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Purification, crystallization and preliminary X-ray diffraction analysis of a novel keto-deoxy-D-galactarate (KDG) dehydratase from *Agrobacterium tumefaciens*

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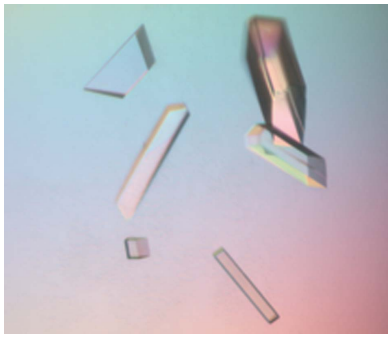
D-Galacturonic acid is the main component of pectin. It could be used to produce affordable renewable fuels, chemicals and materials through biotechnical conversion. Keto-deoxy-D-galactarate (KDG) dehydratase is an enzyme in the oxidative pathway of D-galacturonic acid in *Agrobacterium tumefaciens* (*At*). It converts 3-deoxy-2-keto-L-*threo*-hexarate to α-ketoglutaric semialdehyde. *At* KDG dehydratase was crystallized by the hanging-drop vapour-diffusion method. The crystals belonged to the monoclinic space group *C*2, with unit-cell parameters $a = 169.1$, $b = 117.8$, $c = 74.3$ Å, $\beta = 112.4^\circ$ and an asymmetric unit of four monomers. X-ray diffraction data were collected to 1.9 Å resolution using synchrotron radiation. The three-dimensional structure of *At* KDG dehydratase will provide valuable information on the function of the enzyme and will allow it to be engineered for biorefinery-based applications.

1. Introduction

One of the main components of plant cell walls is pectin, a heteropolysaccharide. It can be found in, for example, citrus peel and sugar beet pulp, which are usually considered as waste but could be a valuable source of raw materials for the biotechnical production of fuels and chemicals. Pectin is rich in D-galacturonic acid, whose catabolism in microbes has been widely studied (Ashwell *et al.*, 1958; Richard & Hilditch, 2009; Chang & Feingold, 1970). There are two routes for D-galacturonic acid degradation in bacteria: the isomerase and the oxidative pathways. The latter was shown to be active in *Agrobacterium tumefaciens* (Zajic, 1959) and *Pseudomonas syringae* (Kilgore & Starr, 1959). In this pathway, uronate dehydrogenase (EC 1.1.1.203) first oxidizes D-galacturonic acid to galactaro-1,4-lactone, with galactaro-1,5-lactone as an enzyme-bound intermediate (Boer *et al.*, 2010; Parkkinen *et al.*, 2011). Galactarolactone cycloisomerase (EC 5.5.1.–) then catalyses ring opening and the formation of 3-deoxy-2-keto-L-*threo*-hexarate (Andberg *et al.*, 2012). The 3-deoxy-2-keto-L-*threo*-hexarate is converted by a decarboxylating hydrolyase (EC 4.2.1.41) into α-ketoglutaric semialdehyde (Jeffcoat *et al.*, 1969; Aghaie *et al.*, 2008). In the final step α-ketoglutaric semialdehyde is oxidized by a dehydrogenase to α-ketoglutarate, which is a metabolite of the TCA cycle (Watanabe *et al.*, 2007).

In *A. tumefaciens* (*At*), the conversion of 3-deoxy-2-keto-L-*threo*-hexarate is catalysed by keto-deoxy-D-galactarate (KDG) dehydratase, an enzyme belonging to the dihydrodipicolinate synthase (DHDPS) family. The crystal structures of several DHDPS family members have been solved. However, *At* KDG dehydratase shares little sequence identity with these enzymes. According to the PDB, its sequence identity to, for example, DHDPS from *Bacillus clausii* (PDB entry 3e96; New York SGX Research Center for Structural Genomics, unpublished work), DHDPS from *B. anthracis* (PDB entry 3hij; Voss *et al.*, 2010) and DHDPS from *Mycobacterium tuberculosis* (PDB entry 1xxx; Kefala *et al.*, 2008) is 29, 27 and 29%, respectively. Intriguingly, the members of this enzyme family, which share a similar three-dimensional TIM-barrel structure (Theodossis *et al.*, 2004), catalyse different types of reactions, for example dehydration, condensation (Dobson *et al.*, 2004) and retro-aldol condensation (Eaton, 1994).



Structure–function studies on *At* KDG dehydratase are crucial for a comprehensive understanding of the metabolic pathways of D-galacturonic acid, which can then be manipulated to develop microbial strains for the production of platform chemicals from plant biomass. In this study, we present the crystallization and preliminary X-ray data analysis of *At* KDG dehydratase.

2. Materials and methods

2.1. Expression and purification of *At* KDG dehydratase

The open reading frame of the KDG dehydratase gene (*At kdg*) was amplified by PCR using primers containing the *Nco*I and *Hin*dIII restriction sites and genomic DNA isolated from *A. tumefaciens* as the template. To create the *Nco*I site, the Asn residue following the N-terminal methionine was changed to an Asp. The genes were ligated into the pBAT4 vector (Peränen *et al.*, 1996) using the *Nco*I and *Hin*dIII restriction sites. *Escherichia coli* strain BL21(DE3) was transformed with the generated plasmid, pBAT4-*At kdg*. A *Strep*-tag II (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) was added to the C-terminus of *At kdg* by PCR amplification using the following primers: Fwd_*At*KDG, 5′-GATATATCCATGGACCCTGAACAAATCAAG-3′ and Rev_*At*KDG, 5′-GACGAGAAGCTTCATTTTTCAAACT-GCGGATGAGACCAGGCTTTGCGCTTGTGTG-3′. The tagged gene was ligated into the pBAT4 vector as described above and sequenced.

For purification of the *At* KDG dehydratase, 3 l *E. coli* BL21(DE3) cells containing the plasmid described above were grown at 310 K and 250 rev min^{-1} in LB medium supplemented with 100 µg ml^{-1} ampicillin to an OD_{600} of 0.6–0.8. After the addition of 1 m*M* of isopropyl *β*-D-1-thiogalactopyranoside to induce the expression of the *At* KDG dehydratase, the culture was further grown at 303 K and 250 rev min^{-1} overnight. The cells were harvested by centrifugation for 15 min at 4000*g* at 277 K, resuspended in 50 m*M* sodium phosphate buffer pH 8 containing 300 m*M* NaCl, 1 m*M* DTT, protease inhibitors (Complete EDTA-free, Roche) and lysozyme (Sigma–Aldrich), and lysed by sonication. After centrifugation at 38 000*g* for 30 min at 277 K, the cell-free extract was loaded onto a *Strep*-Tactin Superflow column (Qiagen) equilibrated with 50 m*M* sodium phosphate pH 8, 300 m*M* NaCl buffer. After washing with equilibration buffer, the target protein was eluted with 2.5 m*M* desthiobiotin in 50 m*M* sodium phosphate pH 8, 300 m*M* NaCl buffer.

The purity of the protein was analysed by SDS–PAGE (Fig. 1) using the 10% Criterion Stain-Free gel-imaging system (Bio-Rad), and the fractions containing *At* KDG dehydratase were pooled and concentrated using a Vivaspin 20 centrifugal concentrator (molecular-weight cutoff 10 kDa; Sartorius AG, Göttingen, Germany). The buffer was replaced with 50 m*M* Tris–HCl pH 7.5 by gel filtration on a PD-10 column (GE Healthcare Life Science, Uppsala, Sweden). The purified enzyme was stored at 193 K. The activity of the purified *At* KDG dehydratase enzyme was assayed at pH 7.5 in a coupled assay using *Acinetobacter baylyi* ADP1 *α*-ketoglutarate semialdehyde dehydrogenase (Aghaie *et al.*, 2008).

2.2. Crystallization

Initial crystallization conditions were found by screening with the commercial PEGRx 2 screening kit (Hampton Research). Crystallization experiments were performed manually using the hanging-drop vapour-diffusion method at 294 K. In the crystallization droplets, 1 µl protein solution (4.6 mg ml^{-1}) was mixed with an equal

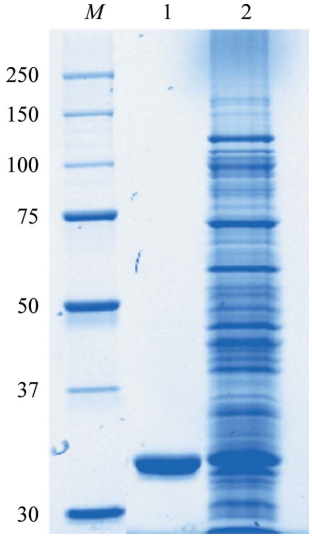


Figure 1
SDS–PAGE analysis of purified *At* KDG dehydratase. Lane *M* contains molecular-weight markers (labelled in kDa), lane 1 contains purified *At* KDG dehydratase and lane 2 contains a crude *E. coli* cell extract of the *At* KDG dehydratase-producing strain. The proteins were visualized using the 10% Criterion Stain-Free gel-imaging system (Bio-Rad).

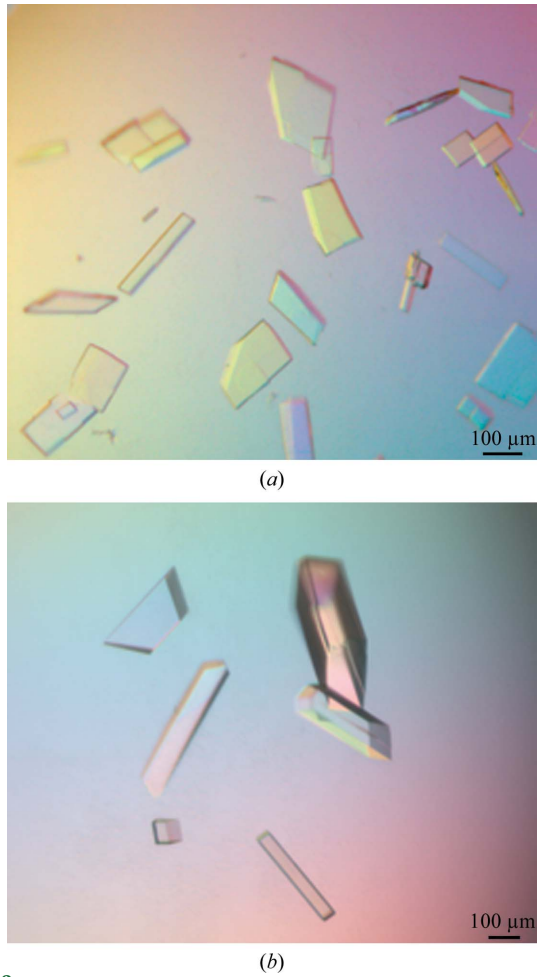


Figure 2
At KDG dehydratase crystals (*a*) from the initial screening and (*b*) after optimizing the crystallization conditions. The optimized crystallization conditions consisted of 0.1 *M* Bicine pH 8.5, 0.2 *M* sodium formate, 15%(*w*/*v*) PEG MME 5000 and the crystals grew in 3–4 d.

Table 1
Data-collection statistics for *At* KDG dehydratase.

Values in parentheses are for the outermost resolution shell.

Beamline	ID29, ESRF
Resolution (Å)	50–1.9 (2.00–1.90)
Wavelength (Å)	0.97625
Temperature (K)	100
Space group	*C*2
Unit-cell parameters (Å, °)	a = 169.1, b = 117.8, c = 74.3, $\alpha = \gamma$ = 90.0, β = 112.4
Total No. of reflections	245256 (34896)
No. of unique reflections	95879 (13473)
Multiplicity	2.6 (2.6)
$R_{\rm merge}$† (%)	4.7 (33.7)
Average $I/\sigma(I)$	12.75 (2.88)
Completeness (%)	94.9 (95.3)
Mosaicity (°)	0.15

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ represent the diffraction-intensity values of the individual measurements and the corresponding mean value, respectively.

volume of reservoir solution on siliconized cover slides (Hampton Research). The cover slides were inverted over the wells of a 24-well plate (Greiner Bio-One) containing 500 µl reservoir solution. The wells were sealed vapour-tight with high-vacuum grease (Dow Corning). The droplets were then equilibrated against the reservoir solution, and plate-like crystals (Fig. 2*a*) formed in a few days. The original conditions were composed of 0.1 *M* Bicine pH 8.5, 0.2 *M* sodium formate, 20%(*w*/*v*) polyethylene glycol monomethyl ether (PEG MME) 5000.

Crystals suitable for X-ray analysis were obtained by optimizing the crystallization conditions by varying the pH (7.6–9.0) and the concentration of the buffer (0.05–0.25 *M*) and the concentrations of precipitant (5–30%) and salt (0.05–0.30 *M*). Therefore, the optimized crystallization conditions were 0.1 *M* Bicine pH 8.5, 0.2 *M* sodium formate, 15%(*w*/*v*) PEG MME 5000. Three-dimensional crystals of several different shapes (Fig. 2*b*) grew to dimensions of approximately 0.4 × 0.2 × 0.2 mm in 3–4 d.

2.3. Data collection and processing

Before data collection, the crystals were quickly soaked in a cryoprotectant consisting of the crystallization solution with an additional 15%(*v*/*v*) glycerol and were then mounted on nylon loops. The crystals were flash-cooled by plunging them quickly into liquid nitrogen. Data were collected on beamline ID29 (de Sanctis *et al.*, 2012) at the ESRF (Grenoble, France) at a wavelength of 0.97625 Å. The diffraction images were collected with a 0.1° oscillation range using a Pilatus 6M pixel detector (Dectris). The distance between the crystal and the detector was 370.10 mm. The crystals diffracted to 1.9 Å resolution. The diffraction data set was processed using *XDS* and scaled with *XSCALE* (Kabsch, 1993).

3. Results and discussion

At KDG dehydratase was expressed in *E. coli* with a C-terminal *Strep*-tag to facilitate affinity purification of the protein. From 1 l of cell culture, approximately 3 mg of *At* KDG dehydratase could be purified to a purity of >95%, as judged by SDS–PAGE analysis (Fig. 1). The *Strep*-tagged *At* KDG dehydratase migrated as a 33 kDa protein on the SDS–PAGE gel, which is in good agreement with the calculated mass of the protein (33 526.4 Da). The purified *At* KDG dehydratase was found to be active towards 3-deoxy-2-keto-L-*threo*-hexarate, with a specific activity of 15.1 µmol min^{-1} mg^{-1}.

The initial screenings produced crystals under many conditions, but after optimization the best quality crystals were obtained using the condition 0.1 *M* Bicine pH 8.5, 0.2 *M* sodium formate, 15%(*w*/*v*) PEG MME 5000 found with the PEGRx 2 screening kit (Hampton Research). Three-dimensional crystals (Fig. 2*b*) grew in 3–4 d to approximate dimensions of 0.4 × 0.2 × 0.2 mm. The crystal diffracted to 1.9 Å resolution at the ESRF (Table 1) and belonged to space group *C*2, with unit-cell parameters a = 169.1, b = 117.8, c = 74.3 Å, β = 112.4°.

The calculated Matthews coefficient (Matthews, 1968) was 2.62 Å^3 Da^{-1}, with a solvent content of 53%, which corresponds to the presence of four monomers in the asymmetric unit. The phase problem was solved with molecular replacement using *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP*4 suite (Winn *et al.*, 2011). Based on the sequence identities of available structures for the DHDPS enzyme family, the structure of dihydropicolinate synthase from *B. clausii* (PDB entry 3e96) was used as a search model. The correlation coefficient for the molecular replacement was 0.178. The structure is currently under refinement using *PHENIX* (Adams *et al.*, 2010). The structure of this novel KDG dehydratase and structures of its complexes will provide significant information on the reaction mechanisms of the oxidative pathway of D-galacturonic acid and may be used to improve the properties of the enzyme for the manufacture of renewable products.

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