PERGAMON

Phytochemistry 54 (2000) 657–666

PHYTOCHEMISTRY

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

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Received 3 January 2000; received in revised form 15 May 2000

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/μg 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/μg protein) which was not observed for the low amounts of enzyme isolated from *Rauvolfia* cells. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Rauvolfia serpentina*; Apocynaceae; Plant cell suspension culture; Raucaffricine-*O*-β-D-glucosidase; Heterologous expression; Substrate specificity

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 (vincaleucoblastine 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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PII: S0031-9422(00)00175-8

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 monoterpenoid 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 deglucosylation 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.

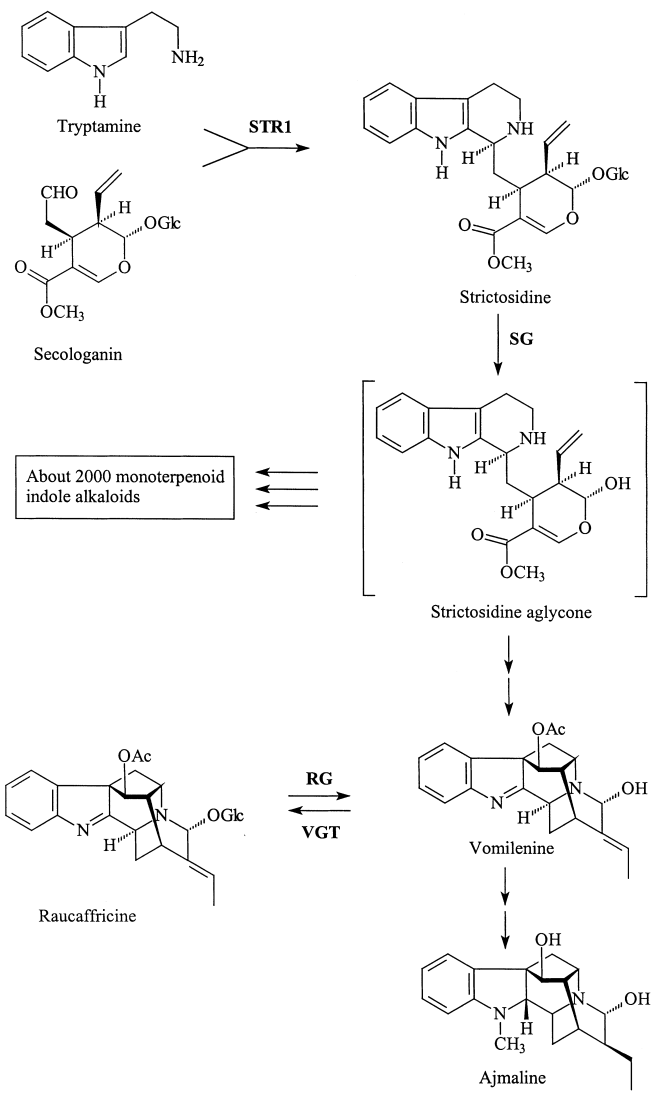

Fig. 1. Schematic representation of ajmaline biosynthesis in *Rauvolfia serpentina*. Double arrows are indicating several enzymatic reactions. Enzymes are: STR1, strictosidine synthase; SG, strictosidine glucosidase; VGT, vomilenine glucosyl transferase; RG, raucaffricine glucosidase.

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 deglucosylated 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 deglucosylated 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-

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 NH_2-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 NH_2-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 deglucosylation of raucaffricine could be measured in the enzyme extract from pSE-RG2. This result strongly suggested that the first ATG is the translation

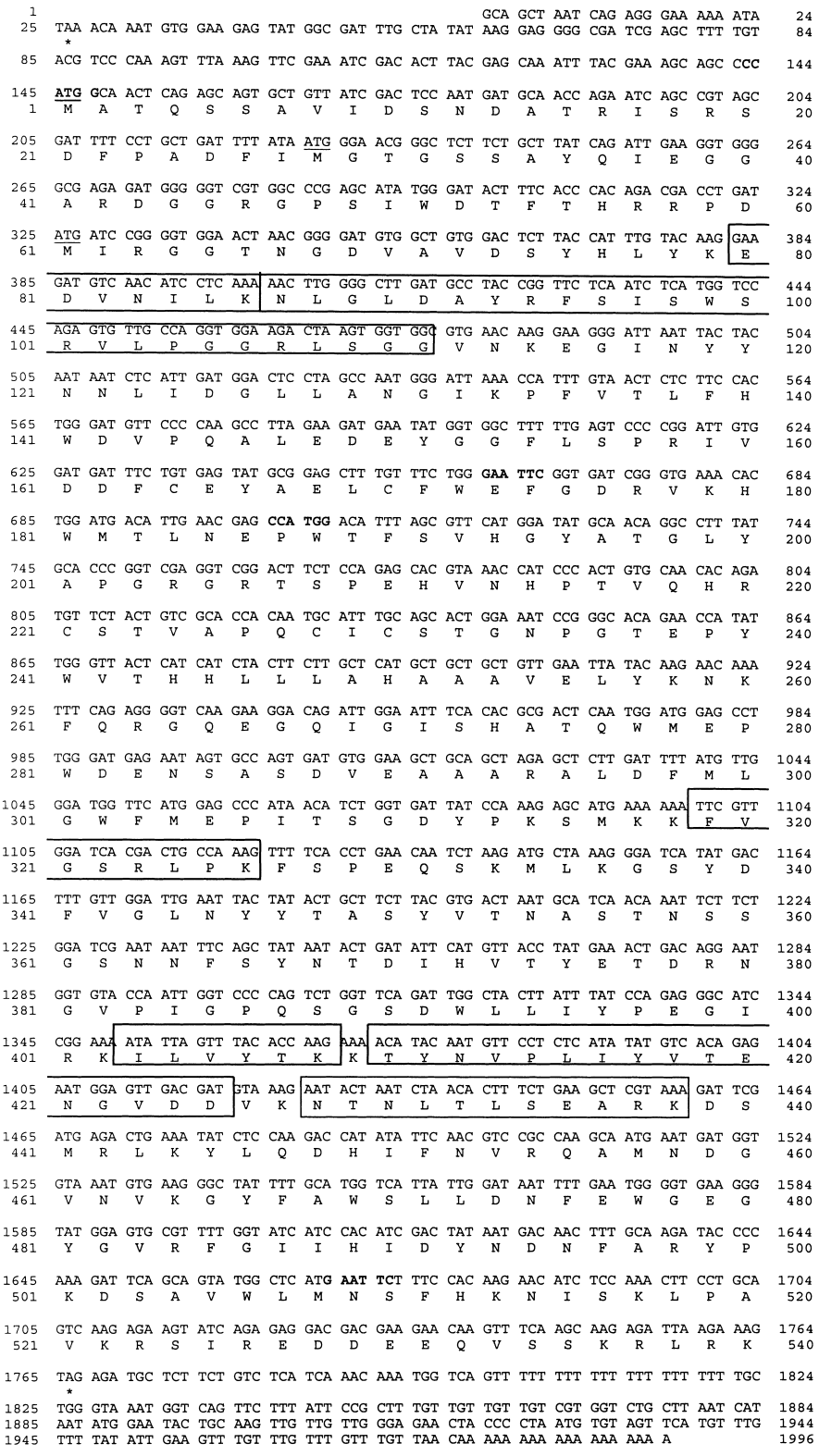

Fig. 2. Nucleotide sequence of the RG cDNA clone and its predicted amino acid sequence. Recognition sequences for *Eco*RI, located at 661 and 1668; *Nco*I, at 143 and 703; are in bold letters. Putative start codons are underlined. Boxed areas represent the peptide sequences obtained from microsequenzing 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 deglucosylation of raucaffricine and the aglycone was identified using mass spectrometry and ^{1}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, 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 deglucosylated strictosidine, as well as its derivate 5α-carboxystrictosidine.

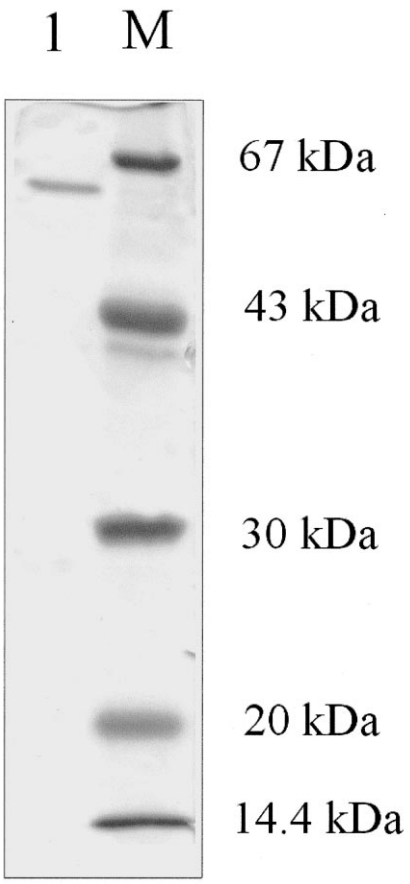

Fig. 3. SDS-PAGE of heterologously expressed raucaffricine glucosidase purified from *E. coli*. Proteins were separated on a 11% gel and coomassie stained. M, marker; 1, fraction with RG activity after Superdex 75 column.

Table 1
Homologies of RG with other plant glucosidases[a]

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

[a] Enzymes identified by their GenBank accession numbers: RG, raucaffricine 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 at al., 1986). This may be due to the higher amount of RG that was expressed in the *E. coli* used in this study. Moreover, 5α-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 3α(*S*) stereochemistry was determined to be essential for RG activity since the 3β(*R*) epimer 5α-carboxyvincoside was not deglucosylated. 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/μg) and for strictosidine (K_M 1.8 mM, V_{max} 2.6 pkat/μg) 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 $\leq$20 μM 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 deglucosylation 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 occurence 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-Dihydroraucaffricine (2 mM)	82.7	70.9
1-Methyl-1,2-dihydroraucaffricine (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
5α-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 μg protein.
[c] The incubation mixture contained 0.08 μg protein.

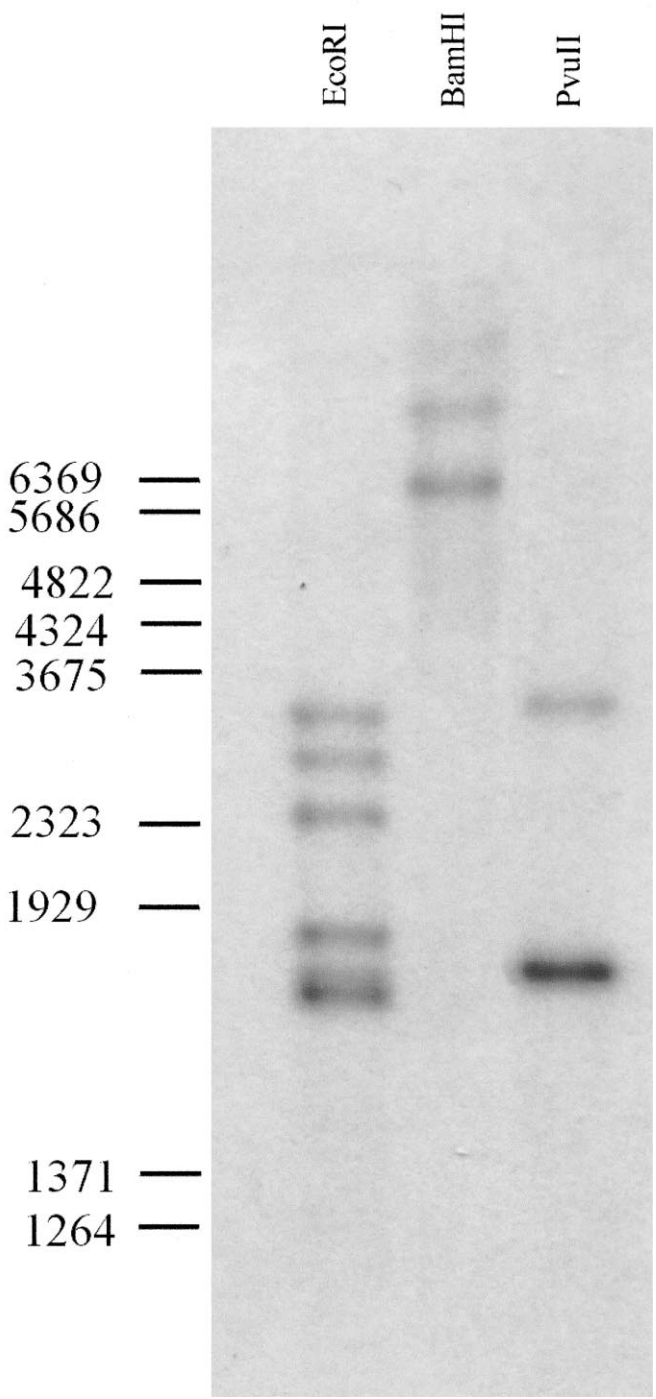

Fig. 4. Southern blot with DNA of *R. serpentina* cell suspension culture. Genomic DNA (10 μg per lane) was digested with restriction enzymes and resolved by agarose gel electrophoresis. ^{32}P-labled 1000 bp RG cDNA fragment obtained from *Eco*RI digestion was used as hybridization probe. While in the cDNA two recognition sites for *Eco*RI 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±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±2°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 ^{32}P-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 Bioinformatics 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: CGATCTCGAGCTACTTTCTTAATCTCTTG) 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 NaH_2PO_4, 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, $(NH_4)_2SO_4$ powder was added to the supernatant over a period of 1 h (4°C, continously stirring) up to a salt concentration of 30%. After centrifugation (30 min, 10,000 × *g*, 4°C) $(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$). After washing the column with 0.8 CV (CV 30 ml) of buffer E, proteins were eluted with a linear $(NH_4)_2SO_4$ gradient (10 CV, 1–0 M $(NH_4)_2SO_4$) prepared from buffers E and C at a flow rate of 10 ml/min. RG activity eluted at $(NH_4)_2SO_4$ 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 ^{1}H-NMR investigations, the product was extracted with CH_2Cl_2 and, after drying, dissolved in DMSO-d_6 MS and ^{1}H-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).

Acknowledgements

This work was supported by a grant of the Deutsche Forschungsgemeinschaft (DFG, Bonn) and by the Fonds der Chemischen Industrie (Frankfurt/Main). We thank Dipl. Biol. Yuri Sheludko for providing samples of alkaloids and Mrs Laura Shevy (Boyce Thompson Institute for Plant Research, Tower Road, Ithaca, New York) for her kind help in correcting the English version.

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