Expression, purification, crystallization and crystallographic study of the *Aspergillus terreus* aromatic prenyltransferase AtaPT

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Prenylated aromatics are produced by aromatic prenyltransferases during the secondary metabolism of bacteria, fungi and plants. The prenylation of nonprenylated precursors can lead to great chemical diversity and extensive biological properties. *Aspergillus terreus* aromatic prenyltransferase (AtaPT), which has recently been discovered and characterized, is such an enzyme and is responsible for the prenylation of various aromatic compounds. Here, recombinant AtaPT was overexpressed in *Escherichia coli*, purified and crystallized. Diffraction data were collected to a resolution of 1.71 Å and the crystal belonged to space group $P2_12_12$, with unit-cell parameters $a = 96.2$, $b = 135.8$, $c = 69.5$ Å, $\alpha = \beta = \gamma = 90^\circ$. Analysis of the calculated Matthews coefficient and the self-rotation function suggested that there are two AtaPT molecules in the asymmetric unit.

1. Introduction

Aromatic prenyltransferases are important enzymes responsible for transferring prenyl moieties to aromatics, resulting in the biosynthesis of hybrid natural products by an electrophilic alkylation mechanism. The prenylated products usually show enhanced chemical activity and improved biological properties compared with their nonprenylated precursors (Li, 2010; Botta *et al.*, 2005). Therefore, exploring biocatalysts that produce diverse prenylated secondary metabolites in bacteria, fungi and plants provides potential tools for the production of various bioactive compounds.

Recently, significant progress has been made in the biochemical and structural investigations of aromatic prenyltransferases. Many prenyltransferases have been identified from bacteria and the crystal structures of some of them have been solved; these prenyltransferases were classified into an aromatic prenyltransferase (PTase) superfamily named ABBA, which shares a common three-dimensional structure called the PT fold with five repetitive $\alpha\beta\alpha$ secondary elements that form a barrel (Kuzuyama *et al.*, 2005; Tello *et al.*, 2008; Saleh *et al.*, 2009). Another well investigated superfamily is the dimethylallyltryptophan synthase (DMATS) family from fungi, which share sequence similarities with the dimethylallyltryptophan synthase involved in the biosynthesis of ergot alkaloids (Yu & Li, 2012). The prenyltransferases in the DMATS superfamily normally use tryptophan derivatives as

Table 1
Sequence identity of AtaPT to other PTases.

	AnaPT	FtmPT1	FgaPT2	CdpNTP
PDB entry	4ld7	3o24	3i4z	4e0t
UniProt accession code	A1DN10_NEOFI	FTMB_ASPFU	DMAW_ASPFU	D1D8L6_ASPFM
Identity to AtaPT (%)	42	28	29	28

Table 2
Overproduction of recombinant AtaPT.

Source organism	<i>A. terreus</i>
DNA source	cDNA library
Forward primer	5'-GCCGGATCCCGGCCCTGGCAGATCC
Reverse primer	5'-GCCCTCGAGCACACGTGGCAGATT
Cloning vector	pGEX-6P-1
Expression vector	pGEX-6P-1
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced†	<u>MSPILGYWKIKGLVQPTRLLLEYLEEKYEELHYE-</u> RDEGDKWRNKKFELGLEFPNLPYYIDGDVKLT- QSMATIRYIADKHNLGGCPKERAISMLEGA- VLDIRYGVSRISYKDFETLKVDFLSKLPEML- KMFEDRLCHKTYLNGDHVTHPDFMLYDALDVV- LYMDPMCLDAFPKLVCFKKRIEAIPIQIDKYLK- SSKYIAWPLQGWQATFGGDDHPPKSDLEVLFQ- GPLGSRPWQILSQALGFNPYDQELWQNTAET- LNRVLEQCDYSVHLQYKYLAFYHKYILPSLGP- FRPGVEPEYISGLSHGGHPLEISVKIDKSKT- ICRLGLQAIGLAGTARDPLNSFGDRELLKNL- ATLLPHVDLRLFDHFNAQVGLDRAQCAVATTK- LIKESHNVCTSLDLKDEGVIKPYVFTIPKG- LVTETPLFDLTFAAIEQMEVYHKDAPLRTALS- SLKDFLRPRVPTDASITPPLTGLIGVDCIDPM- LSRLKVYLATFRMDLSLIRDYWTGLGLLTDAG- TMKLEMVETLAKTLKLGDEACETLDAERLPF- GINYAMKPGTAELAPPQIYFPLLGINDGFIAD- ALVEFFQYMGWEDQANRYKDELKAKFPNVDIS- QTKNVHRWLGVAYSETKGPSMNIYYDVVAGNV- ARV

† The sequence of the upstream glutathione *S*-transferase (GST) tag is underlined. PreScission protease can recognize the amino-acid sequence LEVLFQGP and break the peptide bond between Q and G.

Table 3
Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well plate
Temperature (K)	289
Protein concentration (mg ml ⁻¹)	15
Buffer composition of protein solution	25 mM bis-tris pH 6.0, 100 mM NaCl
Composition of reservoir solution	100 mM bis-tris pH 6.0, 200 mM ammonium sulfate, 17% (w/v) PEG 3350
Volume and ratio of drop	1 µl:1 µl:0.2 µl protein:reservoir:3.3% DDM
Volume of reservoir (µl)	200

their preferred substrates (Yu & Li, 2012), and include AnaPT (Yin *et al.*, 2009), FtmPT1 (Jost *et al.*, 2010), CdpNTP (Yin *et al.*, 2007), CdpC3PT (Yin *et al.*, 2010), FgaPT2 (Unsöld & Li, 2005) and 7-DMATS (Kremer *et al.*, 2007). Although the members of the DMATS superfamily share low sequence identities to those of the ABBA family, their structures are similar, with a common PT fold (Metzger *et al.*, 2009; Jost *et al.*, 2010; Schuller *et al.*, 2012; Yu *et al.*, 2013).

Although many genes encoding aromatic prenyltransferases have been found, there are still many prenyl-

transferases responsible for the prenylation of new aromatic compounds that are awaiting further exploration. Recently, we discovered a unique aromatic prenyltransferase from *Aspergillus terreus*, named AtaPT, which shows significant sequence variation in comparison with the previously studied PTases (Table 1) as calculated by *BlastP* 2.2.31 (Altschul *et al.*, 1997, 2005) and can catalyze the prenylation of diverse novel aromatic compounds (for example, genistin and honokiol), including tryptophan derivatives. Here, we report the expression, purification, crystallization and preliminary crystallographic analysis of recombinant AtaPT.

2. Materials and methods

2.1. Protein production

The gene coding for AtaPT (GenBank accession No. KP893683) was amplified by polymerase chain reaction (PCR) from an *A. terreus* strain A8-4 cDNA library. For crystallization purposes, we deleted the first ten residues at the N-terminus using the primers given in Table 2. The amplified DNAs were digested using the restriction endonucleases BamHI and XhoI (NEB) using 1× buffer 4 (NEB) at 37°C. The digested fragments were the introduced into BamHI/XhoI-digested pGEX-6p-1 vector (GE Healthcare), which encodes an upstream glutathione *S*-transferase (GST) tag followed by a PreScission protease (GE Healthcare) cleavage site. The recombinant plasmid was then transformed into *Escherichia coli* strain BL21 (DE3) for overexpression of AtaPT.

The transformed cells were first picked from a plate and pre-cultured at 37°C for growth in 2×YT medium with 100 µg ml⁻¹ ampicillin and then inoculated into the main culture. When the optical absorption density OD₆₀₀ of the cell culture reached 1.0, 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and the culture was incubated for a further 16–20 h at 16°C for protein expression. Finally, the cells were harvested by centrifugation at 6000 rev min⁻¹ for 10 min at a temperature of 4°C.

The harvested cells were resuspended in 1× PBS lysis buffer at pH 7.4 consisting of 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 140 mM NaCl, 2.7 mM KCl, 1 mM DTT and were disrupted using the high-pressure method at 120 MPa. After centrifugation at 16 000 rev min⁻¹ for 45 min at 4°C, the supernatant containing the target protein fused to a GST tag was loaded onto a glutathione Sepharose 4B affinity column (GE Healthcare) pre-equilibrated with 1× PBS lysis buffer. The resin was then washed using 1× PBS lysis buffer containing an additional 1 M NaCl and washed again with 1× PBS lysis buffer. The GST-fusion protein on the column was

Figure 1

Purification of recombinant AtaPT^{11–424}. (a) Anion-exchange chromatography using a RESOURCE Q column. (b) Size-exclusion chromatography using a Superdex 200 10/300 HiLoad gel-filtration column. (c) SDS-PAGE analysis of AtaPT. The concentration of the gel is 12% (w/v). Lane 1, protein molecular-weight marker (Fermentas; labelled in kDa); lane 2, protein after GST affinity chromatography; lane 3, protein after anion-exchange chromatography; lane 4, protein after size-exclusion chromatography.

Table 4

Data collection and processing.

Values in parentheses are for the highest resolution shell.

Diffraction source	BL17U, SSRF
Wavelength (Å)	0.97907
Temperature (K)	100
Detector	ADSC Q315R
Crystal-to-detector distance (mm)	250
Rotation range per image (°)	0.2
Total rotation range (°)	180
Exposure time per image (s)	1
Space group	<i>P</i> 2 ₁ 2 ₁ 2
Unit-cell parameters (Å, °)	<i>a</i> = 96.2, <i>b</i> = 135.8, <i>c</i> = 69.5, <i>α</i> = <i>β</i> = <i>γ</i> = 90
Mosaicity (°)	0.7
Resolution range (Å)	48.57–1.90 (2.00–1.90)
Total No. of reflections	354138
No. of unique reflections	71707
Completeness (%)	98.7 (100.0)
Multiplicity	4.9 (5.0)
<i>I</i> / <i>σ</i> (<i>I</i>)	5.8 (2.1)
<i>R</i> _{meas} [†] (%)	9.8 (46.4)
Overall <i>B</i> factor from Wilson plot (Å ²)	20.7

[†] *R*_{meas} is the redundancy-independent merging *R* factor. Here, *R*_{meas} is estimated by multiplying the conventional *R*_{merge} value by the factor $[N/(N-1)]^{1/2}$, where *N* is the data multiplicity (Diederichs & Karplus, 1997).

digested overnight using PreScission protease (GE Healthcare) at 4°C. The AtaPT released from digestion of the fusion protein was first exchanged into a buffer consisting of 25 mM bis-tris pH 6.0, 100 mM NaCl on a HiTrap Desalting column (5 ml, GE Healthcare). The protein was concentrated by ultrafiltration (30 kDa cutoff) to about 15 mg ml^{−1} based on a molar extinction coefficient of 1.26 M^{−1} cm^{−1} (calculated using ProtParam; <http://web.expasy.org/protparam/>) for crystallization. Further purification was performed by anion exchange on a RESOURCE Q column (GE Healthcare) using elution buffer consisting of 25 mM bis-tris pH 6.0, 1 M NaCl at a flow rate of 1 ml min^{−1} and gel filtration on a Superdex 200 column (10/300, GE Healthcare), which had previously been equilibrated with buffer consisting of 25 mM bis-tris pH 6.0, 100 mM NaCl, at a flow rate of 0.5 ml min^{−1}.

Figure 2

Quaternary-structure determination of recombinant AtaPT^{11–424} using the size-exclusion chromatography with multi-angle light-scattering (SEC-MALS) method. The experiment was carried out to calculate the molecular weight of AtaPT^{11–424} in solution, and the resulting value was 174.8 kDa. AtaPT^{11–424} is therefore a tetramer in solution.

To test whether the purified recombinant AtaPT^{11–424} had any prenyltransferase activity, we carried out enzymatic assays towards 5-methyltryptophan, a tryptophan derivative, using dimethylallyl diphosphate (DMAPP) as a prenyl donor. The reaction mixture (200 μ l) consisted of 50 mM Tris–HCl pH 7.4, 0.4 mM 5-methyltryptophan substrate, 0.4 mM DMAPP substrate and 45 μ M AtaPT^{11–424}. After incubation for 16 h at 37°C, the reaction was terminated by the addition of 400 μ l methanol and 2 min of vortexing. The protein was removed by centrifugation at 12 000g for 30 min. The supernatant was directly analyzed by HPLC.

2.2. Crystallization

All crystallization experiments were carried out by the hanging-drop vapour-diffusion method at 16°C using 24-well plates with a protein concentration of 15 mg ml^{−1} (Table 3). The commercial crystallization kits Index, PEG/Ion, PEG/Ion

2, Crystal Screen, Crystal Screen 2 and Natrix from Hampton Research were screened to find the initial conditions for crystal growth. Through initial screening and several rounds of optimization, single crystals were obtained by mixing 1 μ l protein solution with 1 μ l reservoir solution consisting of 100 mM bis-tris pH 6.0, 200 mM ammonium sulfate, 17% (v/v) polyethylene glycol (PEG) 3350 with 3.3% *n*-dodecyl- β -D-maltoside (DDM) as an additive.

2.3. Data collection and processing

Before data collection, crystals were soaked in a cryoprotectant solution [0.1 M bis-tris pH 6.0, 0.2 M ammonium sulfate, 30% (m/v) PEG 3350] and flash-cooled in a liquid-nitrogen stream. Diffraction data were collected on beamline BL17U at Shanghai Synchrotron Radiation Facility (SSRF) with an ADSC Q315r CCD detector using radiation of wavelength 0.97907 Å with 80% attenuation of the intensity. A

Figure 3
Crystals grown during the optimization process. (a) Crystals grown from initial conditions consisting of 100 mM bis-tris pH 6.5, 200 mM ammonium sulfate, 25% (w/v) PEG 3350. (b) One of the twinned crystals grown after the first round of optimization by adjusting the pH to 6.0 and the concentration of PEG 3350 to 17% (w/v). (c) In the presence of 3.3% (w/v) DDM, most of the crystals that grew were still twinned when the protein buffer contained 25 mM Tris pH 8.0, 100 mM NaCl. (d) After a second round of optimization of additive screening, single crystals grew when 3.3% (w/v) DDM was selected as an additive and the protein buffer contained 25 mM bis-tris pH 8.0, 100 mM NaCl.

complete data set consisting of 900 images was collected with a crystal-to-detector distance of 250 mm, an oscillation of 0.2° and an exposure time of 1 s per image. The diffraction spots were indexed and integrated using *iMosflm* v.7.0.9 (Battye *et al.*, 2011) and were scaled using *SCALA* from the *CCP4* program suite (Winn *et al.*, 2011). An *L*-test was carried out to verify the presence or absence of twinning in the data set. Statistics of data collection and processing are summarized in Table 4.

2.4. Self-rotation function calculation

A self-rotation function was calculated using *MOLREP* (Vagin & Teplyakov, 2010) in the resolution range 45.34–2.06 Å and the integration radius was set to 57 Å.

3. Results and discussion

Recombinant AtaPT^{11–424} from *A. terreus* strain A8-4 was expressed and purified for crystallization in a three-step purification that included GST affinity purification, anion-exchange chromatography (Fig. 1*a*) and gel-filtration chromatography (Fig. 1*b*). The protein purity in each step was examined by SDS–PAGE analysis and reached 95% after gel-filtration chromatography (Fig. 1*c*). The quaternary structure of recombinant AtaPT^{11–424} was assayed using the size-exclusion chromatography with multi-angle light-scattering method, showing a tetrameric form in solution with a molecular weight of 174.8 kDa (Fig. 2).

HPLC–MS analysis of the enzymatic assay showed that purified recombinant AtaPT^{11–424} exhibited obvious prenyl-transferase activity towards 5-methyltryptophan with DMAPP as the prenyl donor (Supplementary Fig. S1). The molecular-weight increase of 68 Da of the product indicated the addition

of one prenyl moiety to 5-methyltryptophan. We are now working to determine the positions at which prenylation occurs on the 5-methyltryptophan skeleton.

To begin with, we cloned and expressed the full-length form of AtaPT, AtaPT^{1–424}, with a His tag at the C-terminus. The Index screen was used to find initial conditions that yielded crystals. The crystals belonged to space group *P1* and most diffracted to 6 Å resolution on beamline BL5A at the Photon Factory. Most importantly, SDS–PAGE and Western blot analysis of these crystals revealed that AtaPT^{1–424} was degraded at the N-terminus (data not shown). We therefore discontinued working with the AtaPT^{1–424} form and selected another approach. Based on the protein secondary-structure predictions of *Phyre2* (Kelley & Sternberg, 2009), we deleted the first ten residues at the N-terminus that may be degraded and may prevent higher order arrangement of the protein. Initial screening using the crystallization kits listed above gave rise to one crystallization condition for the new construct AtPT^{11–424}, 100 mM bis-tris pH 6.5, 200 mM ammonium sulfate, 25%(w/v) PEG 3350, and the diffraction limit of the crystals was improved to 4 Å resolution on a Rigaku X-ray generator. After a first round of optimization by adjusting the pH value of the bis-tris buffer to 6.0 and the concentration of PEG 3350 to 17% with a protein concentration of 15 mg ml^{−1}, twinned crystals appeared with a further enhanced diffraction resolution of about 2.6 Å on beamline BL17U at SSRF. Another round of optimization by additive screening was then carried out to obtain single crystals, and 5%(w/v) DDM was added to yield single small crystals. Through a last round of optimization of the DDM concentration, 3.3%(w/v) DDM was found to be useful to yield larger crystals that could diffract to higher than 2 Å resolution. It is important to note that in the presence of DDM the pH value of the protein buffer needs to be lowered to 6.0 in order to obtain single crystals. The final

Figure 4
Diffraction of the AtaPT^{11–424} crystal and self-rotation function analysis. (*a*) A typical diffraction pattern of an AtaPT crystal. (*b*) Self-rotation function of the AtaPT crystal plotted at $\chi = 180^\circ$ and contoured at 20.0σ . Latitude (θ angle) and longitude (φ angle) grid lines are drawn at 10° intervals. The noncrystallographic twofold axes, which are symmetrically related, are indicated by black arrows.

Figure 5
An *L*-test for twinning revealed an absence of twinning in the data set.

condition for AtaPT^{11–424} crystal growth was 100 mM bis-tris pH 6.0, 200 mM ammonium sulfate, 17% (w/v) polyethylene glycol 3350, 3.3% (w/v) DDM. Pictures of crystals grown during the optimization process are shown in Fig. 3.

A complete data set was collected on the BL17U beamline at SSRF. The crystal diffracted to a resolution of 1.71 Å (Fig. 4a). The crystal belonged to space group *P*₂₁₂₁₂, with unit-cell parameters *a* = 96.2, *b* = 135.8, *c* = 69.5 Å, $\alpha = \beta = \gamma = 90^\circ$. Statistics of data collection and processing are summarized in Table 4. Based on the unit-cell parameters and the molecular weight of AtaPT (46.8 kDa), the Matthews coefficient (Matthews, 1968) was calculated to be 2.52 Å³ Da^{−1} with a solvent content of 49.76%, suggesting the likely presence of two molecules within the single asymmetric unit (Supplementary Table S1). This observation is consistent with a self-rotation function analysis, which showed the existence of noncrystallographic twofold axes (Fig. 4b). The results of an *L*-test revealed an absence of twinning in the data set (Fig. 5).

Molecular replacement with *Phaser* (McCoy *et al.*, 2007) was used to solve the crystal structure of recombinant AtaPT^{11–424}, using the crystal structure of FgaPT2 (PDB entry 3i4z; Metzger *et al.*, 2009), which has a sequence identity of 29% with AtaPT, as the initial model.

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