

Purification, cloning, functional expression and characterization of perakine reductase: the first example from the AKR enzyme family, extending the alkaloidal network of the plant *Rauvolfia*

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Abstract Perakine reductase (PR) catalyzes an NADPH-dependent step in a side-branch of the 10-step biosynthetic pathway of the alkaloid ajmaline. The enzyme was cloned by a “reverse-genetic” approach from cell suspension cultures of the plant *Rauvolfia serpentina* (Apocynaceae) and functionally expressed in *Escherichia coli* as the N-terminal His₆-tagged protein. PR displays a broad substrate acceptance, converting 16 out of 28 tested compounds with reducible carbonyl function which belong to three substrate groups: benzaldehyde, cinnamic aldehyde derivatives and monoterpenoid indole alkaloids. The enzyme has an

extraordinary selectivity in the group of alkaloids. Sequence alignments define PR as a new member of the aldo-keto reductase (AKR) super family, exhibiting the conserved catalytic tetrad Asp52, Tyr57, Lys84, His126. Site-directed mutagenesis of each of these functional residues to an alanine residue results in >97.8% loss of enzyme activity, in compounds of each substrate group. PR represents the first example of the large AKR-family which is involved in the biosynthesis of plant monoterpenoid indole alkaloids. In addition to a new esterase, PR significantly extends the *Rauvolfia* alkaloid network to the novel group of peraksine alkaloids.

The nucleotide sequences reported in this article have been submitted to the Gene Bank under Accession No: AY766462.

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Abbreviations

AKR	Aldo-keto reductase
DEAE	Diethylaminoethyl
KPi	Potassium phosphate
NTA	Nitrilo-tri-acetic acid
PAGE	Polyacrylamide gel electrophoresis
PR	Perakine reductase
RACE	Rapid amplification cDNA ends
R _t	Retention time
RT-PCR	Reverse-transcription PCR
SDS	Sodium dodecylsulphate
TLC	Thin layer chromatography

Introduction

In the past two decades, systematic investigation of the enzymes of plant alkaloid biosynthesis has been carried out for a handful of alkaloid groups. For instance, extensive

research has been conducted on single enzymes participating in the complex pathways leading to isoquinoline alkaloids such as morphine or berberine (see Kutchan 1998; Zenk 1995 for reviews; Unterlinner et al. 1999). In addition, a set of enzymes and genes involved in the biosynthesis of the diterpenoid taxus alkaloid taxol has been described in detail (see Kaspera and Croteau 2006; Croteau et al. 2006, and cited literature). In this context, the monoterpene-derived alkaloids, including the monoterpene-derived isoquinolines and monoterpene-derived indoles, are of specific interest. For the monoterpene-derived isoquinolines, two enzymes of *Ipecacuanha* alkaloid biosynthesis have previously been detected and characterized (De-Eknamkul et al. 1997, 2000). However, almost all enzymes and several genes of a separate multi-step biogenetic pathway have recently been detected and cloned: those pertaining to the generation of the *Rauvolfia* alkaloid ajmaline and a number of its derivatives which possess the typical sarpagan and ajmalan skeleton types (for reviews see Stöckigt 1995; Ruppert et al. 2005a). This entailed comprehensive alkaloid profiling of in vitro systems, in particular, cell cultures of the Indian medicinal plant *Rauvolfia serpentina* Benth. ex Kurz (Apocynaceae), was employed in this experimental setting for the last 15 years. The resulting knowledge has allowed description of one of the largest and most complicated metabolic networks of plant natural product biosynthesis: with particular focus on alkaloid biosynthesis, it further represents the first, in depth functional characterization of individual *Rauvolfia* enzymes.

The broad alkaloidal metabolome of *Rauvolfia* is due to the existence of several side-routes branching from the ajmaline biosynthetic pathway, the latter encompassing ten enzyme-catalyzed steps (Fig. 1). In one of these branches, the indolenine alkaloid vomilenine serves as the precursor of the aldehydic perakine, which in turn is reduced at the expense of NADPH to raucaffrinoline, a typical *Rauvolfia* alkaloid (Sabino et al. 2006). This enzyme, detected in crude protein mixtures of *Rauvolfia* cell suspensions, has been designated as perakine reductase (PR): expression, preliminary characterization (Stöckigt 1995) and crystallization of a methylated PR triple mutant (Rosenthal et al. 2006) have been carried out for this protein. The current paper describes the purification, “reverse genetics”-mediated cloning, and functional expression of His₆-tagged wild type PR in detail. Sequence analysis groups PR as a novel member of the aldo-keto reductase (AKR) enzyme family. Extensive investigation of the substrate specificity of PR not only points to multi-functionality of the enzyme, which has not been observed for any other *Rauvolfia* enzyme known to date, but also links and significantly extends the large *Rauvolfia* alkaloid metabolome to the recently detected novel group of peraksine alkaloids (Sheludko et al. 2002).

Materials and methods

Chemicals

Benzaldehyde, phenylacetaldehyde, phenylpropionaldehyde, 2-nitrobenzaldehyde, 4-hydroxy-3-nitrobenzaldehyde, 4-nitrobenzaldehyde, 2-hydroxy-5-nitrobenzaldehyde were bought from Acros (New Jersey, USA), *p*-anisaldehyde, coniferyl aldehyde from Sigma-Aldrich (Steinheim, Germany), cinnamic aldehyde from Roth (Karlsruhe, Germany), glucose and pyridoxal from AppliChem (Darmstadt, Germany). *p*-Coumaryl-, coniferyl-, sinapyl- and caffeyl aldehyde were gifts of Prof. Norman G. Lewis (Washington State University, USA). The synthetic cinnamic aldehyde derivatives, 3-(3,4,5-trimethoxy-phenyl)-propenal, 3-(3,4,5-tris-methoxy-methoxy-phenyl)-propenal and 3-[2-bromo-3,5-dimethoxy-4-(3,7,11-trimethyl-dodeca-2,6,10-trienyloxy)-phenyl]-propenal were synthesized by Jingxu Gong, Lihong Hu, Hongbin Zou (Zhejiang University, China). Gardner was kindly provided by Profs. Mariko Kitajima and H. Takayama (Chiba University, Japan), 3-oxotabersonine by Prof. Bruno Danieli (University of Milan, Italy) and vallesine by Prof. Carl Djerassi (Stanford University, USA). The remaining alkaloids were taken from our alkaloid collection at the Department of Pharmaceutical Biology.

Cell suspension cultures and purification of PR

Plant cell suspension cultures of *R. serpentina* were grown as described previously (Warzecha et al. 1999). Extraction and purification of PR was carried out by procedures previously applied to *Rauvolfia* enzymes (Ruppert et al. 2005b). In brief, 1.7 kg of *R. serpentina* cells were extracted with 2 l of Tris/HCl buffer (0.1 mM, pH 7.5). Following filtration and centrifugation of the resulting slurry, proteins in the supernatant were precipitated with (NH₄)₂SO₄ in two consecutive fractions (0–30% and 30–70% (NH₄)₂SO₄ saturation, respectively). The protein pellet obtained in the second fraction was re-dissolved in extraction buffer, dialyzed and subjected to three subsequent activity-guided separation steps via ion exchange by diethylaminoethyl (DEAE) sepharose, AcA54 size exclusion and ion exchange chromatography on a MonoQ column. After the final purification step, samples of the fraction exhibiting reductase activity were subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE) (as described by Ornstein 1964). Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), showed several protein bands.

Determination of protein concentrations and partial amino acid sequence analysis

Protein concentrations were measured according to Bradford (1976) using bovine serum albumin as the reference protein.

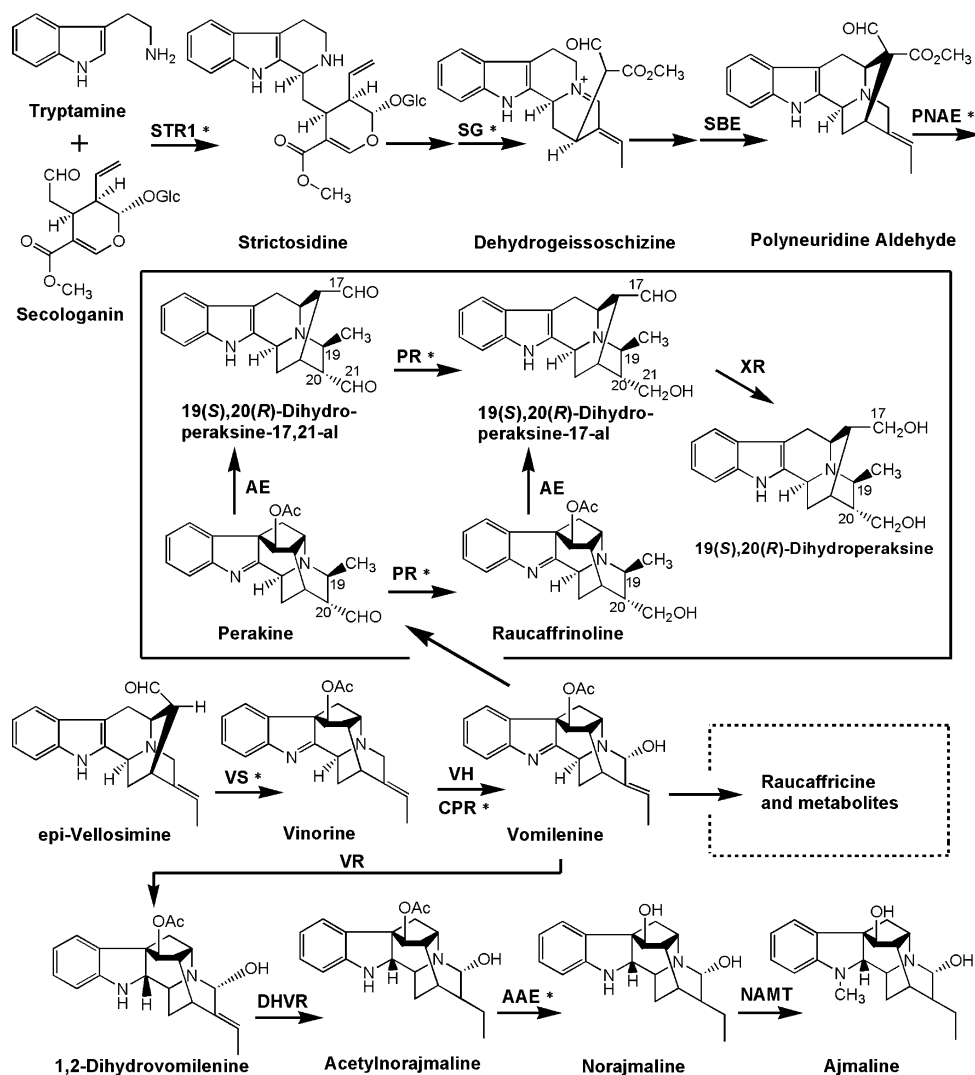


Fig. 1 Part of the alkaloidal network of the *Rauwolfia* plant. The 10-step biosynthetic route to the monoterpenoid indole alkaloid ajmaline is connected at the stage of the intermediate vomilenine with perakine. Vomilenine can be isomerized into perakine under acidic conditions. The corresponding enzyme has not yet been detected. Perakine reductase (PR) and acylesterase (AE) are the central enzymes for the metabolic scheme to the peraksines—a novel group of *Rauwolfia* alkaloids recently detected (framed). XR represents an unknown reductase. Other enzymes of this network are strictosidine

synthase (STR1), strictosidine glucosidase (SG), sarpagan bridge enzyme (SBE), polyneuridine aldehyde esterase (PNAE), vinorine synthase (VS), vinorine hydroxylase (VH), cytochromeP450 reductase (CPR), vomilenine reductase (VR), 1,2-dihydrovomilenine reductase (DHVR), acetylnorajmaline esterase (AAE) and norajmalan methyltransferase (NAMT) (* means heterologously expressed enzymes). A further branch of the main pathway leads to raucaffricine alkaloids and derivatives (framed by dashed line, structures not illustrated)

From the Coomassie-brilliant blue-stained SDS-PAGE, major protein bands were digested in situ with proteinase LysC for sequence analysis and further processed as reported (Ruppert et al. 2005b). Partial peptide sequences obtained from the main protein band (~38 kDa) of the putative PR, were (a) QIYFRIEALAQK and (b) FGISLDFSGLFAK, and were used for cloning.

Isolation of a full-length cDNA of PR

Total cDNA was obtained from *R. serpentina* cell suspensions by reverse-transcription PCR (RT-PCR) (Qiagen,

Hilden, Germany). The oligo peptides mentioned above were used for primer design. The primer sequences were as follows: PR (b)-forward, TTY GGI ATH WSI GAR YTI GAY TTY WSI GG; PR (a)-reverse, TTY TGI GCI ARI GCY TCD ATI CWR AAR TA; R = A/G, S = C/G, W = A/T, Y = C/T, D = A/G/T, H = A/C/T, I = Inosine. PCR was performed using the following conditions: 1 × (94°C 5 min), 4 × (94°C 1 min, 41°C 1 min, 72°C 2 min), 30 × (94°C 1 min, 46°C 1 min, 72°C 2 min), 1 × (72°C 10 min). A 510 bp DNA fragment was obtained and cloned into the pGEM-Teasy vector (Promega, Mannheim, Germany).

The DNA fragment was sequenced and the following primers were designed to identify the sequence of the complete 3'- and 5'-ends by rapid amplification of cDNA ends (RACE-PCR). The following primers were used: 5'-RACE-PR CT CCA GAC TAT GTC CGC TCC TGC TGT GAG G, 3'-RACE-PR CAT CAC ATC CCA GAT TTG TTG GGG AGA ACC. cDNA of the *R. serpentina* cell suspension culture, produced with the GeneRacer Kit (Invitrogen, Karlsruhe, Germany), was used as template. The conditions were 1 × (94°C 5 min), 5 × (94°C 30 s, 71°C 2 min), 5 × (94°C 30 s, 69°C 30 s, 72°C 2 min), 20 × (94°C 30 s, 67°C 30 s, 72°C 2 min), 1 × (72°C 10 min).

The full-length cDNA was amplified by PCR using the following primers: PR for-pQE-2 GCA TGC GCA AAT GCC AAG AGT TAA GCT GGG, PR rev-pQE-2 CCG CGG TTA CTT CAA TGG TGG TGT ATT. The template mentioned above was used. These primers generated restriction sites (*Sph*I and *Sac*II) at the ends of the full-length clone for sub-cloning into the pQE-2 expression vector (Qiagen, Hilden, Germany). The following PCR conditions were used: 1 × (94°C 5 min), 33 × (94°C 1 min, 55°C 1 min, 72°C 3 min), 1 × (72°C 10 min).

Sequence alignments

Multiple sequence alignments were performed with CLUSTALW and BLAST for database searches.

Expression and purification of PR

Full-length PR cDNA (1014 bp; Gene Bank accession number AY766462) from *R. serpentina* was cloned into the pQE-2 vector and transformed in the M15 *Escherichia coli* strain (Qiagen, Hilden, Germany), resulting in a PR fusion protein harboring six histidine residues at its N-terminal. Bacteria were grown at 37°C in LB-medium in 500 ml batches (1000 ml Erlenmeyer flasks) with 50 µg/ml ampicillin and 30 µg/ml kanamycin up to a cell density of OD₆₀₀ ~ 0.8. Expression was induced by addition of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) to the medium. Following 24 h growth at 25°C, bacteria were harvested by centrifugation and the bacterial pellets stored at -20°C.

For enzyme purification, in a typical experiment, 10 g of the harvested bacteria were resuspended in 50 ml extraction buffer (50 mM KPi, 300 mM NaCl, pH 8.0) containing 10 mM imidazole and 1 mg/ml lysozyme. Following bacterial sonication (4°C) and centrifugation (14,000g, 30 min, 4°C) the supernatant was added to a 5 ml column containing 2 ml Ni-nitrilo-tri-acetic acid (Ni-NTA) superflow (Qiagen, Hilden, Germany). The column was then washed with 70 ml of extraction buffer

containing 50 mM imidazole. PR protein was eluted with 250 mM imidazole in the above mentioned buffer. Enzyme containing fractions (1 ml each) were combined and dialyzed for 12 h at 4°C against 4 l of 50 mM KPi buffer (pH 7.0) containing 10 mM of EDTA.

Enzyme assay and substrate specificity studies of PR

For measurement of PR activity during enzyme isolation and purification, 21-hydroxyraumacline and NADPH were used in excess as substrates. In brief, aliquots of fractions from chromatographic separations of the crude protein (after (NH₄)₂SO₄ precipitation) were incubated in a total of 200 µl KPi buffer (40 mM, pH 8.5) in the presence of 0.5 mM 21-hydroxyraumacline and 0.6 mM NADPH at 37°C for 60 min (the alkaloid 21-hydroxyraumacline was used as a substrate instead of perakine, because the latter was only available in trace amounts). The reaction was terminated by adding 200 µl methanol. After centrifugation (14,000g, 5 min), 30 µl of the supernatant was subjected to HPLC analysis.

Purified His₆-tagged PR enzyme (5 µg enzyme for benzaldehyde derivatives, 100 µg for cinnamic aldehyde derivatives and for alkaloids) was incubated in 200 µl KPi buffer (50 mM, pH 7.0) in the presence of 0.8 mM substrate and 4.0 mM NADPH at 30°C for 15 min. Following addition of 200 µl methanol and centrifugation (14,000g, 5 min), 50 µl of the supernatant was subjected to HPLC analysis. The reverse reaction was measured under the same conditions in the presence of NADP⁺ instead of NADPH. All assays were performed in triplicate with controls in the absence of NADPH or NADP⁺.

Kinetic data of PR

Enzyme kinetics were measured by monitoring product formation rates by HPLC and 6–8 different substrate concentrations were used under the same conditions as above, but in the presence of excess NADPH (25 mM). The enzyme amounts were between 1 and 25 µg.

The optimum temperature of PR was determined in the forward direction between 10 and 70°C in 10°C intervals, and the pH optimum between 5.5 and 9.0 in intervals of 0.5 units.

Analysis and identification of PR products

For quantitation of products by HPLC, peak areas were determined; wavelengths of 280 nm were used for cinnamic aldehyde derivatives, and 254 nm for alkaloids and benzaldehyde derivatives respectively. A Lichrospher 60 RP-select B column (250 × 4 mm, Merck, Darmstadt,

Germany) was used and the HPLC conditions were: 20% acetonitrile and 80% water, pH 2.3 and 1 ml/min flow.

The identity of enzymatic reaction products was proven by mass spectrometry (Finnigan MAT 44S quadrupole instrument (Bremen, Germany)) in EI-MS (electron impact) mode at 70 eV (ion source 200°C, evaporation temperature was 30–320°C with a gradient of 100°C/min). A five-fold incubation mixture was extracted with CHCl₃. The organic layer was evaporated, the residue purified by thin layer chromatography (TLC) and the appropriate product measured by EI-MS.

Analysis and identification of *R. serpentina* cells for occurrence of cinnamic aldehydes by TLC, HPLC, GC-MS

To check for the presence of cinnamic aldehydes, 200 g freeze dried (4 kg fresh weight) *R. serpentina* cells were suspended in 2 l of MeOH (HPLC grade, Roth, Karlsruhe, Germany) and divided into two equal parts. 20 mg of coniferyl aldehyde and 20 mg of cinnamic aldehyde was added to one part as a positive extraction control. The two parts were then separately processed under the same conditions. After extracting with MeOH (22°C) for 24 h, the two parts were centrifugated (10,000g, 30 min, 4°C), and the supernatant allowed to evaporate under vacuum at 40°C. The residue obtained was resuspended in 50 ml H₂O (pH 2.0) and extracted four times with 50 ml CHCl₃. The organic fractions were combined and evaporated under vacuum at 40°C. Using coniferyl-, and cinnamic aldehyde as control, the CHCl₃ fraction was analysed by TLC, HPLC, and GC-MS.

TLC plates (20 × 20 cm, Silica gel 60 F254, Merck, Darmstadt, Germany) were developed in a solvent system consisting of CHCl₃/MeOH/formic acid 150:5:0.2, and analysed by UV and spraying with aldehyde reagent (2,4-dinitrophenylhydrazine solution; ethanol and concentrated H₂SO₄).

HPLC conditions for detecting the cinnamic aldehyde derivates were: Lichrospher 60 RP-select B column (250 × 4 mm, Merck, Darmstadt, Germany); mobile phase (A = acetonitrile, B = water pH 2.3), 0–15.0 min 30% A and 70% B, 15.1–18.0 min 50% A and 50% B, 18.1–20.0 min 70% A and 30% B, 20.1–25.0 min 30% A and 70% B; wavelength 280 nm; 1 ml/min flow.

GC-MS analyses were performed on a Viking 573 instrument (Bruker Daltonics, Billerica, MA, USA) in EI mode, using a Restec XTI-5 column (30 m × 0.25 mm I.D., 0.25 μm film thickness). The injection port and the transfer line were maintained at 270°C, the MS transfer line at 285°C, and the GC-oven temperature from 80 to 270°C (at 10°C/min). EI (70 eV) ionization was used and mass spectra were collected full scan over the range

40–400 *m/z* with ion trace filtering on molecular ions M⁺ of the phenylpropanoids cinnamic aldehyde (M⁺ 132 *m/z*) and coniferyl aldehyde (M⁺ 178 *m/z*).

Site directed mutagenesis for generation of PR mutants

Mutagenesis was performed with the QuickChange™ Site-Directed Mutagenesis kit (Stratagene, La Jolla, USA). The PR-pQE-2 plasmid was used as a template for PCR with Phusion-Polymerase (NEB, Frankfurt, Germany). For the generation of the four single mutants: Asp52Ala, Tyr57-Ala, Lys84Ala, His126Ala the following primers were used (exchanged triplet in bold):

Asp52Ala_for 5'-A ACA TTC TTT **GCT** ACA TCA G-3'; Asp52Ala_rev 5'-ATC TGA TGT **AGC** AAA GAA TG-3'; Tyr57Ala_for 5'-TCA GAT ATT **GCT** GGC GAG AAC-3'.

Tyr57Ala_rev 5'-C GTT CTC GCC **AGC** AAT ATC TG-3'; Lys84Ala_for 5'-TG GGA ACG **GCG** TTT GGT ATA C-3'; Lys84Ala_rev 5'-T ACC AAA **CGC** CGT TCC CAC C-3'; His126Ala_for 5'-TAC ATA **GCT** CGC ATA GAT AC-3'; His126Ala_rev 5'-TC TAT GCG **AGC** TAT GTA GAA G-3'.

Results

Enrichment of PR from *Rauvolfia* cells and isolation of a full length PR cDNA

Perakine reductase could be purified according to the protocol developed for another *Rauvolfia* enzyme, the raucaffricine glucosidase (Warzecha et al. 1999). However, as judged by SDS-PAGE, after the third chromatographic step (MonoQ), the fractions showing reductase activity still contained several proteins. To assign the reductase activity to one of the protein bands visible on SDS-PAGE, the enzyme solution was subjected to native PAGE in duplicate: proteins from sections of one gel lane were eluted and tested for enzyme activity. In parallel, proteins in the second lane were subjected to SDS-PAGE. PR activity was found to correspond to the presence of a ~38 kDa protein in SDS-PAGE. Peptides obtained from in situ digestion of the respective protein band were sequenced and yielded two partial amino acid sequences. Degenerate primer pairs based on these fragments were designed and generated a ~500 bp PR cDNA fragment by PCR. Following ligation into the pGEM-T vector, cloning in *E. coli* and sequencing, the PCR product was found to contain the coding region for both peptide fragments indicating that the correct fragment corresponding to PR cDNA had been isolated. RACE-PCR resulted in isolation of the full-length cDNA of 1014 bp (accession number AY766462). cDNA translation yielded a

polypeptide of 337 amino acids with a calculated molecular mass of 37159.8 Da and a theoretical isoelectric point of 5.9.

PR sequence alignment

The highest amino acid sequence identities for PR were found in the group of hypothetical plant oxidoreductase proteins e.g. from *Oryza sativa* (acc. number gb EAY78505.1 and gb EAZ16093.1, each 71% identity), an

auxin-induced protein from *O. sativa* (dbj BAD61512.1, 66% identity), for the F8A5.21, At1g60710 and F8A5.24 protein from *Arabidopsis thaliana* (gb AAB71981.1, gb AAM20506.1, both 56% identity, gb AAB71982.1, 55%) and an AKR from *Manihot esculenta* (gb AAX84672.1, 53%) (see also Fig. 2).

Alignment with sequences of all members of the plant AKRs (<http://www.med.upenn.edu/akr/>; Hyndman et al. 2003) revealed that PR is related to aldose reductase from

Fig. 2 Alignment of the perakine reductase amino acid sequence with the sequences of proposed Aldo-keto reductases from various plant species and with plant AKR's with known function. The predicted catalytic residues are marked red (Asp52, Tyr57, Lys84, His126).

Identical amino acids are marked with * and homolog amino acid exchanges are marked with : or : PR: perakine reductase from *Rauvolfia serpentina* (gbAAX11684.1) (100%); AIOS: putative auxin-induced protein, from *Oryza sativa* (dbjBAD61512.1) (66%); AIAT: auxin-induced protein, putative, from *Arabidopsis thaliana* (gbAAM20506.1) (56%); AKRME: Aldo-keto reductase AKR, from *Manihot esculenta* (gbAAX84672.1) (53%); 2A1: NADP⁺-dependent sorbitol-6-phosphate reductase from *Malus domestica* (sp P28475) (14%); 4A2: chalcone polyketide reductase from *Medicago sativa* (pir S48851) (15%); 4B3: codeinone reductase from *Papaver somniferum* (gb AAF13736) (14%); 4C5: aldose reductase from *Digitalis purpurea* (emb CAC32834) (15%). Identities of the amino acid sequences towards PR is given in brackets

PR	-----MPRVKLGTQGLEVSKLGFSGMGLSGDYNLPEEQGIAVIKEAFNCGITFF	TSDI	GENG
AIOS	MAEQAPLAPMPRVKLGTRGLEVSKLGFSGMGLTGAYNSPLDDDGAAVIAHAFRRGVTF	TSDV	G-PL
AIAT	---MAEACGVRRMKLGSQGLEVSAQGLGCMGLSAFYGAPKENEALIHHAHISGVTL	TSDI	G-PE
AKRME	----MAGAAVKRIKLGSSQGLEVSAQGLGCMSSAFYGPPEMIALIHHAINTGVTF	TSDV	G-PH
2A1	-----MSTVTLSSGYEMPVIGLGLWR---	LEKDELKEVILNAIKIGYRHF	CAAHKSEA
4A2	-----MGSVEIPTKVLNTSSQLKMPVVGMSAPDFTCCKD--KDAIIEAIKQGYRHF	TAA	GSEQ
4B3	-----MESNGVPMITLSSQIRMPALGMGTAEATMVKGTEREKLAFLKAIIEVGYRHF	TAA	QTEE
4C5	-----MAEEIRFFKLNFGAKIPSVGLGTWQSSPG---	DAAQAVEVAIKCGYRHF	GARLGENE
		: : : :	*: * : * : *
PR	SNEELLGKALKQLPREKI---QVGT	FGIHEIGFSGVKAKGTPDYVRSCEASLKR	LDVDYIDLFI
AIOS	ANEILLGKALKQLPREQV---QVAT	FGIRRGADGVRACGRPEYVRACCEASLGR	LGVDYIDLYYQ
AIAT	TNEVLLGKALKDGVREKV---ELAT	FGISYA-EGKREVRGDPYVRAACEASLKR	LDIACIDLYYQ
AKRME	TNEILLGKALKGDIRKKV---ELAT	FAINLK-DGKREIRGDPAYVRAACEASLKR	LDVDCIDLYYQ
2A1	DVGEALAEAFKTLGLVK-REELFIT	TIWNSDHG-----HVVEACKNSLEKLQ	IDYLDLYL
4A2	ALGEALKEAIELGLVT-RDELFTS	LWVTENH-----PHLVI	PALQKSLKTLQ
4B3	CLGEAIAEALQLGLIKSRDELFTS	LWCADAHAD-----LVL	PALQNSLRNKL
4C5	EIGVVLKFLFDDGVVK-REDLFTS	LWSTDH-----APE	DVPVALDKTLED
		: *	* . . : * * : : * : *
PR	-----IDTTVPIEITMG-----	ELKKLVEEGKIKYVGLSEAS	PDTIRRAHAVHP
AIOS	-----VDTTIPIEDTIG-----	ELKKLVEEGKVRYIGLSEAS	PDTIRRAHAVHP
AIAT	-----VDTRVPIEITMG-----	ELKKLVEEGKIKYIGLSEAS	ASTIRRAHAVHP
AKRME	-----VDTSVPIEIVTVG-----	ELKKLVEEGKIKYIGLSEAS	ASTIRRAHAVHP
2A1	MPTKHNAIGKTASLGDVLDIVT	ISLQQTWEGMEKTVSLGLVRSIGLSNYEL	FLTRDCLAYSK
4A2	LSS---QPGKFTFPIDVADLLPFDVKG	---	VWESMEESLKLGLTKAIGVSNFSVKLENLLSVATVLP
4B3	VSL---KPGKQVNEIPKDHILPMDYKS	---	VWAAMEECQTLGFTRAIGVSNFSCKRLQELMETANSPPV
4C5	VRL---KKG--SVGLDPENFIPTDIPG	---	TWKAMEALYDSGKARAIIVSNFTLKKLSDLLDVARIPPA
		: :	: * : : * : :
PR	QIEYSLWTRDIEDEIVPLCRQLGIGIVPYSPIGRGLFA-GKAIKESLPENS	SVLTSHPRFVGENLEKNKQI	
AIOS	QMEWSLWARDIEPEIVPLCRELGIGIVPYSPIARGFFG-GRGVTEQLS	AESNLQGHPRFSAENLEKNKQL	
AIAT	QIEWSLWTRDVEEIIPTCRELGIGIVAYSPLGRGFFASGPKLVENLEK	DKDFRKALPRFQENLDHKNKIV	
AKRME	QLEWSLWTRDVEEIVPTCRELGIGIVAYSPLGRGFFSSGPKLVETLSE	DFRKYLPFRFQENLEHNKHL	
2A1	VSQFETHPHYFQRDLSVKFCMKHGLVPTAHTPLGGAAANKDMFGSVS	PLDD-----	
4A2	VNQVEMNLAWQQKLRREFCNAHGIVLTAFSPLRKG-ASRGN	-----EVMEN-----	
4B3	VNQVEMSPTLHQKLNREYCKANNIMITAHSVLGAVGAAWGTN	---	AVMHS-----
4C5	VNQVGCHPSCAQTKLRAFCKSKGVLHLSGYSPLGSPGTPWVKH	---	DVLEN-----
		: . : *	: : :
PR	YYRIEALSQKHGCTPVQLALAWLHQGEDVVP	IPGTTKIKNLHNNVGALKVKLT	KEDLKEISDAVPL
AIOS	YLKTEELAKKHQCSPAQLALAWLHQGDDVVP	IPGTTKIKNLDANIDSLKVNLT	DDDLKEISSQVREEDV
AIAT	YEKVCALISEKKGCTPGQLALAWVHHQGDVCP	IPGTTKIKNLQNIQALSVKLT	PEEMTELEAIAPGFV
AKRME	FERVNEIAARKQCTPSQLALAWVHHQGDVCP	IPGTTKIKNFQNIQALSVKLT	PEDMAELASASAV
2A1	-PVLNDVAKKYGKVAQICLRWGIQRKTAVIPK	--SSKIQRLENLEVLQSLSD	EDMQLIYSIDRKYR
4A2	-DMLKEIADAHGKVAQISLRWLYEQGVTFVPK	--SYDKERMNQLRIFDW	SLTKEDHEKIDQIKQNR
4B3	-KVLHQIIVARGKVAQVSMRWVYQOGASLVVK	--SFNEARMKENLKI	FDWELTAEDMEKISEIPQSR
4C5	-PILVDVAEKLKGTPAQVAIRWGLQMGHSLVLPK	--SVHESRIKENIDV	FSWIPDLDLFAKFSIEIQVSPG
		: :	: * : * . . : . . : * : : : :
PR	AGESIHEVIAVTNWKFANTPPLK-----		
AIOS	AGGRQYTSFAHYTWKYADTPPK-----		
AIAT	KGDR--YSNMIPTFKNAETPPLSAWKA		
AKRME	KGGR--YGSMDGTYKDSPTPPLSSWKAV		
2A1	-----TSLPSKTWGLDVYA-----		
4A2	-----LIPGP-TKPLGLNDLYDD-----		
4B3	SAAFLLSPTG-PFKTEEFWDEKD----		
4C5	KPEFPVHPEISQYKTVPEEMWDGGI----		

Digitalis (~15% identity), followed by chalcone polyketide reductase of various plant origins with 14% identity, deoxy-mugineic acid synthase1 of *Zea*, *Oryza* and *Hordeum* (12–14% identity), and codeinone reductase from *Papaver somniferum* (~14% identity) (Fig. 2). Comparison of PR amino acid sequence with those of cinnamic aldehyde reductases involved in lignin biosynthesis revealed identities inferior to 13%.

Heterologous expression and properties of His₆-PR

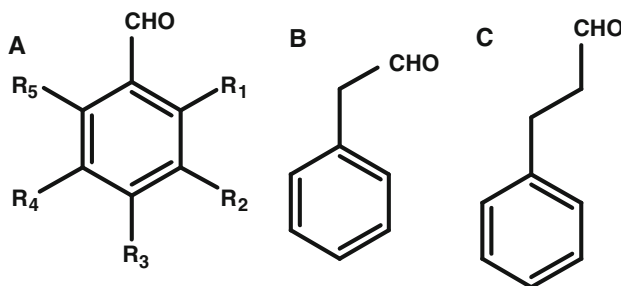
In order to simplify enzyme purification, full-length PR cDNA was ligated into *SphI* and *SacII* restriction sites of the expression vector pQE-2, resulting in synthesis of a fusion protein harboring an N-terminal 6 histidine tag. The vector harboring the putative PR sequence was expressed in *E. coli* M15 cells yielding approximately 10 mg homogeneous protein per liter of bacterial culture, after Ni²⁺-NTA chromatography (Fig. 1, supplementary material). In an NADPH-dependent reaction, the native His₆-PR converted the aldehyde perakine into raucaffrinoline. This compound was identified by TLC and by mass spectrometry on the basis of its molecular ion M⁺ 352 *m/z* and fragmentation pattern, which was identical to a reference sample of raucaffrinoline (Fig. 2, supplementary material). The enzyme was found to exhibit a pH optimum centered at pH 7.0 with half maximal activity at pH 6.5 and 8.5, and the temperature optimum was at 50°C. As shown for a range of substrates, NADPH could not

be replaced by NADH, and in presence of the oxidized cofactor NADP⁺ the reverse reaction of the reductase was low, with <0.5% relative activity of the forward reaction only.

Substrate acceptance of PR

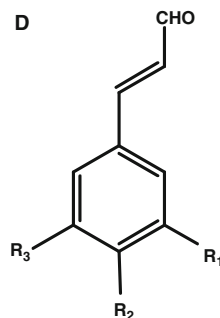
Since the PR sequence showed homology to other enzymes belonging to the AKR superfamily, we investigated its substrate specificity. In order to clearly define the specificity of this novel recombinant plant enzyme, a comprehensive substrate study was carried out. Three main groups of compounds were investigated: six benzaldehyde derivatives (A1–A6), phenylacetaldehyde (B), phenylpropionaldehyde (C), including pyridoxal, glucose and progesterone (Table 1); nine cinnamic aldehyde derivatives (D1–D9) (Table 2), and nine indole alkaloids with reducible carbonyl functions (Tables 3a, 3b). With the exception of benzaldehyde and 2-nitrobenzaldehyde, the enzyme accepted all the other benzaldehyde derivatives (Table 1). 4-Nitrobenzaldehyde and phenyl-propionaldehyde were found generate the highest relative enzyme activity (100% and 38.9% respectively), whereas 4-hydroxy-3-nitrobenzaldehyde was reduced at a very low rate (0.5%). In the second group (Table 2), the naturally occurring cinnamic aldehyde derivatives were generally well accepted. Sinapyl-, 5-hydroxy-coniferyl- and *p*-coumaryl aldehyde were converted at rates of 100%, 76.5% and 36.8%, respectively. Synthetic derivatives of

Table 1 Substrate specificity of PR (benzaldehyde derivatives)



	R ₁	R ₂	R ₃	R ₄	R ₅	Substrate	Relative activity (%)
A1	H	H	NO ₂	H	H	4-Nitrobenzaldehyde	100
A2	OH	H	H	NO ₂	H	2-Hydroxy-5-nitrobenzaldehyde	1.5 ± 0.1
A3	H	NO ₂	OH	H	H	4-Hydroxy-3-nitrobenzaldehyde	0.5
A4	H	H	OCH ₃	H	H	<i>ρ</i> -Anisaldehyde	6.8 ± 1.0
A5	H	H	H	H	H	Benzaldehyde	n.d.
A6	NO ₂	H	H	H	H	2-Nitrobenzaldehyde	n.d.
B						Phenylacetaldehyde	17.7 ± 0.8
C						Phenylpropionaldehyde	38.9 ± 5.8

100% relative activity corresponds to 2.07 U mg⁻¹ (34.51 nkat mg⁻¹). Relative activity of reverse reaction to the forward reaction is lower <0.5%. n.d.: not detectable (detection limit = 0.1% conversion). The typical AKR substrates pyridoxal, glucose and progesterone were not converted

Table 2 Substrate specificity of PR (cinnamic aldehyde derivatives)

	R ₁	R ₂	R ₃	Substrate	Relative activity (%)
D1	OCH ₃	OH	OCH ₃	Sinapyl aldehyde	100
D2	OH	OH	OCH ₃	5-Hydroxy-coniferyl aldehyde	76.5 ± 2.0
D3	OCH ₃	OCH ₃	OCH ₃	3-(3,4,5-Trimethoxy-phenyl)-propenal	76.1 ± 5.5
D4	H	OH	H	<i>ρ</i> -Coumaryl aldehyde	36.8 ± 1.9
D5	H	OH	OCH ₃	Coniferyl aldehyde	36.0 ± 2.0
D6	H	OH	OH	Caffeyl aldehyde	17.3 ± 1.3
D7	H	H	H	Cinnamic aldehyde	9.4 ± 0.4
D8	OCH ₂ OCH ₃	OCH ₂ OCH ₃	OCH ₂ OCH ₃	3-(3,4,5-tris-Methoxy-methoxy-phenyl)-propenal	8.3 ± 0.3

100% relative activity corresponds to 0.0747 U mg⁻¹ (1.25 nkat mg⁻¹). Cinnamic aldehydes with large substituents, like (D9) 3-[2-bromo-3,5-dimethoxy-4-(3,7,11-trimethyl-dodeca-2,6,10-trienyloxy)-phenyl]-propenal (not shown in Table 2) were not accepted. Relative activity of reverse reaction to the forward reaction is lower <0.5%. n.d.: not detectable (detection limit = 0.1%)

this group with large substituents were not reduced, e.g. D9 (see the legend of Table 2). Of the nine alkaloids comprising the third group (Tables 3a, 3b), the natural substrate of PR, perakine and 19(*S*),20(*R*)-di-hydroperaksine-17,21-al were reduced, whereas all remaining alkaloids did not act as enzyme substrates. Typical substrates of enzymes of the AKR superfamily, such as pyridoxal, progesterone and glucose, were also not accepted as substrates by the PR.

Analysis of *Rauwolfia* cells for presence of cinnamic aldehydes

As cinnamic aldehydes and their derivatives are converted in favor of the indole alkaloids by recombinant PR (see Tables 2, 3a), it cannot be excluded that PR also acts in the metabolism of phenylpropanoids at their aldehydic stage. Whether this PR exercises this function in vivo during

Table 3a Substrate specificity of PR (indole alkaloids)

Substrate	Structure	Relative activity (%)
Perakine		17.2 ± 0.9
19(<i>S</i>),20(<i>R</i>)-Dihydro-peraksine-17,21-al		16.9 ± 1.2
19(<i>S</i>),20(<i>R</i>)-Dihydro-peraksine-17-al		n.d.

100% relative activity corresponds to 0.0747 U mg⁻¹ (1.25 nkat mg⁻¹). Relative activity of reverse reaction to the forward reaction is lower <0.5%. n.d.: not detectable (detection limit = 0.1% conversion)

Table 3b Six alkaloids with reducible carbonyl groups not accepted by PR as substrate

Alkaloid	Structure
Gardneral	
3-oxo-Tabersonine	
Picalinal	
Dregamine	
Ajmalidine	
Vallesine	

n.d.: not detectable (detection limit = 0.1% conversion)

Rauvolfia cell growth is uncertain. Although alkaloid formation in these cell cultures has been investigated in the past in significant detail, the presence of cinnamic aldehydes was not previously proven. *Rauvolfia* cells were therefore analyzed for the presence of coniferyl- and cinnamic aldehyde by three analytical techniques (TLC, HPLC and GC-MS) all of which resulted in good separation of both aldehydes: coniferyl- and cinnamic aldehyde (TLC R_f 0.4 and 0.6; HPLC R_f 7.34 and 16.4 min; GC-MS R_f 12.12 and 6.20 min). Based on the detection limit under the HPLC and GC-MS conditions described ($\sim 10^{-5}\%$ and $\sim 10^{-6}\%$ cell dry weight, respectively) neither of the two aldehydes could be identified as constituents of *Rauvolfia* cell cultures (data not shown).

Kinetic data of the recombinant enzyme and site-directed mutagenesis

K_m and V_{max} were measured in order to characterize the enzyme by its kinetic properties (Table 4). K_{cat} values were calculated from K_m , V_{max} and enzyme's molecular mass. In addition to the alkaloid perakine, one representative substrate from each group was selected, i.e. 4-nitrobenzaldehyde and coniferylaldehyde. Both were reduced by PR with high relative activity. Table 4 shows that apparent K_m values were in the same range for each substrate tested. The lowest K_m was determined for 4-nitrobenzaldehyde, whereas for perakine and coniferyl aldehyde the values were 3 and 4.6 times higher, respectively. The catalytic efficiencies (K_{cat}/K_m) strongly differed, resulting in ~ 1750 and ~ 900 -fold efficacy for 4-nitrobenzaldehyde compared to perakine and coniferyl aldehyde, respectively.

In order to determine catalytically active amino acids of PR, the residues belonging to the putative tetrad, as deduced from sequence alignment of plant AKR enzymes (Fig. 2), were substituted by a non-reactive alanine residue. The mutants generated by directed mutagenesis were Asp52Ala, Tyr57Ala, Lys84Ala, His126Ala. These were well expressed in *E. coli* at comparable levels to the wild-type (data not shown), purified, and their activity for direct comparison determined under the same conditions as those used for recombinant, wild type PR. All four mutants showed a loss of activity in the range between ~ 98 and $\sim 100\%$ (Table 5), which clearly indicates that the amino acids selected represent the functionally important PR residues.

Discussion

Alkaloid biosynthesis in *Rauvolfia* cell suspension cultures is well understood at the enzymatic level for the major pathway leading to ajmaline. Side reactions of this pathway have, to date, only been subject of cursory investigations. Knowledge of these pathways would allow additional exploration and likely extension of the metabolic and genetic network of this medicinal plant. The formation of raucaffrinoline by reduction of perakine, the former alkaloid having been isolated several times from *Rauvolfia* plants (Kahn and Siddiqui 1972; Subhadhirasakul et al. 1994; Sabino et al. 2006), is an important side reaction which, until now, remained unexplored at the enzymatic level. During NADPH-dependent reduction-based substrate-consuming chromatography of PR, the use of unnatural 21-hydroxyraumacline was favored over the natural substrate perakine for two reasons. Firstly, the raumacline derivative can easily be obtained from ajmaline by biotransformation, whereas perakine is a very rare

Table 4 Kinetic parameters of recombinant PR

Substrate/Cofactor	K_m (mM)	V_{max} (pKat/ μ g)	K_{cat} (s^{-1})	K_{cat}/K_m ($M^{-1} s^{-1}$)
4-Nitrobenzaldehyde	0.28	217.39	8.26	29,500
Perakine	0.83	0.368	0.014	16.87
Coniferyl aldehyde	1.29	1.11	0.042	32.56
NADPH	<0.003	–	–	–
NADH	n.d.	–	–	–

Kinetic constants are mean values of three independent experimental series. n.d.: not detectable (detection limit = 0.1% conversion). Coniferyl aldehyde was chosen from the group of cinnamic aldehydes because of its commercial availability

Table 5 Activity of mutants of PR generated by exchange of residues of the putative catalytic tetrad

Relative activity (%) Substrate	Wild type	Asp52Ala	Tyr57Ala	Lys84Ala	His126Ala
4-Nitrobenzaldehyde	100	0.75	0.4	n.d.	2.2
Coniferyl aldehyde	100	n.d.	n.d.	n.d.	n.d.
Perakine	100	n.d.	n.d.	n.d.	n.d.
Phenyl-propionaldehyde	100	n.d.	n.d.	n.d.	1.5

n.d.: not detectable (detection limit = 0.1% conversion)

alkaloid. Secondly, 21-hydroxyraumacline mimics the reducible sub-structure of perakine and can be used as an unnatural substrate for PR (for details see Fig. 3). A “reverse genetic” approach, applying sequence-based, degenerate primers for PCR combined with RACE-PCR, allowed isolation of a novel full-length cDNA from a *Rauvolfia* cell suspension derived cDNA library. Since the

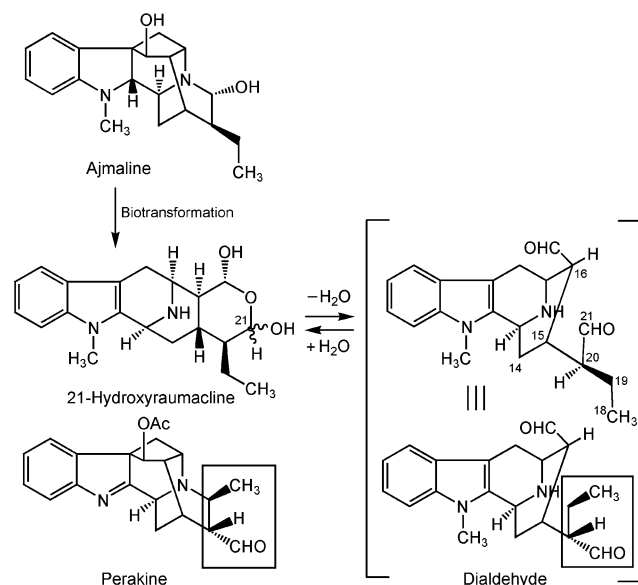


Fig. 3 Generation of 21-hydroxyraumacline from ajmaline and conformational relationship of the structural part important for reduction by PR (see the framed part of the molecules explaining why the raumacline derivative could be used as substrate during purification of PR from *Rauvolfia* cells)

isolated clone showed significant similarities to enzymes of the AKR superfamily, it may constitute the first example of this enzyme family identified in *Rauvolfia* species. If so, it would also be a novel member of the large AKR superfamily (Bashir et al. 2006; Gavidia et al. 2002, 2007) which, at present, consists of >5000 members (InterPro database family IPR001395, November 2007).

Expression of the N-terminal His₆-tagged protein allowed the protein to be functionally analyzed. Protein-dependent conversion of perakine to raucaffrinoline was ascertained, and the result provided clear evidence that the correct clone was functionally expressed. In addition to a recently heterologously expressed cytochrome P450 reductase (our unpublished data), the PR described here constitutes the second example of an over-expressed reductase from *Rauvolfia* cells and seems to be involved in alkaloid metabolism.

An amino acid alignment of PR performed with UniProt confirmed its relationship to plant AKR enzymes, with the three closest AKR relatives (identities of up to 71%) being of plant origin (Fig. 2), from *Oryza*, *Arabidopsis* and *Manihot*. However, in contrast to the PR described here, the biochemical functions of these proteins are yet to be determined.

Enzymes in plant natural product biosynthesis usually have rather limited, or even exclusive, substrate specificity. In *Rauvolfia* for instance, acetylajmalan esterase accepts only two out of six closely structurally related alkaloids as substrates (Ruppert et al. 2005b) and vinorine hydroxylase converts only two of ten structurally highly related alkaloids (Falkenhagen et al. 1995). Similarly, polyneuridine

aldehyde esterase of the ajmaline pathway (Fig. 1) exclusively hydrolyzes its natural substrate (polyneuridine aldehyde), even though a range of 13 esters including eight naturally occurring alkaloid esters, have been tested (Dogru et al. 2000). Most other functionally investigated enzymes of the *Rauvolfia* system likewise exhibit remarkable substrate selectivity (Ruppert et al. 2005a).

Close homology of PR to the AKR family prompted the current study, which represents one of the most comprehensive substrate studies carried out to date in this enzyme family. The AKR proteins are ubiquitous enzymes which are known for their low substrate specificity (Jez et al. 1997; Jez and Penning 2001). From a total of 28 compounds, 16 were found to be accepted by PR, indicating a remarkably low substrate-specific behavior. The putative substrates included some compounds which are usually regarded as typical AKR substrates, in particular nitrobenzaldehydes and other benzaldehyde derivatives. Moreover, a variety of cinnamic aldehydes and a group of indole alkaloids structurally related to perakine were tested. Though to varying degrees, nearly all nitrobenzaldehydes served as substrates (Table 1). These are highly reactive compounds that, due to “activation” of the aldehyde group by the electron-withdrawing nitrogroup in positions 2- and 4-, can be easily reduced chemically. Surprisingly however, even though it represents a typical AKR substrate and has been shown to be an excellent substrate of the AKR YakC protein from *Schizosaccharomyces* (Morita et al. 2002), 2-nitrobenzaldehyde was not converted. The selectivity observed for the nitrobenzaldehydes may exclude electronic effects. Other typical AKR substrates such as pyridoxal, glucose and progesterone, were also not accepted by PR, demonstrating a relatively high degree of AKR-related substrate intolerance. The selectivity of PR may in large part depend on a combination of steric effects and characteristics of its binding pocket.

The cinnamic aldehyde group and structurally related derivatives were tested for two reasons. Firstly, they are significantly larger in size than the benzaldehydes, clearly needing a bigger binding pocket. Secondly, they serve as biosynthetic precursors in the metabolism of phenylpropanoids in higher plants. Without exception, all naturally occurring cinnamic aldehydes assayed are accepted as substrates by PR. However, substrate selectivity is different, in that sinapyl aldehyde is effectively reduced and cinnamic aldehyde is the weakest substrate. The results indicate a surprisingly low overall selectivity of the enzyme in this particular group of aldehydes. It is obvious that the three-carbon chain makes all these phenylpropanoid aldehydes acceptable to the site of reduction, which is likely not to be located on the enzyme surface, but deep in the PR structure. Increasing enzyme activity with extension

of the side chain as observed for benzaldehyde, phenylacetaldehyde, phenylpropionaldehyde (Table 1) support such an assumption.

The most pronounced substrate selectivity was, however, observed in the alkaloid group, because only two out of nine alkaloids belonging to the same family (monoterpene indoles) exhibiting reducible carbonyl functions could be converted, including the natural substrate perakine. Interestingly, of the structurally related 19(*S*),20(*R*)-dihydroperaksine alkaloids, the dialdehyde 19(*S*),20(*R*)-dihydroperaksine-17,21-al alone is reduced, giving rise to the recently isolated 19(*S*),20(*R*)-dihydroperaksine-17-al from *Rauvolfia* hairy roots (Sheludko et al. 2002). The second aldehyde group, located on the opposite side of the molecule at C17, was not reduced by the enzyme. Identification of 19(*S*),20(*R*)-dihydroperaksine alkaloids with both reduced aldehyde functions points to the putative existence of additional reductases in the *Rauvolfia* metabolome, involved in 19(*S*),20(*R*)-dihydroperaksine alkaloid conversion (XR, see Fig. 1).

These substrate studies make the classification of PR as a typical AKR protein difficult, but the AKR characteristic tetrad Asp52, Tyr57, Lys84 and His126 are also found in the PR sequence (Fig. 2), which were finally identified as the functional amino acids by way of site-directed mutagenesis experiments (Table 5). PR must therefore be unambiguously regarded as a novel member of the AKR enzyme superfamily.

Based on the cofactor requirements, the AKR family may be divided into three sub-groups: the major group which is predominantly NADPH-dependent, a small subgroup with dual NADPH/NADH specificity and very few examples which show exclusive specificity for NADH (Di Luccio et al. 2006). Given its cofactor dependence, PR clearly belongs to the first of these three subgroups.

It must however be regarded as an enzyme with an inherently low substrate specificity (Tables 1–3), with pronounced preference for the small nitrobenzaldehydes over medium-sized cinnamic aldehydes, and the structurally bulky alkaloids. Though a large binding site is required, additional structural requirements necessary for PR to convert such structurally diverse substrates remain undefined.

Moreover, PR represents the first example of this enzyme family which possesses a functional relationship to the indole alkaloid biosynthetic network. However, multifunctionality such as the detoxification of reactive aldehydes seen in human AKRs (Jin and Penning 2007) or in the metabolism of phenylpropane aldehydes, may not be excluded for the *Rauvolfia* cells. Since sequence analysis of PR shows no significant identity (<13%) or homology to well known cinnamic aldehyde reductases linked to lignin biosynthesis (Kim et al. 2004; Zhang et al. 2006), the

enzyme may however exemplify a novel component of phenylpropanoid metabolism. However, extensive analysis of *Rauvolfia* cells by TLC, HPLC and GC-MS clearly indicates the absence of cinnamic and coniferyl aldehyde, in contrast to the known presence of the indole alkaloid perakine and its reduction product, a finding which favors a significant role of PR in alkaloid biosynthesis. Further alkaloid profiling of *Rauvolfia* resulted in the detection of the novel peraksines (Sheludko et al. 2002) and an additional, new *Rauvolfia* enzyme, acetyl esterase (AE), was identified (see Fig. 1) (Gerasymenko et al. unpublished). In conclusion, it is very likely that the both enzymes, PR and AE, significantly expand the alkaloidal network of *Rauvolfia*, now resulting in one of the biggest and best understood metabolomes in plant natural product biosynthesis.

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