56. Partial Purification and Characterization of Raucaffricine β-D-Glucosidase from Plant Cell-Suspension Cultures of *Rauwolfia serpentina* BENTH.

by **Helmut Schübel** and **Joachim Stöckigt***

Institut für Pharmazeutische Biologie der Universität München, Karlstr. 29, D-8000 München

and **Richard Feicht** and **Helmut Simon**

Lehrstuhl für Organische Chemie und Biochemie der Technischen Universität München, Lichtenbergstr. 4, D-8046 Garching

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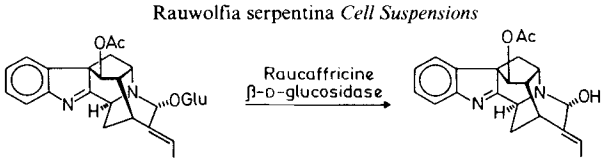
A novel highly substrate-specific *Rauwolfia* enzyme, raucaffricine β-D-glucosidase, was isolated from cell-suspension cultures of *R. serpentina*. The enzyme has been purified *ca.* 1350-fold, its major characteristics such as $M_r = 66600 \pm 5\%$, pH optimum 5.1, temperature optimum 38°, and inhibition of its activity by glucose and fructose were investigated. Its limited distribution in different cell cultures and differentiated plants indicates that the enzyme is present in significant amounts exclusively in cultured *Rauwolfia* cells.

Introduction. – The structure of the rare *Rauwolfia* glyco-alkaloid raucaffricine (**1**) has been recently revised to the β-D-glucoside of vomilenine (**2**) [1] instead of the earlier proposed α-D-galactoside structure [2] [3]. This revision was based on spectroscopic evidence, mainly on 2D-NMR, as well as on enzymatic experiments with a crude protein mixture isolated from *Rauwolfia serpentina* BENTH. cell suspension cultures. This cell-free extract efficiently hydrolysed raucaffricine into vomilenine and glucose pointing to the presence of a new *Rauwolfia* enzyme named here raucaffricine β-D-glucosidase (EC 3.2.1.?). The aglycone of raucaffricine, vomilenine, has been proved to occupy a central position in the biosynthetic pathway to the therapeutically useful ajmaline [4]. On one hand, vomilenine (**2**) is an important substrate of the 'late' enzymes of the ajmaline route – vomilenine reductase(s) or acetylajmalan esterase, proteins which still have to be purified and characterised. On the other hand, **2** is a highly reactive branchpoint intermediate offering a biomimetic approach to the synthesis of a variety of new sarpagine/ajmaline-type compounds. Therefore, we isolated and characterized raucaffricine β-D-glucosidase, which is necessary for the formation of vomilenine (**2**) from raucaffricine (**1**) under mild conditions using cell-suspension cultures of *R. serpentina*. In this paper, we report the partial purification and some properties of this novel plant enzyme.

Scheme. *Formation of Vomilenine* (**2**) *from Raucaffricine* (**1**), *Catalysed by Raucaffricine* β-D-*Glucosidase from* Rauwolfia serpentina *Cell Suspensions*



1 Raucaffricine **2** Vomilenine

Results and Discussion. – The enzyme was isolated and purified in the following manner. Cell material grown under conditions as previously reported [1] was used as a source for the isolation of raucaffricine β-D-glucosidase. From 100 g of tissue, a crude enzyme extract was obtained by a usual workup procedure for *Rauwolfia* enzymes [5], showing a specific activity of 275 pkat/mg protein (9.2 nkat total activity). For quantification of raucaffricine glucosidase, a simple assay was applied, based on the determination of liberated glucose by a glucose oxidase test system. This assay allowed us to monitor the individual purification steps during the enrichment of the enzyme. The physical characteristics of the glucosidase as well as its activity in different cell-material and enzyme-inhi-

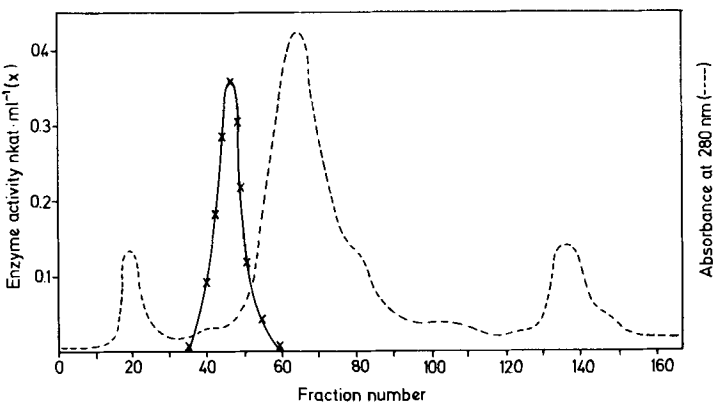


Fig. 1. *Elution profile of raucaffricine β-D-glucosidase on a* TSK 55-S *column* (gel chromatography). Proteins were eluted with potassium phosphate (KPi) buffer (0.1M). Fractions of 3 ml were collected and assayed for protein (– – –) and glucosidase (–×–×–).

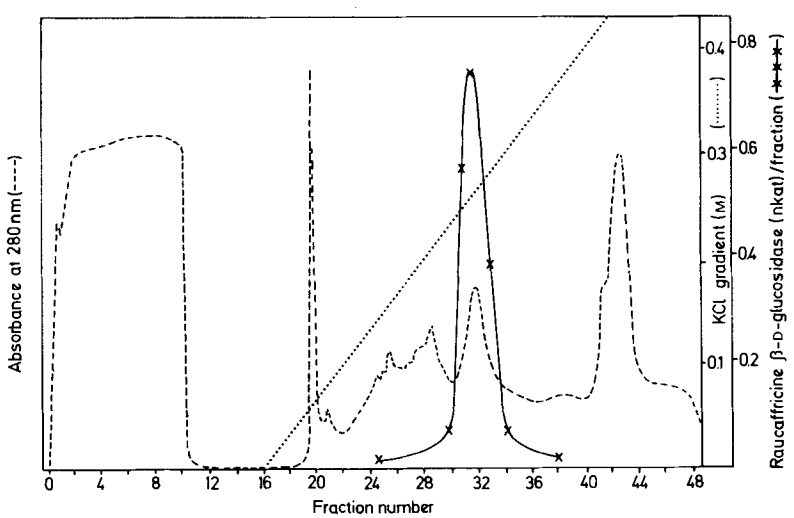


Fig. 2. *Enrichment of prepurified raucaffricine β-D-glucosidase* (from *TSK 55-S* column) *by ion-exchange chromatography* (FPLC, *Mono-Q* column) *and a KCl gradient* (· · · · ·). Fractions of 1 ml were collected and analysed for the protein content (– – –) and enzyme activity (–×–×–).

Table 1. *Purification Steps of Raucaffricine β-D-Glucosidase from 0.1 kg of Fresh* R. serpentina *Cells*

Purification step	Total volume [ml]	Total protein [mg]	Total activity [nkat]	Specific activity [nkat/mg]	Yield [%]	Purification
Crude extract chromatographed on						
1. *Sephadex G-25*	8	33.6	9.24	0.275	100	1.0-fold
2. Gel chromatography on *TSK 55-S*	30	2.1	8.40	4.0	90.9	14.8-fold
3. FPLC on *Mono Q*	12	0.0192	7.20	375	77.9	1364-fold

bition studies were determined by HPLC analysis measuring the decrease of raucaffricine and/or the increase of aglycone concentration. After removing endogenous glucose by gel chromatography *(Sephadex G-25)*, the crude protein mixture was separated by gel chromatography on a *TSK 55-S* column which resulted in a *ca.* 15-fold enrichment of the enzyme. This procedure turned out to be highly reproducible and gave a noteworthy recovery of enzyme activity (> 90%). As depicted in *Fig. 1*, the enzyme is separated from most of the protein which was determined spectrophotometrically at 280 nm. As the second purification step, 'fast protein liquid chromatography' (FPLC) was applied using a basic ion exchanger *('Mono Q')*. The glucosidase was eluted by a salt gradient at 275 mM KCl as a symmetrical peak *(Fig. 2)*. Under these conditions, a significant purification (*ca.* 90-fold) of the enzyme was achieved corresponding to a *ca.* 1350-fold total enrichment in comparison with the original crude raucaffricine-glucosidase-containing protein extract. This is the highest value which we have so far obtained with *Rauwolfia* enzymes. Moreover, the yield of active enzyme again was exceptional, because only 22% of the total activity was lost during the whole protein purification *(Tab. 1)*.

In contrast to HPLC (*TSK G-3000 SW* column) as the second purification step, where 78% of the enzyme activity was lost and only a 3.1-fold enrichment could be achieved, the

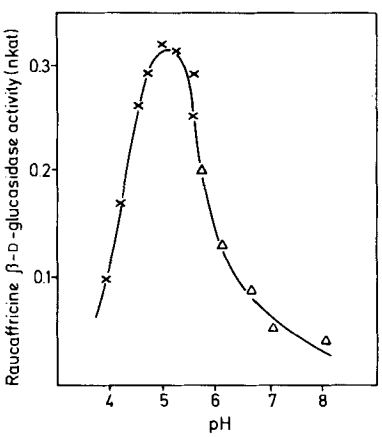


Fig. 3. *Dependence of the catalytic activity of raucaffricine β-D-glucosidase on pH*. The assay was performed in presence of 0.1M KPi/citrate buffer (–×–×–) and 0.1M KPi buffer (–△–△–).

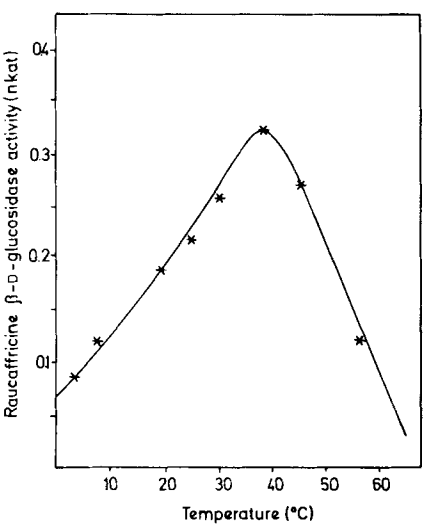


Fig. 4. *The effect of temperature on the reaction rate of raucaffricine β-D-glucosidase*

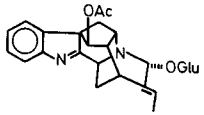
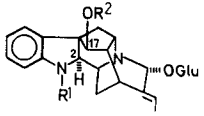
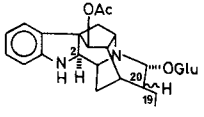
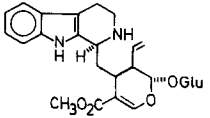
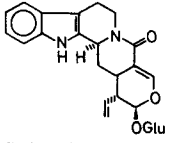
FPLC procedure is clearly more efficient in this particular case, concerning both enzyme recovery and purification. However, it should be pointed out that the unusually good results of this partial enzyme purification were due to the remarkable stability of the glucosidase, which is well documented by the fact that a crude enzyme extract filtered on *Sephadex G-25* (without 2-mercaptoethanol) could be stored at 4° for 6 months losing only 30% of its activity. In addition, such an enzyme mixture can also be freeze-dried, retaining 75% of its original catalytic activity. This feature of raucaffricine glucosidase offers the possibility of handling the crude enzyme like a 'chemical reagent' for the production of the labile aglycone vomilenine (**2**), at least on a laboratory scale of several grams.

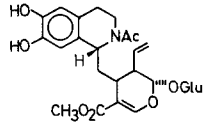
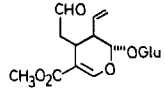
The major properties of the raucaffricine-hydrolysing enzyme were determined at the stage of the 14.8-fold purified preparation. As outlined in *Fig. 3*, the glucosidase exhibits a broad pH tolerance from pH 4–7 with half maximal activity at pH 4.2 and 6.0. The optimum was found to be at pH 5.1, a value which is typical for many β-glucosidases. The temperature optimum *(Fig. 4)* was found to be at 38°, and 50% of the enzyme activity was still measured at 55 °C after 30 min of incubation. The isoelectric point (IP) of the glucosidase corresponds to a pH of 5.8 ± 0.2. This value matches those determined for other enzymes of the biosynthesis of ajmaline/sarpagine alkaloids (*e.g.* polyneuridine-aldehyde-esterase IP 5.0, vinorine synthase IP 4.4, vellosimine reductase IP 5.0). In most cases [6], IP data were found for plant glucosidases in the same range, although much higher values also were reported, *e.g.* for coniferin-hydrolysing enzymes [7] [8]. The relative molecular weight of the enzyme described here was measured by gel chromatography using a calibrated *AcA-54* low-pressure column and by HPLC with a *Fractogel-TSK G-3000-SW* column. Assuming a globular shape of the enzyme molecule, we found a relative mol.wt. (M_r) of 66600 ± 5% applying *AcA-54* chromatography and 65100 using HPLC.

From the biosynthetic point of view, one of the most interesting characteristics of a new enzyme is its acceptance of different substrates. We, therefore, investigated in detail the substrate specificity of the raucaffricine glucosidase. Glucosidases usually were believed to be enzymes of low substrate specifity. During the last ten years, however, an increasing number of relatively highly substrate-specific glucosidases from plant cells have been described. Prominent examples of these enzymes are *e.g.* coniferin β-D-glucosidase from *Picea abies* [7] or from *Cicer arietinum* [8], enzymes which hydrolyse coumarin glucosides [9] or flavonoid glucosides [6]. Strictosidine β-D-glucosidases especially are extraordinarily selective regarding glucosidic substrates. Strictosidine, the immediate precursor of about 1500 monoterpenoid indole alkaloids, was the only natural substrate accepted by the enzymes when 8 different glucosides of several types were tested [10].

It, therefore, seemed to be interesting to clarify the substrate specificity of the raucaffricine glucosidase described here. As depicted in *Table 2*, 15 glycosidic compounds were investigated for their role as putative enzyme substrates. These compounds included complex raucaffricine derivatives, glucosides of monoterpenoid origin (secologanin), simple natural glucosides (amygdalin, sinigrin), disaccharides, and a typical unnatural substrate often used for monitoring glucosidase activities (nitrophenyl glucoside). Besides raucaffricine, only its four derivatives **3**–**6** were hydrolysed, but all of them are new synthetic alkaloids which have so far not been isolated from natural sources. Comparison of the data of *Table 2* shows raucaffricine (**1**) to be the preferred substrate (K_m 1.4 mM, V_{max} 560 pkat) and that the hydrolysis of the remaining ajmalan glucosides is catalysed

Table 2. *Substrate Specificity of Raucaffricine β-D-Glucosidase. Enzyme Assay a* (s. *Exper. Part*), spec. activity 0.6 nkat/mg protein.

Glycoside	Rel. activity [%]	K_m [mM]	V_{max} [pkat]
			
Raucaffricine (**1**)	100	1.4	560
			
1,2-Dihydroraucaffricine (**3**; R^1 = H, R^2 = Ac)	98	1.5	550
17-*O*-Deacetyl-1,2-dihydroraucaffricine (**4**; $R^1 = R^2$ = H)	95	1.7	530
1,2-Dihydro-1-methylraucaffricine (**5**; $R^1 = CH_3$, R^2 = Ac)	36	1.4	200
			
1,2,19,20-Tetrahydroraucaffricine (**6**)	87	2.0	490
			
Strictosidine	0	–	–
			
Strictosidine lactam	0	–	–

Glycoside	Rel. activity [%]	K_m [mM]	V_{max} [pkat]
			
Ipecoside	0	–	–
			
Secologanin	0	–	–
Amygdalin	0	–	–
Sinigrin	0	–	–
Vanillin-β-D-glucoside	0	–	–
Sucrose	0	–	–
Lactose	0	–	–
p-Nitrophenyl β-D-glucoside	0	–	–

with similar efficiency, except 1,2-dihydro-1-methylraucaffricine (**5**; K_m 1.4 mM, V_{max} 200 pkat). All these synthesised derivatives of the ajmalan group have the unnatural (2*R*)-configuration. This configuration is only known (for ajmalans) from related plant alkaloids of the quebrachidine group bearing a methoxycarbonyl group at C(16). The ajmalan skeleton seems to be recognized *in toto* by the active site of the enzyme. Raucaffricine glucosidase, therefore, provides the second example of a highly selective plant glucosidase for which only one natural substrate has been detected. The relatively broad substrate acceptance *within* the group of synthetic ajmalan glucosides is interesting from the synthetical point of view, because simple chemical transformations at the raucaffricine stage followed by enzyme-catalyzed hydrolysis would lead to several aglycones which we expect to be useful for further enzymatic studies and especially for biomimetic syntheses.

Not only this remarkable substrate specificity of raucaffricine β-D-glucosidase points to its exclusive action in the transformation of raucaffricine, but also the occurrence of

Table 3. *Distribution of Raucaffricine β-D-Glucosidase in Cell-suspension Cultures of* Rauwolfia *Species and Different Genera of the* Apocynaceae

Species	pkat Raucaffricine glucosidase/ mg protein	Species	pkat Raucaffricine glucosidase/ mg protein
Rauwolfia serpentina	538	*Rhazya stricta*	0
Rauwolfia caffra	253	*Uncaria gambir*	0
Rauwolfia verticillata	136	*Voacanga africana*	0
Rauwolfia mannii	52	*Tabernanthe iboga*	0
Rauwolfia verticillata (leaves)	< 0.1	*Tabernaemontana divaricata*	0
Rauwolfia verticillata (roots)	< 0.1	*Ochrosia elliptica*	0
Catharanthus roseus	0	*Catharanthus roseus* (leaves)	0

the enzyme in different cell-suspension cultures points to its biogenetic significance. *Table 3* clearly leads to the conclusion that the enzyme is present exclusively in different species of the genus *Rauwolfia*, although the specific enzyme activity ranges between 538 and 52 pkat/mg protein for *R. serpentina* and *R. mannii*. Conversely, leaves or roots of *R. verticillata* contain only traces of the enzyme. However, at this point it must be realized that raucaffricine has not been isolated from the latter plant species. Analysis of *Rauwolfia* cultures as well as of intact plants for the content of raucaffricine or for the enzyme discussed here indicate that the occurrence of the glucosidase is clearly correlated with the presence of the gluco-alkaloid. Cultures or plants which are not able to synthesize raucaffricine obviously do not produce any of the appropriate glucosidase. The same correlation of plant species, alkaloid production, and enzyme synthesis have been found for all the presently characterized *Rauwolfia* enzymes.

Up to now, all our attempts to find evidence for the reverse reaction, the glucosylation of the aglycone vomilenine (**2**), have failed. Under cell-free conditions, even with *Rauwolfia* enzyme preparations free of the highly active glucosidase, raucaffricine formation from vomilenine could not be detected. Assuming that the glucosylation reaction is carried out by a different enzyme, and to obtain more insight into the catalysed reaction, we examined a variety of compounds for their potency to inhibit the glucosidase activity. One of the well known glucosidase inhibitors is δ-gluconolactone [11]. Surprisingly,

raucaffricine glucosidase is not inhibited by this lactone in a wide range of concentrations (0 to 1M). The catalytic activity of the enzyme was also not influenced by a typical hydrolase inhibitor, phenylmethylsulfonyl fluoride (0 to 80 mM), nor was the enzyme reaction inhibited by EDTA (0 to 80 mM) nor in the presence of up to 80 mM iodoacetamide. The results suggest that divalent metal ions or SH groups most probably do not play an essential role for the enzyme activity. Moreover, the latter point is consistent with the high stability of the glucosidase in the absence of SH-protecting compounds, *e.g.* 2-mercaptoethanol, as described above.

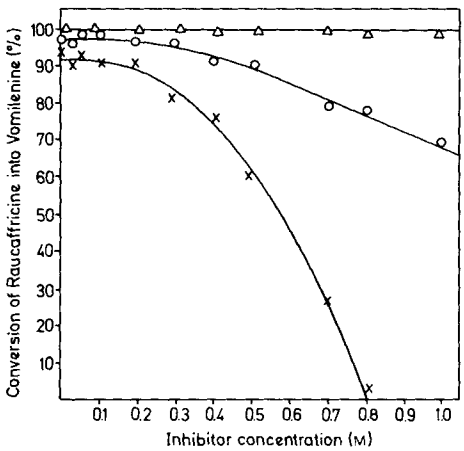


Fig. 5. *Inhibition of raucaffricine β-D-glucosidase by glucose* (–x–x–) *and fructose* (–○–○–). Effect of mannitol: –△–△–.

Similarly, strictosidine or amygdalin, which are not hydrolysed by the enzyme, have no influence on its catalytic activity in the 0 to 50 mM range and are obviously not bound to the active site of the protein. As expected, the reaction can be suppressed in the presence of the end-product glucose. *Fig. 5* demonstrates that complete inhibition is reached with 0.8M glucose. A similar, but less effective influence could be observed when increasing amounts of fructose were included in the test system (30% inhibition with 0.95M fructose). The possibility of an isomerisation of fructose into glucose under these conditions could be excluded. By employing the glucose-oxidase assay, no glucose could be found. However, the sugar alcohol mannitol had no effect when added in concentrations up to 1M indicating that inhibition of hydrolysis observed in the presence of large amounts of glucose was not due to the high viscosity of the incubation mixture. On the basis of these experiments, investigations are now under way to search for the transferase in crude enzyme preparations containing raucaffricine β-D-glucosidase, utilizing vomilenine and an activated glucose as substrates.

In summary, a novel, highly specific glucosidase acting on glucosidic ajmaline congeners has been detected in cell suspensions of *Rauwolfia serpentina*. The extraordinary stability of the enzyme allowed an efficient and short purification procedure. The formation of 1.2 g of raucaffricine (**1**) per l medium [12] is presently the sixth highest value reported for a single secondary metabolite synthesized in plant cell cultures. A sufficient amount of the enzyme and its substrate provides a simple method for the prepara-

tion of the aglycone vomilenine (**2**) and appropriate derivatives. Vomilenine is a close biogenetic precursor of ajmaline. Because of its antiarrhythmic activity, the latter alkaloid has an application in the treatment of heart disorders [13] and is, therefore, of pharmaceutical interest.

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Experimental Part

General. Cell-suspension cultures of *Rauwolfia serpentina* were grown under standard conditions in 1-l *Erlenmeyer* flasks on a gyratory shaker at 100 rpm and 25° in an alkaloid-production medium [14]. After a growth period of 20 days, the cell material was filtered off (360 g of fresh weight/l medium), frozen with liq. N_2, and stored at −25°. Plants were grown under normal greenhouse conditions. TLC: *Polygram Sil G/UV*$_{254}$ plates (*Macherey* and *Nagel*). ^{1}H-NMR: *Bruker AM 200* instrument. MS: *Finnigan MAT 44 S* instrument in EI mode (70 eV).

*Isolation and Partial Purification of Raucaffricine β-*D*-Glucosidase.* In a typical procedure, 100 g of frozen *R. serpentina* cells were stirred at 0° with 200 ml of potassium phosphate (KPi) buffer (0.1M, pH 7.0, 10 mM 2-mercaptoethanol) for 60 min. The obtained slurry was filtered through cheesecloth and the filtrate centrifuged at 20000 × g for 20 min. During 40 min, $(NH_4)_2SO_4$ was added to the supernatant reaching a final salt concentration of 70%. After centrifugation for 20 min at 20000 × g, the precipitated protein was dissolved in 5 ml of 0.1M KPi buffer (pH 7.0) and filtered through *Sephadex G-25* (column 2.8 × 30 cm, flow rate 5 ml/min). After centrifugation at 10000 × g for 10 min, the supernatant was chromatographed on a *TSK-55-S* column (gel chromatography) by the following procedure: 8 ml of the crude protein mixture (33.6 mg of protein, 275 pkat/mg) were applied to the column (2.6 × 85 cm) and eluted with 0.1M KPi buffer at a rate of 20 ml/h collecting 3-ml fractions. The enzyme was found to be in *Fractions 40–50,* which were pooled. Of the total amount of glucosidase, 90.9% were recovered showing a specific activity of 4.0 nkat/mg (14.8-fold enrichment of enzyme activity). This prepurified enzyme was applied in three portions (10 ml each with 0.7 mg of protein) to a 'fast protein liquid chromatography' (FPLC; instrument from *Pharmacia,* Freiburg) on a basic ion-exchanger column, type *Mono Q HR 515.* The enzyme was eluted with *Tris*-HCl buffer (pH 7.5, 20 mM) and a KCl gradient (0 to 0.5M KCl) at a flow rate of 2.0 ml/min (48 fractions, 1.0 ml each). The glucosidase-containing *Fractions 31–34* were combined. This step enriched the enzyme activity 93-fold, and 85.7% of the enzyme activity was recovered with a spec. activity of 375 nkat/mg. The total purification yielded 77.9% of the raucaffricine glucosidase, enriched by a factor of 1364.

HPLC Purification of the Enzyme. A *Spectra Physics* instrument (*SP 8440* UV/VIS detector, *SP 4100* computing integrator, *SP 8700* solvent delivery system, and *ASI 150* automatic sample injector) was used in combination with an *ULTRA Pac TSK-G-3000 SW* column (0.75 × 60 cm). To the column, 0.2 ml (0.84 mg of protein, 2.7 nkat/mg) of an *AcA 54* eluate were applied, and the enzyme was eluted with 0.1M KPi buffer/0.1M NaCl (*v/v,* pH 6.0) at a flow rate of 0.2 ml/min and a pressure of 12 bar. Fractions of 0.9 ml were collected and the enzyme activity was observed in *Fractions 18–22,* which after combining yielded 0.06 mg of enzyme (spec. activity 8.3 nkat/mg) corresponding to 22% of the applied enzyme activity. The enzyme enrichment was 3.1-fold.

Enzyme Assays (Glucosidase Activity). a) *Glucose Oxidase Assay.* The liberated glucose was measured with a coupled enzyme system using glucose oxidase (GOD) and peroxidase (POD). The incubation mixture of the standard assay contained 1.5 mM **1**, 50 µl of GOD (16.67 nkat), 50 µl of POD (666.8 nkat), 50 µl of guaiacol (diluted 1:20 with EtOH), and 0.4 ml of KPi buffer (0.1M, pH 6.0, incubation time 30 min). The reaction was started by addition of the enzyme fraction to be tested. Incubations were carried out at 30° with intermittent shaking. The reaction could be visualized by observing the absorbance of the red oxidation product of guaiacol at 540 nm. Strict absence of reducing agents (*e.g.* 2-mercaptoethanol) was necessary. A parallel incubation without **1** was treated in an identical manner. The detection limit of this assay was 5 pkat.

b) *HPLC Method: Determination of the Decrease of Raucaffricine* (**1**) *or Increase of Vomilenine* (**2**) *Concentration.* HPLC analyses were performed with an *RP-18* column *(Hibar LiChrocart®, Merck)* employing a linear gradient of CH_3CN/10 mM $(NH_4)_2CO_3$ 5:95 to 62:38 in 40 min: **1** t_R 19.5 min; **2**, t_R 25.2 min. The incubation mixture contained 1.5 mM **1**, 0.8 ml of KPi buffer (0.1M, pH 6.0), and raucaffricine glucosidase; incubation temp. was 30°. The enzymatic cleavage of **1** was followed by diluting an aliquot of the reaction mixture with MeOH (100 µl + 300 µl MeOH), centrifuging the denatured protein, and running an HPLC analysis. The detection limit

was 0.1 pkat. Protein was quantified by the method of *Bradford* [15] using a calibration curve obtained with bovine-serum albumin.

Determination of Enzyme Properties. The following characteristics were measured with the 14.8-fold enriched enzyme. The pH optimum of the enzyme was determined in 0.1M KPi/citrate buffer (pH 4–5.5) and 0.1 KPi buffer (pH 6-8) employing the glucose-oxidase assay in presence of 0.1 nkat glucosidase at a concentration of 1.5 mM raucaffricine. The total volume was 0.8 ml.

The temp. optimum was measured under the same assay conditions.

The relative mol.wt. (M_r) of the enzyme was estimated by gel chromatography on an *AcA 54* column (2.3 × 92 cm, 0.1M KPi buffer (pH 7.0), flow rate 20 ml/h) which was calibrated with cytochrome c (M_r = 12500), trypsin inhibitor (M_r = 21500), ovalbumin (M_r = 45000), and bovine-serum albumin (M_r = 68000). The enzyme activity was eluted at 158 ml of the eluent corresponding to a M_r of 66600 ± 5%. Determination of M_r by HPLC was performed with a *TSK-G-3000-SW* column (0.75 × 60 cm), calibrated with chymotrypsin (M_r = 25000), albumin (M_r = 68000), and catalase (M_r = 240000). The elution of the enzyme corresponded to a M_r of 65100.

For the determination of the stability of the enzyme, 0.6 ml (2.52 mg protein, spec. activity 0.4 nkat/mg) of the *G-25* eluate were kept in 6 portions at 4° in a refrigerator for 6 months, and each month the activity was determined by *Assay b*. Moreover, 10 ml of enzyme solution (0.4 nkat/mg) were freeze-dried and the spec. activity determined by the above assay after 6 months.

K_m and V_{max} values were determined in presence of 0.9 mg of protein (0.6 nkat/mg) using *Assay a*.

Determination of the Glucosidase Activity in Different Cell Materials. Crude enzyme mixtures were used to monitor the glucosidase activity in different cell-suspension cultures or intact plants. In all experiments, 50 g of the tissue were used as an enzyme source. Enzyme isolation was carried out as described above, but desalting was performed by dialysis (24 h, KPi, pH 6.0). The enzyme activities were measured by HPLC *(Assay b)*.

Determination of the Isoelectric Point of the Raucaffricine Glucosidase. Isoelectric focussing was performed by the method of *Righetti* and *Gelfi* [16] using a linear pH gradient (pH 4.0–10) generated in acrylamide gel (10 × 10 × 0.5 cm) by an Immobiline Kit *(LKB)*. To the gel, 100 µl (0.4 mg) of the glucosidase (0.3 nkat/mg) were applied. After separation of the proteins, glucosidase activity was determined by cutting the gel in 0.5-cm bands from which enzyme activities were eluted and determined by *Assay a*.

Synthesis of Substrates. 1,2-Dihydroraucaffricine (**3**). To a soln. of 50 mg (98 µmol) of **1** in 2 ml of KPi/citrate buffer (pH 5.0), 40 mg (0.63 mmol) of $NaBH_3CN$ were added. After 2 h, the mixture was subjected to TLC using $CHCl_3$/MeOH 4:1. The product (R_f 0.25) was eluted with MeOH: 34 mg (68%). ^{1}H-NMR (C_5D_5N; configuration at C(2)): 4.7 (*dd*, *J* = 9, 5.5, H–C(3)); irradiation at 4.1 (H–C(2))→4.7 (*d*, *J* = 9, H–C(3)). EI-MS (aglycone): 352 (23, M^+), 297 (9), 222 (20), 185 (100), 184 (34), 169 (36), 168 (50), 143 (28), 131 (49), 130 (53).

17-O-Deacetyl-1,2-dihydroraucaffricine (**4**). A soln. of **3** (30 mg, 58 µmol) in 2 ml of 10N KOH was left for 24 h. TLC of the mixture using $CHCl_3$/MeOH/Et_2NH 4:1:0.1 resulted in 15 mg (54%) of **4** R_f 0.2. EI-MS (aglycone): 310 (23, M^+), 185 (100), 184 (20), 180 (55), 169 (38), 168 (59), 159 (27), 156 (23), 152 (21), 144 (32), 143 (44), 131 (61), 130 (64).

1,2-Dihydro-1-methylraucaffricine (**5**). To a soln. of **1** (50 mg, 98 µmol) in 2 ml of 1M KPi/citrate buffer (pH 5.0), 0.5 ml of 35% HCHO and 40 mg (0.63 mmol) of $NaBH_3CN$ were added. The mixture was allowed to stand for 30 min at 25° and was then directly separated by TLC ($CHCl_3$/MeOH/Et_2NH 50:20:0.1) yielding 34 mg (66%) of **5**, R_f 0.4. EI-MS (aglycone): 366 (28, M^+), 199 (78), 198 (29), 183 (24), 182 (47), 181 (21), 170 (22), 167 (18), 158 (26), 157 (43), 156 (19), 145 (69), 144 (100), 143 (30), 131 (37), 130 (16).

1,2,19,20-Tetrahydroraucaffricine (**6**). To a soln. of 40 mg (78 µmol) of **3** in 1 ml of MeOH and 0.5 ml of 2N HCl, 10 mg of 10% Pd/C were added, and the mixture was stirred for 20 h under H_2. The mixture was filtered and purified by TLC ($CHCl_3$/MeOH/Et_2NH 4:1:0.1) yielding 21 mg (52%) of **6** R_f 0.3. EI-MS (aglycone): 354 (28, M^+), 295 (11), 185 (62), 184 (29), 169 (33), 168 (54), 167 (32), 163 (32), 156 (24), 143 (100), 131 (77), 130 (83).

Enzyme Inhibition Studies. Using the above HPLC assay for raucaffricine β-D-glucosidase, the following compounds were tested for an inhibitory effect: δ-gluconolactone (up to 1M), phenylmethylsulfonyl fluoride, EDTA, iodoacetamide (up to 80 mM), strictosidine or amygdalin (up to 50 mM); total volume was 0.5 ml (KPi buffer 0.1M, pH 6.0). Each compound was preincubated in the presence of 100 pkat glucosidase for 20 min and the reaction started with 0.5 µmol of **1**. After 30 min of incubation, the degree of enzymatic hydrolysis was determined by adding 1.5 ml of MeOH and HPLC analysis of the mixture after separation of the denatured protein by centrifugation.

Influence of Glucose, Fructose, and Mannitol on the Enzymatic Raucaffricine Hydrolysis. Each of the three compounds was incubated with increasing concentrations for 30 min with 100 pkat raucaffricine glucosidase in 1 ml of 0.1M KPi buffer (pH 6.0), and the enzymatic reaction was started with 0.5 µmol of **1**. After 30 min, the reaction was terminated and analysed as above.

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