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Heterologous expression, purification, crystallization and preliminary X-ray analysis of raucaffricine glucosidase, a plant enzyme specifically involved in *Rauvolfia* alkaloid biosynthesis

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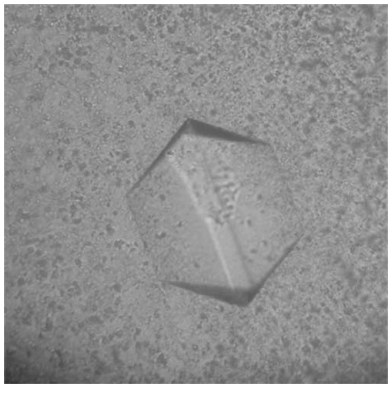


Raucaffricine glucosidase (RG) is an enzyme that is specifically involved in the biosynthesis of indole alkaloids from the plant *Rauvolfia serpentina*. After heterologous expression in *Escherichia coli* cells, crystals of RG were obtained by the hanging-drop vapour-diffusion technique at 293 K with 0.3 *M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.6 buffer and 11% PEG 4000 as precipitant. Crystals belong to space group *I*222 and diffract to 2.30 Å, with unit-cell parameters a = 102.8, b = 127.3, c = 215.8 Å.

1. Introduction

In recent years much knowledge has been accumulated on the biosynthesis of alkaloids in higher plants and especially on the enzymes involved in the biosynthetic pathways. This is particularly true for the two largest alkaloid families, the isoquinolines and the monoterpenoid indole alkaloids, which consist of about 6000 and 2000 structurally different members, respectively. In the first family several examples have been investigated: for example, the biosynthesis of berberine or morphine (Zenk, 1995; Poeaknapo *et al.*, 2004; Boettcher *et al.*, 2005). The antiarrhythmic *Rauvolfia* alkaloid ajmaline together with its derivatives is the best representative of the indole alkaloid family (Ruppert, Ma *et al.*, 2005). The pathway leading to the latter alkaloids has been almost completely unravelled at the enzymatic level, which together with a number of side reactions describes a great deal of the *Rauvolfia* alkaloid metabolism. About half of the enzymes involved in the biosynthesis of ajmaline have previously been functionally overexpressed in *Escherichia coli*, yeast and more recently developed but highly effective plant expression systems such as *Nicotiana benthamiana* (Marillonnet *et al.*, 2004, 2005; Ruppert, Woll *et al.*, 2005). Heterologous expression resulted in highly purified protein in milligram quantities suitable for systematic crystallization of the *Rauvolfia* enzymes and elucidation of their three-dimensional structures. For instance, the structure of strictosidine synthase (STR1), the enzyme generating the ultimate biogenetic precursor to all the monoterpenoid indole alkaloids, has recently been crystallized and solved in complex with its substrates (Ma *et al.*, 2004; Koepke *et al.*, 2005). In fact, this was the first example from the novel STR1 enzyme family to be structurally analysed in detail. Another enzyme, vinorine synthase (VS), located in the middle of the multistep pathway to ajmaline, has also recently been structurally characterized in detail (Ma *et al.*, 2005). The acetylCoA-dependent VS may now serve as a structural basis for three-dimensional analyses of other members of this enzyme family. Many enzymes of this particular family are known to take part in the biosynthesis of clinically important alkaloids such as morphine, taxol or vinblastine (St-Pierre *et al.*, 1998; Walker & Croteau, 2000; Grothe *et al.*, 2001). In addition, the enzyme strictosidine glucosidase (SG) which follows directly after the synthase STR1 in indole alkaloid formation has also recently been overexpressed, crystallized and X-ray analysis was carried out (Gerasimenko *et al.*, 2002; Barleben *et al.*, 2005). SG is one of two major glucose-hydrolysing enzymes

participating in *Rauvolfia* alkaloid metabolism. The second enzyme is raucaffricine glucosidase (RG), which catalyses the deglucosylation of raucaffricine (the substrate of RG). This hydrolysis leads to aglycone vomilenine, a direct intermediate on the route to the target compound ajmaline (Fig. 1). Although the amino-acid sequence and the substrate acceptance of the two glucosidases differ greatly, RG can hydrolyse strictosidine (the substrate of SG); however, SG is unable to hydrolyse raucaffricine (Warzecha *et al.*, 2000; Gerasimenko *et al.*, 2002).

Therefore, it is necessary to determine the three-dimensional structure of RG in order to compare both enzymes in detail from both a molecular and structural point of view in order to understand the basis of substrate specificity.

Here, we report the construction of an *E. coli* expression system for the production of *R. serpentina* RG, as well as the purification, crystallization and preliminary X-ray analysis of the recombinant enzyme.

2. Experimental

2.1. Protein expression and purification

In order to facilitate the purification procedure to obtain pure RG protein suitable for crystallization purposes, the RG gene (AAF03675) from *R. serpentina* was cloned into the pQE-2 vector as a fusion protein with an N-terminal 6×His tag and was expressed in *E. coli* strain M15 (Qiagen, Hilden, Germany). A pSE-RG1 construct was used as a template for PCR (Warzecha *et al.*, 2000). The following primers were applied to amplify the RG cDNA: RG-for-Xa-*Sph*I (5′-GCATGCGATCGAGGGAAGGATGGCAACTCAGAGCAG-TGC-3′, forward) and RG-rev-*Sac*II (5′-CCGCGGTTACTTTCT-TAATCTCTTGCTTGAAAC-3′, reverse). PCR conditions were 368 K for 2.0 min, 31 cycles of 368 K for 0.5 min, 326 K for 1.0 min and 345 K for 2.5 min and finally 345 K for 10 min. The PCR product was ligated into the *Sph*I and *Sac*II sites of the pQE-2 expression vector (Qiagen) and the resulting construct was transformed into the M15 bacteria strain.

The bacteria were grown in Luria–Bertani medium (in 10 × 500 ml batches, each in 1000 ml Erlenmeyer flasks) containing 60 µg ml^{-1} ampicillin and 30 µg ml^{-1} kanamycin at 310 K. After a cell density of OD_{600} = 0.5 had been reached, expression of RG was induced by adding 2 m*M* isopropyl *β*-D-thiogalactopyranoside (IPTG). The bacteria were then grown at 298 K for 36 h. Harvesting and protein isolation from the M15 cells was performed as described previously (Barleben *et al.*, 2005). RG protein was purified using an Ni–NTA column and the purification protocol previously described for strictosidine synthase was followed (Ma *et al.*, 2004). The enzyme was concentrated to 6 mg ml^{-1} in 20 m*M* Tris–HCl pH 7.5 and 20 m*M* *β*-mercaptoethanol.

2.2. Crystallization

Crystallization experiments were carried out with the His tag-containing protein. Crystals were obtained by the hanging-drop vapour-diffusion technique. Drops containing 2 µl protein solution (6 mg ml^{-1}) and 2 µl precipitant buffer were equilibrated against 700 µl precipitant buffer. The best crystals were observed using 0.3 *M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.6 and 11% PEG 4000 as precipitant buffer at a temperature of 293 K. Crystals appeared in 1–3 d and reached maximum dimensions of approximately 0.2 × 0.15 × 0.05 mm (Fig. 2).

2.3. Diffraction data collection and processing

Prior to X-ray data collection, the crystals were treated with cryoprotectant [10 m*M* calcium acetate, 10 m*M* Tris pH 7.5, 7% DMSO, 20%(*v*/*v*) glycerol] and flash-cooled to 100 K. Diffraction data were then collected on beamline X11 of EMBL Hamburg, Germany. Data could be indexed in the *I*-centred orthorhombic space group *I*222, with unit-cell parameters *a* = 102.8, *b* = 127.3, *c* = 215.8 Å. 720 images were measured with 0.5° rotation per frame using a MAR CCD detector at a wavelength of 0.8051 Å. A complete data set was

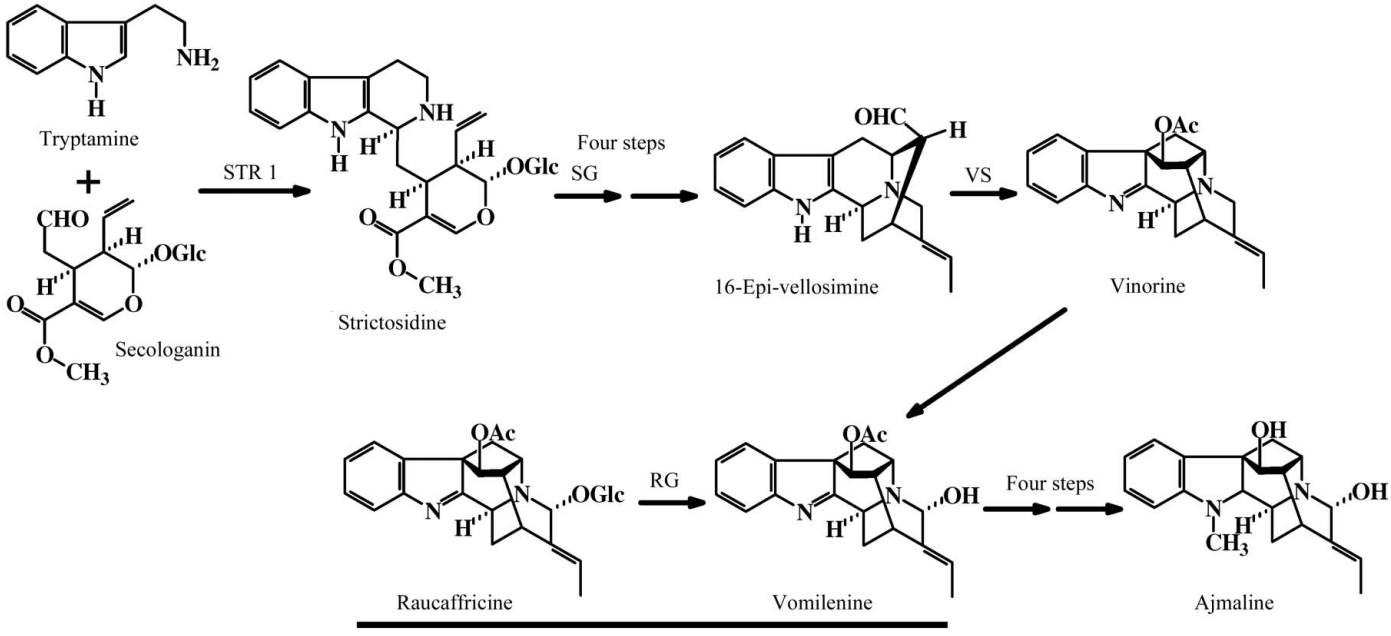


Figure 1
The multistep biosynthetic pathway in *R. serpentina* plant cells leading to the antiarrhythmic ajmaline *via* the intermediate vomilenine. This intermediate can be formed by deglucosylation of the glucoalkaloid raucaffricine, which is catalyzed by raucaffricine glucosidase (RG). Other enzymes involved in the pathway and whose three-dimensional structures have recently been elucidated are strictosidine synthase (STR1), strictosidine glucosidase (SG) and vinorine synthase (VS).

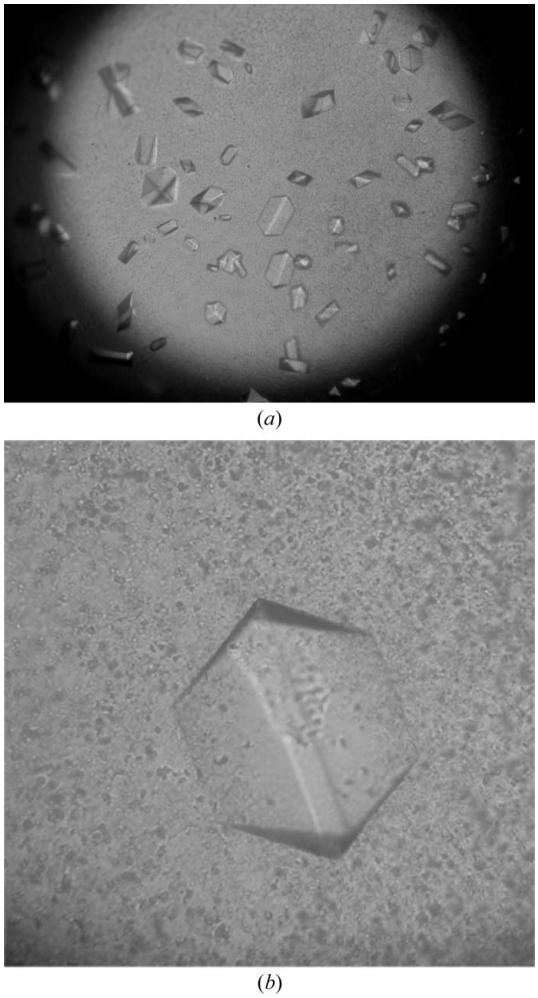
collected to 2.30 Å resolution. The collected data were indexed and processed with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The data-set statistics are summarized in Table 1.

3. Results and discussion

A self-rotation analysis of the native data performed using the program *POLARRFN* (Collaborative Computational Project, Number 4, 1994) showed twofold rotational non-crystallographic symmetry of RG molecules; assuming two molecules per asymmetric unit, the Matthews coefficient (V_M) was calculated to be 2.9 Å^3 Da^{-1}, corresponding to a solvent content of 57.2%. The structure of RG was solved using the molecular-replacement technique with the structure of SG from *R. serpentina* (unpublished data) as a search model. RG shares a sequence identity of 56% with SG. Using the programs *MOLREP* from the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994), two significant peaks in the rotation and translation functions could be found which led to a plausible crystal packing for two molecules per asymmetric unit. A full structure analysis is in progress.

Detailed X-ray analysis of RG will allow further investigation of the molecular recognition of substrates, the reaction mechanism and inhibition of catalysis. The obtained results together with those from SG (Barleben *et al.*, 2005) will provide a rare example of the three-dimensional structures of two different enzymes that catalyze basically the same reaction type (deglycosylation) in the same metabolic pathway (indole alkaloid biosynthesis), originating from the same plant (*R. serpentina*) but exhibiting a remarkable difference in substrate specificity.

Therefore, substrate recognition and evaluation of both glucosidase structures will hopefully provide a better understanding of the deglucosylation reaction in the ajmaline biosynthetic pathway, a reaction type which is common in nature.



(*a*)

(*b*)

Figure 2
Raucaffricine glucosidase (RG) crystals from *R. serpentina*. (*a*) RG crystals obtained after 3 d; (*b*) a close-up view of a single crystal of octagonal shape with dimensions 0.2 × 0.15 × 0.05 mm.

Table 1
X-ray data-collection statistics.

Values in parentheses correspond to the highest resolution shell.

Beamline	X11
Wavelength (Å)	0.8051
Space group	*I*222
Unit-cell parameters (Å)	a = 102.8, b = 127.3, c = 215.8
Measured reflections	956481
Unique reflections	62515
Redundancy	15.3
Resolution range (Å)	20–2.30
$I/\sigma(I)$	28.3 (6.2)
R_{sym}† (%)	10.0 (49.3)
Completeness (%)	99.3 (98.8)
Mosaicity (°)	0.43

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

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