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Expression, purification and crystallization of a plant type III polyketide synthase that produces diarylheptanoids

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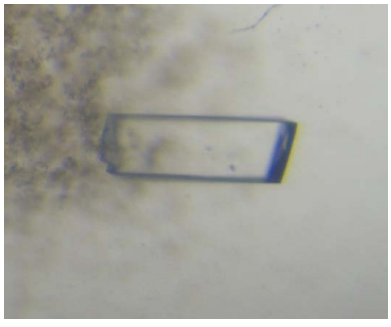

Curcuminoid synthase (CUS) from *Oryza sativa* is a plant-specific type III polyketide synthase that catalyzes the one-pot formation of bisdemethoxycurcumin by the condensation of two molecules of 4-coumaroyl-CoA and one molecule of malonyl-CoA. Recombinant CUS was expressed in *Escherichia coli* and crystallized by the sitting-drop vapour-diffusion method. The crystals belonged to space group $P2_1$, with unit-cell parameters a = 72.7, b = 97.2, c = 126.2 Å, $\alpha = \gamma = 90.0$, $\beta = 103.7^\circ$. A diffraction data set was collected in-house to 2.5 Å resolution.

1. Introduction

The members of the chalcone synthase (CHS) superfamily of type III polyketide synthases (PKSs) are involved in the backbone assembly of pharmaceutically and biologically important structurally divergent natural polyketides such as flavonoids, stilbenes and phloroglucinols (Austin & Noel, 2003; Abe & Morita, 2010). These enzymes are homodimeric proteins consisting of 40 kDa subunits that catalyze the sequential condensation of malonyl-CoA as an extender substrate with an aromatic or aliphatic linked CoA thioester as a starter substrate without the involvement of the acyl-carrier protein required in the type I and type II PKSs. For example, CHS catalyzes the iterative condensation of malonyl-CoA with 4-coumaroyl-CoA to produce naringenin chalcone, which is the biosynthetic precursor of flavonoids. Recent crystallographic analyses have revealed that the functional diversity of type III PKSs is principally derived from modifications of the active-site cavity and have begun to clarify the fine structural details of the enzyme reactions (Austin & Noel, 2003; Abe & Morita, 2010).

Diketide-CoA synthase 1 (DCS1) and curcumin synthase (CURS) from *Curcuma longa*, which share 62% identity with each other, are the key enzymes in the biosynthesis of curcumin, the major component of turmeric from *C. longa* (Katsuyama, Kita, Funa *et al.*, 2009). Thus, DCS1 catalyzes the one-step decarboxylative condensation of malonyl-CoA with feruloyl-CoA to produce the biosynthetic precursor feruloyldiketide-CoA (Fig. 1); CURS then accepts feruloyl-β-keto acid (the hydrolyzed product of feruloyldiketide-CoA) as an extender substrate and converts it to curcumin by condensation with another feruloyl-CoA starter (Fig. 1). Recently, the DCS1 isozymes DCS2 and DCS3 from *C. longa* were discovered (Katsuyama, Kita & Horinouchi, 2009). Although its physiological function has as yet to be elucidated, curcuminoid synthase (CUS) from *Oryza sativa*, which shares 51% identity with DCS1 and 45% identity with CURS, catalyzes the one-pot formation of the C_6-C_7-C_6 curcuminoid bisdemethoxycurcumin. Thus, a single enzyme catalyzes the condensation of two molecules of 4-coumaroyl-CoA as starter substrates and one molecule of malonyl-CoA as the first extender substrate through the formation of the extender unit β-keto acid (Fig. 1; Katsuyama *et al.*, 2007).

We very recently reported analyses of crystal structures of *Rheum palmatum* benzalacetone synthase (BAS; PDB codes 3a5q and 3a5r), which shares 51% identity with CUS and produces benzalacetone from the condensation of one molecule of 4-coumaroyl-CoA and one

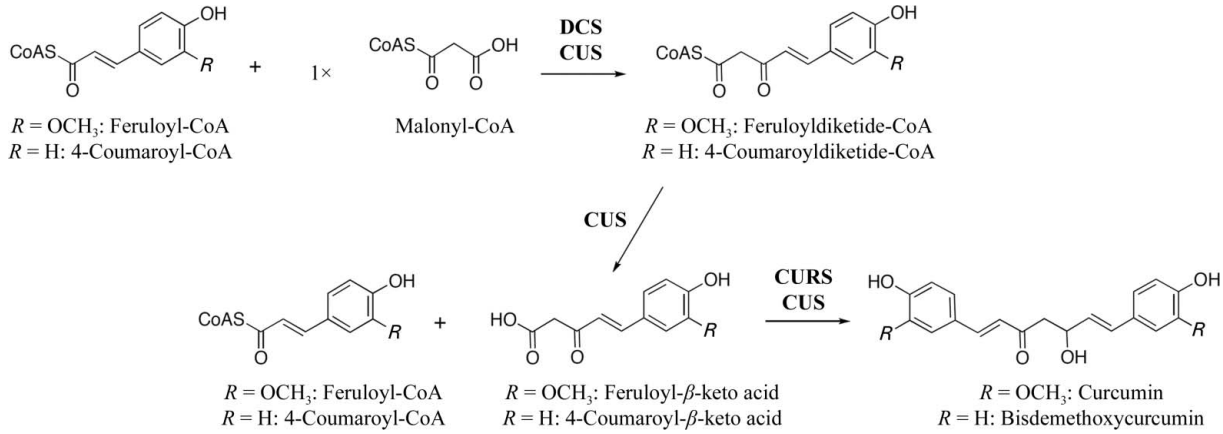

Figure 1
Proposed pathways for the formation of diketide-CoA by DCS1, of curcumin by CURS and of bisdemethoxycurucumin by CUS.

molecule of malonyl-CoA through a 4-coumaroyldiketide acid intermediate (Morita *et al.*, 2010). To clarify the three-dimensional structural details of CUS-catalyzed processes and to elucidate the structure–function relationship of the functionally diverse type III PKS enzymes, we expressed hexahistidine-fused recombinant *O. sativa* CUS in *Escherichia coli* and obtained good-quality crystals of the recombinant enzyme.

2. Experimental

2.1. Construction of expression plasmids

The AK109558 cDNA encoding full-length CUS was purchased from the Rice Genome Resource Center (Tsukuba, Japan) and was used as the template to amplify the CUS gene by PCR using 5′-GCGGGATCCAATGGCACCGACGACGACCATGGGAAGC-ATGGGAAGCGC-3′ as the sense primer, which introduces a *Bam*HI restriction site, and 5′-GCGAAGCTTTTAATTCACATG-AGAGGTGGCGTGCAACAG-3′ as the antisense primer, which introduces a *Hin*dIII restriction site. The amplified DNA fragment was digested with *Bam*HI/*Hin*dIII and ligated into the *Bam*HI/*Hin*dIII sites of the pETDuet-1 vector (Novagen) for expression as fusion protein with a His_6 tag at the N-terminus.

2.2. Expression and purification of His_6-tagged CUS

After confirmation of the sequence, the pETDuet-1 vector encoding full-length CUS was transformed into *E. coli* BL21(DE3)-pLysS. The cells harbouring the plasmid were cultured to an OD_{600} of 0.6 in LB medium containing 100 µg ml^{-1} ampicillin at 303 K. Isopropyl β-D-1-thiogalactopyranoside was then added to a final concentration of 1 m*M* to induce gene expression and the culture was incubated for a further 16 h at 303 K.

All of the following procedures were performed at 277 K. The *E. coli* cells were harvested by centrifugation at 5000*g* and resuspended in 50 m*M* Tris–HCl buffer pH 8.0 containing 0.2 *M* NaCl and 5%(*v*/*v*) glycerol (buffer *A*). The cells were disrupted by sonication and the lysate was centrifuged at 12 000*g* for 30 min. The supernatant was loaded onto an Ni Sepharose 6 Fast Flow column (GE Healthcare) equilibrated with buffer *A*. After washing the resin with 50 m*M* HEPES–NaOH buffer pH 7.0 containing 200 m*M* NaCl and 5%(*v*/*v*) glycerol (buffer *B*), the His_6-tagged CUS was subsequently eluted with buffer *B* containing 500 m*M* imidazole. The protein solution was then diluted fivefold with 50 m*M* HEPES–NaOH buffer pH 7.0 containing 5%(*v*/*v*) glycerol and 2 m*M* DTT (buffer *C*) and applied onto a Resource-Q column (GE Healthcare). The column was washed with buffer *C* containing 100 m*M* NaCl and the protein was subsequently eluted using a linear gradient of 100–500 m*M* NaCl. The protein solution was further purified to homogeneity by gel-filtration chromatography on Superdex 200HR (10/300GL; GE Healthcare) and was concentrated to 10 mg ml^{-1} in 20 m*M* HEPES–NaOH pH 7.0 buffer containing 100 m*M* NaCl and 2 m*M* DTT.

Dynamic light-scattering (DLS) analysis was performed using a DynaProMSXTC molecular-sizing instrument (Protein Solutions Inc.). After centrifugation using a 0.22 µm Ultrafree-MC filter (Millipore) to remove particulate material from the protein solution, the solution properties of the purified protein were monitored. Data were acquired from 50 scattering measurements at 278 K; three sets of data were analyzed using the *DYNAMICS* software package (Protein Solutions Inc.) and averaged.

2.3. Crystallization and X-ray data collection

Initial crystallization attempts and optimization of the crystallization conditions were performed at 293 K using the sitting-drop vapour-diffusion method by mixing 0.5 µl of the purified His_6-tagged CUS solution with an equal volume of reservoir solution and equilibrating against 100 µl reservoir solution using a 96-condition crystallization screen originally designed by Mitsubishi Chemical Corporation. Clusters of needle-like crystals were observed a month later in crystallization solution consisting of 100 m*M* HEPES–NaOH pH 7.5 and 1995 m*M* ammonium sulfate. Further crystallization was

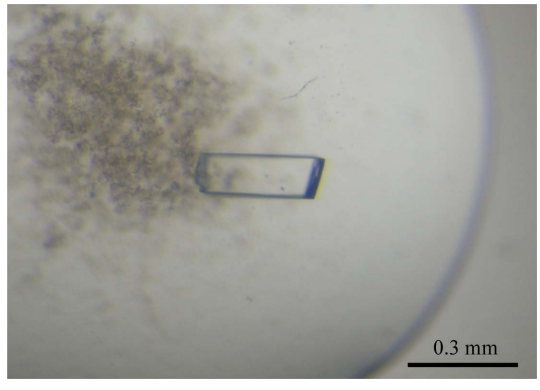

Figure 2
Crystal of CUS. The dimensions of the crystal were approximately 0.3 × 0.1 × 0.1 mm.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters	
a (Å)	72.7
b (Å)	97.2
c (Å)	126.2
β (°)	103.7
Resolution (Å)	50.0–2.50 (2.59–2.50)
Unique reflections	54813
Redundancy	3.3 (3.2)
Completeness (%)	93.0 (88.6)
$\langle I/\sigma(I)\rangle$	21.7 (5.2)
$R_{\rm merge}$† (%)	8.9 (32.5)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection hkl, $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl.

attempted using Additive Screen (Hampton Research) at various pH values together with the use of 1995 m*M* ammonium sulfate as a precipitant. Diffraction-quality crystals were finally obtained at 293 K in 100 m*M* HEPES–NaOH pH 7.8 containing 1995 m*M* ammonium sulfate, 3% dimethylsulfoxide and 2 m*M* CoA-SH using the sitting-drop vapour-diffusion method (Fig. 2).

The crystals were transferred to a cryoprotectant consisting of 100 m*M* HEPES–NaOH pH 7.8, 1330 m*M* ammonium sulfate, 2 m*M* CoA-SH, 12%(*v*/*v*) glycerol and 14%(*w*/*v*) PEG 400. After a few seconds, the crystals were picked up in a nylon loop and flash-cooled at 100 K in a nitrogen-gas stream. X-ray diffraction data sets were collected using a Rigaku RA-Micro7 with a Cu target and a Rigaku R-AXIS VII imaging-plate area detector, with a 200 mm distance between the crystal and the detector. A total of 180 frames were recorded with 1° oscillation angle and 5 min exposure time. The data were indexed, integrated and scaled using the *HKL*-2000 program package (Otwinowski & Minor, 1997).

3. Results and discussion

His_6-tagged recombinant CUS was expressed in *E. coli*. The recombinant enzyme exhibited CUS activity (Katsuyama *et al.*, 2007) and the typical yield was about 3 mg per litre of culture. The purified His_6-tagged CUS migrated as a single band with a molecular mass of 45 kDa on SDS–PAGE, which agrees well with the calculated value of 44.7 kDa for His_6-tagged CUS. In contrast, a gel-filtration experiment using the recombinant CUS yielded a molecular mass of 112 kDa, suggesting that the recombinant CUS exists as a homodimeric or trimeric enzyme. Furthermore, DLS analysis of the recombinant CUS after gel filtration revealed a monomodal distribution, with a polydispersity value of 25.2% and an estimated molecular mass of 74 kDa, suggesting that the recombinant protein is a homodimeric enzyme, as in the cases of other known type III PKSs (Austin & Noel, 2003; Abe & Morita). A GST construct was also made but was abandoned owing to the inactivity of the product even though the GST tag had been removed.

Crystals appeared reproducibly within two weeks and the largest crystal grew to dimensions of approximately 0.3 × 0.1 × 0.1 mm (Fig. 2). A complete data set was collected to 2.5 Å resolution. Detailed data-processing statistics are shown in Table 1. It should be noted that the data were only processed to the edges of the square detector. Thus, higher resolution data may be obtained in the future. Based on the diffraction data, the space group was determined to be $P2_1$, with unit-cell parameters a = 72.7, b = 97.2, c = 126.2 Å, $\alpha = \gamma = 90.0$, $\beta = 103.7°$. With four monomers in the asymmetric unit, the Matthews volume ($V_{\rm M}$; Matthews, 1968) was calculated to be 2.5 $Å^3\ Da^{-1}$ and the estimated solvent content was 47.9%, which is in the range normally observed for protein crystals. A self-rotation function analysis calculated on the data in the resolution range 15.0–4.0 Å using *CNS* (Brünger *et al.*, 1998) indicated the presence of three twofold noncrystallographic symmetry axes. Structure determination by the molecular-replacement method with the *CNS* program and using the crystal structure of *R. palmatum* BAS (PDB code 3a5q; Morita *et al.*, 2010), which shares 51% identity with CUS, as a search model is now in progress. Simultaneously, we are also attempting to crystallize His_6-tagged CUS complexed with substrate and product analogues. These structural analyses will provide valuable insights into not only the structure–function relationship and catalytic mechanism of curcuminoid-producing enzymes, but also the functional diversity of the type III PKSs.

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References

Abe, I. & Morita, H. (2010). *Nat. Prod. Rep.* **27**, 809–838.
Austin, M. B. & Noel, J. P. (2003). *Nat. Prod. Rep.* **20**, 79–110.
Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* D**54**, 905–921.
Katsuyama, Y., Kita, T., Funa, N. & Horinouchi, S. (2009). *J. Biol. Chem.* **284**, 11160–11170.
Katsuyama, Y., Kita, T. & Horinouchi, S. (2009). *FEBS Lett.* **583**, 2799–2803.
Katsuyama, Y., Matsuzawa, M., Funa, N. & Horinouchi, S. (2007). *J. Biol. Chem.* **282**, 37702–37709.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Morita, H., Shimokawa, Y., Tanio, M., Kato, R., Noguchi, H., Sugio, S., Kohno, T. & Abe, I. (2010). *Proc. Natl Acad. Sci. USA*, **107**, 669–673.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.