



Molecular cloning and functional bacterial expression of a plant glucosidase specifically involved in alkaloid biosynthesis[☆]

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Abstract

Monoterpenoid indole alkaloids are a vast and structurally complex group of plant secondary compounds. In contrast to other groups of plant products which produce many glycosides, indole alkaloids rarely occur as glucosides. Plants of *Rauvolfia serpentina* accumulate ajmaline as a major alkaloid, whereas cell suspension cultures of *Rauvolfia* mainly accumulate the glucoalkaloid raucaffricine at levels of 1.6 g/l. Cell cultures do contain a specific glucosidase, known as raucaffricine-*O*- β -D-glucosidase (RG), which catalyzes the in vitro formation of vomilenine, a direct intermediate in ajmaline biosynthesis. Here, we describe the molecular cloning and functional expression of this enzyme in *Escherichia coli*. RG shows up to 60% amino acid identity with other glucosidases of plant origin and it shares several sequence motifs with family 1 glucosidases which have been characterized. The best substrate specificity for recombinant RG was raucaffricine (K_M 1.3 mM, V_{max} 0.5 nkat/ μ g protein) and only a few closely related structural derivatives were also hydrolyzed. Moreover, an early intermediate of ajmaline biosynthesis, strictosidine, is a substrate for recombinant RG (K_M 1.8 mM, V_{max} 2.6 pkat/ μ g protein) which was not observed for the low amounts of enzyme isolated from *Rauvolfia* cells. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Glucosides are widely distributed throughout the plant kingdom. An overwhelming number of plant secondary products is reported to occur in glucosylated form, which increases their water solubility and allows their high vacuolar accumulation. In addition to their enhanced solubility, glucosides may have other important features for plant metabolism, but the functions of only a few compounds have been elucidated. It is well

known that the cyanogenic glucosides have important defensive roles against predation or fungal attack (Poulton, 1990) whereas glucosylated auxins act as inactive storage forms of these plant hormones (Cohen and Bandurski, 1982; Brzobohaty et al., 1993).

Alkaloids can also occur in the glucosylated form, although this is not necessary for enhanced solubility since alkaloids will form ion pairs with appropriate organic acids in the plant cell. Of the 2000 monoterpenoid indole alkaloids that have been characterized, a few are used in medicine as cytostatics (vincleucoblastine and vincristine), neuroleptics (reserpine) or as vasodilatives (yohimbine) and only about 40 of these are glucoalkaloids (Ruyter et al., 1988). In the biosynthesis and metabolism of alkaloids of the ajmalan-group, which is characteristic for the traditional ayurvedic medicinal plant *Rauvolfia*, two glucosides are involved. The first is the biosynthetic intermediate

[☆] The cDNA sequence reported herein was deposited in GenBank under accession number AF 149311.

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strictosidine (Fig. 1), formed from tryptamine and secologanin by the enzyme strictosidine synthase (Stöckigt and Zenk, 1977). Strictosidine occupies a central position in the synthesis of all monoterpene indole alkaloids in plant genera such as *Catharanthus*, *Rauvolfia*, *Strychnos*, *Cinchona*, etc. (Kutchan, 1998). In this case, the glucose moiety functions as a protecting group to stabilize the molecule. After deglycosylation of strictosidine by strictosidine glucosidase, the highly reactive and unstable aglycone is directed into different biosynthetic pathways that are species dependent and result in the production of various indole and quinoline alkaloids (Rueffer et al., 1978). This enzyme which was first described 20 years ago (Hemscheidt and Zenk, 1980) was recently cloned

from *Catharanthus roseus* (Geerlings et al., 2000). In *Rauvolfia*, strictosidine is converted by about 10 different soluble and membrane associated enzymes to the structurally complex alkaloid ajmaline, harbouring a six ring system with a total of nine chiral carbon atoms. Ajmaline is an important plant-derived pharmaceutical which is commercially isolated from *Rauvolfia* roots and has been used in the therapy of heart disorders for the past four decades.

Several side reactions occur in cell cultures of *Rauvolfia serpentina* in addition to the major pathway leading to ajmaline. It has been suggested that these side reactions might exhaust the intermediates from the ajmaline biosynthesis (Stöckigt, 1995) and may explain the low ajmaline accumulation rates of cell cultures compared with the intact plant. Greater accumulation in the cultivated cells has important consequences for the biotechnological production of ajmaline.

Cell cultures accumulate raucaffricine, a glucoside of vomilenine which is a direct biosynthetic precursor of ajmaline (Fig. 1). Vomilenine glucosyl transferase (VGT) forms raucaffricine from vomilenine, resulting in cell cultures that accumulate this glucoside up to levels of 1.6 g/l and represents one of the most abundant indole alkaloids to accumulate in a cell culture (Ruyter and Stöckigt, 1991). In fact, on a dry weight basis raucaffricine formation in the cultivated plant cells might exceed that of intact plants by 67-fold. This side product of ajmaline biosynthesis is deglycosylated by another highly specific enzyme from the cultured *Rauvolfia* cells — raucaffricine glucosidase (RG, EC 3.2.1.125). Therefore, raucaffricine biosynthesis and the re-utilization of raucaffricine for the ajmaline biosynthetic pathway could be the crucial and rate limiting steps in the formation of the antiarrhythmic drug ajmaline. For that reason, both enzymes, VGT and RG, are of an interest.

In this article, we describe the cloning of the RG cDNA, the functional expression of the encoded protein in *Escherichia coli*, and its characterization.

2. Results and discussion

2.1. Cloning of partial and full length RG cDNA

While raucaffricine could not be deglycosylated by any commercially available glucosidase, RG was shown to accept only raucaffricine and structurally closely related derivatives as a substrate (Schübel et al., 1986). In order to study this enzyme that is present in *Rauvolfia* cell cultures more thoroughly, we purified the enzyme and sequenced internal peptides (Warzecha et al., 1999).

Two partial amino acid sequences of RG, the pep-

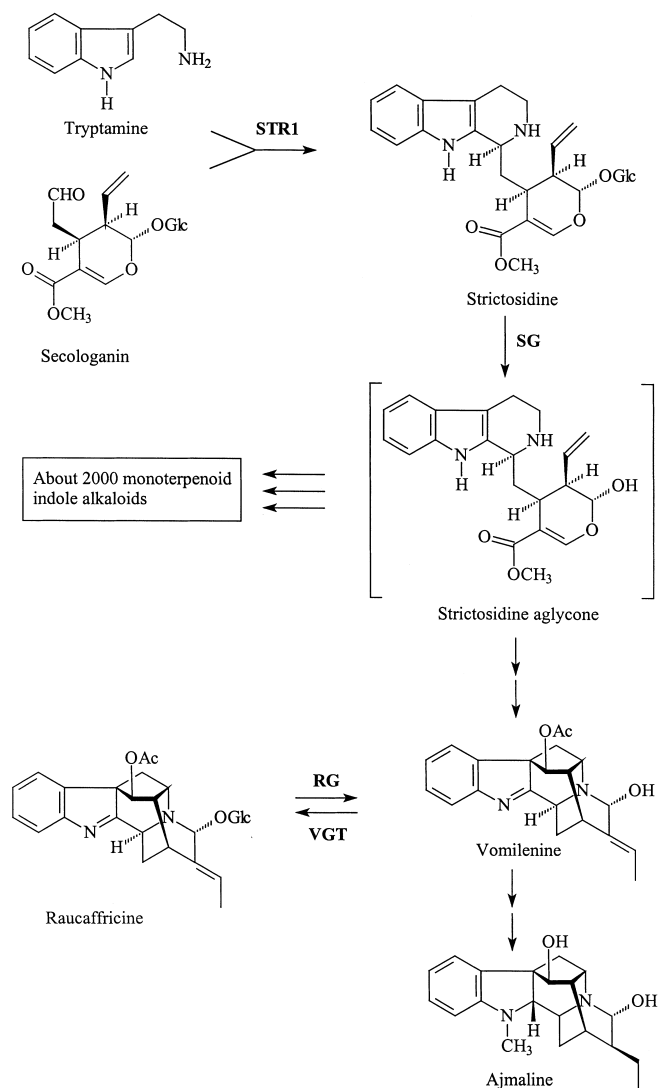


Fig. 1. Schematic representation of ajmaline biosynthesis in *Rauvolfia serpentina*. Double arrows are indicating several enzymatic reactions. Enzymes are: STR1, strictosidine synthase; SG, strictosidine glucosidase; VGT, vomilenine glucosyl transferase; RG, raucaffricine glucosidase.

tide fragments PF 37 and PF 47 showed extensive homology with other glucosidases. As recently described, the sequences of several glucosidases possessing homology with the peptide fragment PF 37 are near the COOH-terminus of the corresponding enzyme, while PF 47 homologues are located near the NH₂-terminus. Both are flanking a region of about 320 amino acids. To amplify a partial cDNA of RG, two degenerate oligonucleotides were synthesized, using one as a sense primer (RG 47s) and the other as an antisense primer (RG 37as). A third degenerate oligonucleotide (RG 47int.s) was derived from a conserved region in other glucosidases, which is located four amino acids downstream of the RG 47-site, and was used for nested PCR to verify that the amplified nucleic acid is indeed a part of a glucosidase cDNA.

RG 47s

5'-CTI GAT GCI TAT CGI TTT TCI ATI TCI TGG-3'
T C C A C AG AG

RG 37as

5'-TC ATC ATC IAC ICC ATT TTC IGT IAC ATA-3'
G G G C G

RG 47int.s

5'-ATI AAT AAA AAA GGI ATI GAA TAT TAT AAT-3'
C G G G G G C C C

Since RG of the *R. serpentina* cell suspension shows its optimum activity after 7 days of growth, RNA was obtained from 6-day-old cells and was used as a template for RT-PCR. With the above selected RG 47s and RG 37as, a PCR product of approximately 970 bp in length was amplified. A second PCR with this template using RG 37as and RG 47int.s as primers resulted in an expected smaller fragment of 940 bp in length. The former PCR product was subcloned into pGEM-T, sequenced and the cDNA was then used as a probe for screening of *R. serpentina* cDNA library. Several clones were isolated which were identical in sequence, but not in length. The longest cDNA contained an ORF of 1623 nucleotides, a 144 nucleotide 5'-flanking region and a 229 bp 3'-flanking region with a poly-A tail. The protein encoded by this sequence consists of 540 amino acids with a calculated molecular weight of 60931 Da (Fig. 2). This value agrees well with the molecular mass of 61 kDa for the native enzyme from plant cell cultures (Warzecha et al., 1999). Additionally, all the six peptide sequences obtained by microsequencing have been found in the deduced amino acid sequence of the cDNA (Fig. 2), confirming that this sequence encodes RG.

2.2. Sequence similarities of RG with other plant glucosidases

Several glucosidases showed great similarity to RG

(up to 60% identity, Table 1), the majority of which were plant-derived. The sequence indicates that RG belongs to family 1 of glucosyl hydrolases (Henrissat, 1991; Henrissat and Davies, 1997). In fact, RG shares several important motifs that are responsible for the catalytic activity of family 1 glucosidases. The first motif is the "Ile/Val-Thr-Glu-Asn-Gly" sequence between residues 418 and 422 found in one of the peptides after microsequencing of RG. The glutamic acid of this motif was previously identified to be the active site nucleophile (Withers et al., 1990; Trimbur et al., 1992). The second residue necessary for cleavage of glucosides might be the aspartic acid in the sequence "Asp-X-X-Arg-X-X-Tyr" between residues 439 and 445 (Trimbur et al., 1992) or the glutamic acid in the "Asn-Glu-Pro/Ile" sequence between residues 185 and 187 (Keresztessy et al., 1994). RG exhibits each of these motifs (in addition to several other conserved regions) and, therefore, belongs to this family of glucosidases.

The comparison of amino acid sequences of RG and of the recently heterologously expressed strictosidine glucosidase (SG) from *C. roseus* (Geerlings et al., 2000) is of special interest because of these enzymes' common involvement in indole alkaloid biosynthesis. The two glucosidases demonstrate relatively a high degree of amino acid and nucleotide identity (52 and 67%, respectively), although the homology of RG to other glucosidases was even more pronounced (see Table 1). Moreover, the analysis of amino acid sequence of SG did not reveal the "Ile/Val-Thr-Glu-Asn-Gly" and "Asp-X-X-Arg-X-X-Tyr" motifs.

RG seems to be not only a new member of family 1 of glucosidases with a unique substrate specificity, but also an excellent example to study substrate specificity of the enzyme, e.g. by mutagenesis. For such investigations, heterologous expression of the active enzyme would be a prerequisite.

2.3. Heterologous expression

Due to the lack of information about the NH₂-terminus of native RG, two possible in-frame ATG codons may function as translation start sites at nucleotides 145 or 226 in this cDNA (Fig. 2). The 1623 bp clone was subcloned into the expression vector pSE280 by the use of the naturally occurring *Nco*I-restriction site (named pSE-RG1), whereas the shorter 1542 bp clone (using ATG at position 226) was generated by PCR (named pSE-RG2). Both constructs were expressed in *E. coli*. While the clone pSE-RG1 showed high glucosidase activity in a crude protein extract, no deglycosylation of raucassicine could be measured in the enzyme extract from pSE-RG2. This result strongly suggested that the first ATG is the translation

1	TAA	ACA	AAT	GTG	GAA	GAG	TAT	GGC	GAT	TTG	CTA	TAT	AAG	GAG	GGG	CGA	TCG	AGC	TTT	TGT	24
25	*																				84
85	ACG	TCC	CAA	AGT	TTA	AAG	TTC	GAA	ATC	GAC	ACT	TAC	GAG	CAA	ATT	TAC	GAA	AGC	AGC	CCC	144
145	ATG	GCA	ACT	CAG	AGC	AGT	GCT	GTT	ATC	GAC	TCC	AAT	GAT	GCA	ACC	AGA	ATC	AGC	CGT	AGC	204
1	M	A	V	S	S	S	I	D	S	N	D	A	T	R	I	S	R	S			20
205	GAT	TTT	CCT	GCT	GAT	TTT	ATA	ATG	GGT	ACG	GGC	TCT	TCT	GCT	TAT	CAG	ATT	GAA	GGT	GGG	264
21	D	F	P	A	D	F	I	M	G	T	G	S	S	A	Y	Q	I	E	G	G	40
265	GCG	AGA	GAT	GGG	GGT	CGT	GGC	CCG	AGC	ATA	TGG	GAT	ACT	TTC	ACC	CAC	AGA	CGA	CCT	GAT	324
41	A	R	D	G	G	R	G	P	S	I	W	D	T	F	T	H	R	R	P	D	60
325	ATG	ATC	CGG	GGT	GGA	ACT	AAC	GGG	GAT	GTG	GCT	GTG	GAC	TCT	TAC	CAT	TTG	TAC	AAG	GAA	384
61	M	I	R	G	G	T	N	G	D	V	A	V	D	S	Y	H	L	Y	K	E	80
385	GAT	GTC	AAC	ATC	CTC	AAA	AAC	TTG	GGG	CTT	GAT	GCC	TAC	CGG	TTC	TCA	ATC	TCA	TGG	TCC	444
81	D	V	N	I	L	K	N	L	G	L	D	A	Y	R	F	S	I	S	W	S	100
445	AGA	GTG	TTG	CCA	GGT	GGA	AGA	CTA	AGT	GGT	GGT	GTG	AAC	AAG	GAA	GGG	ATT	AAT	TAC	TAC	504
101	R	V	L	G	G	R	L	S	G	G		V	N	K	E	G	I	N	Y	Y	120
505	AAT	AAT	CTC	ATT	GAT	GCA	CTC	CTA	GCC	AAT	GGG	ATT	AAA	CCA	TTT	GTA	ACT	CTC	TTC	CAC	564
121	N	N	L	I	D	G	L	L	A	N	G	I	K	P	F	V	T	L	F	H	140
565	TGG	GAT	GTT	CCC	CAA	GCC	TTA	GAA	GAT	GAA	TAT	GGT	GGC	TTT	TTG	AGT	CCC	CGG	ATT	GTG	624
141	W	D	V	P	Q	A	L	E	D	E	Y	G	G	F	L	S	P	R	I	V	160
625	GAT	GAT	TTT	TGT	GAG	TAT	GCG	GAG	CTT	TGT	TTT	TGG	GAA	TTC	GGT	GAT	CGG	GTG	AAA	CAC	684
161	D	D	F	C	E	T	N	G	L	C	F	W	E	F	G	D	R	V	K	H	180
685	TGG	ATG	ACA	TTG	AAC	GAG	CCA	TGG	ACA	TTT	AGC	GTT	CAT	GGA	TAT	GCA	ACA	GGC	CTT	TAT	744
181	W	M	T	L	N	E	P	W	T	F	S	V	H	G	Y	A	T	G	L	Y	200
745	GCA	CCC	GGT	CGA	GGT	CGG	ACT	TCT	CCA	GAG	CAC	GTA	AAC	CAT	CCC	ACT	GTG	CAA	CAC	AGA	804
201	A	P	G	R	G	R	T	S	P	E	H	V	N	H	P	T	V	Q	H	R	220
805	TGT	TCT	ACT	GTC	GCA	CCA	CAAT	TGC	ATC	TGC	AGC	ACT	GGA	AAT	CCG	GGC	ACA	GAA	CCA	TAT	864
221	C	S	T	V	A	P	Q	C	I	C	S	T	G	N	P	G	T	E	P	Y	240
865	TGG	GTT	ACT	CAT	CAT	CTA	CTT	CTT	GCT	CAT	GCT	GCT	GCT	GTT	GAA	TTA	TAC	AAG	AAC	AAA	924
241	W	V	T	H	H	L	L	L	A	H	A	A	A	V	E	L	Y	K	N	K	260
925	TTT	CAG	AGG	GGT	CAA	GAA	GGA	CAG	ATT	GGA	ATT	TCA	CAC	GCG	ACT	CAA	TGG	ATG	GAG	CCT	984
261	F	Q	R	G	E	G	Q	I	S	H	A	T	Q	W	M	E	P				280
985	TGG	GAT	GAG	AAT	AGT	GCC	AGT	GAT	GTG	GAA	GCT	GCA	GCT	AGA	GCT	CTT	GAT	TTT	ATG	TTG	1044
281	W	D	E	N	S	A	S	D	V	E	A	A	A	R	A	L	D	F	M	L	300
1045	GGA	TGG	TTC	ATG	GAG	CCC	ATA	ACA	TCT	GGT	GAT	TAT	CCA	AAG	AGC	ATG	AAA	AAA	TTC	GTT	1104
301	G	W	F	M	E	P	I	T	S	G	D	Y	P	K	S	M	K	K	F	V	320
1105	GGA	TCA	CGA	CTG	CCA	AAG	TTT	TCA	CCT	GAA	CAA	TCT	AAG	ATG	CTA	AAG	GGA	TCA	TAT	GAC	1164
321	G	S	R	L	P	K	F	S	P	E	Q	S	K	M	L	K	G	S	Y	D	340
1165	TTT	GTT	GGA	TTG	AAT	TAC	TAT	ACT	GCT	TCT	TAC	GTG	ACT	AAT	GCA	TCA	ACA	AAT	TCT	TCT	1224
341	F	V	G	L	N	Y	Y	T	A	S	Y	V	T	N	A	S	T	N	S	S	360
1225	GGA	TCG	AAT	AAT	TTC	AGC	TAT	AAT	ACT	GAT	ATT	CAT	GTT	ACC	TAT	GAA	ACT	GAC	AGG	AAT	1284
361	G	S	N	N	F	S	Y	N	T	D	I	H	V	T	Y	E	T	D	R	N	380
1285	GGT	GTA	CCA	ATT	GGT	CCC	CAG	TCT	GGT	TCA	GAT	TGG	CTA	CTT	ATT	TAT	CCA	GAG	GGC	ATC	1344
381	G	V	P	I	G	P	Q	S	G	S	D	W	L	L	I	Y	P	E	G	I	400
1345	CGG	AAA	ATA	TTA	GTT	TAC	ACC	AAG	AAA	ACA	TAC	AAT	GTT	CCT	CTC	ATA	TAT	GTC	ACA	GAG	1404
401	R	K	I	L	V	Y	T	K	K	T	Y	N	V	P	L	I	Y	V	T	E	420
1405	AAT	GGA	GTT	GAC	GAT	ATA	AAG	AAT	ACT	AAT	CTA	ACA	CTT	TCT	GAA	GCT	CGT	AAA	GAT	TCG	1464
421	N	G	V	D	D	V	K	N	T	N	L	T	L	S	E	A	R	K	D	S	440
1465	ATG	AGA	CTG	AAA	TAT	CTC	CAA	GAC	CAT	ATA	TTC	AAC	GTC	CGC	CAA	GCA	ATG	AAT	GAT	GGT	1524
441	M	R	L	K	Y	L	Q	D	H	I	F	N	V	R	Q	A	M	N	D	G	460
1525	GTA	AAT	GTG	AAG	GGC	TAT	TTT	GCA	TGG	TCA	TTA	TTG	GAT	AAT	TTT	GAA	TGG	GGT	GAA	GGG	1584
461	V	N	V	K	G	Y	F	A	W	S	L	L	D	N	F	E	W	G	E	G	480
1585	TAT	GGA	GTG	CGT	TTT	GGT	ATC	ATC	CAC	ATC	GAC	TAT	AAT	GAC	AAC	TTT	GCA	AGA	TAC	CCC	1644
481	Y	G	V	R	F	G	I	I	H	I	D	Y	N	D	N	F	A	R	Y	P	500
1645	AAA	GAT	TCA	GCA	GTA	TGG	CTC	ATG	AAT	TCT	TTC	CAC	AAG	AAC	ATC	TCC	AAA	CTT	CCT	GCA	1704
501	K	D	S	A	V	W	L	M	N	S	F	H	K	N	I	S	K	L	P	A	520
1705	GTC	AAG	AGA	AGT	ATC	AGA	GAG	GAC	GAC	GAA	GAA	CAA	GTT	TCA	AGC	AAG	AGA	TTA	AGA	AAG	1764
521	V	K	R	S	I	R	E	D	D	E	Q	V	S	S	K	R	L	R	K		540
1765	TAG	AGA	TGC	TCT	TCT	GTC	TCA	TCA	AAC	AAA	TGG	TCA	GTT	TTT	TTT	TTT	TTT	TTT	TGC		1824
	*																				
1825	TGG	GTA	AAT	GGT	CAG	TTC	TTT	ATT	CCG	CTT	TGT	TGT	TGT	TGT	CGT	GGT	CTG	CTT	AAT	CAT	1884
1885	AAT	ATG	GAA	TAC	TGC	AAG	TTG	TTG	TTG	GGA	GAA	CTA	CCC	CTA	ATG	TGT	AGT	TCA	TGT	TTG	1944
1945	TTT	TAT	ATT	GAA	GTT	TGT	TTG	TTT	GTT	TGT	TAA	CAA	AAA	AAA	AAA	AAA	AAA	A			1996

Fig. 2. Nucleotide sequence of the RG cDNA clone and its predicted amino acid sequence. Recognition sequences for *Eco*RI, located at 661 and 1668; *Nco*I, at 143 and 703; are in bold letters. Putative start codons are underlined. Boxed areas represent the peptide sequences obtained from microsequencing of RG.

start signal and that the amino acids between positions 1 and 21 are necessary for enzyme activity.

In the crude protein extract of the *E. coli* strain containing pSE-RG1, an enzyme activity of 0.7 nkat/mg protein was measured in the soluble fraction. Although the pSE280 vector has an IPTG-inducible promoter, RG activity could be detected without IPTG-induction. In addition, no pronounced increase of RG activity in crude extracts was noted after induction with IPTG. However, in contrast to the rather difficult and inefficient multistep purification of RG from plant cell cultures, the recombinant enzyme from *E. coli* only required anion exchange (SOURCE 30Q), hydrophobic interaction (SOURCE 15 PHE) and size exclusion (Superdex 75 HR) chromatography to yield homogenous enzyme (Fig. 3).

The purified enzyme catalyzed the deglycosylation of raucassicine and the aglycone was identified using mass spectrometry and $^1\text{H-NMR}$ spectroscopy. This proved that the purified protein was indeed RG.

2.4. Substrate specificity of heterologously expressed RG

In contrast with the inherent low quantities of RG produced in plant cells, the higher amounts of pure enzyme now available from *E. coli* have only recently allowed for an in-depth investigation of the enzyme's substrate specificity. Several glucosides, mainly of plant origin, were tested as putative substrates for RG using the pure enzyme (Table 2). Two different assays were used, one based on the detection of glucose released and the other based on the HPLC resolution of substrate and the aglycone formed after incubation with the enzyme. The results obtained indicate that both the glucosidase from *Rauvolfia* cells and the heterologously expressed enzyme from *E. coli* exhibit very similar substrate specificity. Besides the compounds listed in the Table 2, the heterologously expressed RG did not accept several different glucosides as substrates, e.g. indoxyl- β -D-glucoside, strictosidine lactam,

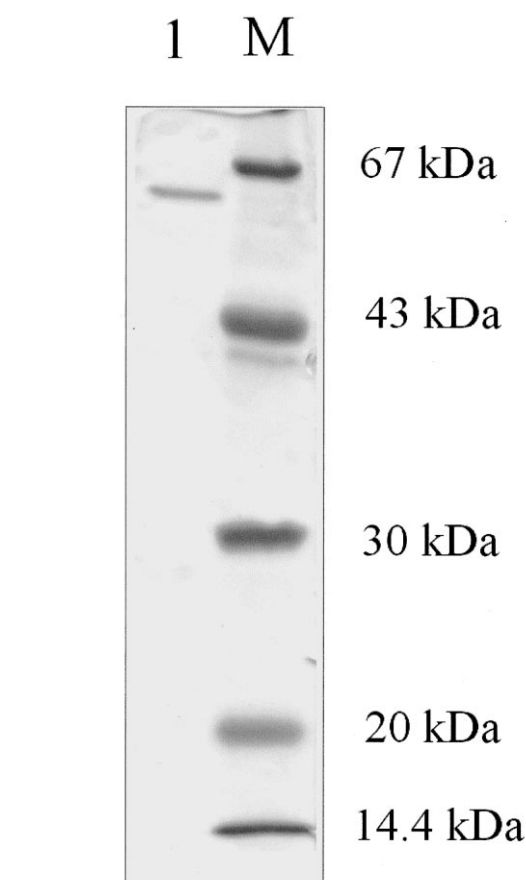


Fig. 3. SDS-PAGE of heterologously expressed raucassicine glucosidase purified from *E. coli*. Proteins were separated on a 11% gel and coomassie stained. M, marker; 1, fraction with RG activity after Superdex 75 column.

vincoside lactam, loganin, secologanin, arbutin, isoquercitrin, vanillin- β -D-glucoside, esculin, fluorescein- β -D-glucoside, 4-nitrophenyl- β -D-glucoside, 6-bromo-2-naphthyl- β -D-glucoside, sinigrin, ipecoside or rutin.

Unexpectedly, however, RG deglycosylated strictosidine, as well as its derivate 5 α -carboxystrictosidine.

Table 1
Homologies of RG with other plant glucosidases^a

	<i>C. roseus</i> SGD	<i>P. serotina</i> AH1	<i>P. avium</i> PH	<i>P. serotina</i> PH	<i>T. repens</i> CG	<i>T. repens</i> nCG	<i>C. speciosus</i> FG
<i>R. serpentina</i> RG	52	55	57	54	60	52	46
<i>C. roseus</i> SGD		49	49	49	52	49	44
<i>P. serotina</i> AH1			72	72	66	59	50
<i>P. avium</i> PH				88	65	57	51
<i>P. serotina</i> PH					62	55	50
<i>T. repens</i> CG						61	50
<i>T. repens</i> nCG							47

^a Enzymes identified by their GenBank accession numbers: RG, raucassicine glucosidase (AF149311); SGD, *Catharanthus roseus* strictosidine glucosidase (AF112888); CG, *Trifolium repens* cyanogenic glucosidase (X56733); nCG, *T. repens* noncyanogenic glucosidase (X56734); AH1, *Prunus serotina* amygdalin hydrolase isoform AH1 (U26025); PH, *P. serotina* prunasin hydrolase (U50201) or *Prunus avium* β -glucosidase (U39228); FG, *Costus speciosus* furostanol glycosid 26-O- β -glucosidase (D83177).

This strictosidine glucosidase activity was not found in the partially purified plant enzyme (Schübel et al., 1986). This may be due to the higher amount of RG that was expressed in the *E. coli* used in this study. Moreover, 5 α -carboxystrictosidine was accepted as a substrate for RG, although this strictosidine derivative plays no role in the biosynthesis of *Rauvolfia* of the ajmalan–sarpagan group (Stöckigt, 1979). The 3 α (S) stereochemistry was determined to be essential for RG activity since the 3 β (R) epimer 5 α -carboxyvincoside was not deglycosylated. Although the data about substrate specificity of heterologously expressed SG (Geerlings et al., 2000) were not reported, the same substrate preference (strictosidine compared to vincoside) was observed for SG from *C. roseus* cell suspension cultures (Hemscheidt and Zenk, 1980; Luijendijk et al., 1998).

A comparison of K_M and V_{max} values for raucaffricine (K_M 1.3 mM, V_{max} 0.5 nkat/ μ g) and for strictosidine (K_M 1.8 mM, V_{max} 2.6 pkat/ μ g) shows raucaffricine to be the preferred substrate. Though similar K_M values suggest comparable binding capacity for both substrates, the much lower V_{max} value for strictosidine indicates that this glucoside is hydrolyzed with significantly lower efficiency. Unfortunately there are no data reported about the enzyme kinetics of heterologously expressed SG (Geerlings et al., 2000). The K_M values for strictosidine determined with SG purified from *C. roseus* cells were much lower (0.2 and 0.1 mM for two isolated SG enzymes reported by Hemscheidt and Zenk, 1980, and ≤ 20 μ M reported by Luijendijk et al., 1998), therefore, demonstrating that this enzyme has a much higher affinity for strictosidine than the heterologously expressed RG. The question whether *C. roseus* SG can also catalyze the deglycosylation of raucaffricine remains unanswered, since the reported studies on its substrate specificity do not include this alkaloid.

Most likely, the hydrolysis of strictosidine is due to secondary activity of RG. This indicates that strictosidine must be cleaved by a different glucosidase in *Rauvolfia* cells which has not yet been identified. The results described here are, therefore, also of special interest for future work to be performed on the important enzyme strictosidine glucosidase, which provides the biosynthetic intermediate for the 2000 naturally occurring monoterpenoid indole alkaloids. In this respect, determination of the gene copy number of RG or of related genes coding for appropriate isoenzymes would be very informative.

2.5. Gene copy number of RG

For Southern blotting, genomic DNA of *R. serpentina* cell suspension culture was hybridized to a 1000 bp *Eco*RI fragment of RG cDNA. Two bands were observed for the *Bam*HI and *Pvu*II digested DNA (6.4 and 8 kb or 1.8 and 3.4 kb, respectively) while *Eco*RI digested DNA showed six bands (between 1.7 and 3.4 kb, Fig. 4). Since RG cDNA has two *Eco*RI restriction sites 1000 bp apart, a hybridization signal corresponding to this size was expected to be found in the *Eco*RI digested DNA. The sole detection of larger fragment signals could be explained, in principle, by the presence of one or more introns in the RG gene. An intron inside the region between the *Eco*RI sites would increase the size of the fragment. Moreover, an intron showing another *Eco*RI restriction site would cause the occurrence of two bands of different size, according to the position of the site and the size of the intron. In this case, the results of the Southern blot indicate the presence of introns in the RG gene. As shown for genes from maize and in several Brassicaceae, the plant glucosidase genes contain up to 11 introns (Esen and Bandaranayake, 1998; Rask et al., 2000). At this

Table 2
Substrate specificity of heterologous expressed raucaffricine glucosidase^a

Substrate tested with raucaffricine glucosidase	Relative activity (%), determined by HPLC	Relative activity (%), determined by glucose assay
Raucaffricine (2 mM)	100 (49.8 pkat) ^b	100 (40.75 pkat) ^b
21-Glucopyranosyl-hydroxysarpagan-17-al (2 mM)	n.d.	77.8
21-Glucopyranosyl-hydroxysarpagan-17-ol (2 mM)	84.9	62.8
1,2-Dihydrraucaffricine (2 mM)	82.7	70.9
1-Methyl-1,2-dihydrraucaffricine (2 mM)	47.4	40.5
Raucaffricine (4 mM)	100 (104.0 pkat) ^c	100 (77.3 pkat) ^c
Acetylrauglucine (4 mM)	n.d.	4.2
Strictosidine (4 mM)	6.5	7.1
5 α -Carboxystrictosidine (4 mM)	23.1	12.8

^a Activity of RG against raucaffricine was set to 100%. Activity was tested by HPLC assay and/or glucose testing. n.d., not determined.

^b The incubation mixture contained 0.02 μ g protein.

^c The incubation mixture contained 0.08 μ g protein.

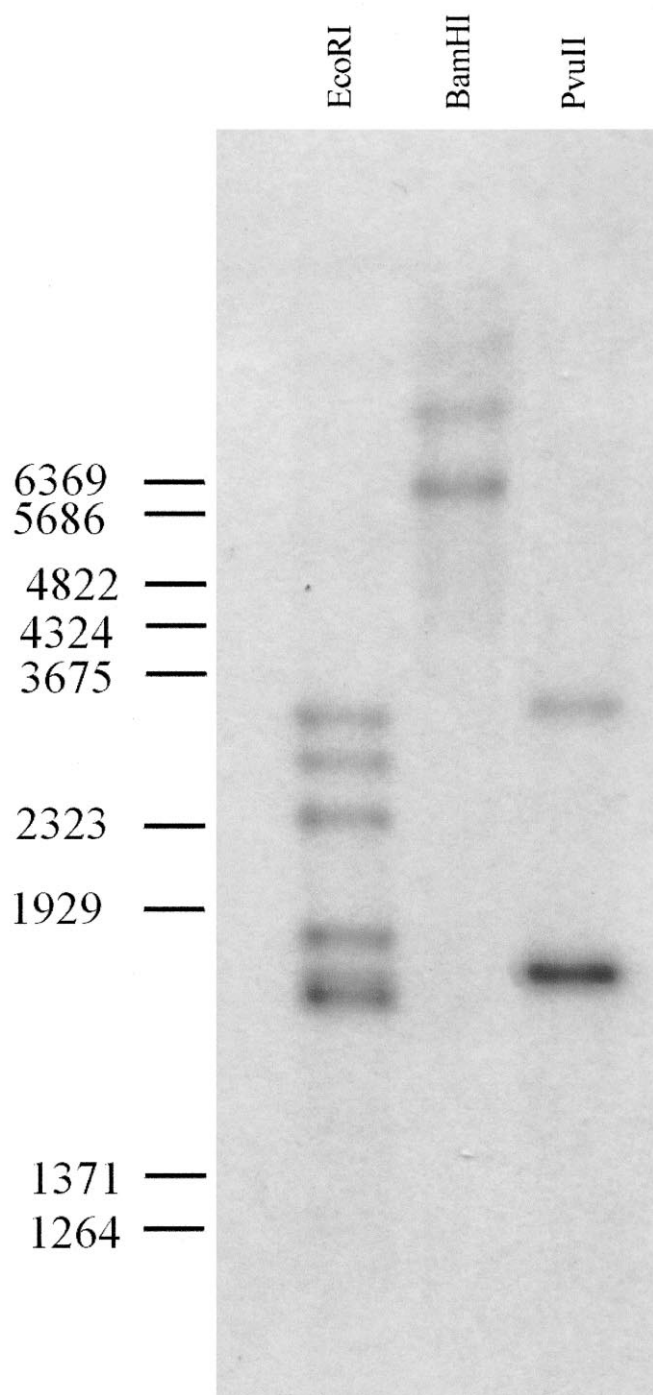


Fig. 4. Southern blot with DNA of *R. serpentina* cell suspension culture. Genomic DNA (10 µg per lane) was digested with restriction enzymes and resolved by agarose gel electrophoresis. 32 P-labelled 1000 bp RG cDNA fragment obtained from *EcoRI* digestion was used as hybridization probe. While in the cDNA two recognition sites for *EcoRI* occur, none of the other restriction endonucleases should hydrolyze within the RG reading frame.

stage it could not be concluded if there is more than one copy of the RG gene in the *Rauvolfia* genome.

In conclusion, RG obtained by heterologous expression in *E. coli* shows similar properties to those of

the native enzyme from the *Rauvolfia* cell suspension culture. The molecular weight of $66.600 \pm 5\%$ determined for the active enzyme by gel chromatography (Schübel et al., 1986) and of 61 kDa by gel electrophoresis (Warzecha et al., 1999) is in agreement with the calculated weight of the complete amino acid sequence. While the identity of the enzyme was unequivocally confirmed by the identification of the enzyme product vomilenine, the former's ability to hydrolyze strictosidine was here detected for the first time. This is perhaps a result of the greater availability of RG from heterologous expression of the protein in *E. coli*. This shows the importance of re-investigating every recombinant enzyme for determining its function, especially when quantities available from plant material are very low and, therefore, limit extensive biochemical characterizations. Although the activity of recombinant enzymes can usually be detected in crude protein extracts, characterization should be carried out with purified enzymes only. Several microorganisms possess the ability to convert plant secondary products like strictosidine (Shen et al., 1998) and it can not be excluded that host enzymes in crude extracts may be responsible for product formation. This fact could distort statements about substrate specificity and the identity of products. Moreover, the structure of the formed products must be confirmed by spectroscopic methods such as MS and NMR to unequivocally verify the functional identity of the enzyme.

The present results do not allow us to conclude whether RG also catalyses the SG activity in *Rauvolfia* and, therefore, other glucosidase clones from this plant are now being isolated, expressed and characterized (Gerasimenko and Stöckigt, in preparation). The isolation and identification of RG will also allow the inhibition or overexpression of this side reaction in cultivated *Rauvolfia* cells and the investigation of the significance of this particular enzyme for the entire alkaloid metabolism in *Rauvolfia*.

3. Experimental

3.1. Plant cell cultures

Cell suspension cultures of *R. serpentina* BENTH. ex KURZ were cultivated in 1 l conical flasks containing 400 ml LS medium (Linsmaier and Skoog, 1965) on a gyratory shaker (100 rpm) at $24 \pm 2^\circ\text{C}$ in diffuse light (600 lx). After 7 days, half of the cell suspension was routinely added to 200 ml of fresh LS medium under aseptic conditions.

3.2. Isolation and analysis of nucleic acids

Total RNA was isolated from plant cell suspension

cultures as described in (Ausubel et al., 1997). Poly(A)⁺ RNA was purified from total RNA using Oligotex[®] beads (Qiagen, Hilden, Germany). DNA was obtained from cell cultures using the DNeasy Kit (Qiagen). Cell suspensions were harvested, immediately frozen with liquid nitrogen and freeze dried. The lyophilized cells were then pulverized and processed according to the manufacturers instructions. For Southern hybridization analyses, genomic DNA was digested with restriction endonucleases, separated by electrophoresis in a 1% agarose gel in TAE buffer (Sambrook et al., 1989), partially hydrolyzed in 0.2 N HCl for 15 min, 30 min in 1.5 M NaCl/0.5 M NaOH, neutralized 2 × 30 min in 1.5 M NaCl/0.5 M Tris-HCl (pH 7.2) and 1 mM EDTA. After transferring the nucleic acids by capillary blotting (Sambrook et al., 1989) to a nylon membrane (Hybond N⁺, Amersham-Pharmacia) and UV crosslinking the DNA to the filter, prehybridization and hybridization were carried out overnight (5× SSPE [0.19 M NaCl, 10 mM NaPi, 1 mM EDTA], 5× Denhardt's, 0.1% SDS, 200 µg/ml salmon sperm, 50% formamide) at 42°C. The filters were washed two times with 2× SSC (0.15 M NaCl, 30 mM Na-citrate) (Sambrook et al., 1989) 0.1% SDS at 42°C and then several times with 0.2× SSC, 0.1% SDS at increasing temperatures (50–65°C). The hybridizing DNA was identified by autoradiography on Biomax films (Kodak). DNA probes were ³²P-labeled with the Random Priming System (Life Technologies, Eggenstein).

A cDNA library was constructed of mRNA from 6-day-old cell suspension culture using the λ ZAP II system (Stratagene). Screening was performed according to the manufacturer's instructions.

3.3. PCR and cloning

Partial cDNA clones encoding RG were generated by PCR using cDNA produced by reverse transcription of total RNA isolated from 6-day-old cell suspension cultures. DNA amplification was performed under the following conditions: 5 min at 94°C, 3 cycles of 94°C for 30 s, 40°C for 2 min and 72°C for 1 min; then 30 cycles of 94°C for 30 s, 50°C for 2 min, 72°C for 1 min. The last step was followed by an additional elongation step at 72°C for 5 min. The amplified DNA was separated by agarose gel electrophoresis. The band of approximately desired size was eluted from the gel (NucleoSpin Extraction, Macherey & Nagel, Düren, Germany) and cloned into the pGEM-T vector (Promega, Mannheim, Germany) for further analysis. pBluescript containing full length clones of RG cDNA (pBlueRG) were isolated from the λ ZAP library and were used for sequencing (Genterprise, Mainz).

For the alignment of the amino acid sequence of RG the Fasta 3 program at EBI (European Bioinfor-

matics Institute) was used. Multiple sequence alignment and calculation of homologies were performed using MegAlign (LASERGENE).

3.4. Heterologous expression

For heterologous expression of RG, the vector pSE280 (Invitrogen) was restriction digested with *Nco*I and *Xho*I. Due to a second internal *Nco*I site in addition to that at the translation start (position 143), a partial digestion of pBlueRG (*Xho*I digested) with *Nco*I was carried out. After electrophoretic separation, the fragment of approximately 1850 bp was purified and cloned into pSE280 (*Xho*I/*Nco*I digested). The plasmids (pSE280RG) were expressed in TOP 10 *E. coli* cells. For use of the second ATG codon as a translation start signal, the cDNA was amplified with two extended deoxy-oligonucleotides as PCR primers (RG2for: ACTAGCCATGGGAACGGGCTCTTC; RG2rev: CGATCTCGAGCTACTTTCTTAA-TCTCTTG) and the product was cloned into pSE280. *E. coli* TOP 10 was cultivated at 37°C in conical flasks (LB medium containing 1% trypton, 0.5% yeast extract, 1% NaCl). For induction of RG expression, isopropyl-β-D-thiogalactopyranoside was added to the cells (OD 0.5) to a final concentration of 1 mM. After induction, the cells were cultivated for an additional 4 h at 37°C in a gyratory shaker (200 rpm).

3.5. Protein purification

To produce a small scale cell lysate of *E. coli* (up to 20 ml culture), the cells were pelleted, resuspended in buffer A (50 mM NaH₂PO₄, pH 8, 0.3 M NaCl) and sonicated 6 × 10 s with 200 W on ice. For larger volumes, the cells were disrupted by the use of a French press. After centrifugation, the supernatant was used for enzyme assays or further purification steps.

For ammonium sulfate precipitation of proteins, (NH₄)₂SO₄ powder was added to the supernatant over a period of 1 h (4°C, continuously stirring) up to a salt concentration of 30%. After centrifugation (30 min, 10,000 × g, 4°C) (NH₄)₂SO₄ was added to the supernatant to a final concentration of 75%. The solution was centrifuged again, and the precipitated protein was dissolved in 100 ml buffer B (20 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol (β-ME)). After another centrifugation, the supernatant was dialyzed against 10 l buffer B overnight.

For anion exchange chromatography, the dialyzed protein was applied to a SOURCE 30Q XK 50/30 column (Pharmacia) equilibrated with buffer C (20 mM Tris-HCl, pH 8.0; 10 mM β-ME). After washing the column with 0.5 column volumes (CV, 240 ml) of buffer C, the proteins were eluted with a linear KCl gradi-

ent (6 CV, 0–0.5 M KCl) prepared from buffers C and D (20 mM Tris–HCl, pH 8.0, 10 mM β -ME, 1 M KCl) at a flow rate of 20 ml/min. RG activity appeared at KCl concentrations of 0.23–0.26 M. Fractions containing enzyme activity were pooled and prepared for the next purification step by adding $(\text{NH}_4)_2\text{SO}_4$ to a resulting concentration of 1 M.

The combined fractions were resolved by hydrophobic interaction chromatography on a SOURCE 15 PHE XK 16/20 column (Pharmacia) which had been equilibrated with buffer E (20 mM Tris–HCl, pH 8.0, 10 mM β -ME; 1 M $(\text{NH}_4)_2\text{SO}_4$). After washing the column with 0.8 CV (CV 30 ml) of buffer E, proteins were eluted with a linear $(\text{NH}_4)_2\text{SO}_4$ gradient (10 CV, 1–0 M $(\text{NH}_4)_2\text{SO}_4$) prepared from buffers E and C at a flow rate of 10 ml/min. RG activity eluted at $(\text{NH}_4)_2\text{SO}_4$ concentrations of 0.59–0.51 M. Fractions containing active RG were pooled and concentrated using Centriprep 10 and Microcon 30 concentrators (Amicon, Witten).

Concentrated fractions were applied to a Superdex 75 HR 10/30 column (Pharmacia) for size exclusion chromatography (CV 30 ml). The proteins were eluted with 20 mM Tris–HCl buffer, pH 7.8, containing 100 mM KCl and 10 mM β -ME at a flow rate of 30 ml/h collecting 0.5 ml fractions.

3.6. Analysis of substrates and products

Glucosidase activity was analyzed with the HPLC assay, as described recently (Warzecha et al., 1999). For the identification of vomilenine, the product of the enzymatic reaction was dissolved in methanol and analyzed by GC–MS. For ^1H -NMR investigations, the product was extracted with CH_2Cl_2 and, after drying, dissolved in $\text{DMSO}-d_6$. MS and ^1H -NMR data were identical with those of a vomilenine standard.

For the testing of glucosidase activity with a broad range of substrates, the glucose liberated was measured using Glucose reagent (Trinder) (Sigma). This system is based on a coupled enzyme system which consists of glucose oxidase and peroxidase. Unless otherwise stated the incubation mixture (total volume 100 μl) contained 4 mM of the corresponding substrate, 10 μl of citrate–NaOH buffer (pH 5.0), and 0.08 μg of RG. Incubations were carried out at 28°C with agitation. The reaction was terminated by addition of 200 μl methanol. Of this mixture, 200 μl were added to 1 ml of Glucose reagent (0.5 mM 4-aminoantipyrine; 20 mM *p*-hydroxybenzene sulfonate; 15,000 U/l glucose oxidase; 10,000 U/l peroxidase; pH 7.0) and after 18 min, the absorbance at 505 nm was recorded. Parallel incubations without RG were treated identically. The detection limit of this assay was 0.5 pkat (20 nM glucose liberated in 12 h). It was shown that RG still

hydrolyzed raucaffricine after 12 h of incubation under the conditions described.

K_M and V_{max} values for raucaffricine were determined in presence of 0.25 μg protein using both the HPLC and the glucose release assay. K_M and V_{max} values for strictosidine were determined in presence of 4 μg protein using the glucose release assay.

3.7. Other methods

Protein electrophoresis, staining, and quantification were performed, as described recently (Warzecha et al., 1999).

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