, a Fast Protein Liquid Chromatography (FPLC) system equipped with a P-500 pump, a UV-1 detector, a 50-ml superloop, and a Frac-100 fraction collector (Amersham Biosciences, Piscataway, NJ, USA) was used. Protein concentration was determined by the Bradford method (Bradford 1976) with BSA as a standard.

Plant material

Seeds of *C. ternatea* cv. Double Blue (DB) were purchased from Sakata Seed Ltd., Yokohama, Japan, and plants were cultivated in a greenhouse. Flower buds were harvested just before blooming (40–45 mm length) and stored at -80°C until use.

Standard assay conditions and analysis of the reaction mixture

For enzymatic assays, delphinidin 3-*O*-(6''-*O*-malonyl)- β -glucoside-3'-*O*- β -glucoside (**2**) was used as the substrate and production of ternatin C5 (**3**) was measured. The standard assay mixture consisted of 2.0 mM of **2** in 40% MeCN containing 0.05 M Trifluoroacetic acid (TFA) (4 μl), 5.0 mM UDP-glucose in distilled water (4 μl), enzyme solution (8 μl), 1.0 M potassium phosphate buffer (pH 7.5, 2 μl), and distilled water (2 μl) in a final volume of 20 μl . The reaction was initiated by adding the anthocyanin solution to the rest of the assay mixture. The reaction was terminated by the addition of 4 μl of 1.0 M HCl. For detection of the activity, the reaction times of 10 min were used. However, for determining the initial velocity, three reaction times of 30, 60, and 90 s were used. Prior to HPLC analysis, the reaction was mixed with 5% MeCN containing 0.05 M TFA (24 μl). The reaction mixtures were analyzed by HPLC with a 14–86% linear gradient of solvent B (40% MeCN in aq. 0.05 M TFA) in solvent A (5% MeCN in aq. 0.05 M TFA) for 20 min at a flow rate of 0.5 ml/min.

Purification of UA3'5'GT

The buffer systems used were: buffer A, 100 mM Tris–HCl, pH 7.5; buffer B, 20 mM Tris–HCl, pH 7.5; buffer C, 20 mM Tris–HCl, pH 7.5, containing 1.0 M NaCl; buffer D, 5.0 mM Mes, pH 7.0; buffer E, 5.0 mM Tris–HCl, pH 8.0, containing 10 mM UDP-glucose; buffer F, 2.0 mM sodium/potassium phosphate, pH 7.5; buffer G, 15 mM sodium/potassium phosphate, pH 7.5; and buffer H, 30 mM sodium/potassium phosphate, pH 7.5. Dithiothreitol (DTT) and *p*-Amidinophenylmethylsulfonylfluoride (*p*APMSF) were added to each buffer just before use to final concentrations of 5.0 mM and 10 μM , respectively (buffer A), or 1.0 mM and 10 μM , respectively (buffers B–H).

All the purification procedures were carried out at 4°C . Powdered frozen petals (1,035 g) mixed with polyvinyl-pyrrolidone (51 g) were extracted three times with buffer A. The extract was stirred with Dowex 1 × 2 (*ca.* 1 kg) (Muromachi Technos Co. Ltd., Tokyo, Japan) to remove pigments. After filtered, the extract was brought to $(\text{NH}_4)_2\text{SO}_4$ precipitation. The 35–70% $(\text{NH}_4)_2\text{SO}_4$ fraction was retrieved and desalted with a Sephadex G-25 (Amersham Biosciences) column (void volume 38 ml). The protein solution (333 ml) in buffer B was brought to DEAE-Toyopearl 650M (Tosoh Co., Tokyo, Japan) anion exchange chromatography (three times, using *ca.* 100 ml at once). The elution with a 0–20% linear gradient of buffer C in buffer B for 45 min at a flow rate of 8 ml/min using an FPLC system could give an active fraction in 6.6–10.2% buffer C. The proteins were retrieved by 90% $(\text{NH}_4)_2\text{SO}_4$ saturation and desalted with the Sephadex G-25 column. Two halves of the protein solution in buffer D (*ca.* 17 ml each) were passed through two columns (10 mm i.d. × 120 mm, containing 10 ml of the resin) of Reactive Yellow 86 resin (Sigma, St. Louis, MO, USA) in parallel. After washing with buffer D (52.5 ml each), an active fraction was eluted with buffer E (50 ml each), and *ca.* 14-times concentrated with Amicon Ultra-15 10K NMWL units (Millipore, Bedford, MA, USA) followed by buffer-exchange to buffer F with PD-10 (Amersham Biosciences) columns. Cellulofine HAp (Seikagaku

Co., Tokyo, Japan) column (10 i.d. \times 75 mm, containing 5 ml of the resin) chromatography gave an active fraction by eluting with buffer H (30 ml) after the column was successively washed with buffers F and G (30 ml each). The active fraction was 60-times concentrated with an Amicon Ultra-15 10K NMWL unit followed by buffer-exchange to buffer B with a NAP-10 column (Amersham Biosciences). A column chromatography with pre-packed Mono Q HR 5/5 column (0.5 i.d. \times 5 cm; Amersham Biosciences) gave an active fraction in 13.1–14.4% buffer C when it was eluted with a 0–25% linear gradient of buffer C in buffer B for 60 min at a flow rate of 1.0 ml/min using an FPLC system. The active fraction was finally concentrated to 2.7 ml by ultrafiltration in an Ultracent-30 unit (Tosoh Co.). UDP-glucose: anthocyanin 3',5'-*O*-glucosyltransferase (UA3'5'GT) was purified several times to obtain enough amount of enzyme for determining enzyme characteristics.

Determination of molecular mass

The molecular mass of the protein was determined by SDS-PAGE and gel filtration. Gel filtration was performed on a calibrated Superdex 75 HR 10/30 column (Pharmacia, Kalamazoo, MI, USA) with 20 mM Tris-HCl buffer of pH 7.5 containing 150 mM NaCl at a flow rate of 0.5 ml/min. The column was calibrated with molecular mass standards of albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease (13.7 kDa).

Preparation and identification of substrates and products

1 (Kazuma et al. 2003), **2**, **3**, and delphinidin 3,3'-di-*O*- β -glucoside (**10**) (Kazuma et al. 2004), cyanidin 3-*O*-(6''-*O*-malonyl)- β -glucoside (**5**) and petunidin 3-*O*-(6''-*O*-malonyl)- β -glucoside (**7**) (Kogawa et al. 2007), and delphinidin 3,3',5'-tri-*O*- β -glucoside (**11**) (Terahara et al. 1990) were prepared in our laboratory. Cyanidin 3-*O*-(6''-*O*-malonyl)- β -glucoside-3'-*O*- β -glucoside (**6**) and petunidin 3-*O*-(6''-*O*-malonyl)- β -glucoside 5'-*O*- β -glucoside (**8**) were prepared from **5** to **7**, respectively, with the glucosyltransferase activities in the crude petal extract of *C. ternatea* cv. DB in the presence of UDP-glucose. Delphinidin 3,5-di-*O*- β -glucoside (**12**) was prepared by alkaline hydrolysis of a crude acidic methanolic extract from flowers of *Verbena hybrida* cv. Tapien Blue-Violet, which accumulates delphinidin 3,5-di-*O*-(6-*O*-acetyl)- β -glucoside (**13**) (Toki et al. 1991). Spectroscopic and other chemical data for **6**, **8**, and **12** were shown in Fig. 1.

Determination of optimum pH, metal ion requirements, effects of chemical reagents, and substrate specificities

The same conditions as those of the standard assay were used unless otherwise stated. For all reactions, the concen-

tration of UDP-glucose was 150 mM, and the purified UA3'5'GT solution (19.6 μ g/ml) was used. Three buffer systems with various pHs (1.0 M acetate buffers of pHs 4.0, 5.0, and 5.5; 1.0 M potassium phosphate buffers of pHs 6.0, 6.5, 7.0, 7.5, and 8.0; 1.0 M Tris-HCl buffers of pHs 7.5, 8.0, 8.5, and 9.0) were used to determine the optimum pH, and three reaction times of 60, 90, and 120 s were used.

To assess the metal ion requirements, 10 mM aqueous solutions of MgCl₂, CaCl₂, FeCl₂, CuCl₂, ZnCl₂, and ethylenediaminetetraacetic acid (EDTA) were prepared. To the standard reaction mixture, 2.0 μ l of each solution were added instead of distilled water. Reaction times of 60, 90, and 120 s were used. To assess the effects of chemical reagents, 10 mM solutions of *p*-chloromercuribenzoic acid (PCMB), *N*-ethylmaleimide (NEM), iodoacetate, diethylpyrocarbonate (DEPC), and dithioerythritol (DTE) were prepared. To the standard reaction mixture, 2.0 μ l of each solution were added instead of distilled water and reaction times of 60, 90, and 120 s were used.

To determine the substrate specificities, 2.0 mM solutions of **1**, **2**, pelargonidin 3-*O*-(6''-*O*-malonyl)- β -glucoside (**4**), **5**, **6**, **7**, delphinidin 3-*O*- β -glucoside (**9**), **10**, **11**, and **12** were prepared, and the reaction times of 30, 60, and 90 s were used.

Detection of UA3'GT and UA3GT activities

The same conditions as those of the standard assay were used except for the substrates. For the detection of UA3'GT activity, **1** was used for the substrate, and the production of **2** was measured. For the detection of UA3GT activity, delphinidin chloride was used for the substrate, and the production of delphinidin 3-*O*-glucoside was measured.

Steady-state kinetic analyses of UA3'5'GT

The reaction conditions were the same as the standard assay conditions except for the reaction time and substrate concentrations. Three reaction times of 30, 60, and 90 s were used to determine the initial velocity. When parameters for **1** or **2** were determined, protein solutions of 20.7 and 19.6 μ g/ml were used, respectively. The dependence of the activity on various concentrations of UDP-glucose (0.1–30 mM) was analyzed at a fixed concentration (400 μ M) of **2**. The UDP-glucose concentration was held constant (30 mM), and the concentration of **2** was varied (10–400 μ M). Then, the UDP-glucose concentration was varied (0.1–30 mM) while holding the concentration of **1** fixed (400 μ M). Finally, the concentration of **1** was varied (10–400 μ M) while holding the UDP-glucose concentration constant (30 mM). Hanes-Woolf plots produced from the data sets were used to calculate apparent K_m and V_{max} values. To calculate turnover numbers, the molecular weight of 50.8 kDa from SDS-PAGE was used.

Results

Purification of UA3'5'GT and molecular mass

UDP-glucose: anthocyanin 3',5'-*O*-glucosyltransferase was purified by monitoring its UA5'GT activity, which converts **2** into **3** (Figs. 1, 2) in the presence of UDP-glucose. It was purified in 1,007-fold purification with the purification steps summarized in Table 1. During the purification, UA3'GT and UA3GT activities were also measured at each purification step. While UA3GT activity was excluded in the early steps, UA3'GT activity remained throughout the steps. Moreover, both activities were similarly concentrated at each purification step, which suggests the same origin expressing both UA3'GT and UA5'GT activities.

SDS-PAGE revealed that the final active fraction showed a single band of 50.8 kDa (Fig. 3). The molecular mass is similar to those of known flavonoid glycosyltransferases (40–59 kDa), however smaller than the UDP-glucose: anthocyanin 3'-*O*-glucosyltransferase (UA3'GT) of *G. triflora* (55 kDa in SDS-PAGE) (Fukuchi-Mizutani et al. 2003). Gel-filtration chromatography showed a molecular mass of 48 kDa.

Substrate specificity

The substrate specificities for several anthocyanins and UDP-sugars were examined by determining the specific activities (Table 2). UA3'5'GT converted **7** to **8**. In addition, **1** and **5** were also the substrates for this enzyme, with **2** and **6** produced, respectively. Therefore, it can transfer a glucosyl group not only to the 5' position of anthocyanins but also to the 3' position, namely, UA3'5'GT has UA3'GT activity as well as UA5'GT activity. Further, the enzyme could use neither **4** nor **6** as a substrate, suggesting that it cannot transfer a glucosyl group to the 5, 7, or 4' OH groups when there are no OH groups at the 3' or 5' positions. UA3'5'GT is highly regioselective to the 3' and 5' OH groups.

Although there is no simple correlation of the enzymatic activity and the acid dissociation constant (pK_a) of acceptor compounds, it was still of interest whether the reaction was accelerated or decelerated by the acidity of the 3' or 5' OH groups. The only structural differences among **1**, **2**, **5**, and **7** are in the substituent group of the B-ring at the *meta* position of the OH group that participates in the reaction: $R_1 = OH$ for **1**, $R_1 = O$ -glucosyl for **2**, $R_1 = methoxy$ for **7**, and no substitution ($R_2 = H$) for **5** (Fig. 1). All of these substituent groups accelerated the reaction 2.3–5.1-fold (in terms of relative activity) compared to **5**. The OH group that participates in the reaction in these anthocyanins might be more acidic than that for **5** because *m*-OH and *m*-methoxy groups generally work as electron-withdrawing groups as seen in resorcinol and *m*-methoxyphenol (Gawron et al.

Table 1 Purification of UA3'5'GT

Purification step	Protein (mg)	UA5'GT			UA3'GT			UA3GT		
		Total activity (pkat)	Specific activity (pkat mg/protein)	Purification (-fold)	Total activity (pkat)	Specific activity (pkat mg/protein)	Purification for each step ^a (-fold)	Total activity (pkat)	Specific activity (pkat mg/protein)	Purification for each step ^a (-fold)
Ammonium sulphate	2,330	87,729	38	1	109,399	47		307,700	132	
DEAE Toyopearl	226	113,980	504	13	67,248	297	6.3	25,733	114	6.3
Reactive yellow 86	4.5	78,962	17,547	462	41,865	9,303	31.3	n.d	n.d	31.3
Cellulofine HAP	0.296	6,817	23,055	607	3,040	10,280	1.1	n.d	n.d	1.1
Mono Q	0.0527	2,017	38,267	1,007	857	16,270	1.6	n.d	n.d	1.6

During purification, UA5'GT, UA3'GT, and UA3GT activities were measured throughout the purification steps

^a Purification was calculated in comparison to the specific activity of the one step former fraction

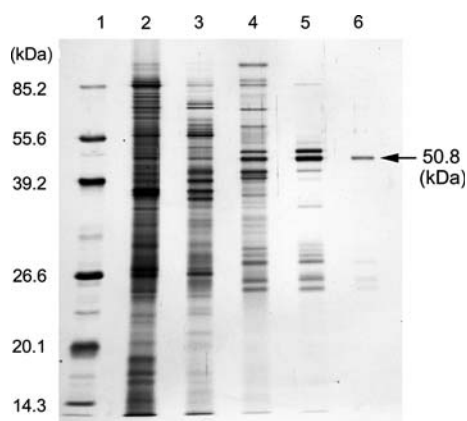


Fig. 3 SDS-PAGE of active fractions during purification of UA3'5'GT. Lane 1, Marker proteins; lane 2, ammonium sulfate (0.75 µg protein); lane 3, DEAE Toyopearl (0.28 µg protein); lane 4, reactive yellow 86 (0.30 µg protein); lane 5, hydroxyapatite (0.13 µg protein); lane 6, Mono Q (0.021 µg protein). Numbers in parentheses indicate the amount of protein applied. The proteins were visualized by silver staining

Table 2 Substrate specificities of UA3'5'GT

Substrate	Glucosyl donor	Products	Specific activity (pkat mg/protein)	Relative activity (%)
2	UDP-glucose	3	138	100
1	UDP-glucose	2	76.8	55.7
6	UDP-glucose	None	n.d	n.d
4	UDP-glucose	None	n.d	n.d
12	UDP-glucose	None	n.d	n.d
10	UDP-glucose	11	4.6	3.3
9	UDP-glucose	10	5.4	3.9
7	UDP-glucose	8	170	123
5	UDP-glucose	6	33.5	24.3
2	UDP-galactose	None	n.d	n.d

Relative activities are expressed as the percent of the specific activity at pH 7.5 for **2**. "None" indicates no products were found, and "n.d" indicates "not detected". For the structures of the compounds, see Fig. 1

1952). A slightly more acidic OH group on the acceptor anthocyanin (**1**, **2**, and **7**) might experience an easier formation of a phenoxide nucleophile than **5**, resulting in acceleration of the reaction.

Finally, none of the demalonylated anthocyanins were favored substrates. **9** and **10** decreased the specific activity by about 30-fold and 26-fold, respectively. In addition, the enzyme could not use **12**. Although UDP-galactose was not used as a donor substrate, several recombinant glucosyltransferases have been reported to use UDP-galactose (Offen et al. 2006).

Optimum pH, ion requirements, and effects of chemical reagents

UDP-glucose: anthocyanin 3',5'-*O*-glucosyltransferase has both UA3'GT and UA5'GT activities. For its UA5'GT activity, it showed an optimum pH of 7.5–8.0 [pH 4.5 (0 nkat/mg), 5.0 (8.8), and 5.5 (26.0) for acetate buffer; pH 6.0 (122.2 nkat/mg), 6.5 (158.5), 7.0 (165.4), and 7.5 (213.9) for potassium phosphate buffer; pH 7.5 (148.7 nkat/mg), 8.0 (213.9), 8.5 (128.7), and 9.0 (88.9) for Tris–HCl buffer].

None of the tested metal ions promoted its activity. The enzyme was completely inhibited by Cu²⁺ ion (0 nkat/mg protein; 0% of the original specific activity of 35.3 nkat/mg protein), but moderately inhibited by Zn²⁺ ion (22.0; 62%) by which many flavonoid glycosyltransferases are strongly inhibited. Other metal ions such as Mg²⁺ (31.1; 88%), Ca²⁺ (28.9; 82%), and Fe²⁺ (25.8; 73%) as well as EDTA (26.3; 75%) reduced the enzymatic activity to some extent.

Several chemical reagents were tested for their effects on the enzyme activity. PCMB completely inhibited the activity (0 nkat/mg protein; 0% of the original specific activity of 141.5 nkat/mg protein). In addition, NEM strongly reduced the activity (22.7; 16%). The same level of inhibition was observed by DEPC (23.2; 16%). Iodoacetate (119.1; 84%) and DTE (117.4; 83%) moderately inhibited the activity as compared to the other reagents tested here.

Finally, for its UA3'GT activity, it similarly affected by the buffer's pHs, the metal ions, and chemical reagents when comparing to UA5'GT activity (data not shown).

Steady-state kinetic analyses of purified UA3'5'GT

The steady-state kinetics was studied for both the glucosyl acceptor (**1** and **2**) and donor (UDP-glucose) substrates (Table 3). First, the k_{cat} value for **2** (5.4 per s) was similar to that for **1** (4.4 per s), which showed nearly the same rate of transfer of glucose from UDP-glucose. This suggested that the two anthocyanins act almost equally as substrates. However, the apparent K_m value for **2** (138 µM) was 3.5 times greater than that for **1** (38.9 µM), making the **1** catalytically more efficient than **2** (in terms of k_{cat}/K_m). This result suggests that the enzyme favors **1**, an intermediate one step upstream of **2**, rather than **2**, when the concentration of the acceptor anthocyanin is low. These kinetic characteristics are reasonable if the enzyme is responsible for the two subsequent biosynthetic processes that convert **1** to **3** via **2** (Fig. 2), because the enzyme cannot use **2** without producing it from **1** first.

A noticeably large apparent K_m value for UDP-glucose (6.18 mM) was obtained when **2** was saturated. This value is much larger than that reported for flavonoid *O*-glycosyltransferases, although values higher than 1 mM have

Table 3 Steady-state kinetic parameters for purified UA3'5'GT

Substrate		V_{\max} (nkat mg/protein)	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\cdot\text{s}^{-1}$)
Varied	Fixed				
2	UDP-Glc	106	1.38×10^{-4}	5.4	3.9×10^4
1	UDP-Glc	87	3.89×10^{-5}	4.4	1.1×10^5
UDP-Glc	2	96	6.18×10^{-3}	4.9	7.9×10^2
UDP-Glc	1	100	1.49×10^{-3}	5.1	3.4×10^3

For the structures of the compounds, see Fig. 1

been reported for several glycosyltransferases from *Vitis vinifera* (1.2 mM; Do et al. 1995) and *Vigna mungo* (1.67 mM; Ishikura and Mato 1993). The apparent K_m value (1.49 mM) for UDP-glucose (when the **1** was saturated) was also greater than 1 mM, although it was 4.1 times lower than that for UDP-glucose (when **1** was saturated). Despite these differences in the apparent K_m values, the k_{cat} values were almost the same. This suggests that the structure of the anthocyanins does not affect the rate of the product formation from the enzyme-substrate (ES) complex, but lowers the rate of UDP-glucose binding to the enzyme by 4.3-fold (in terms of k_{cat}/K_m). The binding of UDP-glucose is very likely to be inhibited by **2** with its 3'-*O*-glucosyl group probably located in the UDP-glucose binding site because **2** is not only a substrate but also a product converted from **1**.

Discussion

UDP-glucose: anthocyanin 5'-*O*-glucosyltransferase has been of interest because it is one of the key enzymes for producing polyacylated anthocyanins with 3',5'-di-*O*-acylglucosyl groups such as lobelinins, delphinidin 3,7,3',5'-tetra-*O*-*p*-coumaroylglucoside, and ternatins which accumulate in the blue flowers of *Lobelia erinus* (Kondo et al. 1989), *Dianella* spp. (Bloor 2001), and *C. ternatea* (Terahara et al. 1998), respectively. Among these anthocyanins, only ternatins of *C. ternatea* have been studied for their biosynthetic pathways. In this study, UA3'5'GT was first purified as UA5'GT, however, it was also found to possess UA3'GT activity as well. Since it had the similar kinetic parameters for the two neighboring intermediates **1** and **2** (Table 3) in the ternatin biosynthetic pathway (Fig. 2), both intermediates are probably the substrates for this enzyme. In addition, the mauve flower line of *C. ternatea* loses not only UA5'GT but also UA3'GT activities (Kazuma et al. 2004), which can be explained by the lack of this bi-functional UA3'5'GT. This enzyme is, thus, crucial for the blue color expression in *C. ternatea*. Bi-functional glycosyltransferases are rare among glycosyltransferases, however, another bi-functional anthocyanidin glucosyltransferase, UDP-glucose: anthocyanidin 5,3-*O*-glucosyltransferase which catalyzes the transfer of two

glucosyl groups to the 5- and 3-OH groups of cyanidin, was identified recently from roses (Ogata et al. 2005).

The bi-functionality is one of the key characteristics of UA3'5'GT. It is an important structural feature that the B-ring of anthocyanin is able to rotate around C2–C1' single bond axis. In addition, the 3' and 5' positions in the B-ring are the positions symmetrical to each other; namely, they cannot be distinguished if the substituents are the same at those positions (Fig. 4a). Because **2** might bind to the enzyme with its 3'-*O*-glucosyl group located in the UDP-glucose binding site (Fig. 4b), the rotation of the B-ring along the C2–C1' single bond can bring the 3'-*O*-glucosyl group away from the catalytic center and turn the 5'-OH group toward the catalytic center (Fig. 4c). Thus, **2** could exist in one of the two rotational isomers in the active site; the product conformation and the substrate conformation. In the product conformation, the 5'-OH group points into the solvent and the 3'-*O*-glucosyl group points into the UDP-glucose binding site (Fig. 4b). In the substrate conformation, the 5'-OH group is directed toward the active site and the 3'-*O*-glucosyl group points into the solvent (Fig. 4c). Thus, the regioselectivity of UA3'5'GT might come from the positional symmetry of 3'- and 5'-OH groups and the rotatability of the B-ring during the formation of the ES complex. However, these two rotational isomers would be identified only when anthocyanin is in its binding site because the rotation is expected to be much more restricted in the substrate binding site than in a solution.

It would be disadvantageous if the inhibition of UDP-glucose binding were mediated by **2** in planta. Thus, there might be a mechanism that cancels the inhibition by the 3'-*O*-glucosyl group: UDP-glucose might bind first and **2** next, resulting in an ordered bi–bi mechanism that can help to prevent product inhibition by **2**. An ordered bi–bi mechanism was reported for a flavonol-ring-B *O*-glucosyltransferase from *Chrysosplenium americanum* (Khouri and Ibrahim 1984).

Finally, UA3'GT of *G. triflora* transfers one glucosyl group to 3'-OH of **12** (Fukuchi-Mizutani et al. 2003) while it was not reported to be able to use the product, delphinidin 3,5,3'-tri-*O*- β -glucoside, as a substrate. UA3'5'GT is unique in terms of reusing the product **2** as a substrate. A possible explanation for the mechanism behind this unique-

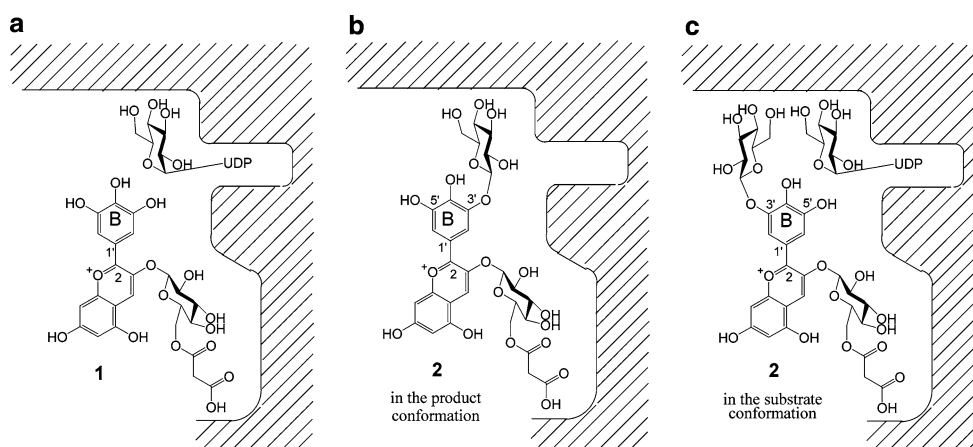


Fig. 4 a–c Proposed substrate binding mode of UA3'5'GT. When **1** (or **2**) binds to the enzyme, its malonyl residue contributes to fix it in the proper position for the reaction in the substrate binding site. **a** When **1** reacts with UDP-glucose, one of the two indistinguishable hydroxyl groups at the 3' and 5' positions participates in the reaction, resulting in the production and release of **2**. **b** When **2** binds to the enzyme as the product of the UA3'5'GT reaction in the product conformation, its 3'-O-glucosyl group is located in the UDP-glucose binding site. **c** However, **2** can also bind to the enzyme in the substrate confor-

mation with its 5'-OH group pointing toward the catalytic center, and its 3'-O-glucosyl group away from the catalytic center by rotating the C2–C1' axis. This is because of an enough space for the 3'-O-glucosyl group at the opposite side of UDP-glucose binding site. The formation of the substrate conformation of **2** is assisted by the ordered bi-bi mechanism (UDP-glucose binds first), which can help to prevent product inhibition by **2**. Then, **2** reacts with UDP-glucose, resulting in the production of **3**. The flavilium cation structure of the delphinidin aglycone is tentatively drawn

ness is that its substrate binding pocket has an enough space for the 3'-O-glucosyl group of **2** at the opposite side of UDP-glucose binding site, which allows **2** to bind to the enzyme in the substrate conformation (Fig. 4c). The three-dimensional structure of UA3'5'GT should enable clarification of mechanism.

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