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Purification, partial amino acid sequence and structure of the product of raucaffricine-*O*-*β*-D-glucosidase from plant cell cultures of *Rauwolfia serpentina*

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Abstract

Plant cell suspension cultures of *Rauwolfia* produce within 1 week ~250 nkat/l of raucaffricine-*O*-*β*-D-glucosidase. A five step procedure using anion exchange chromatography, chromatography on hydroxylapatite, gel filtration and FPLC-chromatography on Mono Q and Mono P delivered in a yield of 0.9% ~1200-fold enriched glucosidase. A short protocol employing DEAE sepharose, TSK 55 S gel chromatography and purification on Mono Q gave a 5% recovery of glucosidase which was 340-fold enriched. SDS-PAGE showed a M_r for the enzyme of 61 kDa. The enzyme is not glycosylated. Structural investigation of the enzyme product, vomilenine, demonstrated that the alkaloid exists in aqueous solutions in an equilibrium of 21(*R*)- and 21(*S*)-vomilenine in a ratio of 3.4:1. Proteolysis of the pure enzyme with endoproteinase Lys C revealed six peptide fragments with 6–24 amino acids which were sequenced. The two largest fragments showed sequences, of which the motif Val–Thr–Glu–Asn–Gly is typical for *β*-glucosidases. Sequence alignment of these fragments demonstrated high homologies to linamarase from *Manihot esculenta* (81% identity) or to *β*-glucosidase from *Prunus avium* (79% identity). Raucaffricine-*O*-*β*-D-glucosidase seems to be a new member of the family 1 of glycosyl hydrolases.

Keywords: *Rauwolfia serpentina* cell culture; Apocynaceae; Raucaffricine-*O*-*β*-D-glucosidase; Purification; Partial amino acid sequence; Vomilenine structure

1. Introduction

Glucosidases belong to a group of enzymes which are of widespread distribution and for a long time have been extensively studied from several points of view. These enzymes were investigated not only because of their significant role in cellulose degradation (Beguin, 1990), their involvement in plant defence reactions (Poulton, 1990) and activation of phytohormones (Brzobohaty et al., 1993), but also to elucidate their still unrevealed reaction mechanisms (Withers & Street, 1988; Sinnot, 1990) or to assign them to the appropriate glycosyl hydrolase families (Henrissat, 1991; Henrissat & Bairoch, 1993).

Consequently many attempts have been directed towards the purification and characterization of glucosidases from quite different origins and especially in order to gain insight into their substrate acceptance as 'unspecific' and 'specific' enzymes. The use of unnatural substrates, like *p*-nitrophenyl glucoside, for the enrichment of glucosidases hampered detailed studies of their real substrate specificities and was criticized several times in the literature (Hemscheidt & Zenk, 1980; Conn, 1993). Very recent work, however, describes a number of more or less substrate specific plant glucosidases (Gus-Mayer, Brunner, Schneider-Poetsch, & Rüdiger, 1994; Falk & Rask, 1995) and among them the well characterized *β*-glucosidases hydrolyzing cyanogenic glucosides (Oxtoby, Dunn, Pancoro, & Hughes, 1991; Li, Swain, & Poulton, 1992).

Although glucosidases are involved in the metabolism of a broad variety of plant natural products it seems that this type of enzyme has no general significance in alkaloid biosynthesis and this is especially true for one of the largest alkaloid groups, containing the so far known 2000 monoterpenoid indole alkaloids. Among them less than 40 are glucoalkaloids (Ruyter, Schübel, & Stöckigt, 1988). From two of these glucoalkaloids only the corresponding glucosidases were detected and could be partially characterized from plant cell suspension cultures of *Catharanthus roseus* G. Don and *Rauwolfia serpentina*

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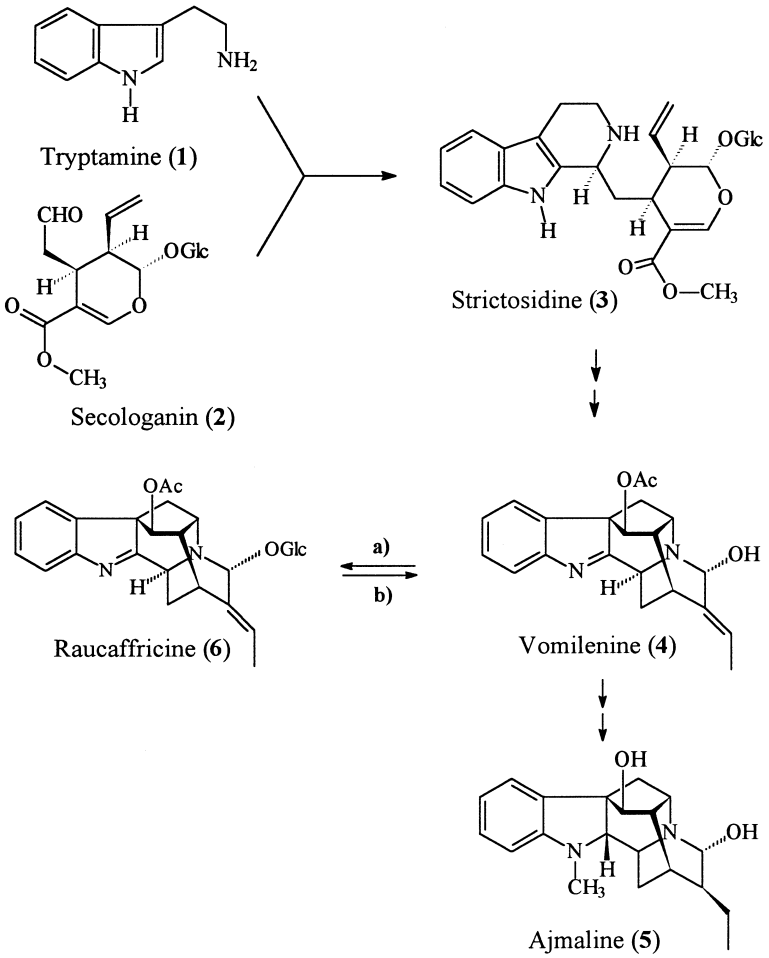

Fig. 1. Biosynthesis of ajmaline. Short description of the overall biosynthetic pathway leading from tryptamine (**1**) and secologanin (**2**) through the intermediates strictosidine (**3**) and vomilenine (**4**) to the antiarrhythmic ajmaline (**5**); (a) is catalyzed by the membrane bound vomilenine-UDP glucose transferase, (b) by the soluble raucaffricine glucosidase (RG).

Benth. ex Kurz (Hemscheidt & Zenk, 1980; Schübel, Stöckigt, Feicht, & Simon, 1986). Both of them, the alkaloidal glucoside strictosidine (**3**) and the alkaloid raucaffricine (**6**) are, however, of great interest (Fig. 1).

Strictosidine (**3**), which is the condensation product of the monoterpene glucoside secologanin (**2**) and the amine tryptamine (**1**), occupies a crucial biosynthetic role (Kutchan, 1993). It is most probably the biogenetic progenitor of all monoterpenoid indole alkaloids in nature. This glucoside is therefore an extraordinary example because glucosides of natural products are usually 'located' at the end of biosynthetic routes as water soluble compounds. **3** is, however, situated at the beginning of pathways and becomes a highly activated and reactive alkaloid precursor on entering these routes after the splitting off the glucosidic bond by strictosidine glucosidase (Hemscheidt & Zenk, 1980).

In contrast, the second glucoside, **6**, might well belong to a group of storage compounds but its precise biosynthetic role is not yet known (Stöckigt, 1995). The deglucosylation of **6** catalyzed by raucaffricine glucosidase (RG; EC 3.2.1.125) leads to its aglycone vomilenine (**4**). The latter alkaloid is a direct intermediate in the biosynthetic pathway of the antiarrhythmic alkaloid ajmaline (**5**) (Stöckigt, 1995). RG, therefore, might have a fascinating biochemical function, e.g. by channeling a biogenetic precursor into the late stages of ajmaline biosynthesis. Moreover the glucosidase has never been detected in other plants or cell suspension cultures than *Rauwolfia* and is obviously unique to the co-occurrence of ajmaline type alkaloids. Therefore, for its further investigation, e.g. the elucidation of its reaction mechanism, a pure enzyme and information on its amino acid sequence would be advantageous. The present paper describes the purification, partial sequencing, the structure of the enzyme product **4** and a comparison of this exceptional enzyme in alkaloid biosynthesis to other plant glucosidases.

2. Results

2.1. Cell growth and glucosidase activity

Cell growth, protein amount and RG activity of cultivated *Rauwolfia* cells were dependent on cultivation time. Enzyme activity (nkat/l cell suspension) and protein content (g/l) increased up to the 5th/6th day from 100 to 270 nkat and 0.3 to 0.6 g protein, respectively. Optimum conditions were around 5–6 days. For cultivation of the *Rauwolfia* cells usually 1 week growth period is applied routinely. Because at that time only slight decrease of enzyme activity from 270 to 250 nkat/l occurs, cells for isolation of RG were harvested after 7 days of growth.

2.2. Purification of RG

The results of a typical purification procedure of RG are shown in Table 1. The combination of five different steps of enzyme enrichment including HPLC chromatography resulted finally in over 1000-fold enzyme purification. The crude protein extract obtained from the *Rauwolfia* cells was subjected to $(NH_4)_2SO_4$ fractionation followed by anion exchange chromatography on DEAE Sephacel (Fig. 2A). At this stage a 3.5-fold enzyme enrichment with an overall yield of 43% was obtained. For further purification the glucosidase-containing fractions were added to a hydroxylapatite column which, after elution with a linear KPi gradient, gave a 10-fold purification of the RG activity with a specific activity of 3.9 nkat/mg.

As Fig. 2(B) illustrates, maximum enzyme activity was separated from the bulk of the protein and this enzyme preparation was then subjected to size exclusion chromatography applying AcA 54 material after it had been concentrated by ultrafiltration. The maximum glucosidase activity was eluted under the applied conditions after 75 ml corresponding to a M_r of 63 kDa $\pm$ 10% (Fig. 2C). The remaining glucosidase fractions did not contain the well known *Rauwolfia* enzyme polyneuridine aldehyde esterase (PNA esterase, data not shown (Pfitzner & Stöckigt, 1983)) indicating its further purification, which corresponded to a 133-fold enrichment. Fractions with the highest enzyme activity were combined, concentrated and were used for the next purification step based on the second ion exchange chromatography in this protocol, on the Mono Q anion exchanger. Although this step did not efficiently resolve the enzyme activity from the major protein peaks (Fig. 2D), the resulting enzyme solution obtained by KCl elution showed a ~600-fold enrichment with a specific enzyme activity of 240 nkat/mg. Compared with the starting protein amount at this stage a yield of ~4% was determined. After concentration of the protein solution, the remaining enzyme could be finally purified by FPLC isoelectric focusing on a Mono P column (Fig. 2E). The maximum catalytic activity of RG corresponds to an isoelectric point of 5.8. In summary, the enzyme was ~1200-fold purified and showed a specific activity of ~470 nkat/mg with a 0.9% recovery from the crude protein extract. Only about 10 μg of the enzyme remained.

Based on these data a shorter purification protocol for RG could be developed with the aim of obtaining sufficient pure enzyme amounts for protein digestion and partial sequencing. This procedure finally was similar to the just described one but only 3 steps of column chromatography were applied; DEAE Sepharose (fast flow), size exclusion on TSK 55 S and Mono Q chromatography resulted in an enzyme preparation with a specific activity of 136 nkat/mg which was 340-fold enriched with a total recovery of 5%.

2.3. Purity, molecular weight and sugar content of RG

The 340-fold enriched enzyme preparation showed on SDS-PAGE (Fig. 3A, lane A) after Coomassie Blue staining several bands with a major protein at 61 kDa. Previous determination of M_r by gel chromatography on Fractogel TSK 3000 indicated 66 kDa ($\pm$5%) for the enzyme (Schübel et al., 1986). In order to finally assign

Table 1
Purification of raucaffricine-o-β-D-glucosidase

Step	Total protein (mg)	Specific activity (nkat[a]/mg)	Total activity (nkat)	Purification (fold)	Recovery (%)
Crude extract	1296	0.40	518		100
$(NH_4)_2SO_4$ (30–75%)	798	0.42	335	1.1	65
DEAE-Sephacel	160	1.4	224	3.5	43
Biogel-HT	31	3.9	121	9.8	23
Ultrogel AcA 54	1.4	53	74.2	133	14
Mono Q	0.09	243	21.9	608	4.2
Mono P	0.01	468	4.68	1170	0.9

[a] 1 kat corresponds to the enzyme amount which catalyzes the hydrolysis of 1 mol raucaffricine under the described assay conditions.

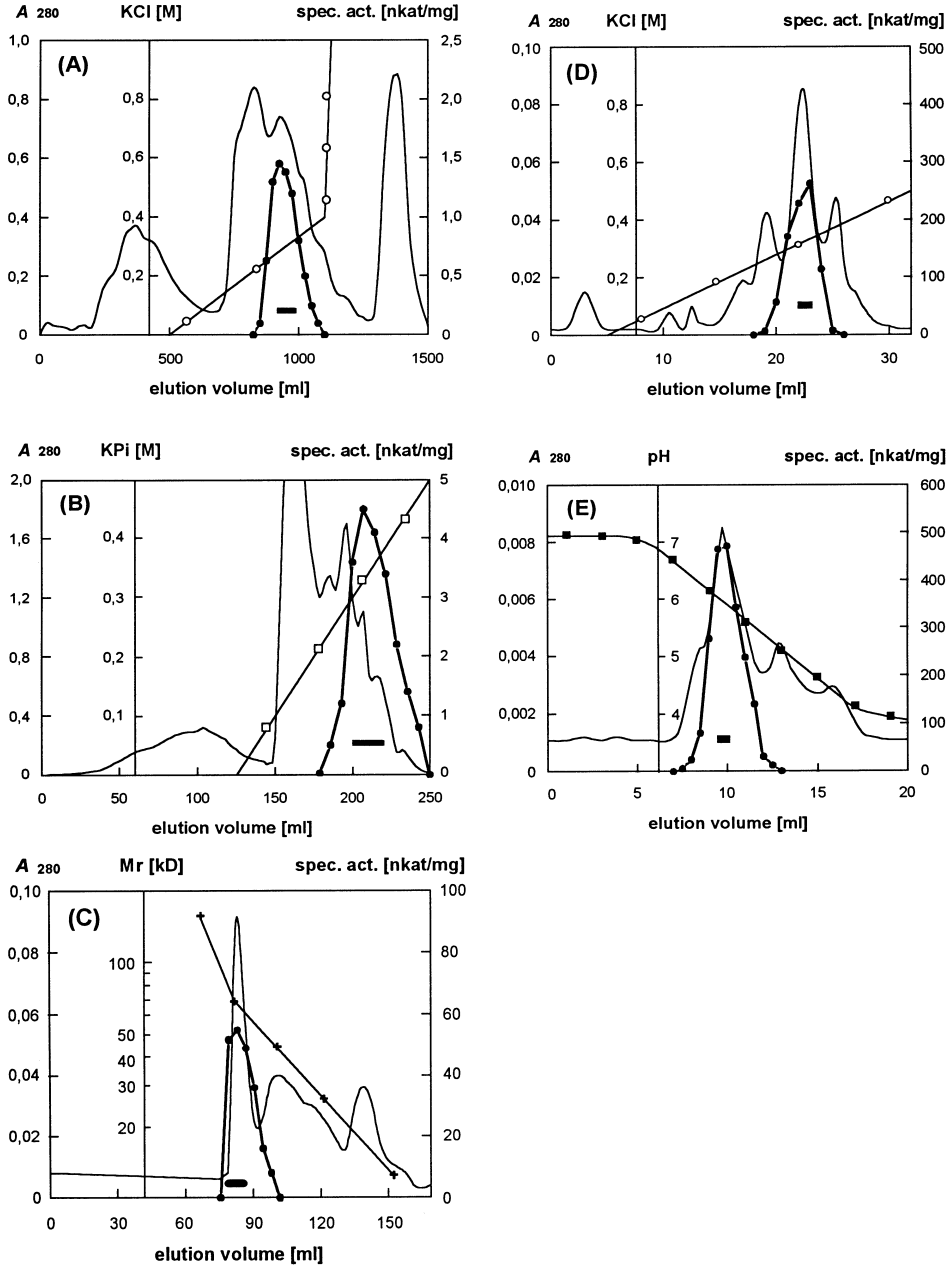

Fig. 2. Purification of raucaffricine-β-D-glucosidase. (A) Elution profile of DEAE-Sephacel anion exchanger with a linear gradient 0–0.4 M KCl; (B) separation on a Biogel HT hydroxylapatite column with a linear gradient of 0.02–0.5 M KPi; (C) enrichment of the enzyme by Ultrogel AcA 54 size exclusion chromatography; (D) elution profile of RG from a Mono Q FPLC anion exchanger column with 0–0.5 M KCl; (E) FPLC-chromatofocusing on a Mono P column starting at pH 7.1 followed by elution with 10% Polybuffer 74, pH 4.0. ———, UV absorption; –·–, specific activity; –◦–, KCl gradient; –□–, KPi gradient; — + —, M_r; –■–, pH value.

the glucosidase to one of the bands on SDS-PAGE, the same enzyme preparation was subjected three times (lane A–C) to basic native electrophoresis (BNE, Fig. 3B). Lane A was separated and stained with Coomassie Blue, indicating three different protein regions, with one major (a) and with two minor protein amounts (b) and (c). From the unstained lane B the corresponding regions (a), (b) and (c) were scraped off and analyzed for enzyme activity. Only the major protein band (a) showed RG activity. The third lane C was treated accordingly, but used finally for SDS-PAGE. After staining, this gel clearly exhibited only one protein band at 61 kDa (Fig. 3A, lane B) which was the same as that dominating in the original SDS-PAGE of the 340-fold enriched enzyme preparation (Fig. 3A, lane A). Silver staining of this gel also showed only one protein band. This protein, therefore, could be clearly assigned as RG.

To obtain knowledge of a glycosylation of this enzyme, an enriched glucosidase fraction (340-fold) was separated on SDS-PAGE with the reference proteins, transferrin as

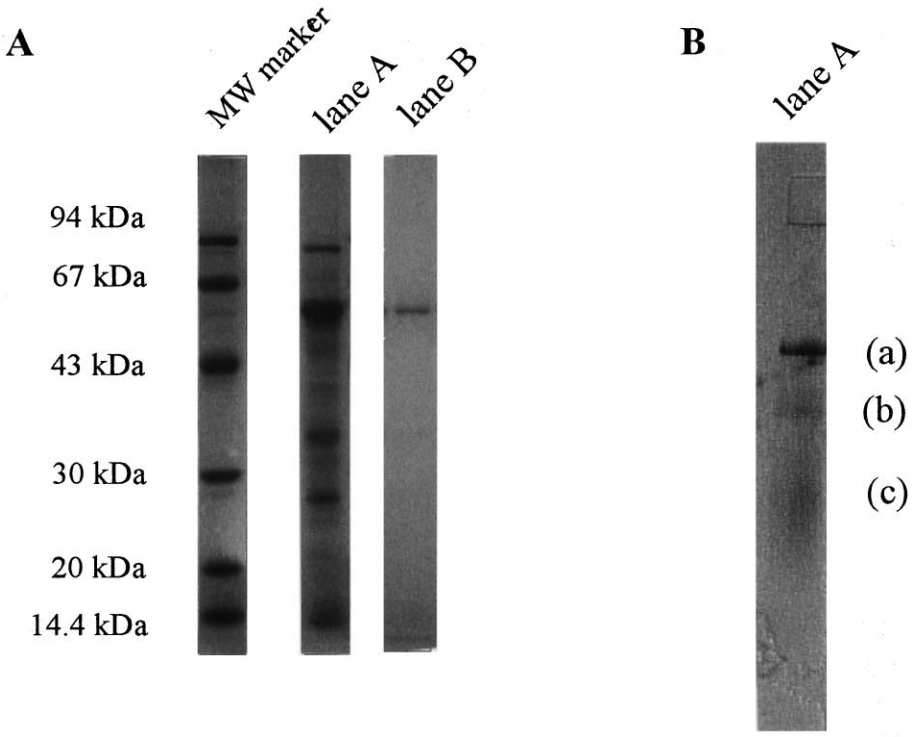

Fig. 3. SDS-PAGE and BNE of RG after a short purification procedure. (A) SDS-PAGE (12%) of 340-fold enriched RG (lane A), compared with enzymatic active fraction after BNE (lane B). (B) One lane of 3 after BNE (10%), stained with Coomassie Blue. Regions of the other lanes (not shown) corresponding to those indicated in the stained lane (a–c) were used for enzyme activity testing and SDS-PAGE.

a glycoprotein and creatinase as a non-glycosidic protein. The gel exhibited, after blotting on nitrocellulose membranes and staining with the Dig Glycan Detection Kit, only staining of the glycoprotein transferrin (data not shown). The RG is therefore most probably a sugar-free enzyme.

2.4. MS and NMR identification of the glucosidase product vomilenine

The enzyme product **4** showed identical MS-fragmentation when analyzed by EIMS and the same behaviour as a reference sample when analyzed by coupled GC–MS. When the ^{1}H NMR spectrum of the purified enzyme product **4** was measured in DMSO-d_6 several protons appeared as double signals in a ratio 3.4:1, like the quartet for H-19, the singlet of H-21 or the singlet of H-17. Vomilenine obviously occurs as a mixture of isomers. Analysis of the NMR data indicates that the mixed spectrum consists of the signals for a 21α(*S*)- and a 21β(*R*)-isomer of **4**, the latter being the main product, but exhibiting the reverse configuration when compared with the enzyme substrate **6** or with **5** and all of its natural derivatives. The stability of **4** can be low due to its carbinol (aminal) structure. Structures could exist in an equilibrium through the open form (aldehyde and free amino group) which leads to the 21α- (**4b**) and 21β-isomers (**4a**), respectively (Fig. 4).

NMR experiments were performed, by measuring ^{1}H of **4** in DMSO-d_6 in the presence of increasing amounts of D_2O. In this series the ^{1}H signals of H-17 and H-21 were measured in DMSO-d_6/D_2O ratios of 100:0, 75:25, 65:35 and 50:50 (Fig. 5). With increasing amounts of D_2O the signal distances of the appropriate proton signals (H-17, H-21) of each isomer decreased and signals overlapped completely at 50% D_2O. In the presence of water a fast equilibrium between both configuration isomers exists and one spectrum only is obtained due to a short life time of the isomers compared to the NMR-time scale.

2.5. Partial amino acid sequence of RG

Degradation of the enzyme by endoproteinase Lys C, HPLC-analysis of the resultant peptide fragments (PF) and their sequencing delivered 6 peptide sequences with 6 to 24 amino acids (Table 2). Comparison of the six peptide sequences against a protein database (SwissProt) showed a high homology of PF 37 and PF 47 with different β-glucosidases from various sources (Table 3) while for the other peptides no significant homologies could be found. PF 37 showed the highest homology (81%) to linamarase from *Manihot esculenta* (Hughes et al., 1992) while PF 47 had a 79% identity to β-glucosidase from *Prunus avium* (Wiersma & Fils-Lycaon, 1995).

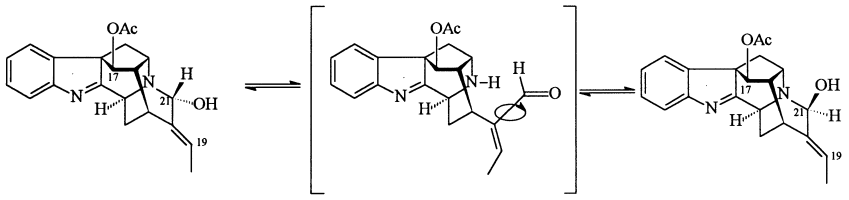

Fig. 4. Structure of 21(*R*)- and 21(*S*)-vomilenine and its equilibrium through the unstable open form of the aminal structure.

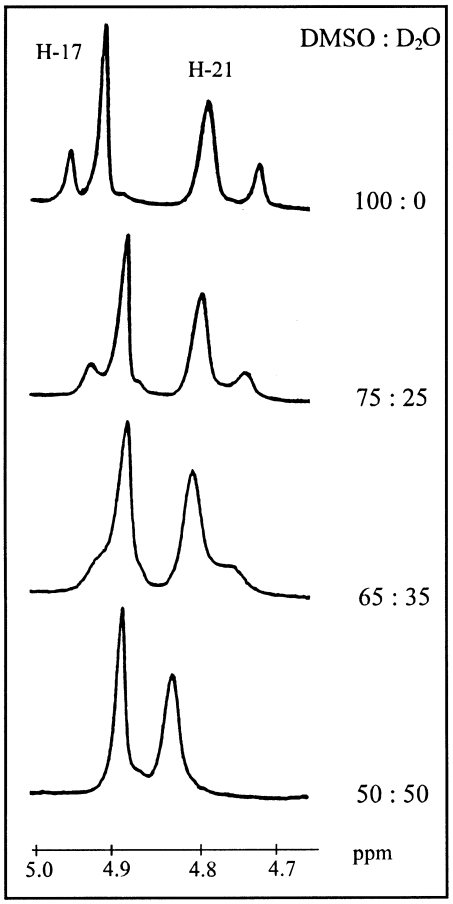

Fig. 5. ^{1}H NMR spectrum of enzymatically formed vomilenine from raucaffricine catalyzed by RG. Only part of the spectrum is shown representing H-17 and H-21 which illustrates the influence of increasing D_2O concentrations. Addition of D_2O leads to a fast equilibrium between 21(*R*)- and 21(*S*)-vomilenine.

3. Discussion

The monoterpenoid *Rauwolfia* alkaloid ajmaline (Gilurytmal®) and its synthetic derivative prajmalium (Neo-Gilurytmal®) are well established in the therapy of heart rhythm disorders. Because of their pronounced quinine related activity they belong to class Ia of antiarrhythmic drugs. Ajmaline (**5**) has a complicated hexacyclic structure possessing nine chiral carbon centers. For commercial use **5** is therefore not synthesized but still isolated from *Rauwolfia* roots. In order to understand the formation of this alkaloid in plant cells we have investigated its biosynthesis in plant cell suspension cultures at the enzyme level and have elucidated the major biochemical reactions of this multi-step pathway (Stöckigt, 1995).

Enzymatic and phytochemical analysis of *Rauwolfia* plants and cell suspension cultures showed greater activities of vomilenine UDP glucose transferase and RG when compared with the differentiated tissue (data not shown) and a much higher content of the glucoalkaloid **6** in the in vitro cell systems exceeding that of the differentiated *R. serpentina* plant by a factor of 67 (Ruyter, Akram, Ilahi, & Stöckigt, 1991). The results obtained up to the present point to the most important branchpoint in the ajmaline pathway involving the intermediate **4**, apart from a number of side routes delivering only minor alkaloid amounts. Both enzymes, the vomilenine glucosylating protein and the glucosidase hydrolyzing raucaffricine (vomilenine-β-D-glucoside), are therefore of extraordinary interest (Fig. 1). To get a more detailed insight into the properties, structure and mechanism of this outstanding glucosidase in the biosynthesis of *Rauwolfia* alkaloids we purified the enzyme, investigated the structure of the enzyme product vomilenine and made

Table 2
Sequences of 6 peptide fragments (PF) after raucaffricine glucosidase digestion with endoproteinase Lys C, separated by HPLC

Peptide fragment (PF)	Amino acid sequence
PF 14	Asn–Thr–Asn–Leu–Thr–Leu–Ser–Glu–Ala–Arg–Lys
PF 15	Ile–Leu–Val–Tyr–Thr–Lys
PF 16	Phe–Val–Gly–Ser–Arg–Leu–Pro–Lys
PF 17	Glu–Asp–Val–Asn–Ile–Leu–Lys
PF 37	Thr–Tyr–Asn–Val–Pro–Leu–Ile–Tyr–Val–Thr–Glu–Asn–Gly–Val–Asp–Asp–Ser–Lys
PF 47	Asn–Leu–Gly–Leu–Asp–Ala–Tyr–Arg–Phe–Ser–Ile–Ser–Trp–Ser–Arg–Val–Leu–Pro–Gly–Gly–Arg–Leu–Ser–Gly

Table 3
Sequence comparison of RG peptide fragments against SwissProt data base using BLAST

Enzymes	Sequences with homologies to PF 37	Identity (%)	Sequences with homologies to PF 47	Identity (%)
RG	**TYNVPLIYVTENGVDD**		**NLGLDAYRFSISWSRVLPGGRLSG**	
ME	403 *TYNDPVIYVTENGVDN* 418	81	76 *MGFNAFRMSISWSRVIPSGR* 95	65
PS	412 *YNDPLIYITENGVDE* 426	80	109 *MGFDAYRFSISWSRVLPNGKVSG* 131	78
PA	404 *YNDPIMYITENGMDE* 418	60	98 DM*GLDAYRFSISWSRLLPNGTLSG* 121	79
SA	417 *YNNPLIYITENGI* 429	76		
TR			95 DMN*LDAYRFSISWPRVLPKGKLSG* 118	75

Enzymes are: ME, linamarase from *Manihot esculenta* (U95298); PS, amygdalin hydrolase from *Prunus serotina* (U26025); PA, β-glucosidase from *Prunus avium* (U39228); SA, myrosinase from *Sinapis alba* (X59879); TR, β-glucosidase from *Trifolium repens* (X56733).Gene bank accession numbers are given in brackets, numbers indicating location of peptides in the enzymes. Identically amino acids are italic.

amino acid sequence alignments of peptide fragments obtained by hydrolyzing the glucosidase.

A relatively long purification protocol employing 5 steps permitted the enrichment of RG to near homogeneity. But in general from 1 kg of fresh *Rauwolfia* cell suspension cells only 10 μg RG remained, an inefficient procedure which did not deliver enough material to allow successful protein sequencing. The whole purification procedure could, however, be optimized with only 3 steps of column chromatography, including the efficient purification on Mono Q material, to yield enough enzyme (∼550 μg protein) but of lower purity. With the higher amount of enzyme several experiments could be performed, like the testing of the enzyme for sugar residues. The result that RG is most probably not glycosylated might be an important prerequisite for a heterologous expression of RG in procaryotic systems in future. Part of the enzyme from an enriched preparation was used to generate the aglycone **4** from **6** which was then analysed by MS and NMR spectroscopy. Our previous isolation of this alkaloid from cell suspensions has pointed to the occurrence of an isomer mixture (Stöckigt, Pfitzner, & Firl, 1981) which is in contrast to the vomilenine structure discussed in earlier literature (Taylor, Frey, & Hofmann, 1962). A careful and complete analysis of the ^{1}H NMR spectrum of **4** (the appropriate data only are shown) suggested two C-21 epimers. The major epimer exhibited the C-21 β-hydroxygroup (*S*-isomer) whereas the minor showed the C-21 α OH. Both (*S*)- and (*R*)-isomers occurred in a constant ratio of ∼3:1 when measured in dry DMSO-d_6 or pyridine-d_5. Since the naturally occurring alkaloids with ajmaline structure are all (*R*)-configurated at C-21, an equilibrium between both vomilenine isomers can be assumed, from which by enzymatic reduction of two double bonds exclusively (*R*)-vomilenine is removed leading finally to **5**. In fact, the existence of such an equilibrium became evident by dynamic NMR experiments. When water (D_2O) was added to the NMR sample the interconversion of both isomers became very fast because the signals corresponding to both C-21 isomers moved toward each other and finally overlapped. It therefore seems quite clear that the existence of the isomeric **4** mixture being in equilibrium has no real influence on ajmaline biosynthesis.

The comparison of the two longest peptide sequences resulted in the detection of surprisingly high sequence homologies (from 73 to 80%) to more than 10 plant β-glucosidases such as linamarase from Cassava (*Manihot esculenta* Crantz, (Hughes et al., 1992)), which is a major crop plant of tropical Africa or the well investigated amygdalin and prunasin hydrolases from black cherry (*Prunus serotina*, (Zheng & Poulton, 1995)) and dhurrinase from mustard (*Sinapis alba*, (Xue, Lenman, Falk, & Rask, 1992)). The amino acid motif Val–Thr–Glu–Asn–Gly in PF 37 is remarkably similar to conserved sequence motifs in β-glucosidases (Withers et al., 1990; Trimbur, Warren, & Withers, 1993). In some cases the glutamic acid in this motif was identified as the active site nucleophil, e.g. in β-glucosidase from *Agrobacterium faecalis* (Trimbur, Warren, & Withers, 1992) or sweet almond (He & Withers, 1997).

From these data, in addition to the catalyzed reaction (chemical identification of the conversion of **6** to **4**), it became evident that the here isolated and purified enzyme was indeed a glucosidase. Moreover its relation to other glucosidases is obvious although the substrate specificity of RG which has been published earlier (Schübel et al., 1986) is completely different to the above mentioned hydrolases. RG is exclusively active towards glucosides derived from **6** like 1.2-dihydro- and 1.2.19.20-tetrahydroraucaffricine (Schübel et al., 1986). Obviously a glucosidic ajmalan-type skeleton is a prerequisite for a glucoside being hydrolyzed by this specific glucosidase. In fact, 15 other glucosides including the alkaloidal glucosides strictosidine, strictosidine lactam, monoterpenoid glucosides or artificial glucosides used frequently for glucosidase purification (nitrophenyl glucosides) were not accepted by RG (Schübel et al., 1986). The appropriate binding site at the enzyme is, however, not yet known.

The obtained data so far suggest that RG is a new member of the family 1 of glycosyl hydrolases for which

the substrate specificity for β-glucosides and the above mentioned amino acid motif found in PF 37 are characteristic.

On the other hand such sequence information might be very useful in the future search for the corresponding gene in *Rauwolfia* plant cells and in obtaining enough enzyme by heterologous expression for a much more detailed analysis of this protein.

In addition it will be of future interest to further investigate not only the molecular structure of RG but also to extent our understanding of the biochemical and physiological function of the soluble RG especially in connection with the membrane bound vomilenine UDP-glucose transferase (Ruyter & Stöckigt, 1991) catalyzing the 'reverse' reaction of the glucosidase. Moreover it would be most interesting to find out whether RG can exert direct influence on the biosynthesis of the target compound ajmaline, e.g. by channelling the aglycone **4** into the biosynthetic pathway or to block the enzyme or the corresponding gene and to analyze the remaining biosynthetic potency of the cells to perform alkaloid biosynthesis (Fig. 1). Appropriate work is now in progress.

4. Experimental

4.1. Cell material

Cell material used in this report was obtained from cell suspension cultures of *R. serpentina* grown in 1 l Erlenmeyer flasks in Linsmair and Skoog medium (Linsmaier & Skoog, 1965) for 7 days under the following conditions; $24 \pm 2^{\circ}C$, under continuous light (600 lx) and shaking (100 rpm). For optimization of enzyme isolation after the transfer of *Rauwolfia* cells to new nutrition medium, the RG activity and the protein content were measured every day for 2 weeks.

4.2. Enzyme assay for RG activity

20 nmol **6** (solved in 10 μl EtOH) and appropriate amounts of enzyme solution (RG with an activity of 1–5 pkat) were incubated in a total of 0.1 ml of citrate/NaOH buffer (0.1 M, pH 5.0) for 30 min at 30°C under shaking (100 rpm). The enzyme reaction was terminated by addition of 0.2 ml MeOH. After centrifugation at $18000 \times g$ for 5 min the supernatant was quantitatively analyzed by HPLC, using a Lichrospher RP-select B column (125×4 mm) equipped with a 4×4 mm pre-column of the same type (Merck, Darmstadt); solvent system was (a) CH_3CN and (b) KPi buffer (20 mM, pH 4.0) with a gradient of 25% (a) at 0 min, 35% (a) at 3.0 min, 80% (a) at 3.5–4.5 min and 25% (a) from 5–7 min, flow rate was 1.5 ml/min and detection was at 258 nm. The substrate **6** ($R_t = 1$ min 44 s) and the enzyme product **4** ($R_t = 2$ min 41 s) were completely separated and quantified using **6** as an external standard. The used **6** was isolated from cell suspension cultures of *R. serpentina* according to a published procedure (Schübel & Stöckigt, 1984).

4.3. Protein determination

Protein concentrations were determined as described by Bradford (1976) using Coomassie solution.

4.4. SDS-Polyacrylamide gel electrophoresis-protein staining

SDS gels were stained with Coomassie solution (0.25% Coomasie Brilliant Blue R-250, 45% MeOH and 9% HOAc in water); destaining of backgrounds were performed with a solution of 5% MeOH and 7.5% HOAc in water. Silver staining was performed as published previously (Heukeshoven & Dernick, 1985). Enzyme enrichment during the purification of the glucosidase was followed by SDS-PAGE under denaturing conditions using 10% acrylamide. Marker proteins used were the LMW marker mixture (Pharmacia).

4.5. Buffers

Buffer A: 0.1 M Tris–HCl, pH 7.5, 1 mM EDTA, 20 mM β-mercaptoethanol (EtSH). Buffer B: as for A but 20 mM Tris–HCl and 10 mM EtSH. Buffer C: as for B but 20 mM KPi. Buffer D: 20 mM Tris–HCl, pH 7.0, 10 mM EtSH. Buffer E: 20 mM Tris–HCl, pH 8.0, 0.1 M KCl, 10 mM EtSH. Buffer F: as for buffer D, but pH was 7.5. Buffer G: 25 mM Bis–Tris set to pH 7.1 with saturated iminodiacetic acid, 10 mM EtSH. Buffer H: 10% polybuffer 74 (Pharmacia) at pH 4.0 adjusted with satd. iminodiacetic acid, 10 mM EtSH. Buffer I: 0.1 M Tris–HCl, pH 7.5, 20 mM EtSH. Buffer K: 20 mM Tris–HCl, pH 8.0, 10 mM EtSH. Buffer L: 39 mM glycine, 48 mM Tris–HCl, 0.0375% SDS, 20% MeOH. Buffer M: 10 mM CAPS, pH 11, 10% MeOH.

4.6. MS and GC–MS

A Finnigan MAT 44S quadrupole instrument was used. Direct inlet mass spectra were obtained under EI-conditions (70 eV) at an ion source temperature of 200°C and a temperature gradient of 50°C/min. GC–MS analyses were performed with the same instrument coupled to a Varian Gaschromatograph type 3700, which was equipped with a $30\ m \times 0.32$ mm Durabond DB1 column. GC–MS analyses for identification of **4** were carried out with a gas flow of 10 ml/min under isocratic conditions (320°C).

4.7. NMR measurements

NMR spectra were recorded on Bruker 360 MHz (360 AM) and 400 MHz (400 ARX) instruments using Bruker standard software and 5 mm NMR tubes with DMSO-d_6; for dynamic NMR spectroscopy mixtures of DMSO-d_6 and D_2O were used.

4.8. Purification of RG

All purification steps were carried out at 4°C.

4.9. Preparation of crude protein extracts

Cells of *R. serpentina* cell suspensions (1.0 kg, fr. wt) were frozen with liquid nitrogen, added to 1300 ml buffer A and the mixture was stirred at 35°C until thawed. The mixture was then homogenized in an ultraturrax for 2 min and filtered through cheese cloth. The resultant solution was centrifuged at $10{,}000 \times g$ for 30 min and the supernatant (1.8 l) used for the next purification step.

4.10. Fractional precipitation with $(NH_4)_2SO_4$

The protein in the resulting supernatant was subjected to $(NH_4)_2SO_4$ precipitation. The precipitated protein between 30–75% saturation was collected after centrifugation (30 min, $10{,}000 \times g$), solubilized in 150 ml buffer B, centrifuged and the supernatant dialyzed overnight against 10 l of buffer B. Centrifugation (20 min, $10{,}000 \times g$) yielded the soluble protein fraction ($\sim$200 ml, 4.2 mg/ml).

4.11. Anion exchange chromatography on DEAE-Sephacel

The protein solution so obtained was added at a rate of 1.5 ml/min to a DEAE-Sephacel column (7.8×5 cm, XK 50/20-column, Pharmacia) which was pre-equilibrated with buffer B. After washing the column with buffer B for 200 min the enzyme activity was fractionated (10 ml per fraction) with a linear KCl gradient (0–0.4 M) prepared from buffer B and KCl and applied with the rate of 1.5 ml/min. Enzyme activity appeared at around 0.3 M KCl. Fractions containing glucosidase activity >1.3 nkat/mg were combined and dialyzed overnight against 5 l buffer C.

4.12. Biogel HT-hydroxylapatite chromatography

The remaining protein solution from the anion exchange chromatography was added to the top of a Biogel HT-column (1.6×15 cm) with a flow rate of 0.5 ml/min. After washing the column for 50 min with buffer D (0.5 ml/min) proteins were eluted with a gradient of KPi (buffer D and 20–500 mM KPi) within 200 min. Fractions of 2.5 ml were collected and assayed for glucosidase activity. Fractions containing >3.5 nkat/mg enzyme were combined (35 ml) and conc. to 5 ml by ultra-filtration (membrane pore size <20 kDa, 0.1–0.2 MPa).

4.13. Gel filtration on Ultrogel AcA 54

For gel filtration the above enzyme solution was chromatographed on a 2×90 cm column containing the AcA 54 material (Pharmacia), which had been equilibrated earlier with buffer E. The same buffer was used for the fractionation of the protein solution (0.33 ml/min, 3 ml/fraction). Fractions exhibiting glucosidase activity of >47 nkat/mg were combined (10 ml total) and conc. to 0.5 ml by filtration through *Centrisat I* filters (20–30 min, $2000 \times g$, pore size <20 kDa).

4.14. Mono Q HR 5/5 ion exchange chromatography

The Mono Q column (0.5×5 cm) was equilibrated with buffer F and the above mentioned enzyme solution added to the column. For elution of the enzyme a KCl gradient (prepared by mixing buffer F and buffer G containing 1 M KCl) was applied (0.5 ml/min): 0–5 ml buffer F:G = 100:0; 0.32 ml buffer F:G = 50:50, 35 ml buffer F:G = 25:75, 36 ml buffer F:G = 100:0. Fractions of 0.75 ml were collected and were combined with specific glucosidase activities of >220 nkat/mg. This enzyme solution was concentrated to 0.25 ml and equilibrated with the starting buffer of the next step (buffer G).

4.15. FPLC-chromatofocusing with Mono P column

The protein solution obtained by chromatography on Mono Q was added to the Mono P HR 515 column. Proteins were fractionated (0.5 ml) with 20 ml buffer H at a flow rate of 0.25 ml/min generating a pH gradient from pH 7.1 to 4.0. The enzyme was eluted after 10 ml of buffer H.

4.16. Short procedure for RG purification

A short procedure for the enrichment of RG in higher concentration for proteinase-catalyzed sequencing included the following steps. From 1 kg frozen *R. serpentina* cells stirred for ca. 60 min in buffer I a crude protein solution of 1.8 l (0.8 mg protein/ml) was obtained with a specific enzyme activity of 0.4 nkat/mg. $(NH_4)_2SO_4$ precipitation (30–75%) and solubilisation of the ppt. in 180 ml buffer F gave a protein concentration of 5.6 mg/ml. After dialysis against 10 l buffer F the final solution of 250 ml contained 4.0 mg/ml protein with a specific activity of 0.49 nkat/mg.

This protein solution was added to a 15.3×5 cm column of DEAE sepharose (fast flow). After washing the column with 900 ml buffer B, proteins were fractionated with a linear KCl gradient (0–0.5 M, 1.5 ml/min). Glu-

cosidase activity appeared between 0.2–0.3 M KCl. The combined active fractions (190 ml) contained 200 mg protein with a specific activity of 1.72 nkat/mg (4.3-fold enrichment).

This solution was concentrated to 6 ml by ultrafiltration (cellulose triacetate membrane <20 kDa, 0.1–0.2 MPa) and was then applied to a TSK 55 S size exclusion chromatography column (2.5×47 cm). Protein was eluted with buffer K at a flow rate of 15 ml/h. Fractions between 95–115 ml were combined and yielded 7.6 mg protein with a specific activity of 18.3 nkat/mg (46-fold enrichment).

After concentration of this solution by centriprep (Amicon/Witten) it was added to a Mono Q column which was eluted as described above. Maximum enzyme activity appeared at 0.28 M KCl. Fractions from 23 to 25.5 ml, showing highest enzyme activity, were combined, resulting in 2.5 ml enzyme solution (0.55 mg glucosidase exhibiting a specific activity of 136 nkat/mg). The total yield of enzyme isolated was 5.2%.

4.17. Basic-native-electrophoresis of the enriched RG

Polyacrylamide gels (*Clean Gel*, Pharmacia) were treated according to the manufacturers instructions. Electrophoresis was carried out under the following conditions: for 10 min continuous 300 V (9 mA) reaching after 60 min 900 V (25 mA). The gel was cut into several bands; one was used for Coomassie staining, one for cutting into small samples which were eluted and concentrated (Microcon 10, Micropur 22; gel nebulizer, Amicon) and some gel strips were used directly for enzyme assay revealing the glucosidase activity. The protein band corresponding to ca. 61 kDa showed RG activity.

4.18. Protein blotting

SDS-gels were blotted by the 'semi dry' blotting procedure (Kyhse-Andersen, 1984) for 60 min at 20 V. For nitrocellulose membrane blotting the transfer buffer L was applied and for blotting on PVDF membranes buffer M was used. The PVDF membranes had been washed first for 1 min with MeOH and then equilibrated with buffer M for 20 min.

4.19. Investigation of RG for glycosylation

A fraction of $\sim$300-fold enriched RG from Mono Q chromatography was separated by SDS-PAGE. The gel was then blotted to a nitrocellulose membrane, which was processed as described in the Dig Glycan Detection Kit (Boehringer Mannheim) for the detection of glycosylated proteins.

4.20. Formation of the enzyme product vomilenine

2 mg (3.9 μmol) of **6** were dissolved in 0.5 ml of 100 mM KPi buffer (pH 8.0). Purified RG (0.01 ml) was added and the mixture was incubated at 37°C for 2 h. The mixture was freeze dried and 1 ml KPi buffer and 1 ml CH_2Cl_2 were added and mixed. The organic solvent was separated and after repeating the procedure and combining the CH_2Cl_2 fractions the organic layer was dried, resulting in 0.9 mg (2.57 μmol, yield 66%) vomilenine.

4.21. Sequencing of RG and peptide sequence alignment

An enriched protein fraction from step three of the short enzyme purification protocol was separated on SDS and blotted on a PVDF membrane by the semi dry blotting method. Using the Edman degradation several attempts to sequence RG were unsuccessful.

The enzyme, therefore, was digested with the endoproteinase Lys C directly in the gel. After separation and fractionation of formed peptide fragments by HPLC on a Supersphere 60 RP select B column (Merck, Darmstadt), the peptides were sequenced.

Two of the obtained peptide sequences, named peptide 37 and peptide 47, of RG were used for screening the SwissProt protein sequences library by the sequence analysis program BLAST.

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