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Crystallization and preliminary X-ray crystallographic analysis of UDP-glucuronic acid:flavonol-3-O-glucuronosyltransferase (VvGT5) from the grapevine *Vitis vinifera*

Grapevine (*Vitis vinifera*) glycosyltransferase 5 (VvGT5) is a UDP-glucuronic acid:flavonol-3-O-glucuronosyltransferase that catalyses the 3-O-specific glucuronosylation of flavonols using UDP-glucuronic acid as a sugar donor to produce flavonol 3-O-glucosides, which are important bioactive phytochemicals. Recombinant VvGT5 expressed in *Escherichia coli* cells was purified and crystallized by the sitting-drop vapour-diffusion method. A full set of X-ray diffraction data was collected to 2.2 Å Bragg spacing from a single crystal using a synchrotron-radiation source. The crystal was hexagonal, belonging to space group *P*6₁22, with unit-cell parameters *a* = *b* = 102.70, *c* = 535.92 Å. The initial phases were determined by the molecular-replacement method.

1. Introduction

From the beginning of human civilization, grapes (*Vitis vinifera*) have been one of the most important fruit crops. Dietary intake of grape-based products gives diverse biological effects that are beneficial to human health, partly owing to polyphenols such as flavonoids (e.g. flavonols and anthocyanins; Corder *et al.*, 2006). Flavonols, such as quercetin and kaempferol, are bioactive flavonoids, which are most abundant as their 3-O-glycosides (i.e., glucoside, glucuronoside and galactoside) in the berries, seeds and leaves of grapevine (Castillo-Muñoz *et al.*, 2009).

From a food-chemistry viewpoint, flavonol glycosides are important phytochemicals for viticulture and oenology, because they define the colour of wine grapes by acting as copigments (Schwarz *et al.*, 2005). They also have important biomedical activities: the anti-atherosclerotic activity of quercetin glucuronides that are specifically incorporated by macrophages (Kawai *et al.*, 2008), the cytoprotective effects of quercetin 3-O-glucoside (isoquercitrin) by induction of cholesterol biosynthesis (Soundararajan *et al.*, 2008) and the anti-depressant effects of quercetin 3-O-glucuronide (miquelianin), quercetin 3-O-glucoside and quercetin 3-O-galactoside (hyperoside) (Butterweck *et al.*, 2000; Juergenliemk *et al.*, 2003).

Glycosylation is performed by UDP-sugar-dependent glycosyltransferases (UGTs), which catalyse the transfer of the glycosyl group from nucleoside diphosphate-activated sugars to acceptor molecules (Wang, 2009). The UGTs that play essential roles in plant secondary metabolism, including flavonoid biosynthesis, have been classified into 'family 1' (Bowles *et al.*, 2005). These UGTs are characterized by a unique, well conserved sequence of ~45 amino-acid residues (called a PSPG box) and a catalytic mechanism that inverts the anomeric configuration of a transferred sugar (Campbell *et al.*, 1997; Wang, 2009).

The grapevine genome contains as many as 240 UGT genes (Jaillon *et al.*, 2007) and, of these, VvGT1 is the only grapevine UGT for which the structure–function relationships have been well characterized by biochemistry (Ford *et al.*, 1998) and X-ray crystallography (Offen *et al.*, 2006). The enzyme catalyses the 3-O-specific glucosylation of anthocyanidin using UDP-glucose as a sugar donor to produce anthocyanidin 3-O-glucoside, and is involved in the coloration of grape skin. Recently, we identified and characterized a

VvGT1-related gene *VvGT5* (molecular mass 49.7 kDa; Ono *et al.*, 2010). The biochemical analyses of the enzyme revealed that VvGT5 is a UDP-glucuronic acid:flavonol-3-*O*-glucuronosyltransferase, catalysing 3-*O*-specific glucuronosylation using UDP-glucuronic acid as the sugar donor instead of UDP-glucose in the case of VvGT1. *VvGT5* and *VvGT1* share 53% primary sequence identity, suggesting that one of these genes arose from the other by gene duplication (Ono *et al.*, 2010).

It is very interesting how these two enzymes establish their UDP-sugar specificities. The three-dimensional structure of VvGT5 will answer this question *via* the comparison of its catalytic site structure with that of VvGT1 and lead us to propose a scenario for the evolution of plant UGTs, illustrating the plasticity that enhances the chemical diversity of plant secondary metabolites after gene duplication. Here we report the crystallization and preliminary X-ray diffraction study of recombinant VvGT5 produced in *Escherichia coli* cells.

2. Materials and methods

2.1. Protein expression and purification

Recombinant VvGT5 was expressed in *E. coli* BL21 Star (DE3) (Invitrogen) as previously described (Ono *et al.*, 2010). Cells were harvested and stored at 193 K. All purification steps were carried out at 277 K. The frozen cells were thawed and disrupted with an EmulsiFlex-C3 homogenizer (Avestin) in buffer A (20 mM HEPES–NaOH pH 7.4, 10 mM imidazole, 1 mM dithiothreitol) containing protease-inhibitor cocktail (Roche). The sample was centrifuged at 100 000g for 40 min. The supernatant was mixed with Ni–NTA Superflow resin (Qiagen) and incubated for 1 h with stirring. The resin was packed into a column and washed with 20 column volumes of buffer A. Proteins were eluted from the column with buffer B (20 mM HEPES–NaOH pH 7.4, 500 mM imidazole, 1 mM dithiothreitol) and the protein solution was buffer-exchanged in buffer C (20 mM Tris–HCl pH 8.0, 1 mM dithiothreitol). The sample was then applied onto a HiTrap Q HP column (GE Healthcare) and eluted with 30 column volumes of a 0–0.5 M NaCl linear gradient in buffer C. The fractions containing the VvGT5 protein were pooled, concentrated using a Vivaspin 20 (Sartorius) and applied onto a HiLoad 16/60 Superdex 200 column (GE Healthcare), which was developed with buffer D (20 mM Tris–HCl pH 8.0, 150 mM NaCl,

Table 1

Diffraction data-collection and processing statistics for VvGT5.

Values in parentheses are for the highest resolution shell.

X-ray source	BL44XU, SPring-8, Japan
Detector	Rayonix MX225HE
Wavelength (Å)	0.90000
No. of crystals	1
No. of images	937
Space group	<i>P</i> 6 ₁ 22
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = 102.70, <i>c</i> = 535.92
Resolution range (Å)	50–2.2 (2.28–2.20)
Total No. of reflections	987270
No. of unique reflections	84389
Multiplicity	11.7 (3.4)
Average <i>I</i> /σ(<i>I</i>)	20.1 (2.8)
Completeness (%)	97.2 (91.4)
<i>R</i> _{merge} † (%)	12.3 (32.2)
No. of molecules in asymmetric unit	3
<i>V</i> _M (Å ³ Da ^{−1})	2.74
<i>V</i> _{solv} (%)	55.1

† $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the value of the *i*th measurement of the intensity of a reflection, $\langle I(hkl) \rangle$ is the mean value of the intensity of that reflection and the summation is over all measurements.

1 mM dithiothreitol). Retention-time analysis of gel filtration suggested that the VvGT5 protein eluted as a monomer. The VvGT5 protein was purified to homogeneity, as judged by SDS–PAGE. The purified protein was concentrated to 7.0 mg ml^{−1} and stored at 193 K until the crystallization experiments. The estimated yield was 2.4 mg of purified protein from 1 l *E. coli* cell culture. The amount of the protein was determined spectrophotometrically based on absorbance at 280 nm, with a calculation of (A₂₈₀/0.90) mg ml^{−1}.

2.2. Crystallization

The frozen VvGT5 was thawed and 0.4 M UDP-glucose was added to the solution to give final concentrations of 5 mM. Initial crystallization screening of VvGT5 was performed using the commercial screening kits Crystal Screen HT, Crystal Screen Lite, Crystal Screen Cryo and PEGRx from Hampton Research, and MemStart, MemSys and MemGold from Molecular Dimensions, and protein crystals appeared under a variety of conditions. After optimizing the conditions, the best crystal was obtained by the sitting-drop vapour-diffusion method on an Intelli-Plate 96 (Art Robbins Instruments) at 293 K. A drop consisting of 1.0 µl protein solution and 1.0 µl reservoir solution [0.1 M MES–NaOH pH 6.3, 14%(w/v) PEG 4000, 1 M

Figure 1

Crystals of VvGT5 appeared in the presence of UDP-glucose (left) and the crystal was soaked with quercetin (right). The dimensions of the largest crystals are approximately 0.3 × 0.2 × 0.1 mm.

NaCl, 0.2 M MgCl₂, 5 mM UDP-glucose] was equilibrated against 60 µl reservoir solution in the deep well. Crystals appeared within 2 d and grew to maximum dimensions of 0.3 × 0.2 × 0.1 mm in one week. The crystals were then soaked in the modified reservoir solution [0.1 M MES–NaOH pH 6.3, 17% (w/v) PEG 4000, 1 M NaCl, 0.2 M MgCl₂, 20 mM UDP-glucose, 2 mM quercetin, 20% ethylene glycol]. Gradually, the colour of the crystals turned to yellow, suggesting binding of quercetin to the catalytic site of VvGT5 (Fig. 1).

2.3. Data collection and processing

A summary of the data statistics is presented in Table 1. X-ray diffraction data were measured from a single crystal on beamline

BL44XU at SPring-8 (Harima, Japan), using a Rayonix MX225HE detector (Fig. 2). The crystal was fished out with a standard nylon loop and flash-cooled in a nitrogen-gas stream at 100 K. Diffraction data were collected from seven parts of a single crystal. To maximize the resolution of diffraction, the crystal-to-detector distance, crystal oscillation angle per image and beam exposure time were adjusted among the seven wedges in the ranges 257.4–273.0 mm, 0.20–0.30° and 1.8–2.7 s, respectively. A complete data set was collected from 937 images covering 200.6° in total.

3. Results and discussion

The data set was processed with the *HKL-2000* program suite (Otwinowski & Minor, 1997). The crystal of VvGT5 was hexagonal, belonging to space group *P*₆122 or *P*₆522, with unit-cell parameters *a* = *b* = 102.70, *c* = 535.92 Å. Assuming three monomers of VvGT5 in the asymmetric unit, the crystal volume per enzyme mass (*V*_M) and the solvent content (*V*_{sol}) were calculated to be 2.74 Å³ Da^{−1} and 55.1%, respectively (Matthews, 1968). These values are within the frequently observed ranges for protein crystals. Molecular-replacement calculations were performed with the program *MOLREP* (Vagin & Teplyakov, 2010) in the *CCP4* program package (Winn *et al.*, 2011), using a structure of VvGT1 (PDB entry 2c1x; Offen *et al.*, 2006) substituted with polyalanine as the search model. Initial rigid-body and restrained refinements with *REFMAC5* (Murshudov *et al.*, 2011) using the *MOLREP* output model revealed that the crystal belonged to space group *P*₆122 and gave an *R*_{work} of 43.0% and an *R*_{free} of 47.7%. Further structural refinement is in progress.

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Figure 2
An X-ray diffraction image (0.20° oscillation) from a VvGT5 crystal with a 2.2 Å resolution limit.

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