Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray analysis of geraniol dehydrogenase from *Backhousia citriodora* (lemon myrtle)

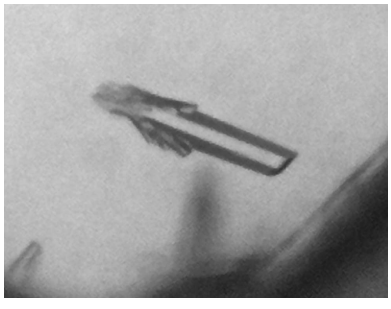
Yosuke Saito,[a]‡ Sohei Ito,[a]*‡ Anna M. Koltunow[b] and Hiroshi Sakai[a]

[a]Department of Food and Nutritional Sciences, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Yada 52-1, Suruga-ku, Shizuoka 422-8526, Japan, and [b]Commonwealth Scientific and Industrial Research Organization, Plant Industry, Glen Osmond, South Australia 5064, Australia

‡ These authors contributed equally to this work.

Correspondence e-mail: itosohei@u-shizuoka-ken.ac.jp

Received 25 January 2011
Accepted 23 February 2011



A recombinant form of geraniol dehydrogenase (EC 1.1.1.183) from *Backhousia citriodora* was overexpressed in *Escherichia coli* and purified and crystallized by the sitting-drop method using polyethylene glycol 3350 as a precipitant. A data set to 2.3 Å resolution was collected from a monocrystal at 98 K using synchrotron radiation on beamline NE3A of the Photon Factory. The crystals belonged to the orthorhombic group $P2_12_12$, with unit-cell parameters $a = 125.00$, $b = 151.01$, $c = 51.18$ Å. The asymmetric unit is expected to contain two BcGEDH molecules, with a corresponding crystal volume per protein weight of 3.1 Å^3 Da^{-1} and a solvent content of 60.6%.

1. Introduction

Backhousia citriodora (lemon myrtle) belongs to the Myrtaceae family and the essential oil, foliage and fruits of this plant have been widely used as ingredients of perfumes, food flavourings and herbal teas (Taylor, 1996). The leaves of *B. citriodora* contain a relatively large amount of oil and its essential oil consists predominantly of citral, which is an isomeric mixture of geranial and neral (Penfold *et al.*, 1951). Citral has a strong 'lemony' scent and has antimicrobial and insect repellent activity (Hayes & Markovic, 2002). *B. citriodora* oil is richer in citral than other citral-rich commercial oils such as lemongrass oils (*Cymbopogon citratus* and *C. flexuosus*) and *Litsea cubeba* oil. Interestingly, we found that *B. citriodora* shows an increased content of oil particularly at the margins of the leaves, where large oil sacs are present (of up to 1000 µm in diameter; Y. Saito, unpublished work). In addition, we also found that *B. citriodora* contains citral in young stems but not in old stems where lignification occurs. Recently, Hanawa and coworkers found that the oral administration of citral provides significant protection against mosquito bites and have published a patent application on citral usage (Hanawa *et al.*, 2010). Citral may have potential as a preventative against mosquito-borne diseases.

Cinnamyl alcohol dehydrogenases (CADs) belong to the medium-chain dehydrogenase/reductase (MDR) superfamily and consist of two identical (~350-residue) subunits containing two zinc ions per subunit (Jörnvall *et al.*, 1999). Within the MDR superfamily, at least eight families have been distinguished. CADs are one of the most important enzymes in plants and can be further classified into two types: class I and class II CADs. CADs in plants, especially class I CADs, catalyze the last step in the biosynthesis of the precursors of lignin, which is the main constituent of plant cell walls (Nordling *et al.*, 2002; Persson *et al.*, 2008). Duplication of CAD genes has been observed in angiosperms and large numbers of CAD, CAD-like or putative CAD genes have been registered in GenBank. Recently, Iijima and coworkers have shown that the monoterpene fraction (which consists mostly of citral) of *Ocimum basilicum* cv. Sweet Dani (sweet basil) is stored in the peltate glands (the average diameter of the peltate glands is 80 µm) found on the leaf epidermis and have identified basil cDNA encoding a class II CAD, namely geraniol dehydrogenase 1 (GEDH1; Iijima *et al.*, 2006). GEDH1 is an enzyme with sequence similarity to CADs and is capable of oxidizing geraniol and nerol. However, the low efficiency of GEDH1 activity and its low expression levels in the peltate glands indicate that GEDH1 is not the key enzyme of citral biosynthesis in sweet basil.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.000
Resolution range (Å)	75.00–2.30 (2.38–2.30)
Space group	$P2_12_12$
Unit-cell parameters (Å)	a = 125.00, b = 151.01, c = 51.18
Measured reflections	178947
Unique reflections	44411
Completeness (%)	99.1 (98.5)
R_{merge} (%)	8.9 (37.2)
Mean $I/\sigma(I)$	14.8 (3.7)

We cloned the CAD genes from *B. citriodora* and found one CAD, namely BcGEDH, which catalyzes the conversion of geraniol to geranial and neral. On the basis of phylogenetic analysis of CADs, BcGEDH can be classified in the class II CAD family. The potential substrate-binding residues are not well conserved in class II CADs. Therefore, the relationship between the active-site structure and the enzymatic mechanism of BcGEDH is of particular interest. In addition, BcGEDH may be valuable for food engineering, *i.e.* the addition of BcGEDH may improve the characteristics of geraniol-containing ingredients. In this paper, we present the first report of the crystallization and preliminary X-ray analysis of geraniol dehydrogenase.

2. Materials and methods

2.1. Gene cloning

cDNA of *B. citriodora* was prepared as described previously (Sugiura *et al.*, 2011). The gene encoding the geraniol dehydrogenase homologous to class II CADs was amplified by PCR and then subcloned into pET-28a vector (Novagen, USA) using the restriction enzymes *Nde*I and *Bam*HI to construct pET-28a-BcGEDH. The sequences of the gene-specific primes used for PCR were as follows: 5′-GCGCGGGCGCGCTAGCATGGCAATGTCGTCAGAGAACGAACATCC-3′ and 5′-CGCCCGCGCGGATCCTTAGAATGGCTTCAACGAATTGGCGATATCG-3′.

2.2. Protein expression and purification

Recombinant BcGEDH was produced in *Escherichia coli* Rosetta (DE3) strain (Novagen, USA). Briefly, a single colony of *E. coli* Rosetta (DE3) harbouring pET-28a-BcGEDH was inoculated into 200 ml Luria–Bertani medium supplemented with 20 µg ml^{-1} kanamycin, 30 µg ml^{-1} chloramphenicol and 20 µ*M* $ZnCl_2$. The culture was grown at 310 K to an OD_{600} of 0.5. After static culture for 30 min at 291 K, isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 0.5 m*M* to induce production of BcGEDH. The culture was grown for 20 h at 291 K with agitation at 100 rev min^{-1}. The supernatant was purified using a HisTrap HP column (GE Healthcare, Japan) according to the manufacturer's protocol. The purified BcGEDH was dialyzed against 20 m*M* glycine–NaOH buffer pH 8.5 containing 10% glycerol, 5 m*M* dithiothreitol and 150 m*M* NaCl and then concentrated to 10 mg ml^{-1} using an Amicon Ultra-15 centrifugal filter (Millipore). The purity of the protein was assessed by SDS–PAGE.

2.3. Crystallization

BcGEDH crystals were grown by the sitting-drop vapour-diffusion method at 285 K. Initial screening was performed using sparse-matrix screens based on the commercially available Wizard I and II crystallization screens (Emerald BioSystems, USA) in CrystalQuick crystallization plates (Greiner Bio-One, Germany). The initial crystallization conditions were refined by optimizing the sample and precipitant concentrations as well as the pH, additive reagents and sample/reservoir-solution volumes. Thus, 1 µl protein solution (5.0–7.5 mg ml^{-1} GEDH1, 1 m*M* $NADP^+$ and 10 m*M* citral) mixed with 1.5 µl reservoir solution was equilibrated against 100 µl reservoir solution [15–18%(*w*/*v*) polyethylene glycol 3350 and 100 m*M* Tris–HCl pH 7.0] to give BcGEDH crystals.

2.4. Data collection and processing

Diffraction data were collected on beamline NE3A of the High Energy Accelerator Research Organization (KEK, Tsukuba, Japan). For data collection under cryogenic conditions, crystals were soaked in a cryoprotectant solution consisting of 22%(*w*/*v*) polyethylene glycol 3350, 18% glycerol, 100 m*M* Tris–HCl pH 7.0 for a few minutes. Crystals were mounted in a nylon loop and flash-cooled to 98 K in a stream of dry nitrogen. Data were indexed, integrated and scaled to 2.3 Å resolution using *DENZO* and *SCALEPACK* from the *HKL*-2000 suite (Otwinowski & Minor, 1997). Data-collection and data-processing statistics are presented in Table 1.

3. Results and discussion

Alignment analysis of the primary sequences of BcGEDH and CAD-family members revealed that potential substrate-binding residues are well conserved in class I CADs but are not conserved in class II CADs. Here, we subcloned GEDH from *B. citriodora* into pET-28a vector. A recombinant form of BcGEDH was overexpressed in *E. coli*, purified and crystallized by the sitting-drop method. The molecular mass of BcGEDH was determined to be 41 kDa by SDS–PAGE, which is in good agreement with the calculated value of 40.87 kDa. Gel-filtration analysis suggests that BcGEDH exists as a dimer in solution (data not shown). Small rod-shaped crystals of BcGEDH were obtained using buffer conditions including polyethylene glycol from the Wizard I and II crystallization screen kits. The initial crystallization conditions were refined by optimizing the sample and precipitant concentrations as well as the pH, additive reagents and sample/reservoir-solution volumes. Before crystallization, $NADP^+$ solution was added to the BcGEDH to a final concentration of 1 m*M*. Typical conditions for the crystallization of

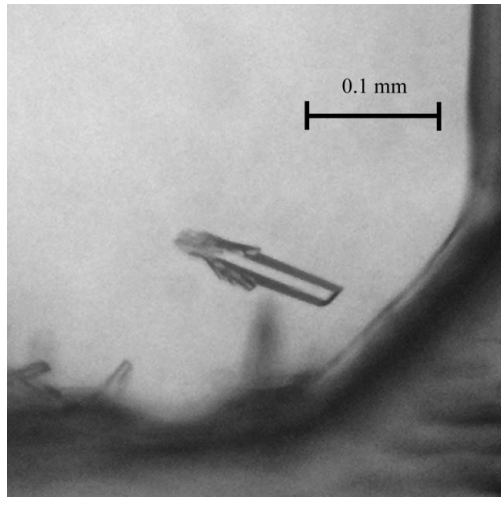


Figure 1
Image of a crystal of *B. citriodora* GEDH. The rod-shaped monocrystal appeared after one week using the sitting-drop vapour-diffusion method. The largest dimension of the crystal was approximately 0.1 mm.

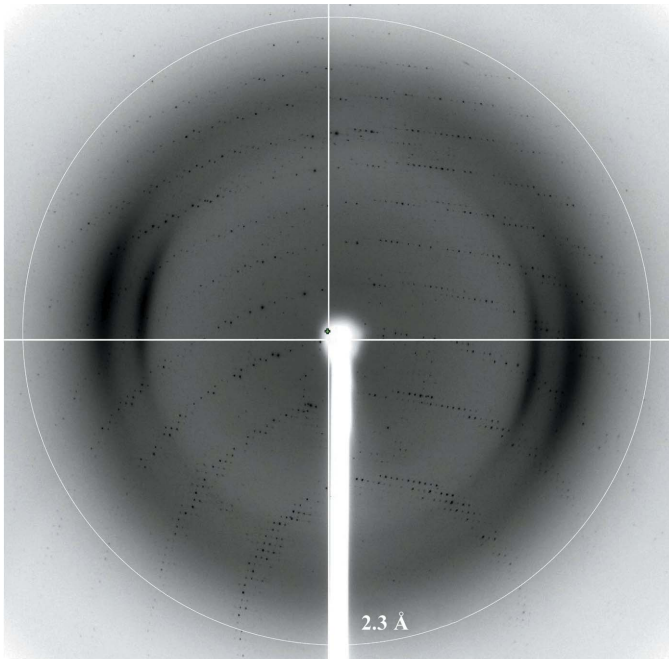


Figure 2
Diffraction pattern of the BcGEDH crystal. The crystal was grown under optimum conditions and then flash-frozen in reservoir solution containing 18% glycerol as a cryoprotectant. The exposure time was 10 s, with an oscillation angle of 1°. The pattern displayed a maximum diffraction resolution of 2.3 Å and belonged to space group $P2_12_12$.

BcGEDH were at 285 K with 5–7.5 mg ml^{-1} protein solution and a reservoir solution consisting of 15–18%(*w*/*v*) polyethylene glycol 3350, 100 m*M* Tris–HCl pH 7.0. Crystals grew within a few days (Fig. 1). The diffraction data sets were collected using 1.0° oscillations with a crystal-to-detector distance of 273 mm. A data set to 2.3 Å resolution (Fig. 2) was collected from a monocrystal at 98 K using synchrotron radiation on beamline NE3A of the Photon Factory. The data were indexed, integrated and scaled to 2.3 Å resolution using *DENZO* and *SCALEPACK* from the *HKL*-2000 suite (Otwinowski & Minor, 1997). The crystals belonged to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 125.00, b = 151.01, c = 51.18 Å. The asymmetric unit is expected to contain two BcGEDH molecules and the V_M value and solvent content were determined to be 3.1 Å^3 Da^{-1} and 60.6%, respectively. Self-rotation function calculations are consistent with the presence of two BcGEDH molecules in the asymmetric unit. The data-collection statistics for the BcGEDH crystal are presented in Table 1. Attempts to solve the structure of BcGEDH by molecular-replacement procedures are in progress.

This study was supported in part by a Grant-in-Aid for Young Scientists (B) in Japan. The data collection was approved by the Photon Factory Advisory Committee (Proposal No. 2010G169).

References

Hanawa, M., Sumita, K., Torizumi, Y. & Nagata, K. (2010). Japan Patent Kokai 2010-001285.
Hayes, A. J. & Markovic, B. (2002). *Food Chem. Toxicol.* **40**, 535–543.
Iijima, Y., Wang, G., Fridman, E. & Pichersky, E. (2006). *Arch. Biochem. Biophys.* **448**, 141–149.
Jörnvall, H., Höög, J. O. & Persson, B. (1999). *FEBS Lett.* **445**, 261–264.
Nordling, E., Jörnvall, H. & Persson, B. (2002). *Eur. J. Biochem.* **269**, 4267–4276.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Penfold, A. R., Morrison, F. R., Willis, J. L., McKern, H. G. & Spies, M. C. (1951). *J. Proc. R. Soc. N. S. W.* **85**, 123–126.
Persson, B., Hedlund, J. & Jörnvall, H. (2008). *Cell. Mol. Life Sci.* **65**, 3879–3894.
Sugiura, M., Ito, S., Saito, Y., Niwa, Y., Koltunow, A. M., Sugimoto, O. & Sakai, H. (2011). In the press.
Taylor, R. (1996). *Rural Res.* **172**, 18–19.