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Expression, purification, crystallization and preliminary X-ray analysis of perakine reductase, a new member of the aldo-keto reductase enzyme superfamily from higher plants

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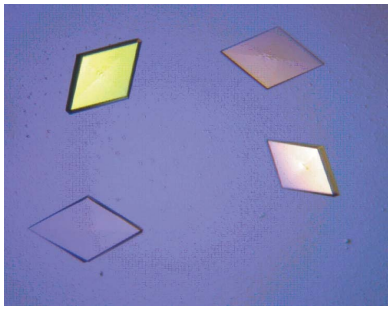

Perakine reductase (PR) is a novel member of the aldo-keto reductase enzyme superfamily from higher plants. PR from the plant *Rauvolfia serpentina* is involved in the biosynthesis of monoterpenoid indole alkaloids by performing NADPH-dependent reduction of perakine, yielding raucaffrinoline. However, PR can also reduce cinnamic aldehyde and some of its derivatives. After heterologous expression of a triple mutant of PR in *Escherichia coli*, crystals of the purified and methylated enzyme were obtained by the hanging-drop vapour-diffusion technique at 293 K with 100 m*M* sodium citrate pH 5.6 and 27% PEG 4000 as precipitant. Crystals belong to space group $C222_1$ and diffract to 2.0 Å, with unit-cell parameters a = 58.9, b = 93.0, c = 143.4 Å.

1. Introduction

A rational engineering of the biosynthetic pathways leading to valuable natural products in higher plants is a great scientific challenge and requires deep understanding of the participating enzymes (Kutchan, 1995). A broad knowledge of such pathways together with biochemical and structural details of the proteins involved is one prerequisite for performing this kind of research. However, biosynthetic knowledge has so far only been accumulated for a few secondary products, in particular examples from the alkaloid field. In this respect, the family of monoterpenoid indole alkaloids, which consists of about 2000 structurally different members, is an important representative. The pathway generating the antiarrhythmic alkaloid ajmaline is the best example from the entire alkaloid family (Ruppert, Ma *et al.*, 2005). The pathway has been almost completely elucidated at the enzymatic level and describes a great deal of the *Rauvolfia* alkaloid metabolism, together with a number of side reactions. Several of the enzymes involved in the biosynthesis of ajmaline have been functionally characterized after overexpression in *Escherichia coli* or using a recently developed highly effective *Nicotiana* plant expression system (Marillonnet *et al.*, 2004, 2005; Ruppert, Woll *et al.*, 2005). Heterologous expression gave for the first time quantities of highly purified *Rauvolfia* proteins suitable for systematic crystallization approaches and elucidation of their three-dimensional structures. Strictosidine synthase (STR1), the enzyme generating the biogenetic precursor (strictosidine) of the entire monoterpenoid indole alkaloid family, has recently been crystallized and its X-ray structure has been determined, along with those of complexes with its substrates (Ma *et al.*, 2004, 2006; Koepke *et al.*, 2005). This was the first example from the novel STR1 enzyme family for which the three-dimensional structure has been elucidated (Ma *et al.*, 2006). A further enzyme, vinorine synthase (VS), located in the middle of the biosynthetic pathway to ajmaline, has also recently been structurally investigated in detail (Ma *et al.*, 2005). VS is the first member of the so-called BAHD enzyme superfamily to have its structure determined and may now serve as a structural basis for three-dimensional analyses of other members of this family (D'Auria,

2006). Many enzymes of this particular family are involved in the biosynthesis of clinically important alkaloids such as vinblastine, taxol and morphine (St-Pierre *et al.*, 1998; Walker & Croteau, 2000; Grothe *et al.*, 2001). An update on the significance of the BADH enzyme family has recently been reported by D'Auria (2006). Moreover, the enzyme strictosidine glucosidase (SG), which follows directly after the synthase STR1 in indole alkaloid biosynthesis, has also recently been crystallized and its preliminary X-ray analysis described (Barleben *et al.*, 2005). SG is one of two glucosidases participating in *Rauvolfia* alkaloid metabolism. The second is raucaffricine glucosidase (RG), which catalyzes the deglucosylation of raucaffricine (Ruppert *et al.*, 2006). This hydrolysis leads to the aglycone vomilenine, which on one hand is a direct intermediate in the route to ajmaline (Fig. 1) and on the other hand serves as the precursor of perakine. The aldehydic alkaloid perakine is reduced by an NADPH-dependent reductase named perakine reductase (PR). Here, we describe the expression and purification of *R. serpentina* PR, which is a novel member of the aldo-keto reductase (AKR) enzyme superfamily from higher plants. We also report the crystallization of the methylated enzyme and its preliminary X-ray analysis.

2. Experimental

2.1. Protein expression and purification

In order to obtain sufficient pure PR for crystallization purposes, the PR cDNA (Gene Bank accession No. AY766462) from *R. serpentina* was cloned into the pQE-2 vector as a fusion protein with an N-terminal 6×His tag. The cDNA was expressed in *E. coli* strain M15 (Qiagen, Hilden, Germany). Transformed *E. coli* cells were grown at 310 K in Luria–Bertani medium in 500 ml batches (1000 ml Erlenmeyer flasks) with 50 µg ml^{-1} ampicillin and 30 µg ml^{-1} kanamycin. After a cell density of $OD_{600} \simeq 0.8$ had been reached, expression of PR was induced by adding isopropyl β-D-galactopyranoside (IPTG) to a concentration of 0.5 m*M*. The bacteria were then grown at 298 K for 24 h. M15 cells were harvested by centrifugation (6000*g*, 8 min, 277 K). The obtained pellet was stored at 253 K if not processed immediately. In a typical experiment for the purification of PR, 10 g of the *E. coli* pellet was sonicated at 277 K in 50 ml buffer *A* (50 m*M* potassium phosphate, 300 m*M* NaCl pH 8.0) containing 10 m*M* imidazole and 1 mg ml^{-1} lysozyme. After centrifugation (14 000*g*, 30 min, 277 K), the supernatant was applied onto a

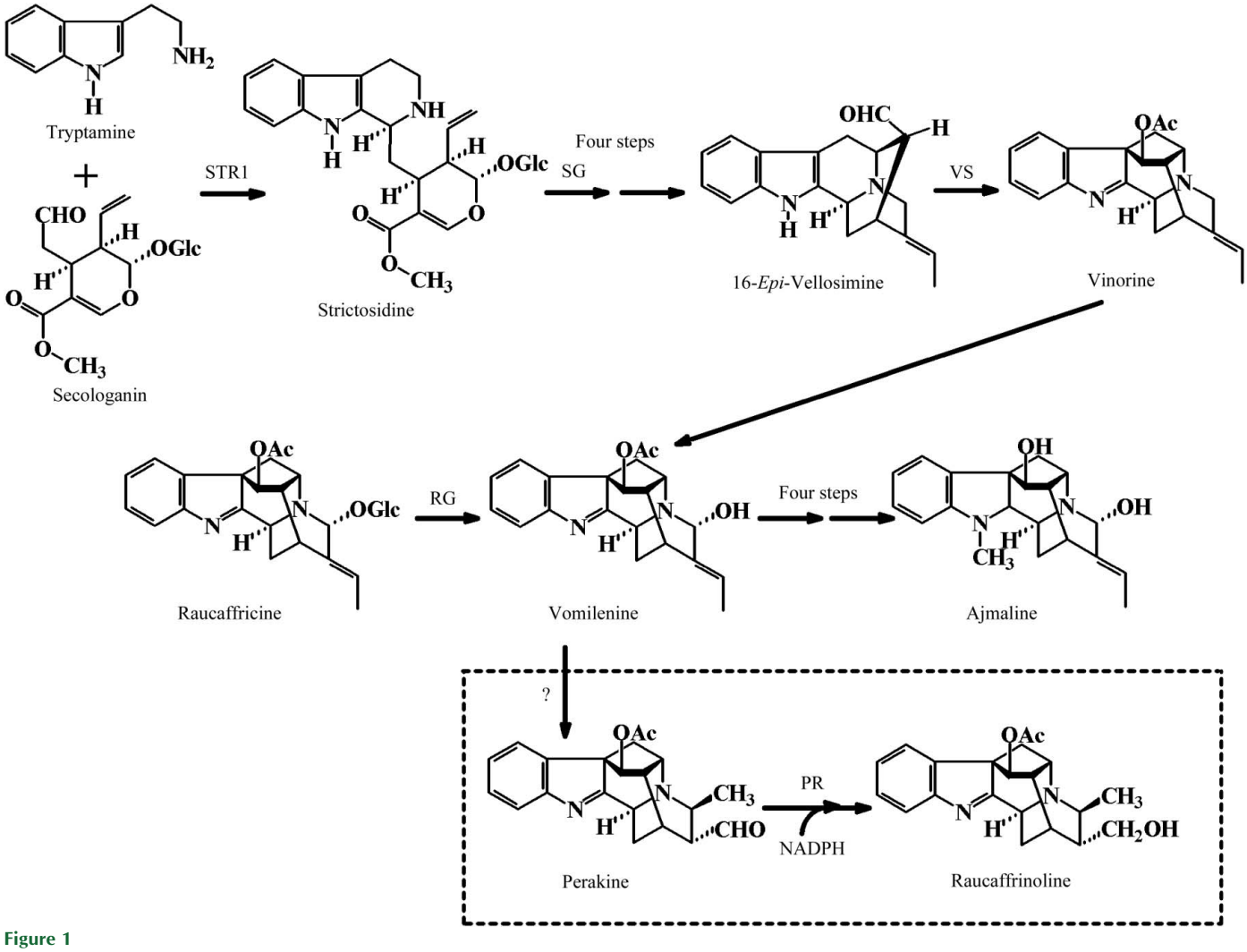

Figure 1
The biosynthetic pathway in *R. serpentina* plant cells leading to the antiarrhythmic monoterpenoid indole alkaloid ajmaline through the indolenine intermediate vomilenine is shown. This intermediate can be formed by deglucosylation of the glucoalkaloid raucaffricine, which is catalyzed by raucaffricine glucosidase (RG). In a side-route of the pathway, vomilenine also serves as a precursor of perakine, which is in turn reduced by the NADPH-dependent perakine reductase (PR), producing the alkaloid raucaffrinoline (shown in the dotted box). The conversion of vomilenine into perakine is easily achieved chemically under acidic conditions, but the enzyme catalyzing this reaction has not yet been detected. Other enzymes involved in the pathway and for which three-dimensional structures have recently been analyzed in detail are strictosidine synthase (STR1), strictosidine glucosidase (SG) and vinorine synthase (VS).

5 ml Ni–NTA column. The column was washed with 70 ml buffer *A* containing 50 m*M* imidazole, followed by elution of PR with 250 m*M* imidazole in the same buffer. Enzyme activity was detected after fractionation (into 1 ml fractions) in the first 3–6 ml, which were combined and dialyzed for 12 h against 4 l buffer *A* pH 7.0 without NaCl but containing 10 m*M* EDTA.

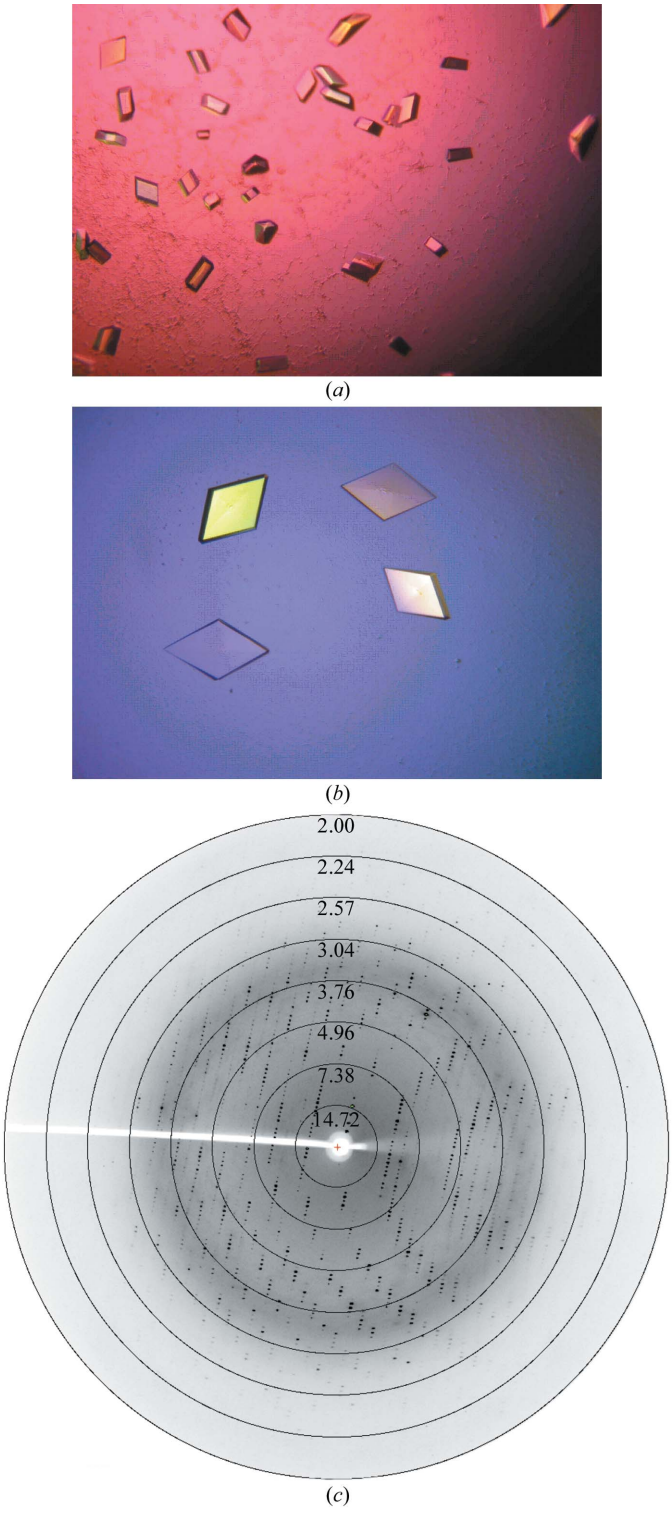

Figure 2
Crystals of the methylated triple mutant of perakine reductase (PR) from *R. serpentina*. (*a*) PR crystals obtained after 14 d growth; (*b*) a close-up view of four single PR crystals of orthorhombic shape with dimensions of 0.12 × 0.12 × 0.03 mm; (*c*) X-ray diffraction image of a PR crystal collected at BESSY, beamline BL14.2, using a MAR Research (Norderstedt, Germany) MAR345-IP imaging-plate detector.

Table 1
X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell.

Beamline	BESSY BL14.2
Wavelength (Å)	0.91841
Space group	$C222_1$
Unit-cell parameters (Å)	a = 58.9, b = 93.0, c = 143.4
Unique reflections	26970
Redundancy	16.6 (14.1)
Resolution range (Å)	20.0–2.00 (2.1–2.00)
$I/\sigma(I)$	25.5 (5.1)
$R_{\rm sym}$† (%)	0.065 (0.51)
Completeness (%)	99.6 (99.3)

† $R_{\rm sym} = \sum_{hkl}\sum|I_i - \langle I\rangle|/\sum\langle I\rangle$, where I_i is the intensity of the ith measurement of reflection hkl and $\langle I\rangle$ is the average intensity of a reflection.

2.2. Enzyme-activity assay

Relative enzyme activity was determined after incubating perakine (or other putative substrates) at 0.2 m*M* in the presence of 0.2 m*M* NADPH and 40 µg enzyme at 320 K in buffer *B* (50 m*M* potassium phosphate pH 7.0) for 45 min. The product was monitored (peak area) by HPLC. Control experiments were performed in a similar way in the absence of NADPH in order to determine nonspecific substrate binding. The isocratic HPLC solvent system used was 30%:70% acetonitrile:H_2O (pH 2.3, adjusted with H_3PO_4) on a Lichrospher 60 RP-select B column (250 × 4 mm, Merck, Darmstadt, Germany) with a flow rate of 1 ml min^{-1}.

2.3. Mutation and methylation of PR

Because of the initial instability of the purified enzyme, we observed severe degradation of the protein within a few days. In order to minimize degradation, the residues Lys98, Lys242 and Lys294 were mutated to Ala by site-directed mutagenesis. The obtained mutant with 24 remaining Lys residues was methylated using the protocol described by Rypniewski *et al.* (1993). The reaction with formaldehyde and dimethylamine–borane complex (DMAB) resulted in the dimethylation of the accessible free amino groups of lysine residues and the N-terminal amino group and offers the opportunity to change the surface properties of the protein, increasing the chances of crystallizing the protein. Briefly, 1 ml PR solution (5–10 mg ml^{-1}) was mixed with 20 µl DMAB (1 *M* in water) and 40 µl formaldehyde (1 *M* in water) was added immediately. After 2 h, the procedure was repeated and the solution was incubated for 15 h after again adding 10 µl DMAB. After adding 100 µl 1 *M* ammonium sulfate and 2 h incubation, the methylated enzyme solution was dialyzed against 10 m*M* Tris–HCl and 1 m*M* dithiothreitol (DTT) pH 7.1. All steps were carried out at 277 K.

2.4. Crystallization

Crystallization experiments were carried out by the hanging-drop vapour-diffusion technique using the methylated His-tagged triple mutant. Drops were prepared by mixing 2 µl protein solution (10 mg ml^{-1}) with 2 µl reservoir solution and were equilibrated against 1 ml reservoir solution at 293 K. The best crystals were observed when 100 m*M* sodium citrate pH 5.6 and 27% PEG 4000 was used as precipitant solution. A similar system with additional 0.2 *M* ammonium acetate and containing 30% PEG 4000 (instead of

Table 2
Substrates accepted by the His_6-tagged perakine reductase mutant.

n.c., no conversion.

Substrate	Relative enzyme activity (%)
Perakine	69 ± 1
Cinnamic aldehyde	100 ± 1
p-Coumaric aldehyde	49 ± 1
3-(3,4,5-Trimethoxyphenyl)propenal	42 ± 2
3-[2-Bromo-3,5-dimethoxy-4-(3,7,11-trimethyl-dodeca-2,6,10-trienyloxy)phenyl]propenal	n.c.
Progesterone	n.c.
Glucose	n.c.

27%) was previously described for the crystallization of xylose reductase (Kavanagh *et al.*, 2002). Crystals appeared after 4 d at 293 K and continued to grow for 14 d, reaching maximum dimensions of 0.12 × 0.12 × 0.3 mm (Fig. 2). The unmethylated His-tagged triple-mutant protein did not crystallize.

2.5. Diffraction data collection and processing

Crystals of methylated PR were cryoprotected in 10% glycerol, 21% PEG 4000, 100 m*M* sodium citrate pH 5.6 for 30 s and flash-cryocooled at 100 K for data collection. Native data were collected to a resolution of 2.0 Å at a wavelength of 0.91841 Å on beamline BL14.2 of BESSY, Berlin, Germany (Table 1). The diffraction data were integrated, scaled and merged with *XDS* (Kabsch, 1993) in the orthorhombic space group $C222_1$, with unit-cell parameters a = 58.9, b = 93.0, c = 143.4 Å, $\alpha = \beta = \gamma = 90^\circ$. The intensities were then converted to structure-factor amplitudes by the *TRUNCATE* program from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Calculation of the Matthews crystal-packing coefficient (V_M = 2.9 Å^3 Da^{-1}) suggests the presence of one molecule in the asymmetric unit, with a solvent content of 57.7% (Matthews, 1968).

Structure solution was attempted by molecular replacement using *MOLREP* (Vagin & Teplyakov, 1997) as well as *Phaser* (McCoy *et al.*, 2005) from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The structure of the NADPH-dependent family 11 aldo-keto reductase from *Bacillis subtilis* (PDB code 1pz0; Ehrensberger & Wilson, 2004) with 32% sequence identity was used as the search model for molecular replacement. However, a satisfactory solution could not be obtained using molecular replacement.

3. Results and discussion

Amino-acid sequence alignments of PR (alignment not shown) revealed that the reductase belongs to the widespread AKR superfamily. The highest sequence identities to five members of the family were between 57 and 72%; these were mostly putative enzymes from the plants *Arabidopsis*, *Oryza* and *Manihot*. However, PR is the first example of an AKR to be detected in the group of enzymes involved in alkaloid biosynthesis. PR seems to have a specific function in *Rauvolfia* alkaloid formation, although it also reduces cinnamic aldehyde and some derivatives, but not ketosteroids such as progesterone. The latter compound is a typical substrate accepted by AKR members (Gavidia *et al.*, 2002). This raises questions on the probable dual functionality of PR, which may not only include alkaloid biosynthesis but may also include function in phenylpropanoid metabolism, generating cinnamic alcohols (Table 2). However, more detailed biochemical and structural data are now required in order to explain the precise metabolic role of PR. *De novo* phasing diffraction experiments on various types of heavy-atom-derivatized PR crystals will be used to determine the structure and to gain a detailed insight into this enzyme of alkaloid metabolism in the Indian medicinal plant *Rauvolfia*.

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