Molecular Cloning, Heterologous Expression and Characterization of Strictosidine Glucosidase from *Rauvolfia serpentina* Cell Suspension Cultures

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LIST OF ABBREVIATIONS

AE	acetyl esterase
bp	base pairs
BSA	bovine serum albumin
CAS	ceric ammonium sulfate reagent
COSY	correlation spectroscopy
CV	column volume
dNTP	deoxyribonucleoside 5'-triphosphate (N=A,T,G,C)
DTT	dithiothreitol
DW	dry weight
EDTA	ethylenediaminetetraacetate
EI-MS	electron impact mass spectrometry
ER	endoplasmic reticulum
EtOAc	ethyl acetate
FAB-MS	fast atom bombardment mass spectrometry
FC	flash chromatography
FD-MS	field desorption mass spectrometry
FPLC	fast protein liquid chromatography
GSP	gene specific primer
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
HR-MS	high resolution mass spectrometry
HSQC	heteronuclear single quantum coherence
IPTG	isopropyl-thio-β-galactoside
JA	jasmonic acid
KPi	potassium phosphate
MeOH	methanol
MJ	methyl jasmonate
Mops	4-morpholinepropanesulfonic acid
MT	methyl transferase
MW	molecular weight
NADP, $NADP^+$, NADPH	nicotinamide-adenine dinucleotide phosphate and its oxidized and reduced forms

NBA	3-nitrobenzylalcohol
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser enhancement
NOESY	NOE spectroscopy
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PNAE	polyneuridine aldehyde esterase
RACE	rapid amplification of cDNA ends
RG	raucaffricine glucosidase
RT	reverse transcription
SBE	sarpagan-bridge enzyme
SDS	sodium dodecyl sulfate
SG	strictosidine glucosidase
SS	strictosidine synthase
SS1-7	solvent system 1-7
TLC	thin layer chromatography
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
V	volume
VGT	vomilenine glucosyltransferase
VH	vinorine hydroxylase
VR	vomilenine reductese
VS	vinorine synthase

I. INTRODUCTION

1. *Rauvolfia serpentina* and its therapeutic valuable alkaloids

Rauvolfia serpentina (L.) Benth. ex Kurz (Fig. 1a) is a tropical bush native to India, where it has been known as "Sarpagandha root" (Sahu, 1979). Its Latin name was given to the plant in honour to the German physician and botanist Leonhardt Rauwolf, who was one of the first to describe for Europeans a large number of Asian and North African plants found during his botanical expeditions. In India *R. serpentina* has been used in medicine since about 3 000 years. Its therapeutic applications included the treatment of snake bites, fevers and insanity (Sahu, 1979). While the action as an antidote for snake poisons could not be proved, other important properties of *R. serpentina* are recognized by modern medicine.

The therapeutically applied constituents of the plant represent the monoterpenoid indole alkaloids. The most important are reserpine, ajmaline and ajmalicine (Fig. 1b). Reserpine is used in cases of mild to moderate hypertension and in treatment of chronic psychoses (Schmeller and Wink, 1998). It decreases peripheral resistance and blood pressure by depletion of peripheral noradrenaline stores. This action is explained through inhibition of a Mg^{2+}-dependent ATPase at the vesicle membrane, which pumps protons into the storage vesicles. The concentration of H^+ decreases and basic substances such as noradrenaline and dopamine cannot be protonated intravesicularly. The remaining noradrenaline follows the concentration gradient and diffuses into the cytoplasm where it is destroyed by intraneuronal monoamine oxidase, so that little or no neurotransmitter remains. Reserpine also causes depletion of catecholamine and serotonine stores in the brain, heart, and many other organs (Mutschler et al., 2001). Ajmalicine, also called raubasine, decreases blood pressure in a way similar to reserpine and is used in treatment of peripheral and celebral

vascular disorders (Schmeller and Wink, 1998). Ajmaline is a class Ia antiarrhythmic agent. It prolongs the refraction phase of the heart through a blockage of Na^+ channels (Mutschler et al., 2001).

Reserpine

Ajmalicine Ajmaline

Fig. 1a. *Rauvolfia serpentina*

Fig. 1b. Examples of pharmaceutical valuable indole alkaloids found in its root bark.

The successful medical use of preparations from *R. serpentina* roots led to a significant depletion of the plant population in India. Slow growth and problems of cultivation in the greenhouse forced the search for an alternative source of pharmaceutically valuable alkaloids. An extensive investigation of other *Rauvolfia* species (Iwu and Court, 1977; Court, 1983) revealed, that *R. vomitoria*, abundantly growing in Africa, has an alkaloid pattern qualitatively similar to that of *R. serpentina*. *R. vomitoria* is used now as an industrial source of reserpine and other indole alkaloids.

2. Cultivation of *R. serpentina in vitro*

Another possibility to obtain *R. serpentina* alkaloids and to study their biosynthesis is the use of an *in vitro* cell culture. The first callus cultures of *R. serpentina* were able to synthesize ajmaline, but grew rather slowly (cultivation period 55 days) (Vollosovich et al., 1967; Ohta and Yatazawa, 1979). The cell suspension culture (Fig. 2a) established in Zenk's laboratory in 1970s was significantly more productive. The first phytochemical investigation resulted in isolation and identification of 12 indole alkaloids belonging to 4 structural groups, namely ajmaline, sarpagine, yohimbine and heteroyohimbine groups (Stöckigt et al., 1981). Further optimization of nutrition medium and growth conditions allowed to achieve a production of a glucoalkaloid raucaffricine (Fig. 2b) up to 1.6 g/l medium during the cultivation period of 18 days (Schübel et al., 1989). Up to now, a total of 30 different indole alkaloids were detected in the *R. serpentina* cell suspension culture (Stöckigt, 1995).

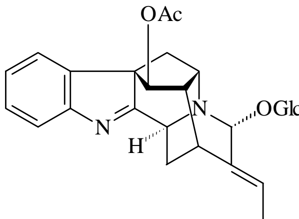


Fig. 2a. *Rauvolfia serpentina* cell suspension culture.

Fig. 2b. Its major alkaloid, raucaffricine.

Different approaches have been used to modify and improve the alkaloid production *in vitro*. The hairy root culture of *R. serpentina*, obtained by transformation with *Agrobacterium rhizogenes*, was shown to synthesize 20 indole alkaloids including 6 novel compounds (Falkenhagen et al., 1993; Sheludko et al., 2000a; Sheludko et al., 2001). Hybrid cell line *RxR*17 of *R. serpentina* and another Apocynaceae species, *Rhazya stricta* Decaisne, was obtained using somatic hybridization techniques (Kostenyuk et al., 1991) and has been proved to produce indole alkaloids from both parental plants as well as compounds previously not detected neither in *R. serpentina* nor in *R. stricta* plants or cell cultures (Kostenyuk et al., 1995; Aimi et al., 1996).

3. Elicitation of plant cell cultures

The elicitation of plant cell cultures has proved to be a successful approach to stimulate plant secondary metabolism. Elicitors are compounds which induce a defense response in plants (Darvill and Albersheim, 1984). Often the preparations of the phytopathogenic fungi or the oligosaccharides derived from their cell walls are used as elicitors. For example, the cell cultures of *Papaver somniferum*, which do not produce the morphinan type of isoquinoline alkaloids under normal conditions, accumulate sanguinarine at level of 2.9% DW after elicitation (Eilert et al., 1985).

The stress signal transduction in the plant cell leading to the defense response involves the secondary messengers, which are synthesized or released after elicitation and can alter the gene expression. These substances can be applied exogeneously to the plant cells instead of elicitors. One of the secondary messengers was identified as jasmonic acid (JA) (Fig. 3). It is synthesized from linolenic acid, which is released from the cell membrane after interaction of an elicitor with a membrane receptor (Creelman and Mullet, 1997). The increase of JA level leads to the triggering of several defense systems including the expression of proteinase inhibitor genes

(Farmer et al., 1992) and, in case of longer exposure to the stress conditions, the general down-regulation of protein biosynthesis due to accumulation of ribosome-inactivating proteins (Reinbothe et al., 1994a). JA is able to alter the gene expression on different levels, such as transcription (Reinbothe et al., 1997; Memelink et al., 2001), post-transcriptional modification (Reinbothe et al., 1993) and translation (Reinbothe et al., 1994b). It was suggested to participate in controlling many processes in plant organism. JA and its methyl ester, methyl jasmonate (MJ), influence seed germination and growth (Creelman and Mullet, 1997), regulate biosynthesis of vegetative storage proteins (Anderson, 1988), promote senescence (Ueda and Kato, 1980) and induce tendril coiling (Falkenstein et al., 1991). Exogeneously applied JA or MJ were shown to stimulate the biosynthesis of different classes of plant secondary metabolites, e.g. benzophenanthridine alkaloids in the cell suspension cultures of *Eschscholtzia californica* (Gundlach et al., 1992) and paclitaxel and baccatin III in the cell suspension culture of *Taxus baccata* (Yukimune et al., 1996).

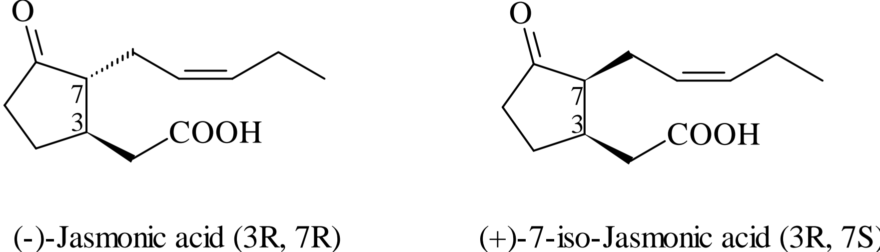


Fig. 3. Biologically active isomers of jasmonic acid.

4. Biosynthesis in *R. serpentina* cell suspension culture

The key intermediate in the biosynthesis of monoterpenoid indole alkaloids is the glucoalkaloid strictosidine (Stöckigt and Zenk, 1977a; Stöckigt and Zenk, 1977b;

Rueffer et al., 1978; Nagakura et al., 1979). It is formed by the condensation of tryptamine, the decarboxylation product of the amino acid tryptophane, and the monoterpene secologanin (Kutchan, 1993) (Fig. 4). The condensation reaction is catalyzed by the enzyme strictosidine synthase (SS), which was characterized and purified from *Catharanthus roseus* and *R. serpentina* cell suspension cultures (Treimer and Zenk, 1979; Hampp and Zenk, 1988; Pfitzner and Zenk, 1989). The cDNA encoding SS was cloned and heterologously expressed in *E. coli* more than 10 years ago (Kutchan et al., 1988; Kutchan, 1989). After deglucosylation of strictosidine catalyzed by strictosidine glucosidase (SG) the unstable aglycone is converted to geissoschizine. The formation of the C5 – C16 bond by the sarpagan-bridge enzyme (SBE) leads to the polyneuridine aldehyde possessing the sarpagan ring system (Schmidt and Stöckigt, 1995). The enzyme catalyzing the next biosynthetic step – the polyneuridine aldehyde esterase (PNAE) – was purified from *R. serpentina* cell suspension culture and the respective cDNA was cloned and actively expressed in *E. coli* (Dogru et al., 2000). The link between the sarpagan and the ajmalan skeleton is catalysed by the enzyme named vinorine synthase (VS). Vomilenine is formed by the vinorine hydroxylase (VH), a membrane bound enzyme consisting of a cytochrome P450 and a cytochrome P450 reductase, and is further converted to 1,2-dihydrovomilenine and 17-O-acetylnorajmaline by two vomilenine reducteses (VR I and VR II). The deacetylation by the acetyl esterase (AE) and the methylation by the N-methyl transferase (MT) leads to ajmaline. The major alkaloid of the *R. serpentina* cell suspension culture, raucaffricine, is a side product formed by the vomilenine glucosyltransferase (VGT) (Ruyter and Stöckigt, 1991). Raucaffricine may be converted back to vomilenine by the raucaffricine glucosidase (RG) (Schübel

Fig. 4. The biosynthetic pathway leading to ajmaline.

SS – strictosidine synthase; **SG** – strictosidine glucosidase; **SBE** - sarpagan-bridge enzyme; **PNAE** – polyneuridine aldehyde esterase; **VS** – vinorine synthase; **VH** – vinorine hydroxylase; **VR I** and **VR II** – vomilenine reductase I and II; **AE** – acetyl esterase; **MT** – methyl transferase; **VGT** – vomilenine glucosyltransferase; **RG** – raucaffricine glucosidase.

and Stöckigt, 1986; Warzecha et al., 1999) and thus enters the main biosynthetic pathway leading to ajmaline.

5. The role of strictosidine glucosidase in the biosynthesis of indole alkaloids

Up to date more than 2 000 of monoterpenoid indole alkaloids are known to occur in higher plants. All these compounds derive from strictosidine. Somewhere downstream of this glucoalkaloid the biosynthetic pathways diverge to lead to different structures. The reaction catalysed by SG is the first point where this biodiversity could be created.

The SG was first characterised from the cell suspension cultures of *Catharanthus roseus* about 20 years ago (Hemscheidt and Zenk, 1980; Luijendijk et al., 1998) and recently it has been cloned from the same source and heterologously expressed in yeast (Geerlings et al., 2000). The deglucosylation of strictosidine by the *C. roseus* enzyme leads through some unstable intermediates to the end product cathenamine (Stöckigt, 1979). The mechanism of this reaction was studied using the partially purified enzyme from *C. roseus* (Stöckigt et al., 1978).

The strictosidine aglycone (Fig. 5) formed after removal of the glucose moiety is unstable. It is supposed to convert spontaneously to a highly reactive dialdehyde. The intramolecular condensation of the C21 aldehyde and Nβ amino groups leads to the formation of 4,21-dehydrocorynantheine aldehyde. The involvement of this compound in the biosynthesis of indole alkaloids in *C. roseus* was proved by conducting the enzymatic reaction in presense of reducing agents, which were expected to reduce the aldehyde groups in the intermediates and thus prevent them from further conversion. As a result, two compounds, sitsirikine and isositsirikine, were isolated (Stöckigt et al., 1978), which represent the reduction products of 4,21-dehydrocorynantheine aldehyde. The 4,21-dehydrocorynantheine aldehyde converts

spontaneously to 4,21-dehydrogeissoschizine (Rueffer et al., 1979) and further to cathenamine. In presence of NADPH and crude enzyme preparation from *C. roseus* containing reductases 4,21-dehydrogeissoschizine can be transformed to ajmalicine-type alkaloids or geissoschizine (Rueffer et al., 1979).

Strictosidine

Aglycone

Dialdehyde

Cathenamine

4,21-Dehydrogeissoschizine

4,21-Dehydrocorynantheine aldehyde

NADPH

$NADP^+$

$NaBH_3CN$ or KBH_4

Geissoschizine

Isositsirikine

Sitsirikine

Fig. 5. Reactions proposed to occur after enzymatic strictosidine deglusosylation.

The aim of the present work was identification and characterization of enzyme(s) involved in strictosidine deglucosylation in *R. serpentina* cell suspension cultures. The strictosidine glucosidase is the fifth enzyme involved in the ajmaline biosynthesis for which the cDNA cloning and heterologous expression has been achieved. These results are an important step towards the heterologous expression of the entire biosynthetic pathway leading from strictosidine to ajmaline. The SG from *R. serpentina* is compared with the analogous enzyme from *C. roseus* and the details of the catalysed reaction are studied in order to answer the question whether the strictosidine glucosidase can influence further conversions of the strictosidine aglycone leading to the diverse groups of monoterpenoid indole alkaloids.

II. MATERIALS AND METHODS

1. Chemicals and solutions

1.1. Standard indole alkaloids

Ajmalicine, ajmaline and reserpine were purchased from Roth (Karlsruhe, Germany). Raucaffricine and strictosidine were prepared according to the published procedures (Schübel and Stöckigt, 1984; Pfitzner and Zenk, 1982, respectively).

1.2. Buffers

Tris/HCl buffers were prepared by dilution of 1 M Tris (AppliChem, Darmstadt, Germany) solution adjusted to the necessary pH value with conc. HCl.

Citrate-phosphate buffers (2x) were prepared by mixing 0.1 M citric acid and 0.2 M NaH_2PO_4 in following proportions and adjusting the pH value using a DIGI 550 pH meter (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany), if necessary:

pH value	3.8	4.2	4.6	5.0	5.4	5.8	6.2	6.6	7.0
NaH_2PO_4, ml	17.7	20.6	23.3	25.7	27.8	30.3	33.1	36.4	43.6
Citric acid, ml	32.3	29.4	26.7	24.3	22.2	19.7	16.9	13.6	6.5

Potassium phosphate (KPi) buffers (10x) were prepared by mixing 1 M KH_2PO_4 and 1 M K_2HPO_4 in following proportions and adjusting the pH value, if necessary:

pH value	5.8	6.0	6.2	6.6	7.0	7.4	8.0
KH_2PO_4, ml	91.5	86.8	80.8	61.9	38.5	19.8	6.0
K_2HPO_4, ml	8.5	13.2	19.2	38.1	61.5	80.2	94.0

2. Biological material

2.1. Bacterium strains

Escherichia coli strains TOP10 (Invitrogen) and ER2566 (New England Biolabs) were cultivated in liquid LB medium at 37 °C on shaker (200 rpm).

LB Medium:

1.0% trypton;

0.5% yeast extract;

1.0% NaCl;

adjust pH to 7.0 with NaOH, sterilize by autoclaving; for preparation of solid medium add 20 g/l agar.

2.2. Plant cell cultures

Cell suspensions cultures were grown in 1 l Erlenmeyer flasks containing 400 ml of LS medium (Linsmaier and Skoog, 1965) in diffuse light (600 lux) on shaker (100 rpm). Cultivation period was 7 days for *Rauvolfia serpentina* cell line T-30 and *R. serpentina x Rhazya stricta* somatic hybrid subculture *RxR*17M. The somatic hybrid subculture *RxR*17K was maintained as callus strain in the dark at 26 °C on solid 4x medium (Gamborg et al., 1968, modified by Sidorov et al., 1985) containing 2.0 mg/l 2,4-dichlorophenoxyacetic acid, 0.5 mg/l indole-3-acetic acid, 0.5 mg/l 1-naphthaleneacetic acid, and 0.2 mg/l kinetin. To establish cell suspension small pieces of calli (0.5-1 cm) were placed in liquid LS medium and cultivated as described above with cultivation period of 14 days. After six months of growing as cell suspension the *RxR*17K somatic hybrid subculture was used for alkaloid analysis. For alkaloid,

nucleic acid or protein extraction the cells and nutrition media (if necessary) were harvested by vacuum filtration.

3. Methyl jasmonate treatment

Methyl jasmonate (Aldrich) was prepared as 0.05 M solution in 70% ethanol and added to 400 ml of cell suspension cultures on the 5th day after inoculation into the fresh medium. The final concentration of MJ in the medium was 100 µM. Equal volumes of 70% ethanol (800 µl) were added to the control cultures. Cells as well as nutrition media were harvested on the 1st and 5th days after treatment in 4 reiterations.

4. Extraction of alkaloids

4.1. Tissue extraction protocol

Freeze-dried cells (0.1 g) were extracted with 10 ml of methanol under sonication during 30 min. After filtering the extract was evaporated to dryness under vacuum at 40°C, dissolved in 0.5 ml of methanol, transferred in an Eppendorf tube and acidified with 35 µl 0.1N HCl. The obtained solution was extracted with 0.125 g of SCX cation-exchanger (ICT, Bad Homburg, Germany) during 30 min on a microshaker (1000 rpm). After centrifugation at 14000 rpm (5 min, Eppendorf centrifuge 5415) the supernatant was removed (giving fraction 1) and the remaining SCX was washed with 250 µl of pure methanol followed by centrifugation under the same conditions. The supernatant was removed and combined with fraction 1 giving fraction 2. SCX was eluted with 1 ml of a mixture MeOH: conc. NH_3 9.5:0.5 during 10 min on the microshaker (1000 rpm). After centrifugation the supernatant was taken out and the procedure was repeated. Both supernatants were combined giving fraction 3. Fraction 2 was extracted with fresh SCX cation exchanger (0.1 g) during 30 min on the microshaker (1000 rpm). After centrifugation at 14000 rpm (5 min) the supernatant was taken out. SCX was eluted as described above and, after centrifugation, the

supernatant was combined with fraction 3 and evaporated under nitrogen, giving a crude alkaloid extract. Crude tissue alkaloid extracts were analyzed by HPLC.

4.2. Medium extraction protocol

Nutrition medium (150 ml) was adjusted to pH level of 2.0 with 30% H_2SO_4 and extracted with an equal volume of ethyl acetate. The water phase was adjusted to pH level of 9.0 with conc. NH_3 and extracted twice with equal volumes and once with half volume of ethyl acetate. Three last organic fractions were combined and evaporated giving crude nutrition medium alkaloid extract used for HPLC analysis.

4.3. Extraction by the method of Smith

For preparative isolation of alkaloids the crude extract was enriched by the modified method of Smith and co-workers (Smith et al., 1987). The dried cells were extracted with MeOH under sonication during 30 min at 40 °C (1 l MeOH/10 g DW). After evaporation of the solvent the residue was taken up in 0.05 M bicarbonate buffer, pH 9.8 (1 l buffer/residue from 5 l methanolic extract) and partitioned three times into equal volumes of EtOAc. Organic fractions were combined and evaporated giving an enriched alkaloid extract.

5. Alkaloid analysis

5.1. Thin layer chromatography

For TLC 0.2 mm silica gel 60 F_{254} plates, 20 x 20 cm (Merck, Darmstadt, Germany) were cut to necessary size and developed with the following solvent systems:

(SS1) $CHCl_3$:MeOH: conc. NH_3 8:2:0.1;

(SS2) EtOAc:MeOH:H_2O: conc. NH_3 7:2:1:0.1;

(SS3) $CHCl_3$:MeOH 8:2;

(SS4) Acetone:Petroleum Ether 2:7;

(SS5) EtOAc:MeOH:H_2O 7:2:1;

(SS6) EtOAc:Benzene 2:1;

(SS7) $CHCl_3$:Acetone 5:4.

Alkaloids were detected by quenching the UV light at 254 nm; the plates were sprayed with ceric ammonium sulfate (CAS) reagent (5% in 85% H_3PO_4) and the colours in visible light as well as fluorescence at 366 nm were recorded.

5.2. Flash chromatography

FC (Still et al., 1978) was carried out under 3-5 kg/cm^2 pressure with silica gel 60 (230-400 mesh) (Merck, Darmstadt, Germany) packed into glass column (∅ 4 cm) using solvent system SS1 and collecting 18 ml fractions. 1 mg of enriched alkaloid extract (see **II.4.3.**) were applied on 50 g of sorbent.

5.3. High performance liquid chromatography

HPLC analyses were performed with a Merck/Hitachi system (L-6200 intelligent pump coupled to AS-2000 autosampler and L-4250 UV/VIS detector) using a CC Nucleosil 100-5 C18 column (0.4 x 25 cm, Macherey-Nagel, Düren, Germany). The solvent system was acetonitrile:39 mM NaH_2PO_4/2.5 mM hexanesulfonic acid buffer (pH=2.5), gradient 15:85 → 20:80 within 5 min → 40:60 within 40 min → 80:20 within 15 min, 1 ml/min flow rate and detection at 255 nm (Gerasimenko et al., 2001a).

5.4. Identification and structure elucidation methods

Melting points were determined using a Büchi apparatus and are uncorrected. Optical rotations were recorded on a Perkin-Elmer 241 MC polarimeter. UV spectra were measured in MeOH using a Perkin-Elmer Lambda 2 spectrophotometer. EI-MS measurements were carried out with a quadrupole instrument (Finnigan MAT 44S) at 70 eV. HR-EI-, HR-FAB-, and FD-MS spectra were recorded on JEOL JMS-700 mass spectrometer. ^{1}H-NMR spectra were measured using AMX 400 and DRX 600

instruments (Bruker, Karlsruhe, Germany) with CD_3OD, $CDCl_3$ and pyridine-d_5 as solvents. The COSY, NOESY, HSQC and HMBC experiments were performed on the DRX 600 instrument. PCMODEL, v. 7.0 (Serena Software, Bloomington, IN) was used for generation of 3-D models and calculation of distances between atoms.

6. Statistical calculations

The error bars in the diagrams represent the confidence intervals for the mean values (at a confidence level of 0.95). Student's t-test was performed (at a significance level of 1%) to determine whether the differences between mean values are significant.

7. Synthesis methods

7.1. Derivatization of alkaloids

For deacetylation 10 mg (19.5 µmol) raucaffricine were dissolved in 200 µl MeOH, 800 µl 1 N NaOH were added and the reaction mixture was incubated on shaker at room temperature (ca. 25 °C) during 1.5 h. The sample was extracted with 1 ml EtOAc and after solvent evaporation the residue was separated by TLC using solvent system SS1. The product located at Rf 0.15 showing grey CAS colour was eluted resulting in 4.6 mg (9.8 µmol) 21-glucohydroxysarpagan-17-al (50% yield).

Reduction of 10 mg (19.5 µmol) raucaffricine was carried out using 7 mg KBH_4 in 500 µl MeOH on ice during 2 h. The reaction mixture extracted with 500 µl EtOAc and after solvent evaporation the residue was separated by TLC using solvent system SS2. The products located at Rf 0.33 (grey CAS colour and blue fluorescence) and 0.37 (orange CAS colour and fluorescence) were eluted giving 2.8 mg (5.9 µmol) of 21-glucohydroxysarpagan-17-ol (30% yield) and 0.9 mg (1.8 µmol) of 1,2-dihydroraucaffricine (9% yield). Otherwise 20.5 mg (40 µmol) raucaffricine were dissolved in 1 ml 0.1 M citrate-phosphate buffer (pH 5.0) and incubated with 16 mg $NaBH_3CN$ on shaker at room temperature (ca. 25 °C) for 2 h. The reaction mixture

was freeze-dried, dissolved in MeOH and separated by TLC using solvent system SS2. As a result, 8 mg (3.8 µmol) of 21-glucohydroxysarpagan-17-ol (9.5% yield) and 6.3 mg (12.3 µmol) of 1,2-dihydroraucaffricine (30.8% yield) were obtained.

For methylation, 10 mg (19.5 µmol) raucaffricine were dissolved in 1 ml 0.1 M citrate-phosphate buffer (pH 5.0) and incubated with 8 mg $NaBH_3CN$ and 200 µl HCHO on shaker at room temperature (ca. 25 °C) during 30 min. The reaction mixture was freeze-dried, dissolved in MeOH and separated by TLC using solvent system SS1. The product located at Rf 0.42 showing red CAS colour and blue fluorescence was eluted giving 6 mg (11.4 µmol) 1-methyl-1,2-dihydroraucaffricine (58.5% yield).

Dolichantoside (Nβ-methylstrictosidine) was synthesized from strictosidine analogously (Achenbach and Benirschke, 1997). For a typical reaction 106 mg strictosidine (200 µmol) were dissolved in 20 ml 0.1 M citrate-phosphate buffer (pH 5.0). 4 ml 37% formaldehyde and 50 mg $NaBH_3CN$ were added. After 30 min incubation at 30 °C with shaking the reaction mixture was freeze-dried and separated by preparative TLC using solvent system SS5. The product at Rf 0.45 displaying brown-greenish CAS colour was isolated resulting in 37 mg of dolichantoside (68 µmol, 34% yield).

For acetylation 10 mg strictosidine (18.9 µmol) were incubated in 500 µl pyridine:acetic anhydride mixture (1:1) over night at room temperature (ca. 25 °C) under shaking. The reaction mixture was separated by TLC using solvent system SS6. The product located at Rf 0.29 showing yellow CAS colour and fluorescence was eluted giving 9.7 mg strictosidine pentaacetate (13.1 µmol, 69.3% yield).

Acetylation before EI-MS measurement was carried out over night by incubation of an alkaloid (1-5 µg) in pyridine:acetic anhydride mixture (1:1).

7.2. Enzymatic deglucosylation of strictosidine and dolichantoside

For synthesis of cathenamine, 1 mg strictosidine dissolved in 100 µl MeOH was incubated in H_2O (total volume 1 ml) with 450 µg crude enzyme preparation from transgenic *E. coli* for 1 h at 30 °C. For control assays the enzyme preparation was heated in a boiling water bath for 20 min. After centrifugation (11 000 xg, 5 min) the pellet (formed after incubation with the active enzyme only) was freeze-dried and analyzed by different MS techniques and ^1H NMR.

For intermediate identification under reducing conditions, 530 µg (1 µmol) of strictosidine were incubated with heterologously expressed SG in presence of 2, 250, 1000, 2000 or 4000 fold excess of $NaBH_3CN$ in 1 ml of 0.1 M citrate-phosphate buffer (pH 5.0). The reaction mixture was extracted with equal volume of EtOAc. Organic phase was dried and the residue analysed by 2D-TLC with solvent systems SS3 and SS4. The products were identified by their EI-MS data. The experiments using KBH_4 or $NaBH_4$ were carried out analogously in 0.1 M KPi or Tris/HCl buffer (pH 8.0).

For synthesis of 3-iso-correantine A, 30 mg dolichantoside (55 µmol) were incubated with 39 µg SG in 30 ml 0.1 M citrate-phosphate buffer (pH 5.0) over night at 30 °C. The reaction mixture was extracted with an equal volume of EtOAc, pH of the water phase adjusted to 8.0 with 25% ammonia and extraction with equal volume of EtOAc repeated. The organic phases were evaporated and chromatographed using solvent system SS3. The product at Rf 0.64 showing blue fluorescence at 366 nm after spraying with CAS was eluted with MeOH:CH_2Cl_2 mixture (3:7) yielding 1 mg 3-iso-correantine A.

8. Protein determination and enzyme assays

8.1. Determination of protein concentration

Protein concentrations were measured by the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) (Merck, Darmstadt, Germany) as standard. Protein solution (100 µl) was mixed with 900 µl Coomassie reagent (50 mg Coomassie Brilliant Blue G250 dissolved in 50 ml EtOH and 100 ml H_3PO_4 (85%), diluted to 1000 ml with H_2O and filtered) and the optical absorbance at 595 nm was recorded on a Perkin-Elmer Lambda 2 spectrophotometer. The calibration line was build for the BSA concentration range 10 – 100 µg/ml. If necessary, the sample solutions were diluted to concentrations fitting into this range.

8.2. Electrophoretic analysis of proteins

8.2.1. Electrophoretic separation of proteins

Polyacrylamide gel electrophoresis (Ornstein, 1964) in sodium dodecyl sulfate (Weber and Osborn, 1969) (SDS-PAGE) was carried out using a vertical apparatus SE 600 (Hoefer) connected to a E 532 power supply (CONSORT, Turnhout, Belgium). The small-pore running gel (ca. 15 ml) was allowed to polymerize between two glass plates (16 x 18 cm, 1.5 mm gel thickness) for at least 1 h. Thereafter it was covered with the wide-pore stacking gel (ca. 5 ml) in which a comb with 15 wells (ca. 120 µl each) was inserted. The polymerization lasted for 45 - 60 min. The protein solutions were mixed with an equal volume of sample buffer, heated to 95 °C during 5 min and after cooling applied in the wells. The electrophoresis was carried out at 4 °C in running buffer at 30 mA until the bromphenol blue dye reached the bottom of the gel.

Running gel:

11.0 ml acrylamide stock solution
(30%, 29% acrylamide :
1% bisacrylamide,
AppliChem, Darmstadt, Germany);
7.5 ml 1.5 M Tris/HCl, pH 8.8;
11.2 ml H_2O;
150 µl 20% SDS;
20 µl TEMED;
150 µl 10% APS

Stacking gel:

2.5 ml acrylamide stock solution
(30%, 29% acrylamide :
1% bisacrylamide,
AppliChem, Darmstadt, Germany);
3.7 ml 0.5 M Tris/HCl, pH 6.8;
8.5 ml H_2O;
75 µl 20% SDS;
10 µl TEMED;
200 µl 10% APS

Sample buffer:

0.2 M Tris/HCl, pH 6.8;
40% (V/V) glycerol;
20% (V/V) β-mercaptoethanol
(omitted for the intein-mediated
SG purification);
8% SDS;
0.02% bromphenol blue

Running buffer:

50 mM Tris;
0.2 M glycine;
0.15% SDS

8.2.2. Coomassie staining of the SDS gels

For Coomassie staining (Blakesly and Boezi, 1977), the gels were shaken in the staining solution for 20 – 30 min and subsequently de-stained during 5-6 h changing the solution each 1-2 h.

Staining solution:

0.25% Coomassie Blue R250;
45% (V/V) MeOH;
9% (V/V) acetic acid

De-staining solution:

45% (V/V) MeOH;
9% (V/V) acetic acid

8.2.3. Silver staining of the SDS gels

For silver staining (Sammons et al., 1981), the gels were shaken subsequently in the fixation solution for 3 x 20 min and in the incubation solution for 1 h. After washing in deionized water (3 x 10 min) the gels were incubated in the silver solution for 20 min (in darkness), rinsed shortly (10 – 15 sec) in deionized water and placed in the developing solution. When the protein bands became visible (after 1 – 10 min), the developing solution was exchanged for 10% acetic acid (V/V) to stop the reaction.

Fixation solution:

30% (V/V) EtOH;
10% (V/V) acetic acid

Incubation solution:

30% (V/V) EtOH;
6.8% sodium acetate;
0.125% glutardialdehyde
(0.5 ml 25% solution/100 ml);
0.2% sodium thiosulfate pentahydrate

Silver solution:

0.2% silver nitrate;
0.02% formaldehyde
(60 µl 37% solution/100 ml)

Developing solution:

2.5% sodium carbonate;
0.01% formaldehyde
(30 µl 37% solution/100 ml)

8.3. Raucaffricine glucosidase activity assay

Raucaffricine glucosidase activity was calculated on the basis of raucaffricine decrease measured by HPLC. A typical assay contained appropriate enzyme activities between 1 and 5 pkat and 20 nmol of raucaffricine in 10 µl EtOH in a total volume of 100 µl 0.1 M citrate-phosphate buffer (pH 5.0) and was incubated for 15 or 30 min at 30°C. The reaction was terminated by addition of 200 µl MeOH. After centrifugation (11 000 xg, 5 min) the supernatant was analyzed by HPLC on 125/4 RP-select B

column (Macherey-Nagel, Düren, Germany) using the following solvent system: acetonitrile: water (pH 2.5), gradient 25:75 → 35:65 within 3 min → 80:20 within 0.5 min → 80:20 for 1 min → 25:75 within 0.5 min → 25:75 for 2 min, 1.5 ml/min flow rate, detection at 250 nm.

8.4. Strictosidine glucosidase activity assay

Strictosidine glucosidase activity was calculated on the basis of strictosidine decrease measured by HPLC. A typical assay contained appropriate enzyme activities between 1 and 8 pkat and 20 nmol of strictosidine in 5 µl MeOH in total volume of 50 µl 0.1 M citrate-phosphate buffer (pH 5.0) and was incubated for 15 or 30 min at 30°C. The reaction was terminated by addition of 100 µl MeOH. After centrifugation (11 000 xg, 5 min) the supernatant was analyzed by HPLC on CC 250/4 Nucleosil 100-5 C18 column (Macherey-Nagel, Düren, Germany) using the following solvent system: acetonitrile: 39 mM NaH_2PO_4 (pH 2.5), gradient 15:85 → 25:75 within 1 min → 40:60 within 6.5 min → 40:60 for 2.5 min → 85:15 within 0.5 min → 85:15 for 4.5 min → 15:85 within 0.5 min → 15:85 for 4.5 min, 1.2 ml/min flow rate, detection at 250 nm.

8.5. Unspecific glucosidase activity assay

To test glucosidase activity with broad range of substrates, the glucose liberated was measured using Trinder glucose reagent (Sigma). The calibration curve was built using glucose (Sigma) as standard. The appropriate quantity of glucose (1 – 400 nmol) was dissolved in total volume of 100 µl 0.1 M citrate-phosphate buffer (pH 5.0) containing 20% MeOH. After addition of double volume of MeOH, 200 ml of this mixture were added to 1 ml of Trinder 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 30 min the optical absorbance at 505 nm was recorded

Ultrospec II – 4050 II spectrophotometer. The optical absorbance was found to be a linear function of glucose concentration ranging from 10 to 100 nmol/sample. Unless otherwise stated, the reaction mixture (total volume 100 µl) contained 400 nmol of the corresponding substrate dissolved in 20 µl MeOH and appropriate enzyme activities (0.08 µg RG or 0.13 µg SG) in 0.1 M citrate-phosphate buffer (pH 5.0). Incubations were carried out over night. The reaction was terminated by addition of double volume MeOH. Of this mixture, 200 µl were added to 1 ml of Trinder glucose reagent and after 30 min the absorbance at 505 nm was measured.

9. Molecular biology methods

9.1. Nucleic acid isolation

9.1.1. Total RNA isolation

All manipulations with RNA were carried out in a sterile flow box treated with RNAse AWAY® spray (Roth, Karlsruhe, Germany). *R. serpentina* cultured cells (T-30 cell line, 6 days old) were frozen in liquid nitrogen and homogenized in peqGOLD RNAPure solution (PEQLAB, Erlangen, Germany) (3 ml/150 mg cells). After centrifugation (10 min, 18 000 xg, 4 °C) the supernatant was placed in new Eppendorf tubes, incubated at RT for 10 min, mixed with 0.6 ml $CHCl_3$, incubated at RT for 5 min and centrifuged (15 min, 18 000 xg, RT). The water phase was mixed with 0.69 ml isopropanol and 0.69 ml high salt solution (1.2 M sodium citrate, 0.8 M NaCl), incubated at RT for 15 min and centrifuged (15 min, 12 000 xg, 4 °C). The pellet was washed two times with 75% EtOH (V/V), dried carefully on air (10 – 15 min) and dissolved in 36 µl sterile H_2O (if necessary, the solution was warmed to 65 °C).

9.1.2. Plasmid DNA minipreparation

For rapid analysis the plasmid DNA was isolated from *E. coli* cells by the method of Birnboim and Doli (Birnboim and Doli, 1979). Bacterium cells from 2 ml of an over

night grown culture were harvested by centrifugation (5 min, 35 000 xg, 4 °C), taken up in 125 µl Resuspension Buffer (25 mM Tris/HCl, pH 8.0; 10 mM EDTA, 50 mM glucose) and incubated at RT for 5 min. For cell lysis 270 µl alkaline SDS solution (0.2 M NaOH, 1% SDS, freshly prepared) were added and the mixture was incubated at RT for 5 min. The solution was neutralized with 200 µl of cold 3 M potassium acetate solution (60 ml 5 M KOAc; 11.5 ml acetic acid; 28.5 ml H_2O) and after 5 min incubation on ice centrifuged for 15 min at 35 000 xg at 4 °C. The supernatant was mixed with equal volume of cold isopropanol and centrifuged for 15 min at 35 000 xg at 4 °C. The pellet was washed with 1 ml 75% EtOH (V/V), dried carefully on air (10 – 15 min) and dissolved in 20 µl RNAse A solution.

RNAse A solution: dissolve RNAse A in 0.01 M NaOAc, pH 5.2 (10 mg/ml), boil for 15 min and let cool down slowly to RT. Add 0.1 volume 1M Tris/HCl, pH 7.4, aliquot 50 ml and store at –20 °C. Before use dilute with TE buffer (10 mM Tris/HCl, pH 7.5; 1 mM EDTA) to 400 µl.

9.1.3. Plasmid DNA isolation for sequencing

For sequencing by the dideoxy chain termination method (Sanger et al., 1977) carried out by GENterprize (Mainz, Germany) the plasmid DNA was isolated and purified using the NucleoSpin Plasmid Kit (Qiagen) according to the manufacturer’s manual.

9.2. Electrophoresis of nucleic acids

Electrophoresis of nucleic acids was carried out in Blue Marine 100 or 200 horizontal apparatus (Serva) connected to PowerPac 3000 power supply (BioRad).

9.2.1. DNA separation

DNA fragments of different size were separated in 1% agarose gel containing ethidium bromide (6 µl 10mg/ml solution/100 ml gel) (Sambrook et al., 1989). The electrophoresis was carried out in TAE buffer (40 mM Tris-acatate, 1 mM EDTA). If necessary, DNA was extracted from agarose gel using the NucleoSpin Extract Kit (Qiagen) according to the manufacturer's manual.

9.2.2. RNA analysis

Total RNA was separated in formaldehyde containing agarose gel (Sambrook et al., 1989). All the manipulations were conducted in the flow box, the electrophoresis apparatus was rinsed subsequently with 3% H_2O_2 and MeOH before use. The gel solution (5 ml 10x Mops buffer, 43 ml H_2O, 0.5 g agarose) was boiled, cooled to 55 °C and supplemented with 2.7 ml 37% formaldehyde. The RNA solution (2 µl) was mixed with 10 µl sample buffer, heated to 65 °C for 10 min and applied in the wells. The electrophoresis was carried out in 1x Mops buffer.

10x Mops Buffer:

200 mM Mops;
50 mM NaOAc;
10 mM EDTA;
adjust pH to 6.5-7 with NaOH,
sterilize by filtration.

Sample buffer (always prepared freshly):

60 µl formamide;
20 µl 10x Mops buffer;
32.5 µl 37% formaldehyde;
12.5 µl H_2O;
12.5 µl ethidium bromide (10 mg/ml);
10 µl glycerol;
10 µl saturated bromphenol blue solution

9.3. Nucleic acid amplification

Nucleic acids were amplified in Genius termocycler (Techne).

9.3.1. Synthesis of first strand of cDNA

Unless otherwise stated, oligoT primer (T_{15}-NNN) and RLM reverse transcriptase (RLM-RT) (Promega) were used for first strand cDNA synthesis. The reaction mixture contained 4 µl 5x 1st Strand Buffer (supplied with RLM-RT), 2 µl 0.1 M DDT, 2 µl dNTP solution (10 mM: 2.5 mM each), 7 µl H_2O, 1 µl primer (10 pmol/µl) and 4 µl total RNA. After 5 min heating at 65 °C followed by 10 min incubation at 37 °C 1 µl RLM-RT was added. The synthesis proceeded for 1h and was terminated by 5 min heating to 95 °C.

9.3.2. Polymerase chain reaction

PCR was carried out with *Taq* DNA polymerase from Gibco under following conditions: 94 °C 5 min → 35x (94 °C 1 min, 60 °C 1.5 min, 72 °C 1 min for fragments ≤ 1 000 bp, 2 min for fragments 1 000 - 2 000 bp) → 72 °C 5 min. The reaction mixture consisted of 1 µl template DNA, 1 µl forward primer (10 pmol/µl), 1 µl reverse primer (10 pmol/µl), 12 µl H_2O and was covered with 20 µl mineral oil. PCR mixture (5 µl) was added after 4 min heating at 95 °C (hot-start PCR).

PCR mixture (for one sample):

2 µl 10x PCR buffer (supplied with *Taq* DNA polymerase)
2 µl 50 mM $MgCl_2$
1 µl dNTP solution (10 mM: 2.5 mM each)
0.2 µl *Taq* DNA polymerase

The full-length cDNA was amplified using Advantage® cDNA polymerase from Clontech under following conditions: 94 °C 1 min → 35x (94 °C 0.5 min, 60 °C 1.5

min, 72 °C 3 min) → 72 °C 5 min. The reaction mixture consisted of 2 µl template DNA, 1 µl forward primer (10 pmol/µl), 1 µl reverse primer (10 pmol/µl), 17.5 µl H_2O, 2.5 µl Advantage polymerase buffer (10x), 0.5 µl dNTP solution (40 mM: 10 mM each), 0.5 µl Advantage DNA polymerase and was covered with 25 µl mineral oil.

9.3.3. Rapid amplification of cDNA ends

The 3'- and 5'-RACE-PCR was carried out using Marathon™ cDNA Amplification Kit and Advantage® cDNA polymerase from Clontech according to the manufacturer's manual. The reaction mixture consisted of 5 µl template DNA, 1 µl gene specific primer primer (20 pmol/µl), 1 µl adapter primer (20 pmol/µl), 14.5 µl H_2O, 2.5 µl Advantage polymerase buffer (10x), 0.5 µl dNTP solution (40 mM: 10 mM each), 0.5 µl Advantage DNA polymerase and was covered with 25 µl mineral oil. The touch-down PCR was conducted under following conditions: 94 °C 1 min → 5x (94 °C 0.5 min, 70 °C 2 min) → 5x (94 °C 0.5 min, 68 °C 2 min) → 25x (94 °C 0.5 min, 66 °C 2 min) → 72 °C 10 min.

9.4. Cloning of DNA fragments

9.4.1. Ligation into a cloning vector

The DNA fragments amplified with Taq DNA polymerase were ligated into pGEM®-T Easy cloning vector (Promega). The reaction mixture consisting of 7 µl insert DNA, 1 µl (50 ng) vector DNA, 1 µl 10x T4 Ligase Buffer (supplied with the enzyme) and 1 µl T4 DNA ligase (Promega) and was incubated at 16 °C over night (12 – 16 h).

9.4.2. Transformation into bacterium cells

E. coli strain TOP10 (Invitrogen) was used for cloning of DNA fragments ligated in pGEM®-T Easy vector. The competent bacterium cells (50 µl, see next chapter) were

allowed to thaw on ice (ca. 5 min) and added carefully to the ligation mixture (10 µl, see previous chapter). After 30 min incubation on ice the sample was warmed to 37 °C for 1.5 min and placed on ice for 2 min. The warm (37 °C) LB medium without antibiotica (450 µl) was added and the cells were cultivated at 37 °C during 1h. Thereafter the cell suspension was streaked over agar plates of solid LB medium supplemented with 50 mg/l ampicillin and cultivated at 37 °C over night (12 – 16 h). The ampicillin resistant single colonies were picked and inoculated into 3 ml liquid LB medium supplemented with 50 mg/l ampicillin.

9.4.3. Preparation of competent *E. coli* cells

The bacterium cells (1 ml of an over night grown culture) were inoculated into 100 ml fresh liquid LB medium without antibiotica and cultivated at 37 °C until the OD_{600} of 0.5 was reached (ca. 2h). The flask was cooled on ice, the cells were sedimentated by centrifugation (5 min, 4 000 xg, 4 °C) and taken up in 30 ml TFB1 buffer. After 90 min incubation on ice the suspension was centrifuged (5 min, 4 000 xg, 4 °C) and the pellet was taken up in 4 ml of TFB2 buffer. The cell suspension was aliquoted in Eppendorf tubes (100 – 250 µl), frozen in liquid nitrogen and stored at –80 °C.

TFB1 Buffer:

100 mM RbCl;
50 mM $MnCl_2$;
30 mM KOAc;
10 mM $CaCl_2$;
15% (V/V) glycerol;
adjust pH to 5.8 with HCl,
sterilize by filtration

TFB2 Buffer:

10 mM Mops;
10 mM RbCl;
75 mM $CaCl_2$;
15% (V/V) glycerol;
adjust pH to 8.0 with KOH,
sterilize by filtration

9.5. Heterologous expression in *E. coli*

9.5.1. Subcloning into expression vectors

The DNA fragments were excised from cloning vectors using restriction enzymes purchased from New England Biolabs. The reaction mixture (total volume 30 µl) contained 5 – 15 µl DNA (500 – 1000 ng), 0.5 µl each restriction enzyme and 3 µl corresponding buffer (supplied with the restrictases) and was incubated at 37 °C for 1h. The excised insert was separated from vector DNA in 1% agarose gel and eluted as described in **II.9.2.1.** The expression vectors (pSE280 from Invitrogen or pTYB1 from New England Biolabs) were digested and prepared in the same way. The insert DNA was ligated into the expression vectors as described in **II.9.4.1.**

9.5.2. Expression of plant glucosidases in bacterium cells

The DNA ligated into pSE280 vector was transformed into *E. coli* strain TOP10 (Invitrogen) as described in **II.9.4.2.** and expressed without IPTG induction.

For expression of proteins in fusion with intein affinity tag, IMPACT™-CN system (New England Biolabs) was used according to the manufacturer's manual. The DNA fragments ligated into pTYB1 vector were expressed in *E. coli* strain ER2566 (New England Biolabs). Preparation and transformation of the competent cells were carried out in the same way as described for TOP10 strain (see **II.9.4.2.** and **II.9.4.3.**). Expression was induced by addition of 0.5 mM IPTG (AppliChem, Darmstadt, Germany) to the bacterium suspension.

10. Sequence analysis

The deduced amino acid sequences were scanned for occurrence of conserved patterns against PROSITE database (Hofmann et al., 1999). For prediction of transmembrane helices the servers HMMTOP (Tusnady and Simon, 1998), TMHMM (Sonnhammer et al., 1998) and SOSUI (Tokyo University of Agriculture &

Technology) were used. The subcellular localization was predicted by PSORT server (Nakai and Kanehisa, 1992).

11. Protein isolation and purification

11.1. Preparative expression of plant glucosidases in *E. coli*

For purification of raucaffricine glucosidase 2 l of bacterium culture (TOP10 *E. coli* strain transformed with pSE280::RG construction) were grown in liquid LB medium supplemented with 50 mg/l ampicillin over night at 37 °C without IPTG induction.

For intein affinity tag mediated purification of strictosidine glucosidase 100 ml of bacterium suspension grown over night at 37 °C (*E. coli* strain ER2566 transformed with pTYB1::SG construction) were inoculated into 2.5 l of fresh LB medium supplemented with 50 mg/l ampicillin and cultivated at 28 °C. When the OD_{600} of ca. 0.5 was reached (after 5-6h), IPTG was added (final concentration 0.5 mM) to induce expression and the cells were cultivated at 28 °C for 13-24h.

11.2. Preparation of crude extracts from *E. coli* cells

To obtain a crude enzyme preparation 100 ml of *E. coli* culture (TOP10 strain transformed with pSE280::RG or pSE280::SG construction) grown in liquid LB medium supplemented with 50 mg/l ampicillin over night at 37 °C without IPTG induction were centrifuged (4 500 xg, 10 min), the cells taken up in 1 ml sterile H_2O and crashed with ultrasonic. The supernatant after centrifugation for 30 min at 35 000 xg was used to test the glucosidase activity.

For protein purification the bacterium cells (from 2 or 2.5 l of an over night grown suspension) were harvested by centrifugation (15 min, 5 000 xg, 4°C) and taken up in 100 ml buffer A (100 mM Tris-HCl, pH 7.5, 20 mM β-mercaptoethanol) or in 50 ml Cell Break Buffer (20 mM Tris/HCl, pH 8.0; 1 mM EDTA; 0.5 M NaCl; 0.1% Triton X-100) for the preparation of RG or SG, respectively. The cells were crashed with

French press and the supernatant after centrifugation for 30 min at 15 000 xg was used for further purification.

11.3. Ammonium sulfate precipitation

For ammonium sulfate precipitation of proteins, $(NH_4)_2SO_4$ powder was added to the clarified extract over a period of one hour (4°C, continuously stirring) up to a salt concentration of 30%. After centrifugation (30 min, 10.000 x 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). After another centrifugation, the supernatant was dialyzed against 10 l buffer B overnight.

11.4. Fast protein liquid chromatography

FPLC was carried out with an Äkta system (Pharmacia Biotech) consisting of P-900 pump connected to UV-900 and pH/C-900 monitors. The results were evaluated using UNICORN® software (Pharmacia Biotech).

11.4.1. Anion exchange chromatography

For anion exchange chromatography, the dialyzed proteins were applied onto a SOURCE 30Q XK 50/30 column (Pharmacia) equilibrated with buffer C (20 mM Tris-HCl, pH 8.0; 10 mM β-mercaptoethanol). After washing the column with 0.5 column volume (CV, 240 ml) of buffer C, the proteins were eluted with a linear KCl gradient (6 CV, 0-0.5 M KCl) prepared from buffers C and D (20 mM Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol, 1 M KCl) at a flow rate of 20 ml/min collecting 12 ml fractions. RG activity appeared at KCl concentrations of 0.23 - 0.26 M. Fractions containing the enzyme activity were pooled.

11.4.2. Hydrophobic interaction chromatography

The combined fractions were prepared for hydrophobic interaction chromatography by adding $(NH_4)_2SO_4$ to a resulting concentration of 1 M and resolved on a SOURCE 15 PHE XK 16/20 column (Pharmacia) equilibrated with buffer E (20 mM Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol; 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 collecting 8 ml fractions. 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, Germany) to the volume of 250 µl.

11.4.3. Size exclusion chromatography

11.4.3.1. Purification of raucaffricine glucosidase

Concentrated fractions after hydrophobic interaction chromatography were applied onto 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-mercaptoethanol at a flow rate of 30 ml/h collecting 0.5 ml fractions.

11.4.3.2. Determination of the molecular weight of strictosidine glucosidase

For molecular weight determination the size exclusion chromatography was conducted using the same column as described in previous chapter. The proteins were eluted with 20 mM Tris/HCl buffer, pH 8.0, containing 2 mM Na_2EDTA, 10% glycerol and 10 mM 2-mercaptoethanol at a flow rate of 24 ml/h collecting 0.1 or 0.5 ml fractions for SG activity test. Total proteins from 6 days old *R. serpentina* cell suspension culture (T-30 cell line) were extracted with the same buffer (1 ml per 1 g

cells). The sample was centrifuged (35 000 xg, 30 min) before loading onto the column.

11.5. Affinity chromatography

For purification of the SG fused to intein affinity tag, IMPACT™-CN system (New England Biolabs) was used according to the manufacturer's manual. The crude extract from *E. coli* cells (see **II.11.2.**) was loaded onto gravity flow column (∅ 3 cm) packed with chitin beads (20 ml) and pre-equilibrated with 200 ml of Column Buffer (20 mM Tris/HCl, pH 8.0; 1 mM EDTA; 0.5 M NaCl). After washing with 150 ml of Cell Break Buffer followed by 150 ml of Column Buffer the column was flashed with 50 ml of Cleavage Buffer (20 mM Tris/HCl, pH 8.0; 1 mM EDTA; 0.5 M NaCl; 50 mM DTT). The flow was stopped and the column kept for 23 h at 4°C for cleavage of intein tag. SG was eluted with Column Buffer (fraction size 0.5 ml). Fractions 3-22 with protein concentration higher than 15 µg/ml were bulked and dialyzed against 2 x 1 l of TE buffer (20 mM Tris/HCl, pH 8.0; 1 mM EDTA).

III. RESULTS

1. Strictosidine accumulation in plant cell suspension cultures

1.1. Alkaloid pattern of two somatic hybrid cell subcultures

The somatic hybrid cell line *RxR*17 between *Rauvolfia serpentina* and *Rhazya stricta* was obtained in 1988 and since 1989 it has been maintained both as cell suspension cultures (*RxR*17M) and as callus strains (*RxR*17K). The indole alkaloid patterns of these two subcultures were studied and compared in order to find out the best source of strictosidine. To stimulate the secondary metabolism of the plant cells the subcultures were treated with 100 µM methyl jasmonate (MJ) (Gundlach et al., 1992).

1.1.1. *RxR*17M cell line

Both cells and nutrition media were harvested on the 1st and 5th days after addition of 100 µM MJ as ethanol solution. Crude alkaloid extracts from the hybrid cells and the cultural media were investigated by HPLC. Control cultures treated with equal volumes of ethanol were analyzed in the same way. The identification of peaks was attained by fractionation of HPLC runs followed by TLC purification of collected alkaloids using the solvent system SS1 and their MS analysis. For quantification of major compounds the calibration curves based on the peak area were built using the standard indole alkaloids.

The major alkaloids found in extracts of the non-treated nutrition medium are represented by the ajmalan (ajmaline, 17-O-acetylajmaline), indolenine (vomilenine), yohimban (yohimbine, reserpine) and heteroyohimbine (ajmalicine) subgroups (Fig. 6a). These compounds and the glucoalkaloids raucaffricine, strictosidine and strictosidine lactam were identified in the crude alkaloid extract of the non-treated cells (Fig. 6b). All these alkaloids were reported earlier to be isolated from *R. serpentina* cell suspension cultures (Stöckigt et al., 1981; Stöckigt et al., 1997). The

treatment of the cell suspension with MJ led to a general increase of alkaloid content (Table 1) and the accumulation of compounds which were found in the control samples in trace amounts only e.g. 17-O-acetylnorajmaline and a mixture of vallesiachotamine isomers (Fig. 6c, d; Fig. 7).

Table 1. The mean alkaloid concentrations in the nutrition medium and the tissue of *RxR*17M hybrid cell line.

Alkaloids	**Control**				**MJ-treated**			
	Tissue (mg/100g DW)		Medium (mg/l)		Tissue (mg/100 g DW)		Medium (mg/l)	
	1st day	5th day	1st day	5th day	1st day	5th day	1st day	5th day
17-O-Acetylajmaline	2.9	8.6	1.0	1.0	15.9	46.3	10.6	5.8
17-O-Acetyl-norajmaline	<1	2.6	0.1	0.1	49.2	111.6	14.7	4.8
Ajmaline	<1	2.2	<0.1	<0.1	2.3	7.8	2.3	0.4
Ajmalicine	2.1	3.7	<0.1	<0.1	3.4	4.3	0.4	0.4
Raucaffricine	33.9	76.4	<0.1	<0.1	139.1	341.6	<0.1	<0.1
Reserpine	1.0	2.0	<0.1	<0.1	2.9	5.5	0.1	0.1
Strictosidine	-	11.1	-	-	-	513.1	-	-

All differences between mean alkaloid concentrations in MJ treated and control cultures (Table 1) are significant with the exception of ajmalicine in the tissue both on the 1st day and on the 5th days after treatment. Under normal growth conditions the concentrations of all seven alkaloids increased in the tissue from the 6th to the 10th day of cultivation (which corresponds to the 1st and the 5th day after MJ treatment for elicited cultures) by a factor of 2-3. The same tendency is observed after treatment with MJ, but the alkaloid concentrations are significantly higher already on the 1st day compared with the untreated cells. On the 5th day the concentrations of strictosidine and 17-O-acetylnorajmaline in MJ treated cells are 46- and 43-fold higher, respectively, than in the control cultures. The content of 17-O-acetylajmaline and raucaffricine surpasses the value of the control cultures by 5.4 and 4.5 times,

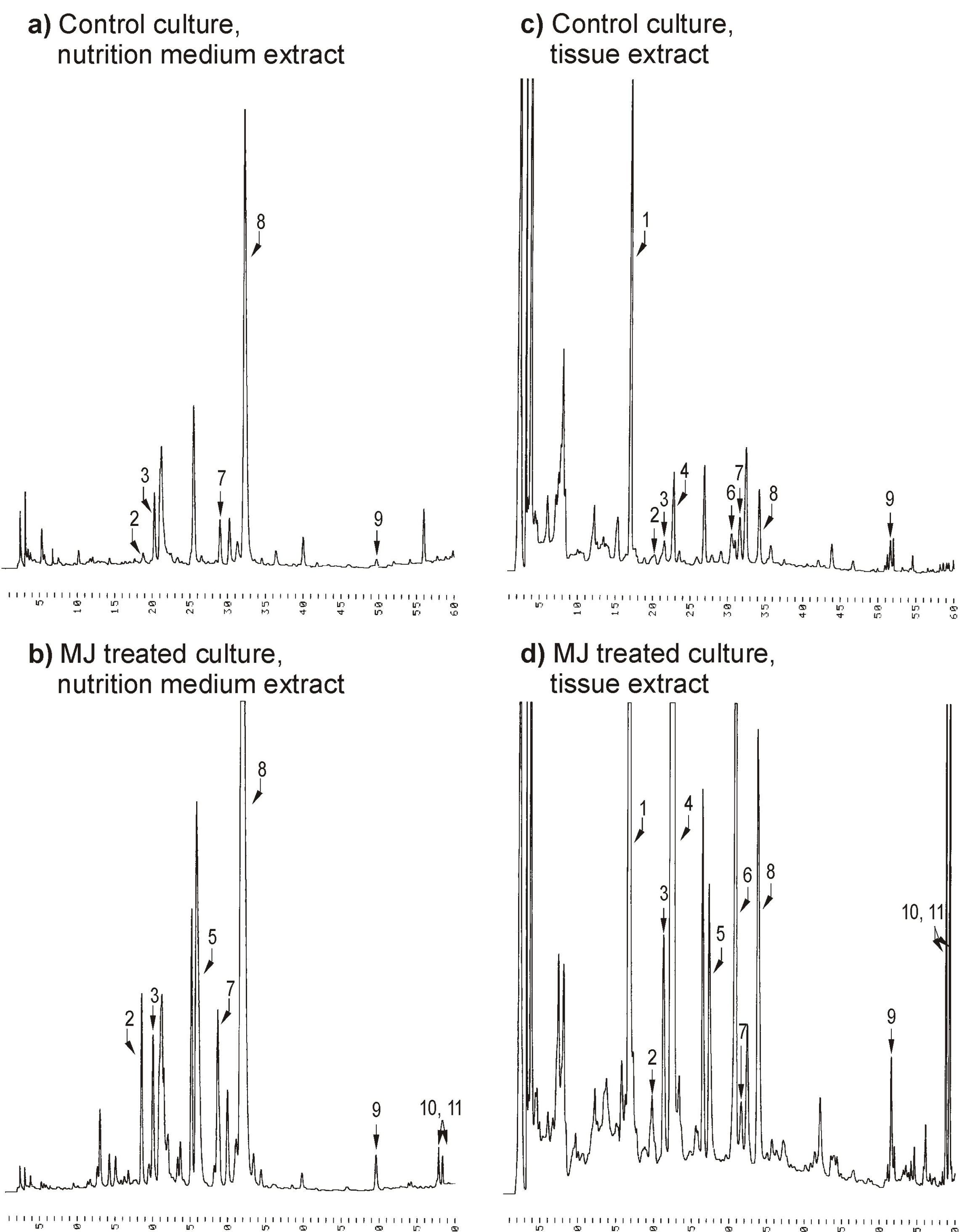


Fig. 6. HPLC separation of the extracts from the nutrition medium and the tissue of *RxR*17M hybrid cell line on the 5th day after treatment with 100 µM methyl jasmonate: 1) raucaffricine; 2) ajmaline; 3) vomilenine, yohimbine; 4) strictosidine; 5) 17-O-acetylnorajmaline; 6) strictosidine lactam; 7) ajmalicine; 8) 17-O-acetylajmaline; 9) reserpine; 10, 11) isomers of vallesiachotamine.

1) Raucaffricine, R=Glc
3) Vomilenine, R=H

2) Ajmaline, R_1=CH_3, R_2=H
5) 17-O-Acetylnorajmaline, R_1=H, R_2=Ac
8) 17-O-Acetylajmaline, R_1=CH_3, R_2=Ac

3) Yohimbine

4) Strictosidine

6) Strictosidine lactam

10,11) Vallesiachotamine isomers

7) Ajmalicine

9) Reserpine

Fig. 7. Indole alkaloids identified in *RxR*17M somatic hybrid cell suspension subculture. Numbers indicate corresponding HPLC peaks in Fig. 6.

respectively. For ajmaline, ajmalicine and reserpine, however, only a slight increase of ~ 2-3 fold was observed. Thus the biosynthesis of the different alkaloids is induced to varying extents.

1.1.2. *RxR*17K cell line

The hybrid cell subculture *RxR*17K was cultivated for about 10 years as callus strain and only six months before phytochemical investigation the cell suspension culture was established. The cells and nutrition media were harvested on the 1st and 5th days after treatment with 100 µM MJ and analyzed by HPLC in the same way as *RxR*17M cell line (Fig. 8). The major compounds identified in the control tissue extracts are tryptamine, stemmadenine, strictosidine, tubotaiwine, strictosidine lactam, 3-oxo-rhazinilam and vallesiachotamine isomers (Fig. 9). Among these, tryptamine is a biogenetic precursor of indole alkaloids; stemmadenine, strictosidine, strictosidine lactam and vallesiachotamine isomers were isolated in past from *R. stricta* plant or cultivated cells (Mariee et al., 1988; De Silva et al., 1971; Pawelka and Stöckigt, 1986). Tubotaiwine was identified earlier in *R. serpentina x R. stricta* somatic hybrid cell culture (Kostenyuk et al., 1995) and was not detected in parent plants. 3-Oxo-rhazinilam is a novel indole alkaloid (Gerasimenko et al., 2001b) structurally related to the cytotoxic rhazinilam known from *R. stricta* (Mukhopadhyay et al., 1981). Thus the secondary metabolite patterns of two somatic hybrid subcultures have diverged during the 10 years long cultivation in undifferentiated state. While the *RxR*17M cell line produces indole alkaloids characteristic for *R. serpentina*, most of the compounds identified in the *RxR*17K cell line are typical for *R. stricta*. The isolation of tubotaiwine and 3-oxo-rhazinilam indicates, that *RxR*17K cell suspension culture displays specific features of secondary metabolism which are probably the consequence of its hybrid nature.

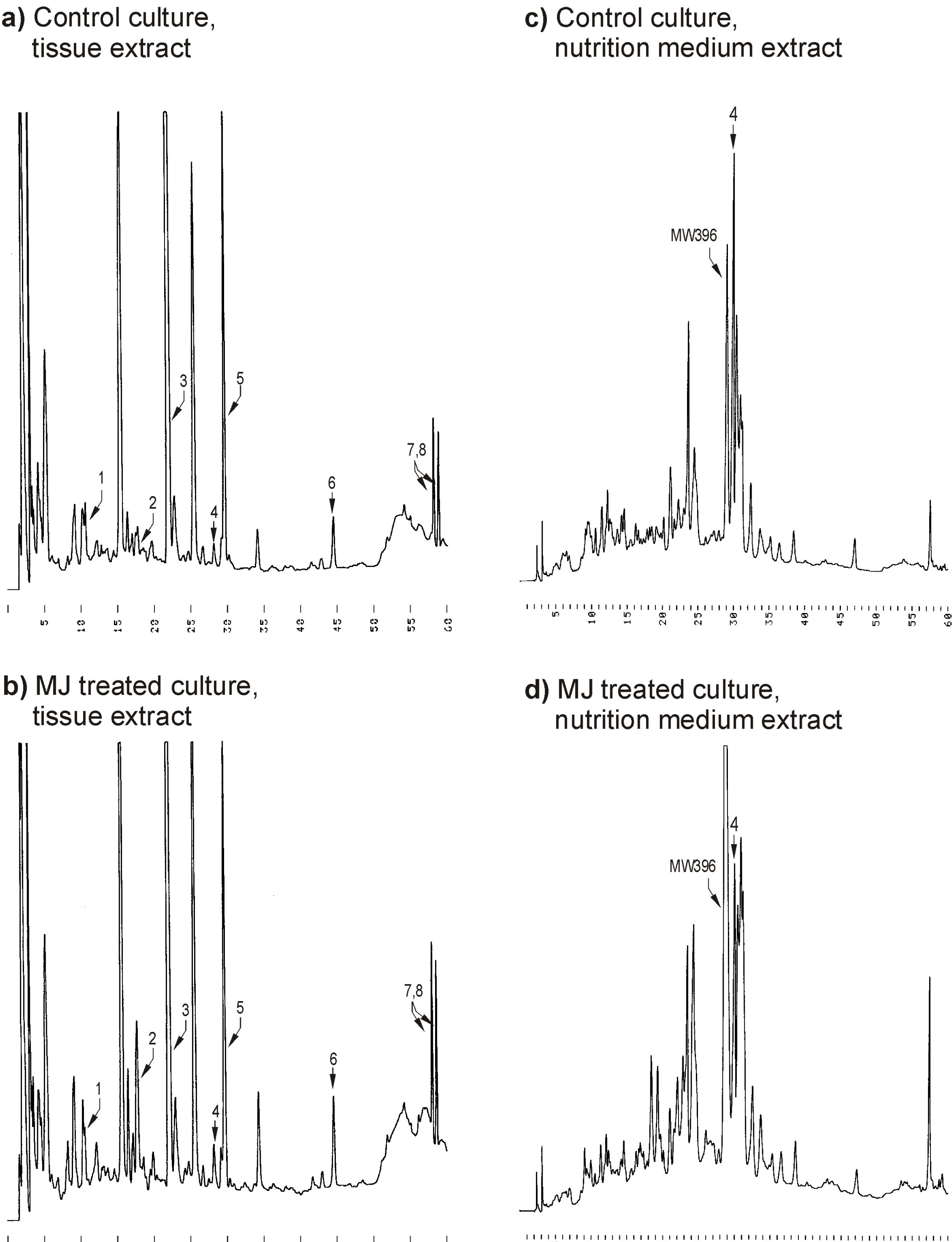


Fig. 8. HPLC separation of the extracts from the nutrition medium and the tissue of *RxR*17M hybrid cell line on the 5th day after treatment with 100 μM methyl jasmonate: 1) tryptamine; 2) stemmadenine; 3) strictosidine; 4) tubotaiwine; 5) strictosidine lactam; 6) 3-oxo-rhazinilam; 7,8) isomers of vallesiachotamine; MW396 – unidentified alkaloid.

1) Tryptamine

2) Stemmadenine

3) Strictosidine

4) Tubotaiwine

5) Strictosidine lactam

6) 3-Oxo-rhazinilam

7,8) Vallesiachotamine isomers

Fig. 9. Compounds identified in *RxR*17K somatic hybrid cell suspension subculture. Numbers indicate corresponding HPLC peaks in Fig. 8.

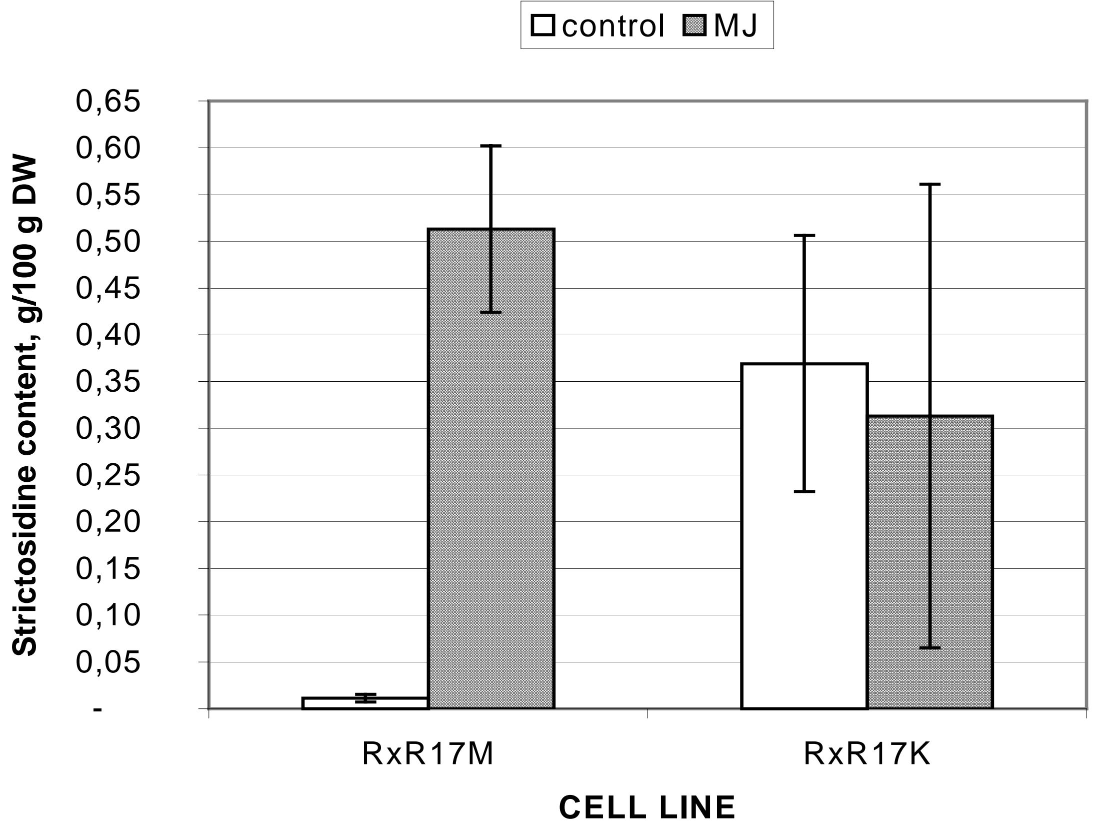


Fig. 10. Strictosidine content in *R. serpentina x R. stricta* somatic hybrid cell lines. Control – non treated cultures, the 10th day of cultivation; MJ – the 5th day after treatment with 100 µM MJ. 100 g DW corresponds to ca. 7 l of cell suspension.

In contrast to *RxR*17M cell line, where MJ treatment led to a significant increase in the indole alkaloid content both in the cells and in the nutrition medium, *RxR*17K hybrid subcultures treated with MJ did not exhibit a substantial alteration of the alkaloid content in the cells as compared with the control samples (Fig. 8a, b). The accumulation of some compounds in the nutrition medium was increased by MJ treatment (for example, the content of unidentified alkaloid with MW 396 surpassed after MJ treatment about 5-fold the content in the control samples, Fig. 8c, d), while the concentrations of other metabolites did not change. It is noteworthy that the concentration of strictosidine on the 10th day of cultivation (corresponding to the 5th

day after MJ treatment for the elicited cultures) in the untreated *RxR*17K cells was much higher than in *RxR*17M hybrid cells (0.369 g/100 g DW and 0.011 g/100 g DW, respectively). The treatment with 100 µM of MJ led to the strong induction of biosynthesis and/or accumulation of strictosidine in *RxR*17M hybrid cells but did not influence the content of this alkaloid in *RxR*17K suspensions (Fig. 10).

1.2. Isolation and purification of strictosidine

The *RxR*17K somatic hybrid cell suspension culture was used for preparative isolation of strictosidine because of high content of this alkaloid without MJ treatment. In addition, the absence of raucaffricine in this cell line simplifies the purification of other glucoalkaloids.

In a typical procedure, dried cells (150 g) were grounded to powder and extracted with 15 l MeOH with sonication. The crude methanol extract was purified by the method of Smith giving 3.33±0.88 g of enriched alkaloid extract. It was separated by flash chromatography using solvent system SS1 and the collected fractions were tested by TLC with the same solvent system. The fractions containing strictosidine were bulked and separated by preparative TLC using solvent system SS2. The substance at Rf 0.40, which displayed a brown-greenish CAS colour and blue fluorescence, was collected yielding 37.7±2.6 mg of strictosidine. The ^{1}H NMR data of the isolated substance were identical with those reported for strictosidine (Patthy-Lukats et al., 1997). The acetylation of the alkaloid resulted in formation of strictosidine pentaacetate, which was identified by comparison of its MS spectrum with literature data (Stöckigt, 1979).

2. Strictosidine hydrolysis by the raucaffricine glucosidase

Raucaffricine glucosidase (RG) converting raucaffricine to vomilenine (Fig. 4) was the first glucosidase involved in indole alkaloid biosynthesis in *R. serpentina* cell suspension culture for which the purification (Warzecha et al., 1999), cDNA cloning and heterologous expression in *E. coli* was achieved (Warzecha et al., 2000). Although the RG enriched from the plant cells was shown not to hydrolyse strictosidine (Schübel and Stöckigt, 1986), the substrate specificity of this glucosidase was tested once more using the sufficient amount of pure enzyme available after heterologous expression in order to find out whether the RG is able to catalyze the strictosidine deglucosylation in *R. serpentina*.

2.1. Subcloning of the cDNA encoding RG

The full-length cDNA encoding RG found after screening of *R. serpentina* cDNA library (Warzecha, 2000) was subcloned from the pBlueScript phagemid to the pSE280 expression vector. pBlueScript phagemid containing the RG cDNA was digested with *Nco*I and *Xho*I restriction enzymes. As the cDNA sequence of the RG reveals an additional *Nco*I recognition site, the partial digestion was carried out in order to obtain the full ORF of 1623 bp. After restriction during different times (2, 5, 8, 12, 15, 20, 40 and 60 min) the resulting DNA fragments were separated in 1% agarose gel. The fragment of necessary size was excised, eluted and ligated into the pSE280 vector digested with *Nco*I and *Xho*I restriction enzymes. The obtained construction was transformed into TOP10 *E. coli* strain and the transformants were selected on LB medium supplemented with 50 mg/l ampicillin. The plasmid DNA isolated from positive clones was digested with *Hind*III restriction enzyme to prove that the fragment of the right size was inserted. The fragment of ca. 1600 bp was

detected in all twelve clones tested. The sequencing of the insert in clone 24 confirmed that it presents the full-length RG ORF without mutations.

2.2. Expression of the RG in *E. coli*

To check the RG activity 50 µl *E. coli* suspension were inoculated into 5 ml LB medium supplemented with 50 mg/l ampicillin. After incubation during 4h at 37 °C IPTG was added (final concentration 3 mM) to induce the expression. After 1h the cells were sedimentated by centrifugation, taken up in 100 µl sterile water and crashed with ultrasonic. The supernatant after centrifugation for 30 min at 35 000 xg was used for the enzyme activity test.

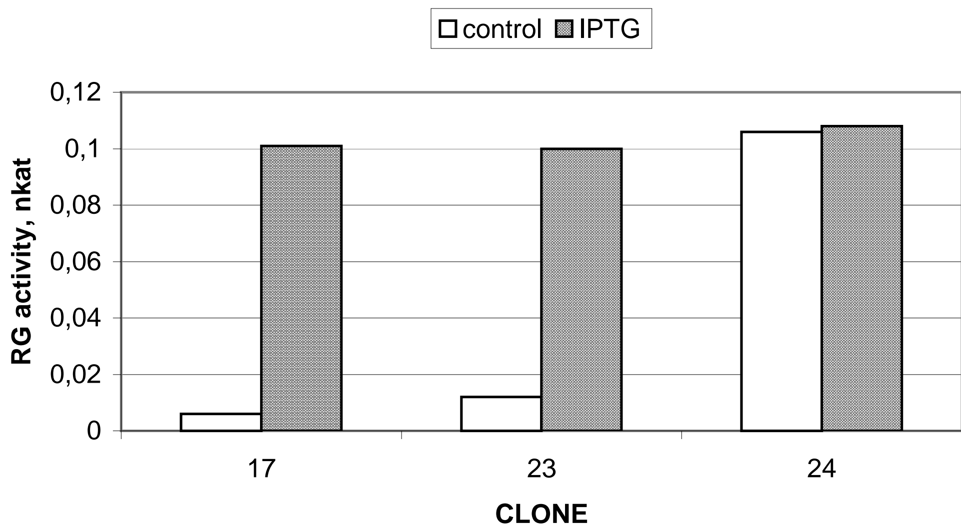


Fig. 11. Raucaffricine glucosidase activity of selected *E. coli* clones transformed with pSE280 vector containing the full-length RG ORF. Control – non induced cells, IPTG – induced with 3 mM IPTG for 1h.

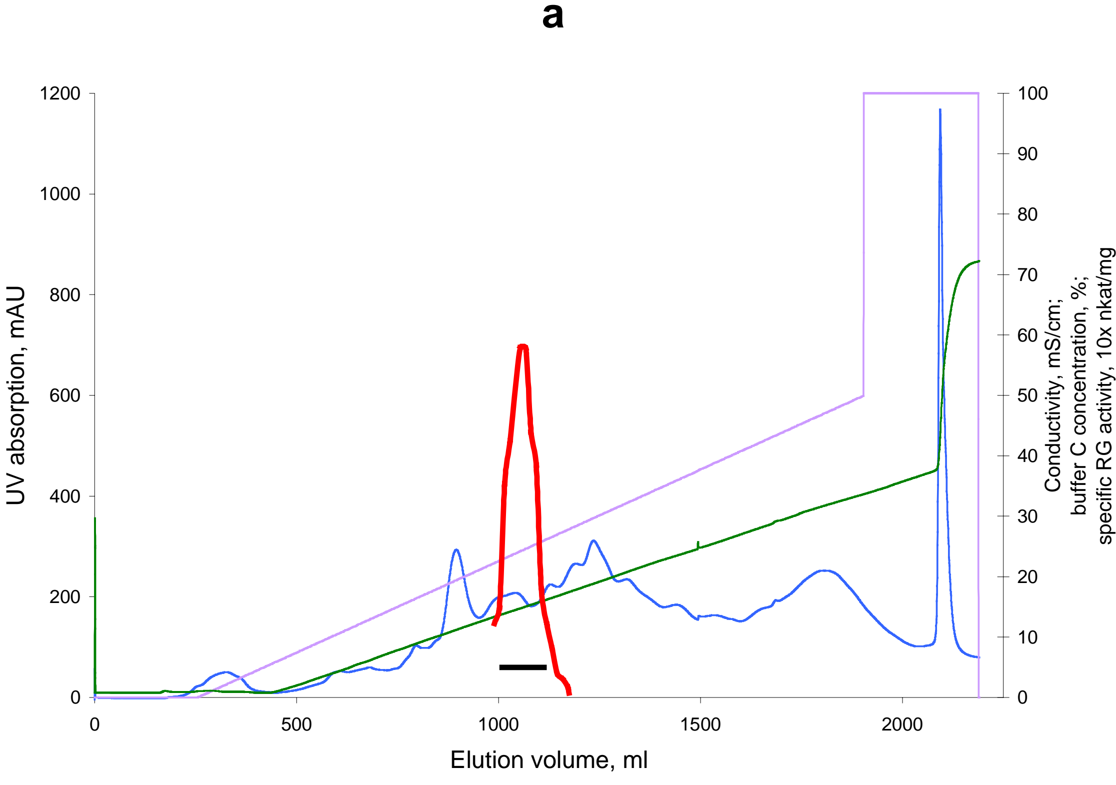
All three clones tested (17, 23 and 24) showed RG activity. Moreover, some amount of active enzyme was present also without IPTG induction (Fig. 11). To verify that

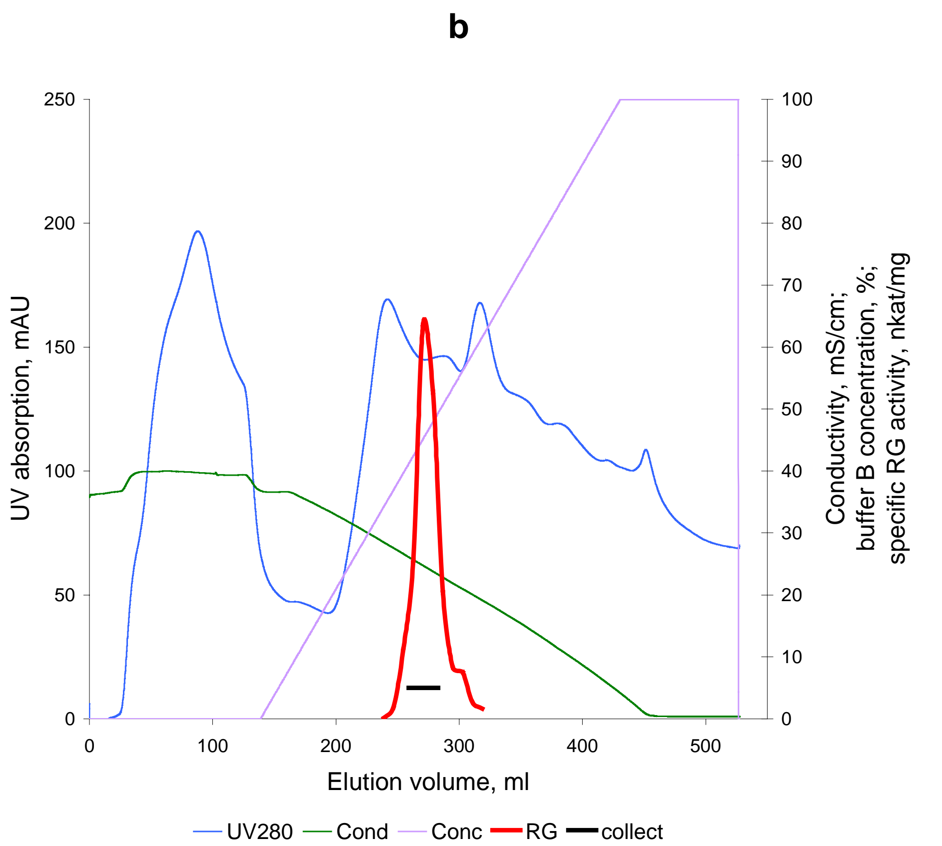
the host *E. coli* strain has no own raucaffricine glucosidase activity, a control clone was tested. This clone harbors a pSE280 vector with partial RG sequence (obtained after complete digestion with *Nco*I and thus lacking the 560 bp at 5'-end) as insert. No RG activity could be detected with these bacteria.

2.3. Purification of the heterologously expressed RG

For preparative purification of the RG clone 24 was used. Bacterium culture (2 l) was grown over night at 37 °C without IPTG induction. The cells were harvested by centrifugation and crashed in French press. After centrifugation the supernatant was fractionated by addition of ammonium sulfate. The proteins precipitated at 30-75% saturation were dissolved, dialyzed and separated by anion exchange chromatography on a Source30Q column (Fig. 12a). The RG activity eluted at 0.23 – 0.26 M KCl. Fractions containing the enzyme were bulked and after addition of ammonium sulfate to the concentration of 1 M the solution was applied onto the hydrophobic interaction column SourcePhe. The highest specific activity was found at 0.59 - 0.51 M $(NH_4)_2SO_4$ (Fig. 12b). The corresponding fractions were bulked, concentrated to the volume of 250 µl and separated by size exclusion chromatography on the Superdex75 column (Fig. 12c). The fractions revealing RG activity were analyzed by SDS-PAGE. One of them (fraction 6) contained homogenous enzyme as shown by Coumassie staining (Fig. 13). This fraction was used for substrate specificity studies.

The developed three-stage purification allowed to enrich the RG 355-fold (Table 2). It should be noted, that the protein amount at the final step of purification was too low to allow the direct measurement of protein concentration by the methods of Bradford or Lowry. The protein concentration in the fraction 6 after size-exclusion chromatography was determined by comparison of the area under the UV absorption curve of this fraction and the following ones, in which the protein content was





(Fig. 12: legend on the next page)

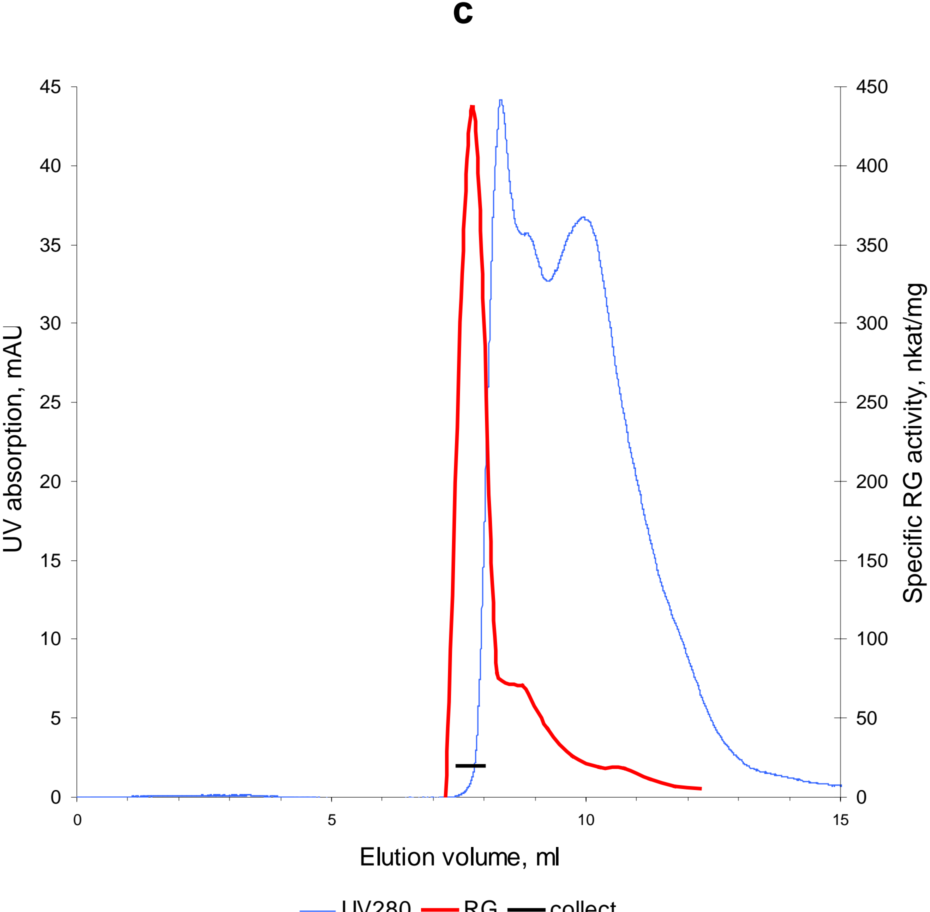


Fig. 12. Purification of heterologously expressed raucaffricine glucosidase: a) anion exchange chromatography on Source30Q column; b) hydrophobic interaction chromatography on SourcePhe column; c) size exclusion chromatography on Superdex75 column.

UV280 – UV absorption profile at 280 nm, mAU; conc – concentration of elution buffer, %; cond – conductivity, mS/cm; RG – specific raucaffricine glucosidase activity; collect – bulked fractions.

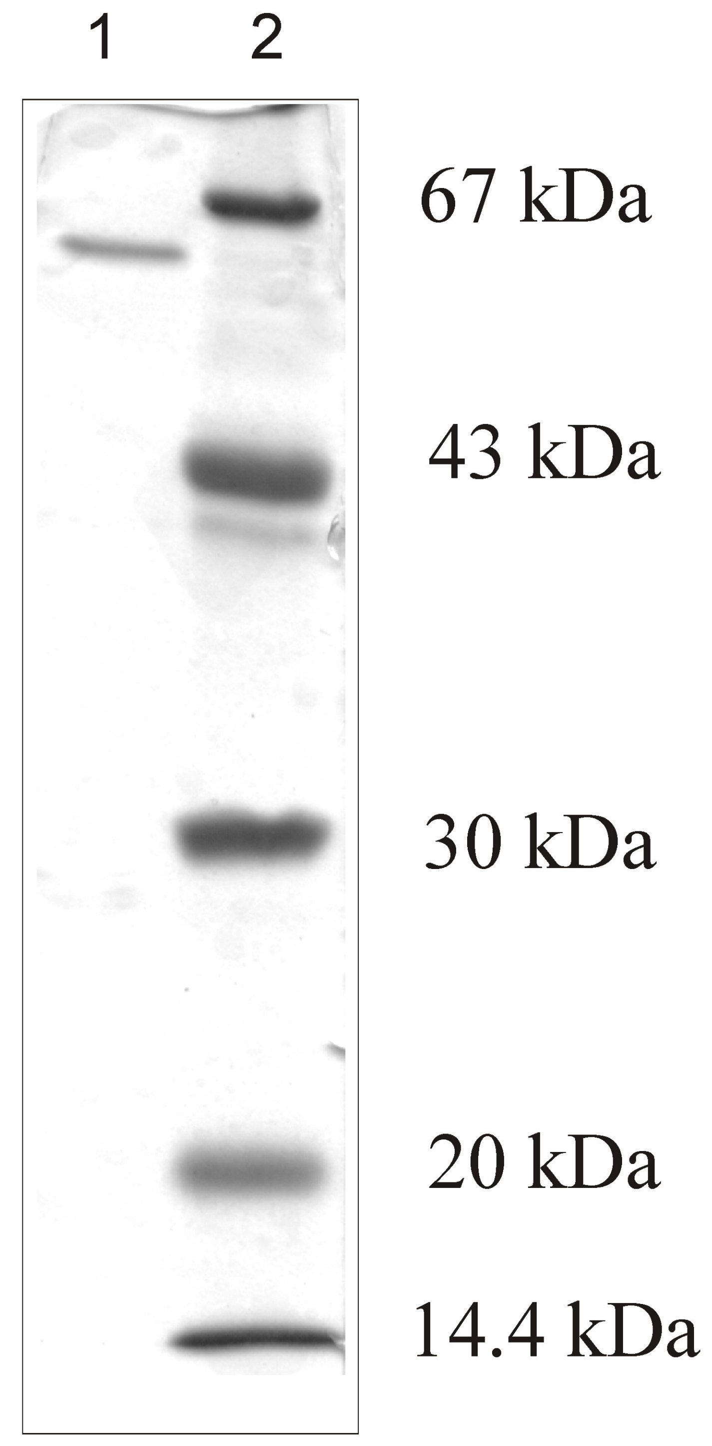


Fig. 13. Coomassie stained SDS-PAGE of purified heterologously expressed raucaffricine glucosidase: **1)** fraction 6 after size exclusion chromatography containing homogenous enzyme; **2)** protein molecular weight marker.

measured by the method of Bradford. Thus the data about the total protein amount and, respectively, the specific activity and enrichment factor after the size-exclusion chromatography should be regarded as approximate estimations only.

Table 2. Purification scheme for the raucaffricine glucosidase heterologously expressed in *E. coli*.

Purification stage	Volume, ml	Total protein, mg	Total RG activity, nkat	Specific activity, nkat/mg	Yield, %	Enrichment factor
Crude extract	94	230.3	283.1	1.23	100	1
Anion exchange chromatography	96	45.1	195.4	4.33	69.0	3.5
Hydrophobic interaction chromatography	24	1.9	89.7	47.21	31.7	38.4
Size-exclusion chromatography	0.5	0.005	2.185	437	0.77	355.3

2.4. Preparation of glucosides structurally related to raucaffricine

In order to investigate the ability of RG to hydrolyze indole glucoalkaloids with different structural features, several derivatives of raucaffricine were prepared.

The deacetylation of raucaffricine leads to the formation of an unstable 17-hydroxyindolenine structure, which converts spontaneously to the 21-gluco-hydroxysarpagan-17-al possessing the sarpagine ring system (Fig. 14a). 10 mg (19.5 μmol) raucaffricine were incubated in 1 N NaOH during 1.5 h. As a result, 4.6 mg (9.8 μmol) 21-gluco-hydroxysarpagan-17-al were isolated (50% yield). The substance was identified by the grey CAS colour and EI-MS data. To ascertain the stereochemical configuration at C-16 the ^{1}H NMR spectrum was measured (Fig. 15). For such type of alkaloids the resonance of the aldehyde proton is expected to appear at ca. 9.7 ppm in case of the exo-position of this group (Pfitzner and Stöckigt, 1983) and at ca. 9.1 ppm in case of the endo-configuration (Sakai et al., 1980).

a)

Raucaffricine → (KOH) → [Deacetylraucaffricine] → 21-Gluco-hydroxysarpagan-17-al

Raucaffricine → (KBH_4 or $NaBCNH_3$) → 1,2-Dihydroraucaffricine

Raucaffricine → ($NaBCNH_3/CH_2O$) → 1-Methyl-1,2-dihydroraucaffricine

21-Gluco-hydroxysarpagan-17-al → (KBH_4 or $NaBCNH_3$) → 21-Gluco-hydroxysarpagan-17-ol

b)

17-O-Acetylrauglucine

Fig. 14. Chemically (a) and enzymatically (b) synthesized glucoalkaloids structurally related to raucaffricine.

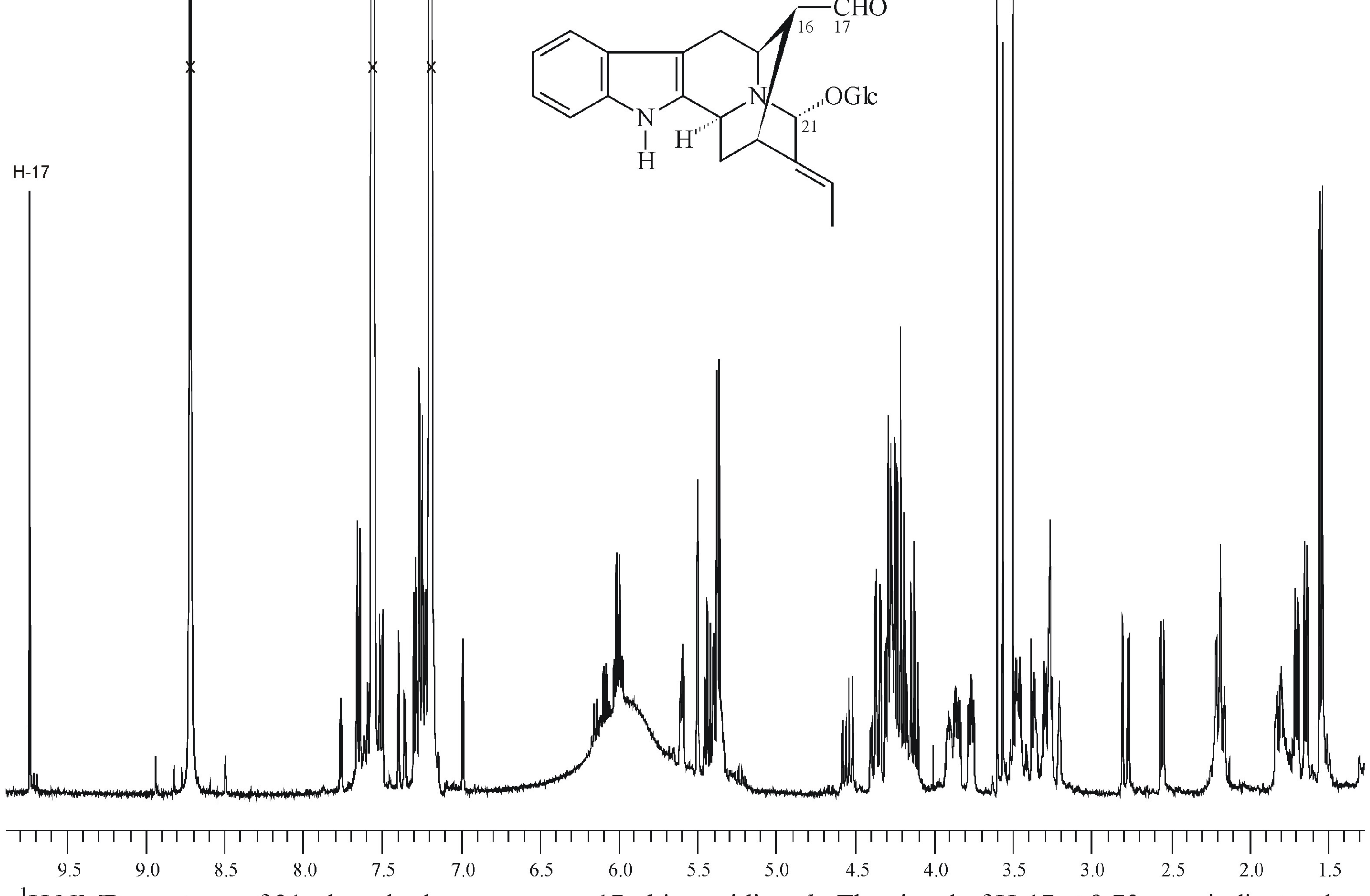


Fig. 15. The ^{1}H NMR spectrum of 21-gluco-hydroxysarpagan-17-al in pyridine-d_5. The signal of H-17 at 9.73 ppm indicates the exo-position of C-17 aldehyde group.

In the ^{1}H NMR spectrum of the obtained 21-gluco-hydroxysarpagan-17-al the signal of the aldehyde proton is found at 9.73 ppm, which means that the C-17 aldehyde group is in the exo-position.

After incubation of raucaffricine with KBH_4 or $NaBCNH_3$ two products were formed. Besides the anticipated 1,2-dihydroraucaffricine (Fig. 14a) recognized by the orange CAS colour, the second substance was detected after TLC separation. It had grey CAS colour, which suggested the sarpagine skeleton (Court and Iwu, 1980). This indicates, that raucaffricine was deacetylated during the reduction procedure. However, the MS spectrum revealed a molecular weight of 472, which shows that further conversion of the 21-gluco-hydroxysarpagan-17-al took place, namely the aldehyde group was reduced resulting in 21-gluco-hydroxysarpagan-17-ol (Fig. 14a). The ratio between this alkaloid and 1,2-dihydroraucaffricine depends on the reducing agent used. Incubation of 10 mg (19.5 µmol) raucaffricine with KBH_4 resulted in 2.8 mg (5.9 µmol) of 21-gluco-hydroxysarpagan-17-ol (30% yield) and 0.9 mg (1.8 µmol) of 1,2-dihydroraucaffricine (9% yield). On the contrary, when 20.5 mg (40 µmol) raucaffricine were incubated with $NaBH_3CN$, 1.8 mg (3.8 µmol) of 21-gluco-hydroxysarpagan-17-ol (9.5% yield) and 6.3 mg (12.3 µmol) of 1,2-dihydroraucaffricine (30.8% yield) were obtained. The cause of this difference may be the more basic pH conditions created after dissolving the KBH_4 in methanol than the $NaBH_3CN$ in 0.1 M citrate-phosphate buffer (pH 5.0).

10 mg (19.5 µmol) raucaffricine were methylated using $NaBH_3CN$ and HCHO giving 6 mg (11.4 µmol) 1-methyl-1,2-dihydroraucaffricine (58.5% yield). As well as 1,2-dihydroraucaffricine, this alkaloid was identified by the MS data. It was shown earlier (Schübel, 1986), that the chemical reduction of raucaffricine using the described

method results in addition of H-2 in α-position. Therefore it was postulated, that both obtained alkaloids have the 2α(S) configuration.

On the contrary, the glucoalkaloids synthesized in *R. serpentina* cell suspension cultures have the 2β(R) configuration (Ruyter et al., 1988). One of these natural alkaloids, the 17-O-acetylrauglucine (Fig. 14b) was isolated in minor amounts from the *RxR*17M somatic hybrid cell line (Sheludko et al., 1999) and used in the RG substrate specificity studies.

2.5. Hydrolysis of various glucosides by the RG

To test the glucosidase activity with different substrates, the glucose formed in the enzymatic reaction was measured using the Trinder glucose reagent. This method is based on a coupled enzyme system which consists of glucose oxidase and peroxidase. The liberated glucose is first oxidised by the glucose oxidase to glucuronic acid and hydrogen peroxide. The peroxidase catalyzes the reaction of the produced hydrogen peroxide with 4-aminoantipyrine and p-hydroxybenzene sulfonate, which results in formation of a quinoneimine dye with an absorbance maximum at 505 nm. The intensity of the colour is directly proportional to the glucose concentration in the sample.

The pure RG (10 µl, ca. 0.08 µg) was incubated with 400 nmol of the corresponding substrate in a total volume of 100 µl at 28 °C for 12 h. After addition of the double volume of methanol to stop the reaction, 200 µl of the resulting mixture were added to 1 ml of Trinder glucose reagent and the absorbance at 505 nm was recorded after 30 min. The control samples without RG were treated in the same way. To prove that the glucosidase was active during all the reaction time, the enzyme was incubated under the same conditions without substrate. After 12 h 400 nmol of raucaffricine were added and the incubation proceeded for 1 h. The liberated glucose was detected with

Trinder glucose reagent, which shows that the enzyme is stable at 28 °C during at least 12 h.

The smallest quantity of glucose that may be measured by the method used is 10 nmol/sample, which means the detection limit for the glucosidase activity assay of 0.23 pkat (10 nmol glucose liberated in 12 h). The activity with raucaffricine was determined to be 77.3 pkat/sample (30 min incubation time). Thus the detection limit for the glucosidase activity amounts to 0.3% of the activity with raucaffricine.

To calculate the relative conversion rate of the raucaffricine derivatives which were fully hydrolyzed by the RG during 12 h (21-gluco-hydroxysarpagan-17-al, 21-gluco-hydroxysarpagan-17-ol, 1,2-dihydroraucaffricine, and 1-methyl-1,2-dihydroraucaffricine), shorter incubations (30 min) in presence of 2.5 µl (ca. 0.02 µg) RG and 200 nmol of substrate were carried out. In this case the activity with raucaffricine was 40.8 pkat/sample.

Raucaffricine was proved to be the preferred substrate for RG (Table 3). The glucoalkaloids with sarpagine ring system were hydrolyzed with high efficiency. The relative glucosidase activity with 21-gluco-hydroxysarpagan-17-al was 77.8%, the alkaloid with reduced C-17 aldehyde group was hydrolyzed at a rate of 62.8%. As well as it has been shown earlier using partially purified plant RG (Schübel and Stöckigt, 1986), 1,2-dihydroraucaffricine with the unnatural 2α(S) configuration is deglucosylated at a sufficient rate of 70.9%, while its methylated analog, 1-methyl-1,2-dihydroraucaffricine, has a significantly lower convertion rate of 40.5%. The stereochemistry at C-2 is important for the enzymatic reaction: 17-O-acetylrauglucine, a glucoalkaloid with 2β(R) configuration, is accepted at a very low rate of 4.2%.

The high amount of the RG available after the heterologous expression allowed to detect the ability of this enzyme to hydrolyze strictosidine, which was not described

Table 3. Substrate specificity of the heterologously expressed raucaffricine glucosidase.

Structure	Substrate	Relative activity
	Raucaffricine	100%
	21-Gluco-hydroxysarpagan-17-al (R = CHO)	77.8%
	21-Gluco-hydroxysarpagan-17-ol (R = CH_2OH)	62.8%
	1,2-Dihydroraucaffricine (R = H; 2H-α)	70.9%
	1-Methyl-1,2-dihydroraucaffricine (R = CH_3; 2H-α)	40.5%
	17-O-Acetylrauglucine (R = CH_3; 2H-β, 19,20-dihydro)	4.2%
	Strictosidine	7.1%
	5α-Carboxystrictosidine (3H-α)	12.8%
	5α-Carboxyvincoside (3H-β)	0
	Strictosidine lactam (3H-α)	0
	Vincoside lactam (3H-β)	0

	Indoxyl-β-D-glucoside	0
	Secologanin	0
	Loganin	0
	Ipecoside	0
	p-Nitrophenyl-β-D-glucoside	0
	Arbutin	0
	6-Bromo-2-naphthyl-β-D-glucoside	0
	Esculin	0
	Fluorescein-β-D-glucoside	0
	Isoquercitrin	0
	Rutin	0
	Sinigrin	0
	Sucrose	0
	Vanillin-β-D-glucoside	0
	Zeatin-β-D-glucoside	0

for the partially purified RG from the *R. serpentina* cell suspension cultures (Schübel and Stöckigt, 1986). The conversion rate of this glucoalkaloid is low, 7.1% compared to raucaffricine. The substrate specificity of the RG concerning the strictosidine type of alkaloids is similar to that of the SG from *C. roseus*. 5α-Carboxyvincoside having the 3β(R) configuration is not accepted, whereas its 3α(S) isomer, 5α-carboxystrictosidine, is hydrolyzed even more effective than strictosidine. The lactamization of strictosidine leads to the loss of its ability to serve as a substrate for RG. The biogenetic precursor of strictosidine, the monoterpene secologanin, is not converted, as well as indoxyl-β-D-glucoside possessing the basic indole unit. Ipecoside, which derives from the condensation of secologanin and dopamine and has a structure similar to strictosidine with an indole moiety exchanged for another ring

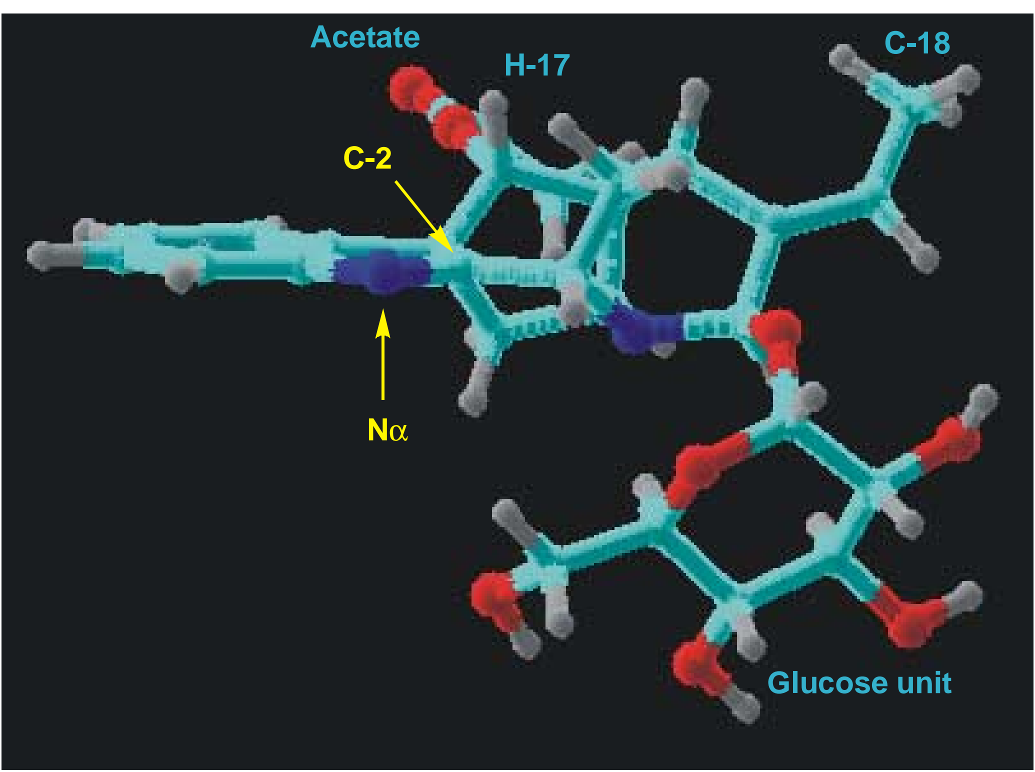


Fig. 16. The 3-D structure of raucaffricine (generated by PCMODEL). The positions most important for the raucaffricine glucosidase activity are indicated by arrows.

system, is not deglucosylated. All other 11 glucosides tested (Table 3) were not accepted as substrates, including the p-nitrophenyl-β-D-glucoside that is often used for detection of unspecific glucosidase activities.

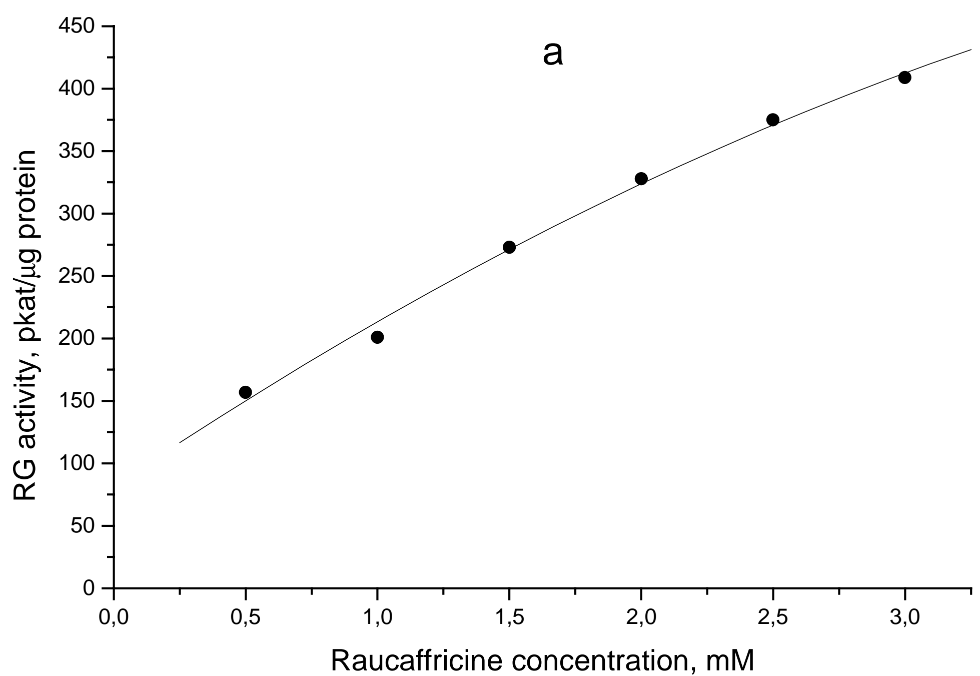
Thus the RG shows a high substrate specificity restricted to monoterpenoid indole alkaloids. The glucosides possessing indole or terpene unit itself are not hydrolysed. Among the accepted substrates, raucaffricine and its derivatives are clearly preferred. The sarpagine type glucoalkaloids with modified β-positioned part of the ring system (Fig. 16) are hydrolyzed with high efficiency, whereas the raucaffricine derivatives with structural changes in the flat indole moiety are converted at lower rates. The deglucosylation rate is significantly decreased if the methyl group at Nα or the proton at C-2 in β-position are present.

2.6. Enzyme kinetics of the RG

The K_M and V_{max} values for raucaffricine and strictosidine were determined by measuring the glucosidase activity in presence of different substrate concentrations.

With raucaffricine 0.25 µg protein were used. The enzyme activity enhanced with the increasing of substrate concentration from 0.5 mM to 3 mM (Fig. 17a). Using the method of Lineweaver and Burk (Fig. 17b) the K_M of 1.22 mM and V_{max} of 0.51 nkat/µg were calculated. The determined K_M is in accordance with the value of 1.4 mM reported for the partially purified RG from the *R. serpentina* cell suspension cultures (Schübel and Stöckigt, 1986).

The enzyme kinetic parameters with strictosidine were defined in presence of 4 µg RG. The normal Michaelis-Menten curve was build for the strictosidine concentration range from 1 mM to 4 mM (Fig. 18a). The K_M value of 1.82 mM and V_{max} of 2.56 pkat/µg were computed from these data (Fig. 18b).



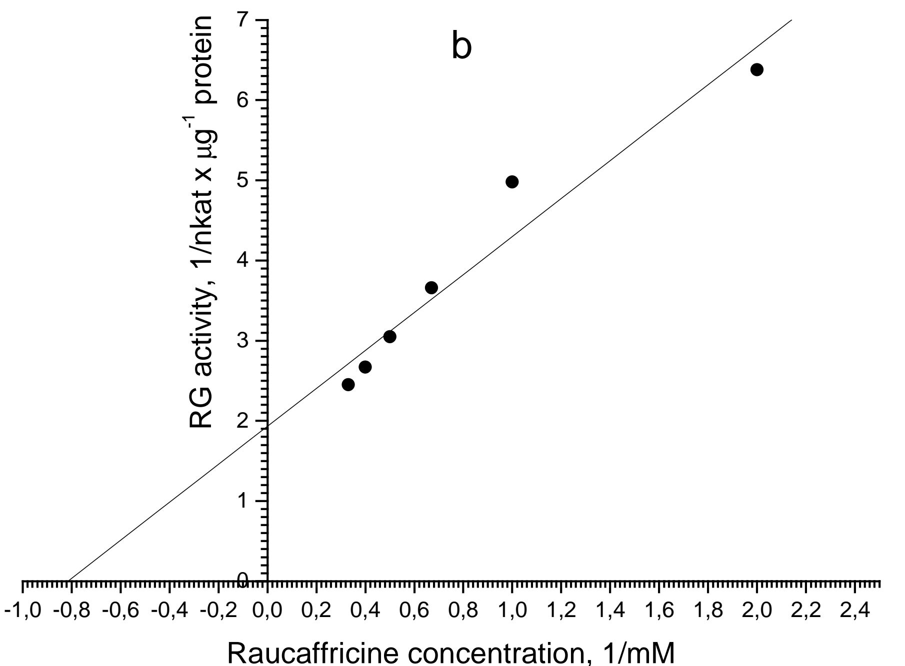
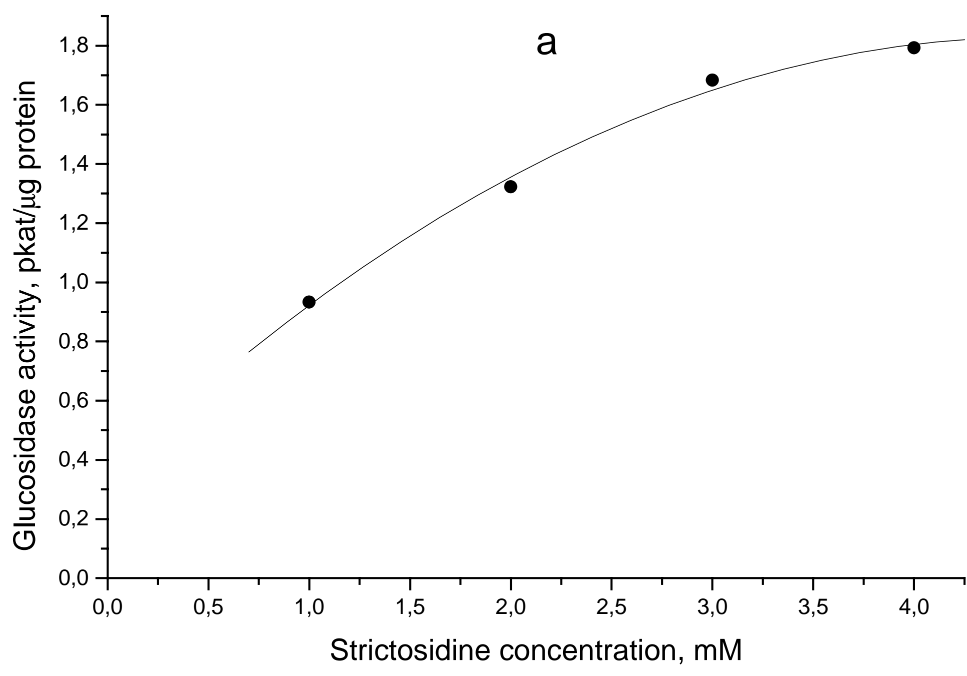


Fig. 17. Determination of enzyme kinetic parameters with raucaffricine: a) dependence of the raucaffricine glucosidase activity on substrate concentration; b) Lineweaver-Burk presentation.



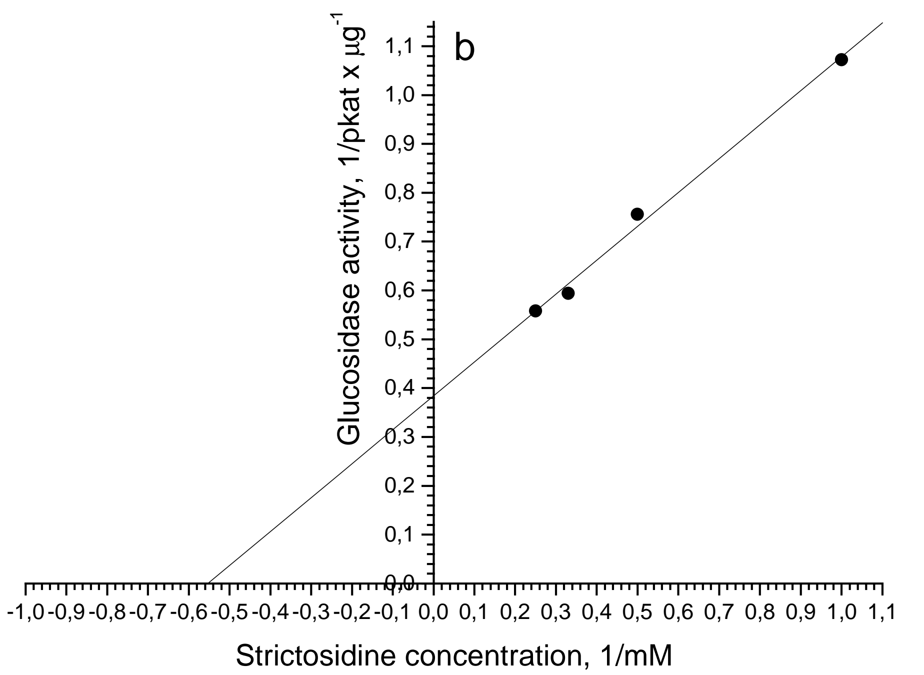


Fig. 18. Determination of enzyme kinetic parameters of raucaffricine glucosidase with strictosidine: a) Michaelis-Menten curve ; b) Lineweaver-Burk diagram.

3. Cloning and heterologous expression of the cDNA encoding the strictosidine glucosidase from *R. serpentina*

3.1. Amplification of the partial SG cDNA

To obtain the strictosidine glucosidase (SG) from *R. serpentina* cell suspension culture the method of homology cloning was applied. This approach is based on the supposition that the proteins executing similar functions share common features of primary structure. Conserved motifs are revealed by the alignment of amino acid or nucleotide sequences of several enzymes catalyzing reactions of the same type. Using the primers designed for such regions, cDNAs encoding new members of the enzyme family may be amplified.

Total RNA was isolated from 6 days old *R. serpentina* cell suspension culture. The first strand of the cDNA was synthesized using the oligoT primer complementary to the polyA tails of the eukaryotic mRNA molecules. To design primers for the subsequent PCR, the cDNA sequences of RG from *R. serpentina* and SG from *C. roseus* were aligned (Fig. 19). These two glucosidases involved in indole alkaloid biosynthesis were expected to have the highest homology to the SG from *R. serpentina*. PCR with a primer pair F5 (forward) and F3 (reverse) resulted in ca. 300 bp long fragment (Fig. 20). This fragment was excised from the gel, eluted and ligated into the pGEM-T Easy vector carrying the ampicillin resistance gene as a marker. After transformation of the obtained plasmid into the *E. coli* strain TOP10 the transformants were selected on solid LB medium supplemented with 50 mg/l ampicillin. Single colonies were picked and their plasmid DNA was digested with *EcoR*I restrictase (Fig. 21). All eight clones analyzed revealed a DNA fragment of necessary size. The sequencing of the inserts showed that in six clones they were identical to the RG. Two clones contained a 319 bp long insert (named F5R3) with

```
                                                                                                                              ----F5----
                                                                                                                ------------F3------------
          250       260       270       280       290       300       310       320       330       340       350
SG_Cr  ⇒TGCATATAATGAAGGCAACCGCGGTCCCAGTATATGGGATACTTTCACAAACCGATATCCAGCCAAAATAGCTGATGGATCTAATGGCAATCAAGCCATCAATTCTTACAATTTGTAC
       ⇒::      :  :: ::    :: :: :: :: :::::::::::::::::  :: ::   :: :  :  ::    : :::: :::: ::  ::    ::  :  : :::::: ::::::::
RG_Rs  ⇒TGGGGCGAGAGATGGGGGTCGTGGCCCGAGCATATGGGATACTTTCACCCACAGACGACCTGATATGATCCGGGGTGGAACTAACGGGGATGTGGCTGTGGACTCTTACCATTTGTAC
           270       280       290       300       310       320       330       340       350       360       370
       -----F5-----                                                                                  ----------F4---------
      360       370       380          390       400       410       420       430       440       450       460       470   ⇒
SG_Cr  AAGGAAGATATCAAGATTATGAAGCAAACA---GGCTTGGAATCATATAGGTTTTCAATTTCATGGTCAAGAGTATTGCCAGGTGGGAATCTATCCGGTGGAGTGAATAAAGATGGTG⇒
       ::::::::: :::: ::   : ::  ::::     :::::: :  : ::  :::: ::::: :::::::: ::::: ::::::::::: :  :::   ::::: ::::: :: :: ::  ⇒
RG_Rs  AAGGAAGATGTCAACATCCTCAA--AAACTTGGGGCTTG-ATGCCTACCGGTTCTCAATCTCATGGTCCAGAGTGTTGCCAGGTGGAAGACTAAGTGGTGGCGTGAACAAGGAAGGGA⇒
       380      390       400         410       420       430       440       450       460       470       480       490   ⇒
                                                  --------------R3-------------
       ⇒600      610       620       630       640       650       660       670       680       690       700       710   ⇒
SG_Cr  ⇒AATTGTGGAAGATTTTACGGAGTATGCAGAATTTTGCTTTTGGGAATTCGGTGACAAAGTAAAATTTTGGACGACTTTCAATGAACCACATACTTATGTTGCAAGTGGATATGCCA⇒
       ⇒ :::::::: :::::    :::::::: ::  :::: :: ::::::::::::::    :: :::   :::: ::: :: :: :: :::   :: : :    :    ::::::::: :⇒
RG_Rs  ⇒GATTGTGGATGATTTCTGTGAGTATGCGGAGCTTTGTTTCTGGGAATTCGGTGATCGGGTGAAACACTGGATGACATTGAACGAGCCATGGACATTTAGCGTTCATGGATATGCAA⇒
       ⇒620      630       640       650       660       670       680       690       700       710       720       730   ⇒
                                                                                                                ----------R2--------//--R1--
      1600      1610      1620      1630            1640      1650      1660      1670      1680      1690      1700      1710
SG_Cr  ⇒ATGGTACAAGAATTTCATTAGTGAAGGATTTGTTACGAA-----TACAGCTAAAAAGAGATTCCGAGAAGAAGATAAACTAGTTGAGTTAGTCAAGAAGCAAAAATACTAAGCAAGCG
       ⇒::::  :: ::::: : ::    ::: :  : :  : ::      : :::  :: :  ::  :: :::: :: ::  ::  :    :  : :  :::::    :: : : : ::    : ::
RG_Rs  ⇒ATGGCTCATGAATT-CTTTCCACAAGAACATCTC-CAAACTTCCTGCAGTCAAGAGAAGTATCAGAGAGGACGACGAAGAACAAGTTTCAAGCAAGAGATTAAGAAAGTAGAGATGCT
       1660      1670       1680      1690       1700      1710      1720      1730      1740      1750      1760      1770
       --------R1-------
          1720      1730      1740      1750      1760      1770      1780      1790      1800      1810      1820      1830
SG_Cr  AATTTGCATCTGATGACCTTTTCTTTTAACTTTTTTTTTCTTCTTTTTAACTCATTTTAATCACTAGATCATTAGATGATCTGAACCGCTCTTTAAGGTACTTCAATAAGCAAAATTGCC
         : ::  ::: :: :          : :  ::::::::: :: :::::
RG_Rs  CTTCTG--TCTCATCAAACAAATGGTCAGTTTTTTTTTTTTTTTTTTTTTGCTGGGTAAATGGTCAGTTCTTTATTCCGCTTTGTTGTTGTTGTCGTGGTCTGCTTAATCATAATATGGA
         1780          1790      1800      1810      1820      1830      1840      1850      1860      1870      1880      1890
```

Fig. 19. The alignment of the cDNA sequences encoding the strictosidine glucosidase from *C. roseus* (SG_Cr) and the raucaffricine glucosidase from *R. serpentina* (RG_Rs). The primers, used for PCR amplification of partial strictosidine glucosidase cDNA from *R. serpentina*, are in boxes, stop codons are highlighted black.

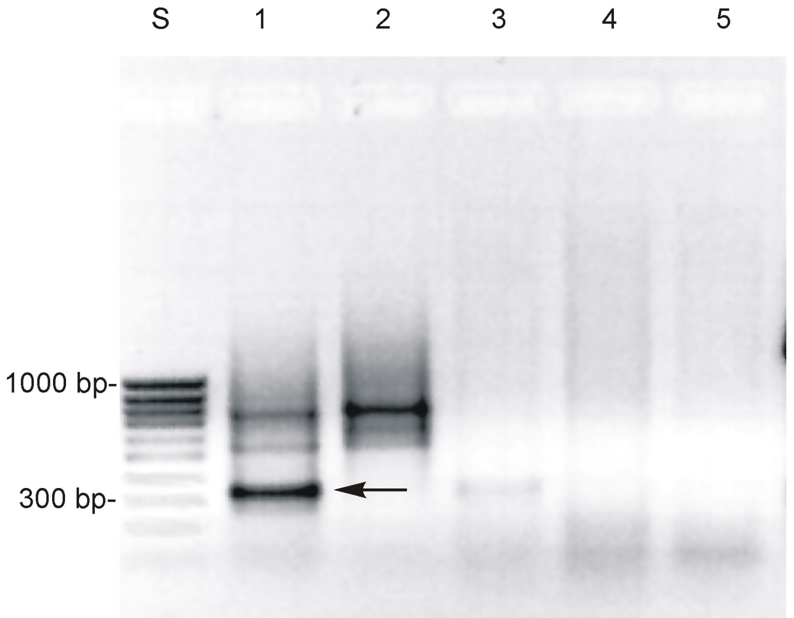


Fig. 20. Results of PCR amplification of *R. serpentina* cDNA using F5 (forward) and R3 (reverse) primers: **S)** DNA size marker; **1)** full PCR assay; **2)** control reaction without F5; **3)** control reaction without R3; **4)** control reaction without both primers; **5)** control reaction without template. The fragment excised is indicated by arrow.

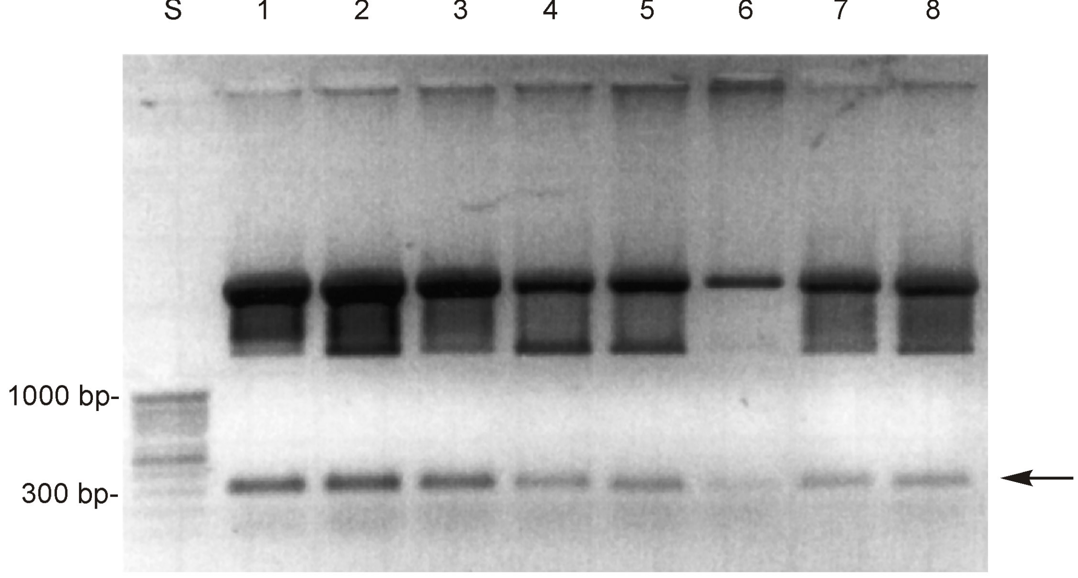


Fig. 21. Screening of clones obtained after transformation with pGEM-T easy vector containing the 300 bp insert. The plasmid DNA was digested with *EcoR*I restrictase: **S)** DNA size marker; **1-8)** restricted plasmid DNA from eight clones. Arrow shows the excised insert.

higher homology to the SG from *C. roseus* (90%) than to the RG (76%), possibly presenting a part of *R. serpentina* SG sequence.

To obtain a longer fragment of the *R. serpentina* SG cDNA, two reverse primers complementary to the 3'-end of the *C. roseus* SG cDNA were designed (Fig. 19). The PCR was carried out using various primer pairs (Fig. 22). A fragment of ca. 1300 bp amplified with F5 (forward) and R2 (reverse) primers was excised, eluted from the agarose gel and after ligation into the pGEM-T Easy vector transformed into *E. coli* TOP10 cells. The ampicillin resistant single colonies were screened by PCR. The DNA fragment of necessary size was amplified with F5 and R2 primers in eleven colonies out of twelve tested (Fig. 23a). However, in colony 7 a band of the same size appeared also when the reverse primer R2 alone was added (Fig. 23b). This clone was excluded from further investigation.

The plasmid DNA was isolated from the clone 2 and the insert (named F5R2) was sequenced. It numbered 1311 bp and showed 79.9% homology to the SG from *C. roseus* and 65.3% homology to the RG from *R. serpentina*. Between the positions 1 and 319 it was identical to the fragment amplified with the primer pair F5/R3, with the exception of 4 nucleotides in the region covered by the R3 primer. One of the reading frames of the F5R2 sequence was open. This fragment was considered to present the partial cDNA encoding the SG in *R. serpentina* cells.

3.2. Amplification of the SG cDNA ends

To obtain the full-length SG cDNA, the method of rapid amplification of cDNA ends (RACE) was applied. This PCR technique allows to amplify the 3'- or 5'-end of the cDNA using only one gene specific primer. The second primer is complementary to the adapter DNA sequence which is ligated to the both ends of the cDNA.

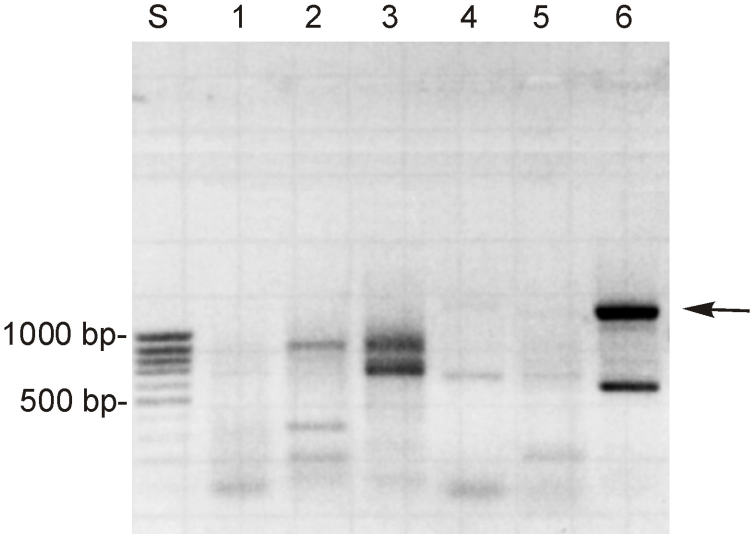


Fig. 22. Results of PCR amplification of *R. serpentina* cDNA using different primer pairs: **S)** DNA size marker; **1)** primers F3 and R1; **2)** primers F4 and R1; **3)** primers F5 and R1; **4)** primers F3 and R2; **5)** primers F4 and R2; **6)** primers F5 and R2. The 1300 bp DNA fragment used in further studies is indicated by arrow.

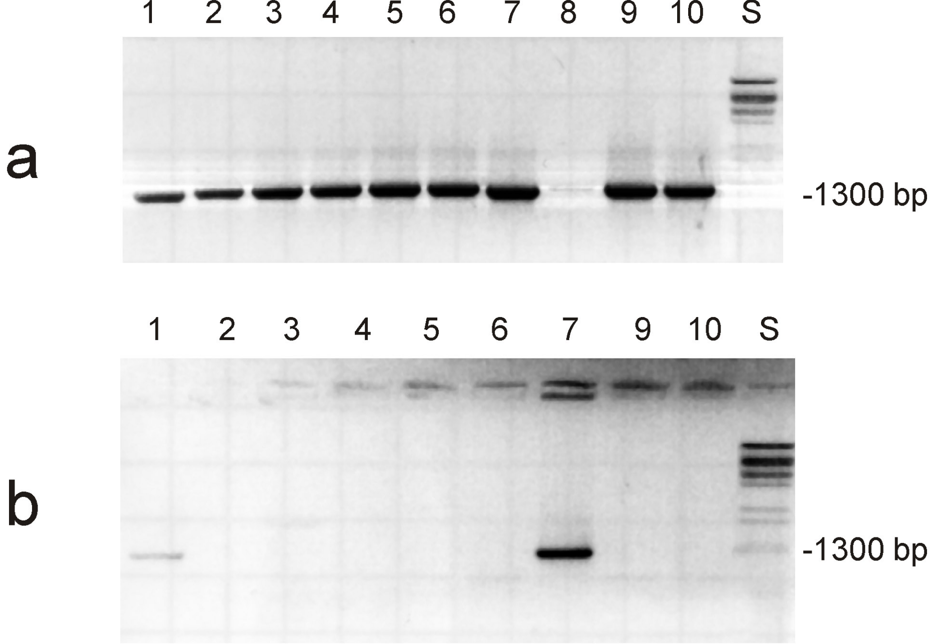
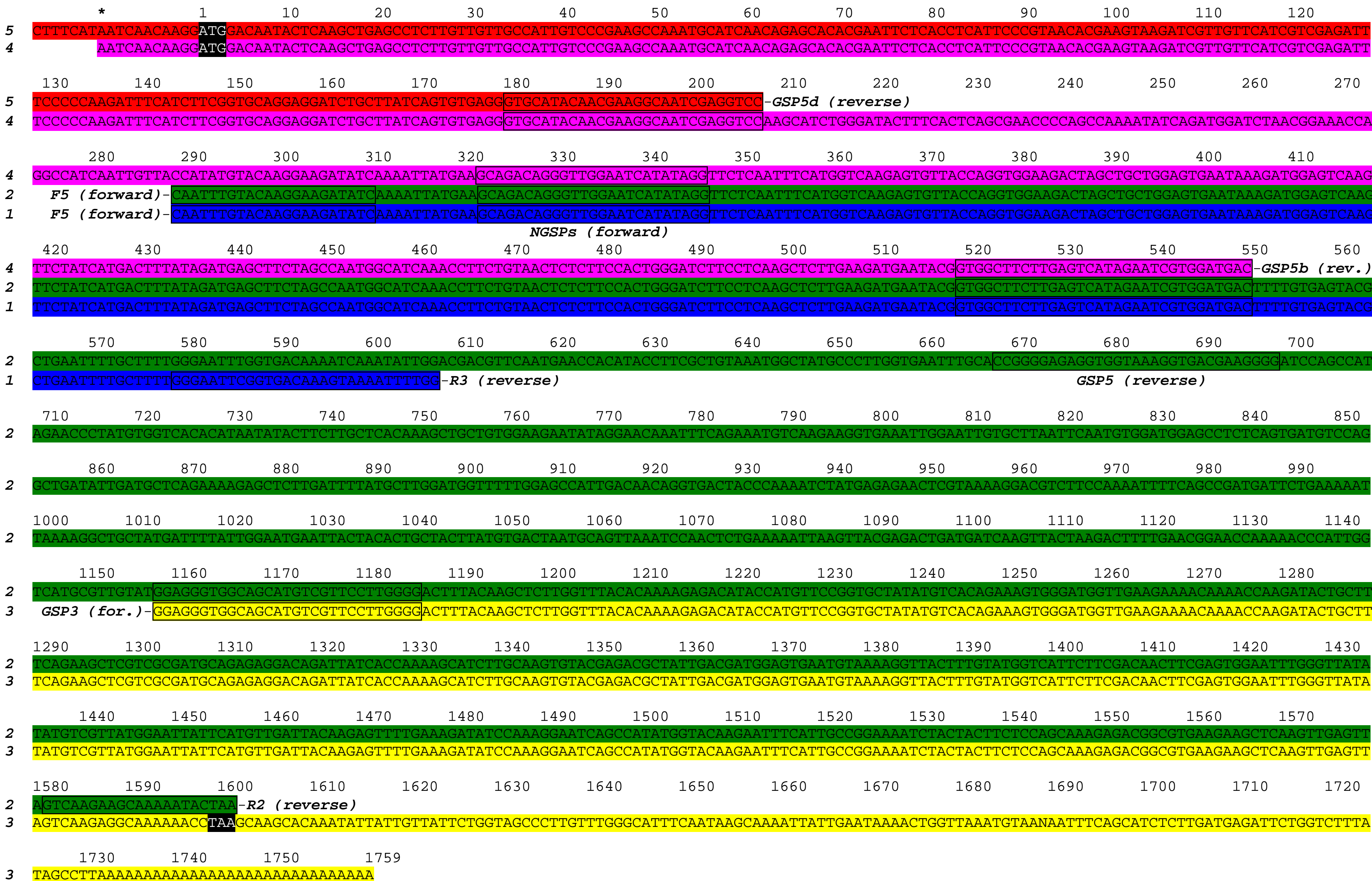


Fig. 23. PCR screening of colonies obtained after transformation with pGEM-T easy vector containing the 1300 bp insert. a) Amplification with both F5 and R2 primers: **1-10)** ten colonies; **S)** DNA size marker; b) control reaction with R2 primer only: **1-9)** nine colonies; **S)** DNA size marker.

The adapter-ligated cDNA library was produced using the mRNA isolated from the 6 days old *R. serpentina* cell suspension culture. The gene specific primers were designed on the basis of the sequence of the F5R2 fragment (Fig. 24).

For the 3'-RACE reaction the sense gene specific primer GSP3 was used. The touch-down PCR with the annealing temperature ranging from 70 °C to 66 °C yielded a band at ca. 700 bp (Fig. 25a), which was excised and eluted from the agarose gel, ligated into the pGEM-T Easy vector and transformed into the *E. coli* TOP10 cells. The screening of the obtained transformants by the digestion of plasmid DNA with *EcoR*I restriction enzyme revealed the insert of necessary size in all three clones tested. The sequencing of the insert showed that it was identical with the F5R2 fragment in 444 bp overlap with the exception of 3 positions in the region covered by the R2 primer. Thus the amplified fragment indeed presented the 3'-end of the SG cDNA. It contained the ochre stop codon and the 130 bp long uncoding region with polyA tail of 30 bp (Fig. 24).

Fig. 24 (next page). Acquiring of the structure of the full-length strictosidine glucosidase cDNA from *R. serpentina* cell suspension culture. Fragments ***1*** (blue, F5R3) and ***2*** (green, F5R2) were amplified by RT-PCR. Sequence ***3*** (yellow) was obtained by 3'-RACE PCR, sequences ***4*** (pink) and ***5*** (red) originate from two subsequent 5'-RACE reactions. The regions used for primer design are in boxes, the start and stop codons are highlighted black, the in-frame stop codon upstream of the start codon is indicated by asterisk.


GSP5d (reverse)
F5 (forward)
F5 (forward)
NGSPs (forward)
GSP5b (rev.)
R3 (reverse)
GSP5 (reverse)
GSP3 (for.)
R2 (reverse)

The 5’-RACE reaction was carried out using the antisense gene specific primer GSP5b. The touch-down PCR under the same conditions as the 3’-RACE reaction allowed to obtain a DNA band at ca. 600 bp (Fig. 25b), which was processed in the same way as the 3’-RACE product. The *EcoR*I digestion of the plasmid DNA isolated from eighteen ampicillin resistant *E. coli* clones revealed in fourteen of them inserts of varying length, which is explained by partial degradation of the mRNA during the cDNA library preparation. To verify whether the obtained fragments present the 5’-end of the SG cDNA, a nestled PCR was carried out using the GSP5b and the sense nestled gene specific primer (NGSPs). The NGSPs was expected to anneal 227 bp upstream of the GSP5b. The fragment of necessary size was amplified in all the twelve clones tested.

The plasmid DNA was isolated from four clones (5, 10, 14 and 16) and the inserts were sequenced. All four obtained sequences were identical to the F5R2 fragment in 262 bp overlap with the exception of 2 positions in the region covered by the F5 primer. The longest fragment (in clone 5) numbered 552 bp and contained an ATG codon which starts an open reading frame (Fig. 24).

To check whether the ATG codon found in the 5’-RACE product 5 indeed functions as the start codon, the additional 5’-RACE reaction was carried out. The gene specific primer for it, GSP5d, was designed on the basis of the obtained sequence of the 5’-end of the SG cDNA. The GSP5d anneals 311 bp upstream of the GSP5b, which simplifies the 5’-RACE PCR because of smaller size of the expected fragment. The amplification was done under the same conditions as with GSP5b. A band at ca. 200 bp was obtained (Fig. 25c) and processed as described above. After screening of the obtained transformants by digestion of the plasmid DNA with *EcoR*I restriction enzyme, the inserts in six clones (1, 2, 3, 6, 12 and 14) were sequenced. The fragment

from clone 14 contained 7 bp more at the 5'-end than the longest product amplified with GSP5b (Fig. 24). The presence of an in-frame stop codon (TAA) upstream of the ATG start codon shows, that the acquired ORF is complete.

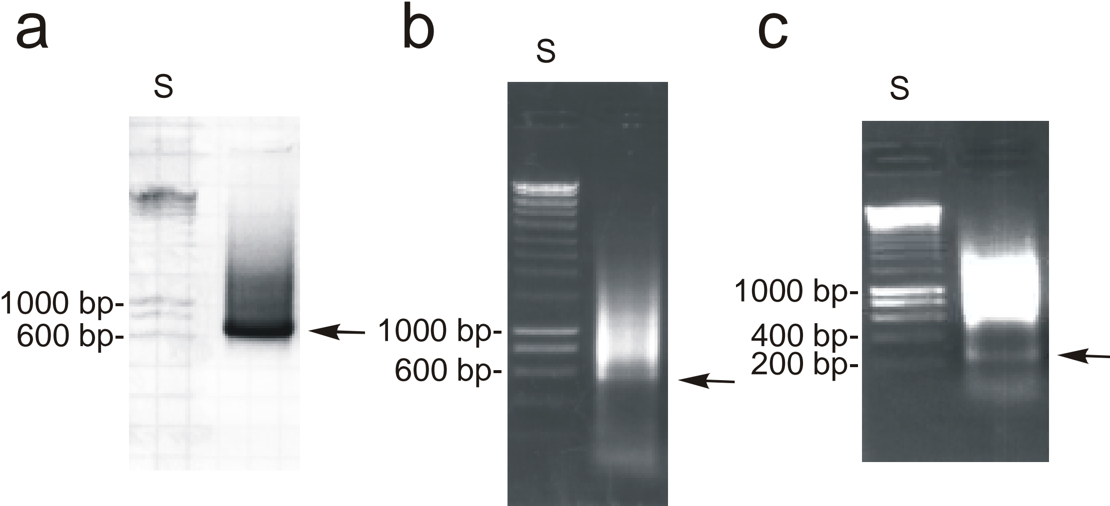


Fig. 25. Results of the RACE PCR. a) 3'-RACE reaction mixture. b) 5'-RACE using gene specific primer GSP5b. c) 5'-RACE using gene specific primer GSP5d. **S)** DNA size marker, arrows show the amplified DNA fragments.

3.3. Synthesis of the full-lenght SG ORF

The full-length SG ORF was obtained by PCR using the Advantage® polymerase mixture from Clontech including along with KlenTaq-1 DNA polymerase (a 5'-exo-minus, N-terminal deletion of *Taq* DNA polymerase) a second DNA polymerase with 3'→5' proofreading activity and TagStart™ Antibody to provide automatic "hot start" PCR (Kellogg et al., 1994). The use of two different DNA polymerases in a PCR reaction allows amplification of significantly longer fragments (Barnes, 1994; Cheng et al., 1994), while the inclusion of a minor amount of a proofreading DNA polymerase results in a considerably lower error rate (Frey and Suppmann, 1995;

Nelson et al., 1995). Primers were designed on the basis of the sequences of the 3'- and 5'-RACE PCR products (Fig. 26). Primers *Nco*Ifor (sense) and *Pst*Irev (antisense) contain the recognition sites for corresponding restriction enzymes, which is necessary for subsequent cloning in the pSE280 expression vector. The recognition sites for *Nde*I and *Sap*I restrictases were introduced using the primer pair *Nde*Ifor (sense) and *Sap*Irev (antisense).

Total RNA was isolated from 6 days old *R. serpentina* cell suspension culture. The first strand of the cDNA was synthesized using the *Pst*Irev antisense primer. Subsequent PCR with primer pair *Nco*Ifor (sense) and *Pst*Irev (antisense) yielded a ca. 1600 bp product. The amplified DNA fragment was ligated into pGEM-T Easy vector and the obtained construction was transformed into *E. coli* TOP10 cells. Both strands of the insert were sequenced by primer walking showing it to be a complete SG ORF.

The SG ORF flanked by recognition sites for *Nde*I and *Sap*I restrictases was synthesized analogously using the primer pair *Nde*Ifor (sense) and *Sap*Irev (antisense).

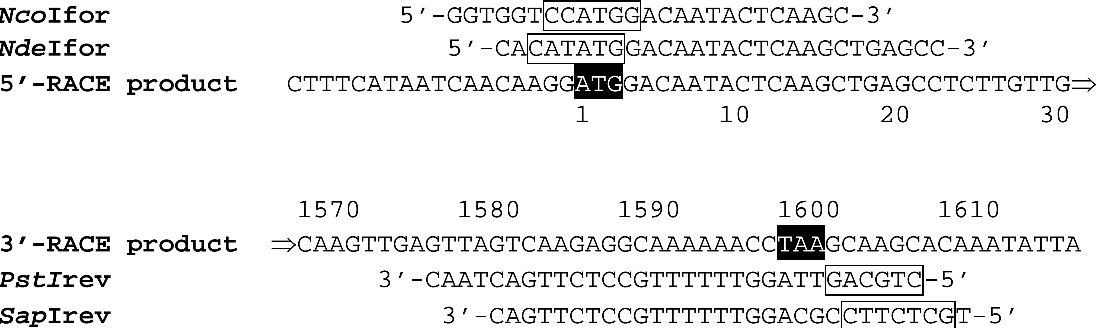


Fig. 26. The primers used for PCR amplification of the full-length ORF encoding strictosidine glucosidase. The recognition sites for corresponding restriction enzymes are in boxes, start and stop codons are highlighted black.

3.4. Expression of the SG in *E. coli*

To verify that the cDNA under investigation indeed encodes the SG, it was expressed in *E. coli*. The ORF was excised from pGEM-T Easy vector using the *Nco*I and *Pst*I restriction enzymes and ligated into the respective sites of pSE280 expression vector digested with the same restrictases. The obtained construction was transformed into *E. coli* TOP10 cells. The transformants were selected on solid LB medium supplemented with 50 mg/l ampicillin and the clones containing the SG ORF in pSE280 plasmid were revealed by digestion of the plasmid DNA with *Nco*I and *Pst*I restriction enzymes. These clones were used for SG activity test.

E. coli suspension (100 ml) grown over night at 37 °C was centrifuged. The sedimentated cells were taken up in 1 ml sterile water, crashed with ultrasonic and centrifuged for 15 min at 14 000 rpm. SG activity was estimated in the supernatant by measuring the decrease of strictosidine HPLC peak area after incubation with *E. coli* preparation. Clones containing the investigated ORF showed SG activity up to 127 nkat/l of bacterium culture (6.2 nkat/mg total protein), whereas with the *E. coli* cells transformed with pSE280 vector without insert no strictosidine conversion could be detected even after long time incubation (Fig. 27). Thus the obtained cDNA was proved to code for the SG in *R. serpentina* cell suspension culture.

3.5. Deduced amino acid sequence of *R. serpentina* SG

The obtained ORF of 1599 bp encodes a protein of 532 amino acids with a molecular weight of 60.881 kDa and calculated isoelectric point of 6.01 (Fig. 28). The deduced amino acid sequence shows highest homology to SG from *C. roseus* (70%) followed by RG from *R. serpentina* (56%) and other plant β-glucosidases. The presence of glycosyl hydrolases family 1 N-terminal signature (F-x-[FYWM]-[GSTA]-x-[GSTA]-x-[GSTA](2)-[FYNH]-[NQ]-x-E-x-[GSTA], position 47-61) allows to assign the SG

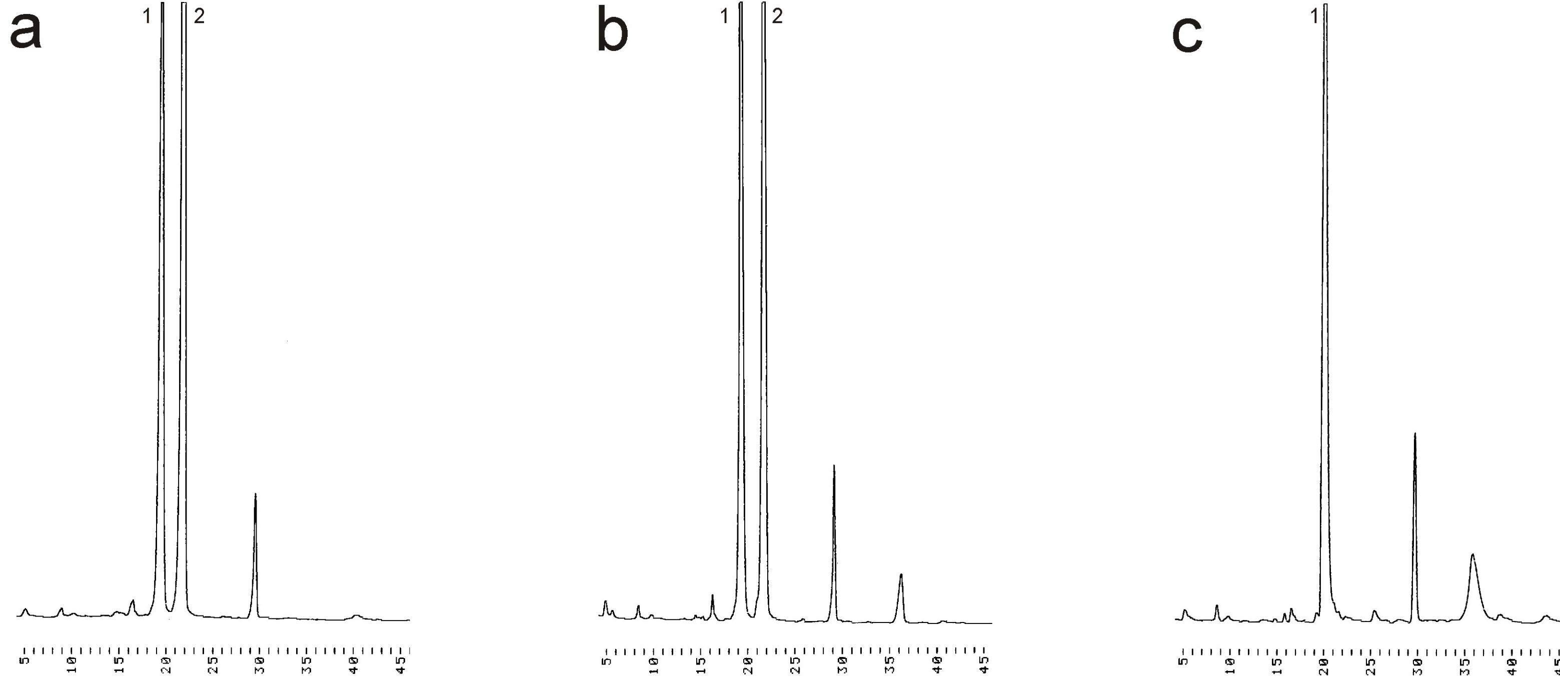


Fig. 27. HPLC based test for strictosidine glucosidase activity (using HPLC system described in **II.5.3.**). Total *E. coli* proteins (130 µg) were incubated with 200 nmol strictosidine **(2)** (ajmaline **(1)** was added as internal standard). a) Control incubation without bacterium preparation. b) 1.5 h incubation with the proteins from *E. coli* cells transformed with pSE280 vector without insert. c) 15 min incubation with the proteins from *E. coli* cells expressing the strictosidine glucosidase ORF.

from *R. serpentina* to this enzyme family (Henrissat and Davies, 1997). In the other signature of glycosyl hydrolases family 1 ([LIVMFSTC]-[LIVFYS]-[LIV]-[LIVMST]-E-N-G-[LIVMFAR], position 412 – 419), which contains the putative nucleophile catalytic glutamic acid (Withers et al., 1990; Trimbur et al., 1992), in position 417 asparagine is exchanged against serine. The second catalytic glutamic acid acting as proton donor is suggested to be located upstream of the nucleophile in the highly conserved motif NEP (position 206 – 208) (Baird et al, 1990; Keresztessy et al., 1994). The sequence DxxRxxY near the C-terminus (position 435 – 441) is also conserved in family 1 of glycosyl hydrolases. Two motifs, NAST, at position 18-21, and NKTK, at position 423-426, may serve as possible N-glycosylation sites. Analysis of the *R. serpentina* SG deduced amino acid sequence revealed no regions which would possibly form transmembrane helices. From the primary structure analysis it can be concluded that SG from *R. serpentina* is a soluble enzyme.

3.6. Purification of the heterologously expressed SG

Simple and efficient purification of the SG was achieved using the IMPACT-CN system. The enzyme was expressed in C-terminal fusion with an intein tag. This tag consists of a chitin binding domain separated from a target protein by a protein splicing element called intein. Intein sequence exhibits the self-cleavage activity in presence of thiols. When the crude extract from transgenic *E. coli* cells is loaded onto chitin column, the recombinant protein binds to it. After extensive washing of the column dithiothreitol (DTT) is added to the buffer to induce the intein cleavage. As a result, the pure target protein without any additional amino acids is eluted, while the chitin binding domain and attached to it intein sequence are left on the column.

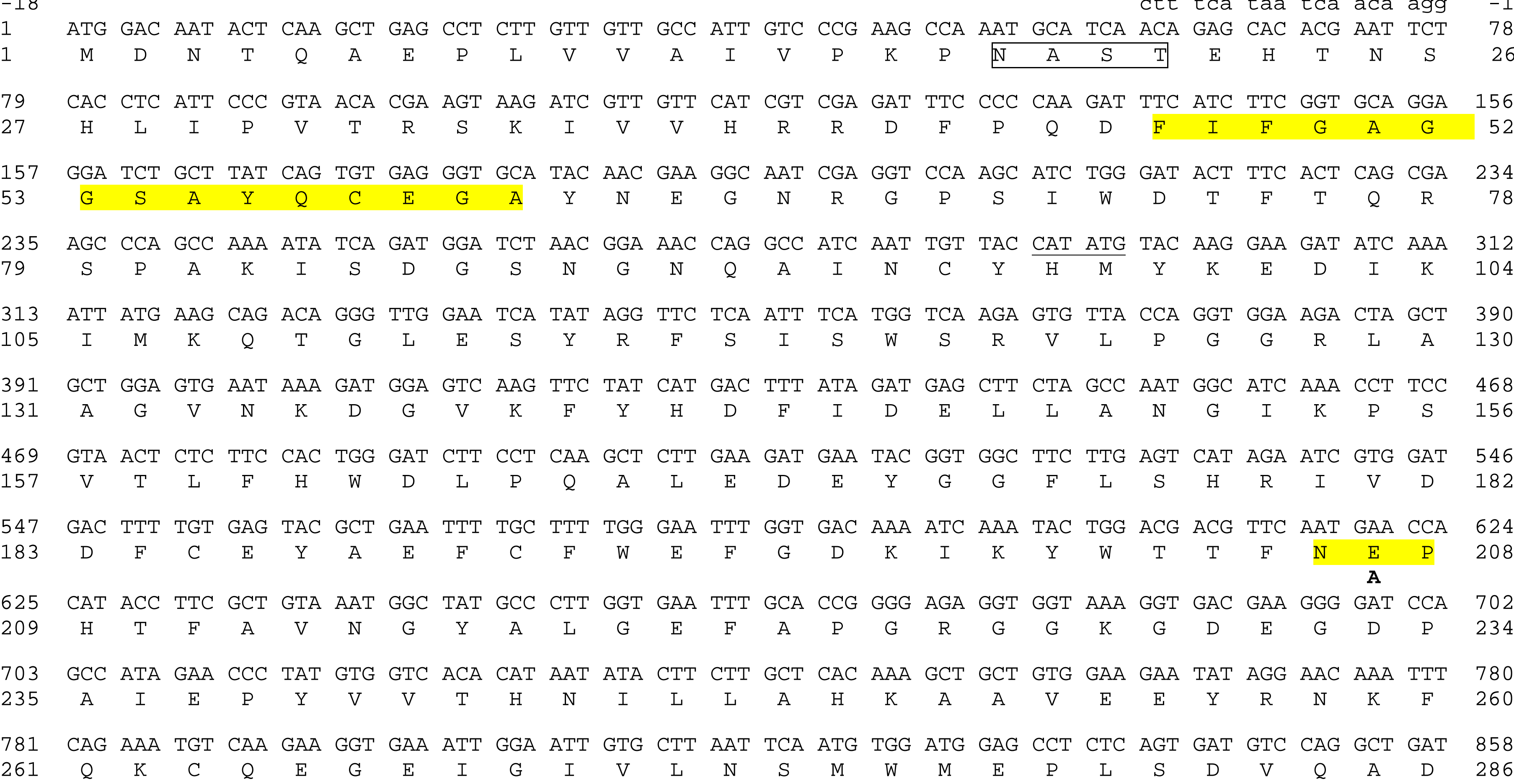


Fig. 28. cDNA Sequence and deduced amino acid sequence of strictosidine glucosidase from *R. serpentina*. Motifs conserved in members of glycosyl hydrolases family 1 are shaded, the putative catalytic glutamate residues are marked: A – proton donor, B – nucleophile. Possible N-glycosylation sites are in boxes, *Nde*I restriction sites are underlined.

```
859  ATT GAT GCT CAG AAA AGA GCT CTT GAT TTT ATG CTT GGA TGG TTT TTG GAG CCA TTG ACA ACA GGT GAC TAC CCA AAA  936
287   I   D   A   Q   K   R   A   L   D   F   M   L   G   W   F   L   E   P   L   T   T   G   D   Y   P   K   312

937  TCT ATG AGA GAA CTC GTA AAA GGA CGT CTT CCA AAA TTT TCA GCC GAT GAT TCT GAA AAA TTA AAA GGC TGC TAT GAT 1014
313   S   M   R   E   L   V   K   G   R   L   P   K   F   S   A   D   D   S   E   K   L   K   G   C   Y   D   338

1015 TTT ATT GGA ATG AAT TAC TAC ACT GCT ACT TAT GTG ACT AAT GCA GTT AAA TCC AAC TCT GAA AAA TTA AGT TAC GAG 1092
339   F   I   G   M   N   Y   Y   T   A   T   Y   V   T   N   A   V   K   S   N   S   E   K   L   S   Y   E   364

1093 ACT GAT GAT CAA GTT ACT AAG ACT TTT GAA CGG AAC CAA AAA CCC ATT GGT CAT GCG TTG TAT GGA GGG TGG CAG CAT 1170
365   T   D   D   Q   V   T   K   T   F   E   R   N   Q   K   P   I   G   H   A   L   Y   G   G   W   Q   H   390

1171 GTC GTT CCT TGG GGA CTT TAC AAG CTC TTG GTT TAC ACA AAA GAG ACA TAC CAT GTT CCG GTG CTA TAT GTC ACA GAA 1248
381   V   V   P   W   G   L   Y   K   L   L   V   Y   T   K   E   T   Y   H   V   P   V   L   Y   V   T   E  416
                                                                                                      B
1249 AGT GGG ATG GTT GAA GAA AAC AAA ACC AAG ATA CTG CTT TCA GAA GCT CGT CGC GAT GCA GAG AGG ACA GAT TAT CAC 1326
417   S   G   M   V   E   E  [N   K   T   K]  I   L   L   S   E   A   R   R   D   A   E   R   T   D   Y   H   442

1327 CAA AAG CAT CTT GCA AGT GTA CGA GAC GCT ATT GAC GAT GGA GTG AAT GTA AAA GGT TAC TTT GTA TGG TCA TTC TTC 1404
443   Q   K   H   L   A   S   V   R   D   A   I   D   D   G   V   N   V   K   G   Y   F   V   W   S   F   F   468

1405 GAC AAC TTC GAG TGG AAT TTG GGT TAT ATA TGT CGT TAT GGA ATT ATT CAT GTT GAT TAC AAG AGT TTT GAA AGA TAT 1482
469   D   N   F   E   W   N   L   G   Y   I   C   R   Y   G   I   I   H   V   D   Y   K   S   F   E   R   Y   494

1483 CCA AAG GAA TCA GCC ATA TGG TAC AAG AAT TTC ATT GCC GGA AAA TCT ACT ACT TCT CCA GCA AAG AGA CGG CGT GAA 1560
495   P   K   E   S   A   I   W   Y   K   N   F   I   A   G   K   S   T   T   S   P   A   K   R   R   R   E   520

1561 GAA GCT CAA GTT GAG TTA GTC AAG AGG CAA AAA ACC TAA gca agc aca aat att att gtt att ctg gta gcc ctt gtt 1638
521   E   A   Q   V   E   L   V   K   R   Q   K   T  stop                          532

1639 tgg gca ttt caa taa gca aaa tta ttg aat aaa act ggt taa atg taa naa ttt cag cat ctc ttg atg aga ttc tgg 1716
1717 tct tta tag cct t (a)30                                                                              1729
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Fig. 28 (continued). cDNA Sequence and deduced amino acid sequence of strictosidine glucosidase from *R. serpentina*. Motifs conserved in members of glycosyl hydrolases family 1 are shaded, the putative catalytic glutamate residues are marked: A – proton donor, B – nucleophile. Possible N-glycosylation sites are in boxes, *Nde*I restriction sites are underlined.

3.6.1. Cloning of the SG cDNA into pTYB1 expression vestor

The SG ORF was excised from pGEM-T Easy vector using the *Nde*I and *Sap*I restriction enzymes. Because of two additional *Nde*I restriction sites in the SG cDNA sequence (Fig. 28) partial digestion was carried out. The *Sap*I enzyme was added to all assays at the beginning of the experiment and was incubated with the DNA for 60 min. The *Nde*I restrictase was added simultaneously or 15, 30, 40 and 50 min later, thus the digestion lasted for 60, 45, 30, 20 or 10 min (Fig. 29). The DNA band at ca. 1600 bp was excised, eluted and ligated into the *Nde*I and *Sap*I cloning sites of pTYB1 expression vector digested with these restrictases. The obtained construction was transformed into *E. coli* TOP10 cells. The transformants were selected on solid LB medium supplemented with 50 mg/l ampicillin and the clones containing the SG ORF in pTYB1 plasmid were revealed by digestion of the plasmid DNA with *EcoR*I and *Pst*I restriction enzymes. The *Nde*I and *Sap*I enzymes could not be used, because the *Nde*I does not digest the plasmid DNA isolated by the method of minipreparation efficiently, and the *Sap*I recognition and restriction sites do not coincide, so the

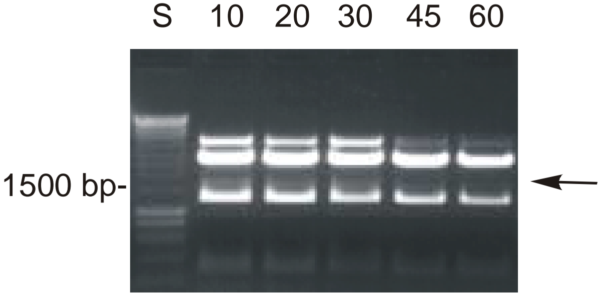


Fig. 29. Partial digestion of the pGEM-T Easy vector containing the strictosidine glucosidase ORF with *Nde*I and *Sap*I restriction enzymes: **10 – 60)** plasmid DNA incubated with *Nde*I restrictase during 10, 20, 30, 45 and 60 min; **S)** DNA size marker. Arrow shows the full-length strictosidine glucosidase ORF.

recognition site is lost after ligation of the insert into the vector. Among three clones tested only one contained the insert of expected size. From this clone the plasmid DNA was isolated and the insert was sequenced, which proved that it presents the SG ORF.

3.6.2. Expression of the SG fused to the intein tag

The pTYB1::SG plasmid was isolated from the TOP10 cells and transformed into *E. coli* strain ER2566. The ampicillin resistant single colonies were selected. 100 ml of bacterium suspension grown over night at 37 °C were inoculated into 2.5 l of fresh medium and cultivated at 28 °C. When the OD_{600} of ca. 0.5 was reached (after 5-6h), IPTG was added (final concentration 0.5 mM) to induce expression. After 24h cultivation at 28 °C the *E. coli* cells were harvested by centrifugation, taken up in 50 ml of Cell Break Buffer and crashed in French press. The clarified crude extract was obtained by centrifugation. The SG activity in the supernatant reached 1.41 nkat/mg total protein.

3.6.3. Purification of the fused SG by affinity chromatography

The clarified extract was loaded onto a gravity flow column packed with chitin bead suspension. The column was washed and rinsed with buffer containing 50 mM DTT. The flow was stopped and the column was incubated for 24h at 4 °C to allow the cleavage of intein. The SG was eluted collecting 0.5 ml fractions. The SG activity ≥ 2.2 nkat/ml was found in fractions 1-30. Fractions 3-22 with protein concentration ≥ 15 µg/ml were bulked and dialyzed against TE buffer yielding ca. 10 ml of a solution with protein concentration of 13 µg/ml and specific SG activity of 345 nkat/mg protein.

The SG became enriched 245-fold (Table 4). The low yield of 5.9% is due to a considerable loss on the stage of binding onto chitin affinity sorbent. The SG activity

in the chitin column flow-through amounted to 215 nkat (23.8%). This may be explained by not sufficient volume of the affinity sorbent used. On the other hand, up to 25% of intein self-cleavage can occur without DTT induction if the C-terminal residue of target protein presents threonine (Chong et al., 1997), as it is in the case of SG.

Table 4. Purification of the strictosidine glucosidase using IMPACT-CN system.

	Volume, ml	Total protein, mg	Total SG activity, nkat	Specific activity, nkat/mg	Yield, %	Enrichment factor
1) Crude extract	50	540	760	1.41	100	1
2) SG eluted from chitin column	10	0.13	44.8	345	5.9	245

The SG eluted from the chitin column presents one band on silver stained SDS-PAGE (Fig. 30). This enzyme solution was used for further studies.

4. Enzyme properties of the *R. serpentina* SG

4.1. Temperature optimum

The catalytic activity of the *R. serpentina* SG was tested at different temperatures ranging from 13 to 60 °C (Fig. 31). The highest enzyme activity was expressed at a temperature of 50 °C.

4.2. pH optimum

The SG was incubated with strictosidine at different pH values. Citrate-phosphate buffer (0.1 M) was applied in the pH range from 3.8 to 7.0, the pH levels from 5.8 to 8.0 were created using the 0.1 M potassium phosphate buffer. The glucosidase showed a pH optimum at 5.2 with activity of ca. 50% of the maximum at pH 4.2 and

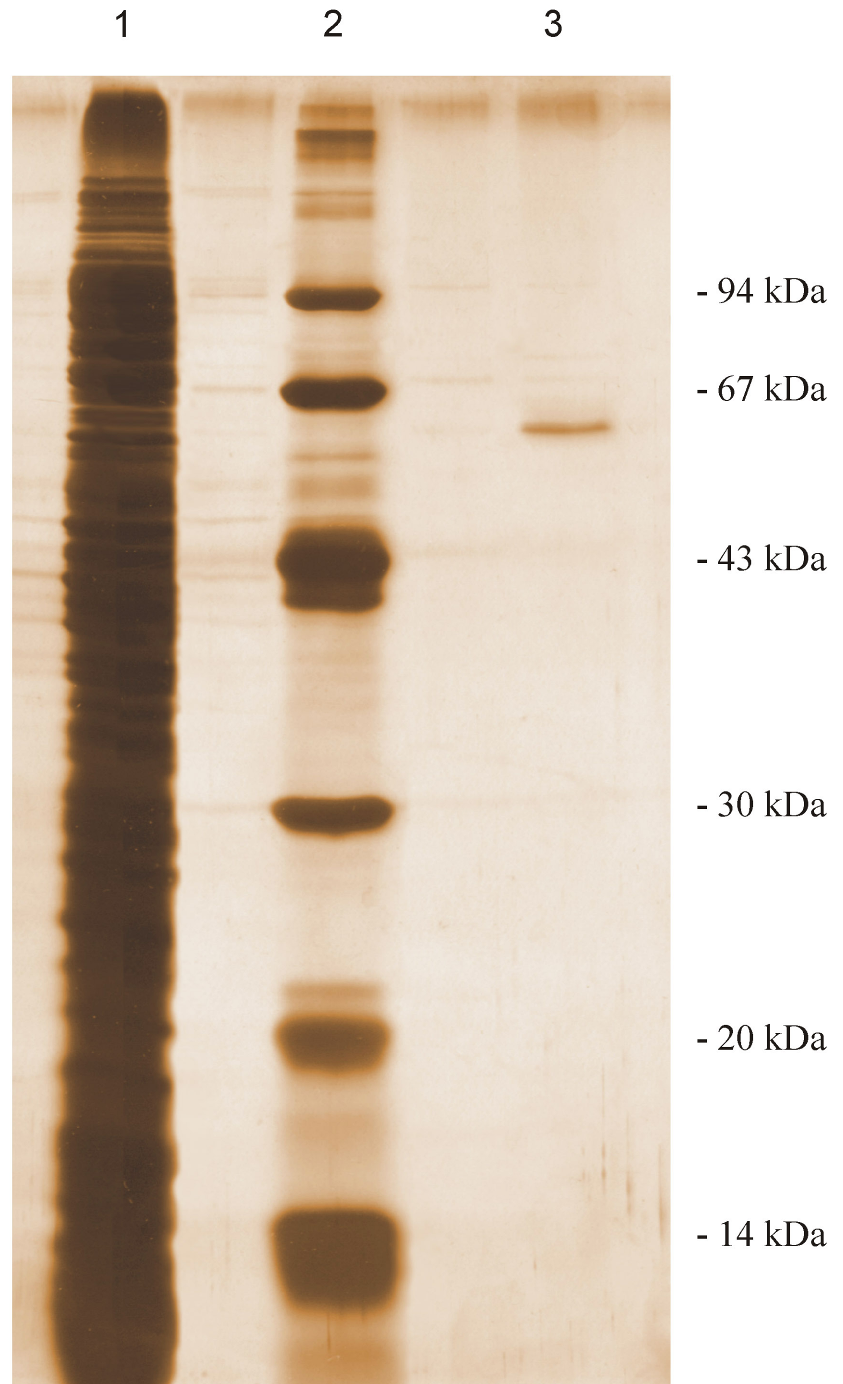


Fig. 30. Silver stained SDS-PAGE of purified strictosidine glucosidase: **1)** crude extract; **2)** protein molecular weight marker; **3)** purified SG, 0.8 μg.

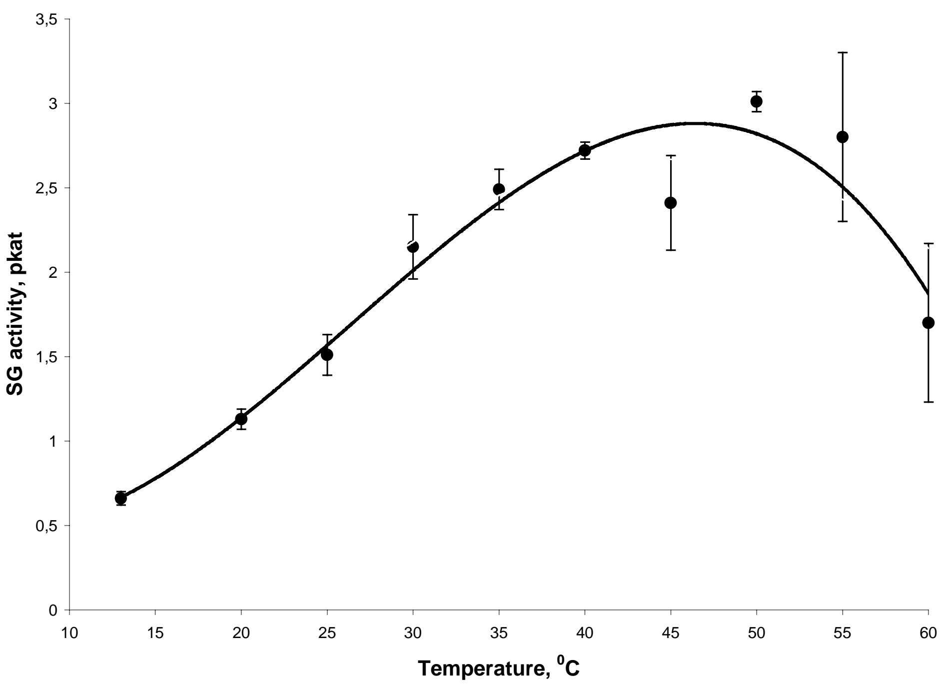


Fig. 31. Activity of purified strictosidine glucosidase at different temperatures.

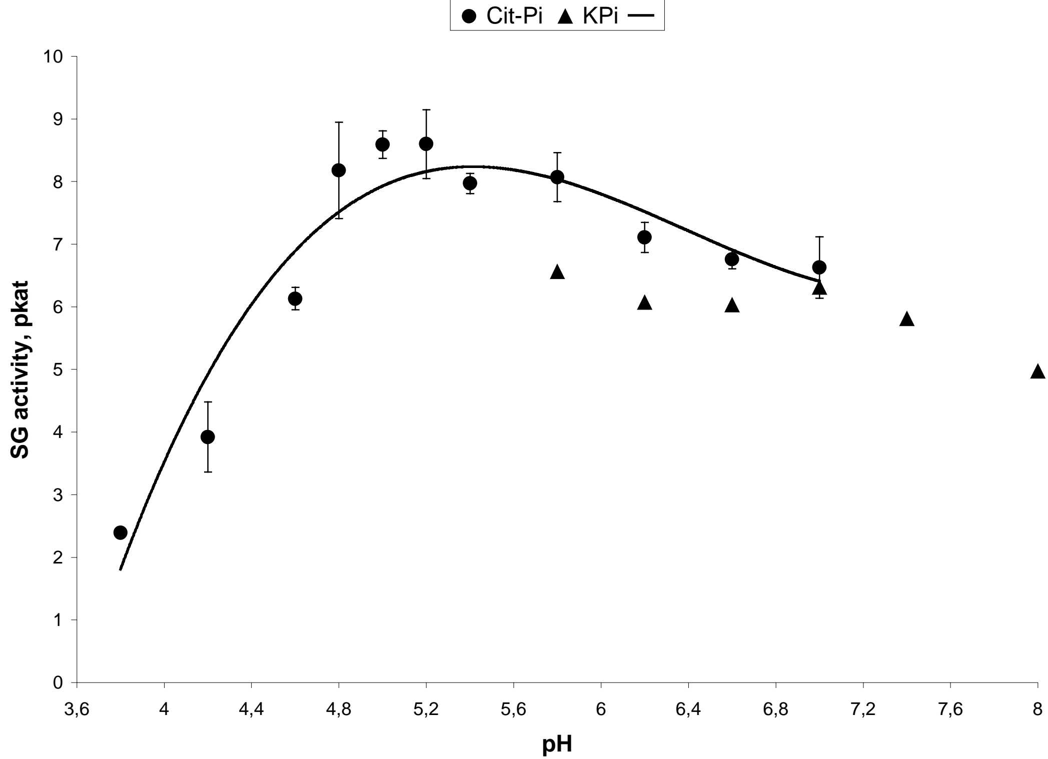


Fig. 32. Strictosidine glucosidase activity at different pH values. **Cit-Pi:** measured in 0.1 M citrate-phosphate buffer, **KPi:** measured in 0.1 M potassium phosphate buffer.

slowly decreasing up to pH 8.0 (Fig. 32). The buffer composition has an influence on the enzyme activity, that is higher in the citrate-phosphate buffer.

4.3. Influence of organic solvents

The typical SG activity assay contains 10% (V/V) of methanol. Increase of the organic solvent concentration to 20% did not lead to the decrease of the enzyme activity (the difference between mean SG activities is not significant) (Fig. 33). The SG was able to hydrolyze strictosidine in presence of up to 50% of methanol, although the glucosidase activity decreased significantly (P=0.95) when the organic solvent concentration was changed from 20% to 30% and from 30% to 40%.

DMF at a concentration of 50% inhibited the SG activity completely.

4.4. Activity in presence of β-mercaptoethanol

The disulfide bonds between cysteine residues were shown to play an important role in the formation of three-dimensional structure of some β-glucosidases belonging to the family 1 of glycosyl hydrolases (Rotrekl et al., 1999). Seven cysteine residues are present in the amino acid sequence of the SG from *R. serpentina*. To estimate their importance for maintenance of the catalytically active enzyme structure, β-mercaptoethanol was added to the SG solution in order to destroy putative disulfide bond(s). β-Mercaptoethanol at 20 mM concentration did not influence the SG activity (the difference between mean enzyme activities in absence and in presence of 20 mM β-mercaptoethanol is not significant), which indicates that cysteine residues do not play a significant role in the formation of the 3-D structure of this glucosidase.

4.5. Inhibition by serpentine and Cu^{2+}

The SG from *C. roseus* was reported to be inhibited for ca. 50% by 1 mM serpentine and 1 mM Cu^{2+} (Luijendijk et al., 1998). The enzyme from *R. serpentina* is also sensitive to these agents, although at a lower degree. The glucosidase activity was

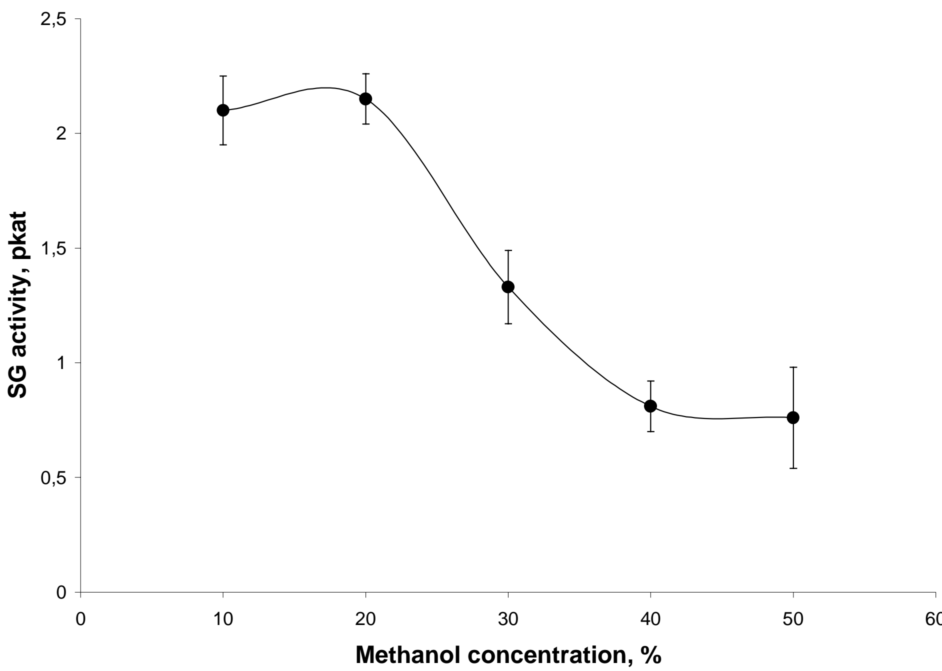


Fig. 33. The influence of different methanol concentrations (V/V) on the activity of strictosidine glucosidase.

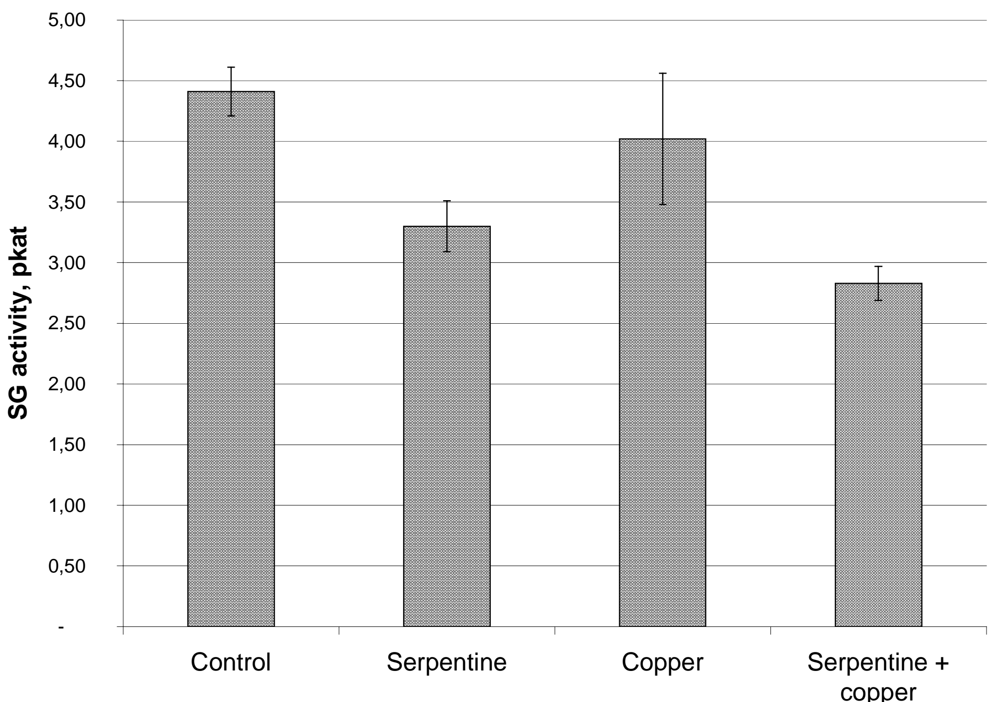


Fig. 34. Inhibition of strictosidine glucosidase activity by 1 mM serpentine (serpentine), 1 mM $CuSO_4$ (copper) and both substances together at 1 mM concentration each (serpentine + copper).

reduced for 25.2% and 8.8% in presence of 1 mM serpentine and 1 mM Cu^{2+}, respectively (Fig. 34). It is noteworthy, that the influence of two inhibitors is additive (35.8% decrease of the enzyme activity in presence of both 1 mM serpentine and 1 mM Cu^{2+}), which suggests that they may have different mode of action.

4.6. Molecular weight under native conditions

Relative molecular weight of native *R. serpentina* SG was determined by size-exclusion chromatography on a Superdex75 column. For calibration five standard proteins were used: ferritin (450 kDa), katalase (240 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa) and egg albumin (45 kDa) (Fig. 35).

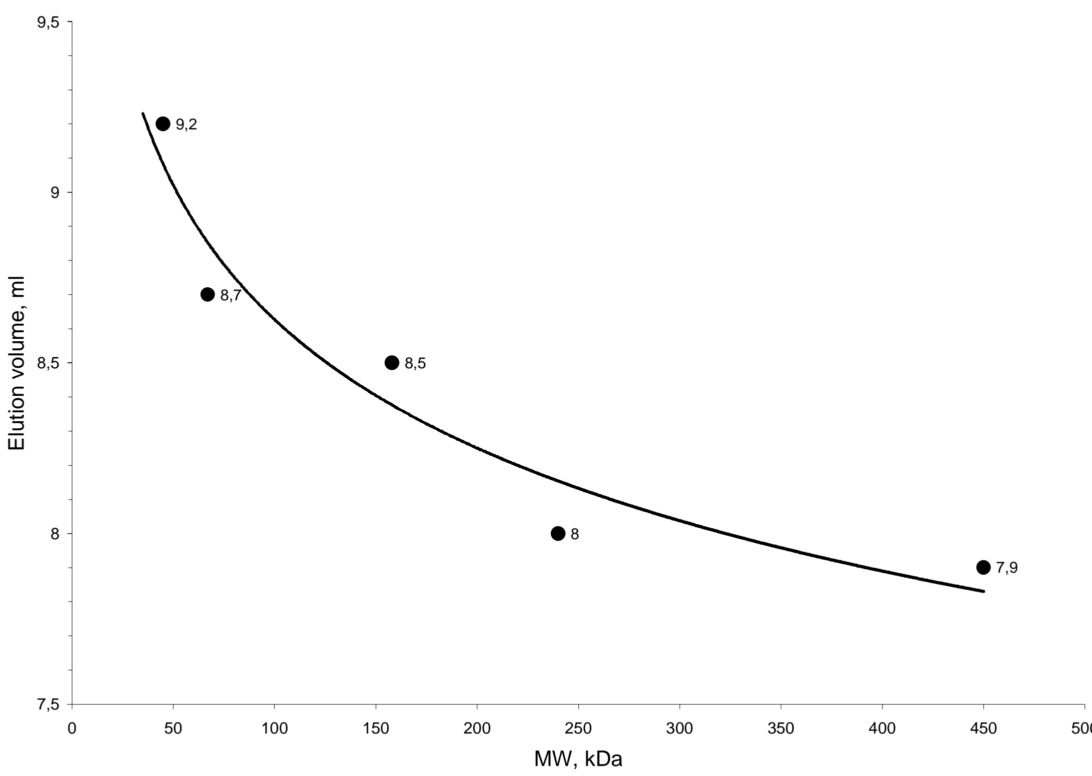


Fig. 35. The calibration curve for Superdex75 size exclusion column (elution buffer: 20 mM Tris/HCl, pH 8.0; 2 mM EDTA; 10% glycerol; 10 mM β-mercaptoethanol; flow rate 24 ml/h).

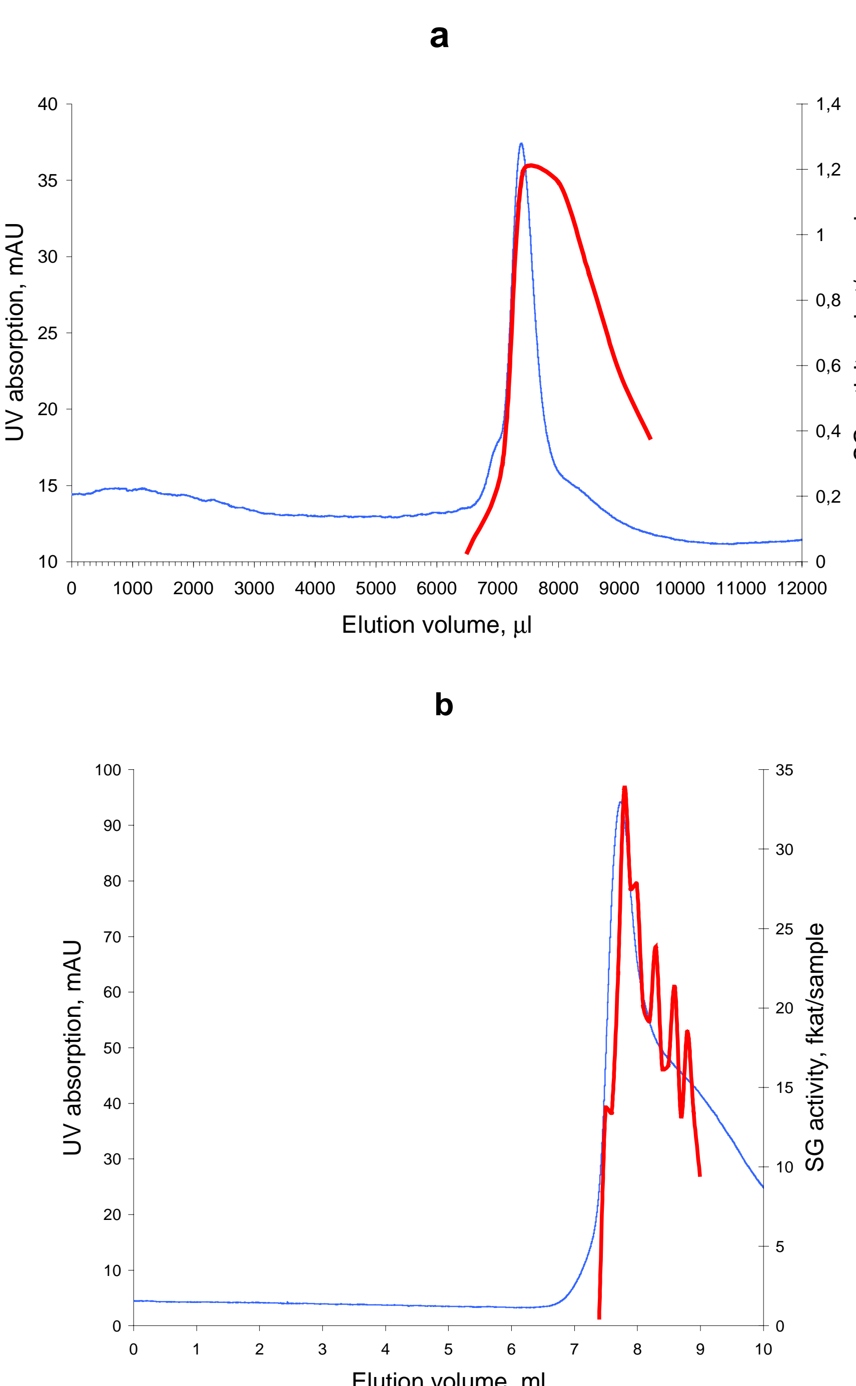


Fig. 36. Determination of molecular weight of strictosidine glucosidase by size exclusion chromatography on Superdex75 column: a) heterologously expressed enzyme; b) enzyme isolated from *R. serpentina* cultured cells. UV280 – UV absorption profile at 280 nm; SG – strictosidine glucosidase activity per sample.

The solution of homogenous heterologously expressed SG (1 ml, 13 μg protein) was concentrated (to 100 ml) and applied on the size-exclusion column. Isocratic elution was carried out collecting 0.5 ml fractions for enzyme activity test. Maximum SG activity was found in fraction 16 (7.64 - 8.14 ml) corresponding to the UV absorption peak at 7.71 ml (Fig. 36a). Thus the molecular weight of the heterologously expressed *R. serpentina* SG is determined to be more than 450 kDa.

Total proteins were extracted from 6 days old *R. serpentina* cell suspension culture with the elution buffer (5 ml per 5 g cells). The sample was centrifuged before loading onto the size-exclusion column. Isocratic elution was carried out collecting 0.1 ml fractions for SG activity test. Maximum enzyme activity was found in fraction 12 (7.74 – 7.84 ml) corresponding to the molecular weight more than 450 kDa (Fig. 36b).

4.7. Substrate specificity

Ability of the SG to hydrolyze different substrates was tested by quantification of the liberated glucose using the Trinder glucose reagent as described for the RG (**III.2.5.**). An exception was the assay containing strictosidine pentaacetate, for which the decrease of the substrate peak was measured by HPLC.

The pure SG (10 μl, 0.13 μg protein, 45.5 pkat with strictosidine) was incubated with 400 nmol of the corresponding substrate in total volume of 100 μl at 30°C for 16 h. After addition of the double volume of methanol to stop the reaction, 200 μl of the resulting mixture were added to 1 ml of Trinder glucose reagent and the absorbance at 505 nm was recorded after 30 min. The control samples without SG were treated in the same way. To prove that the glucosidase was active during all the reaction time, the enzyme was incubated under the same conditions without substrate. After 16 h 20 nmol of strictosidine were added and the incubation proceeded for 1 h. The SG

activity measured by HPLC was 6.54 pkat, which shows that the enzyme is stable at 30°C during at least 16 h.

The smallest quantity of glucose that may be measured by the method used is 10 nmol/sample, which means the detection limit for the glucosidase activity assay of 0.17 pkat (10 nmol glucose liberated in 16 h). Thus the detection limit for the glucosidase activity amounts to 0.4% of the activity with strictosidine.

To calculate the relative conversion rate of the strictosidine derivatives which were fully hydrolyzed by the SG during 16 h, shorter incubations (15 min) in presence of 26 ng (with 5α-carboxystrictosidine) or 65 ng (with 19,20-dihydro- and Nβ-methylstrictosidine) SG and 12.5 nmol of substrate were carried out. The enzyme activity was determined by HPLC measuring the decrease of substrate concentration.

The pure SG was incubated with 34 β-D-glucosides, most of them being natural products of different classes. Five of these compounds were converted at a rate of 0.8–90% compared to strictosidine (Table 5). Except of ipecoside which is deglucosylated at a very low rate of 0.8%, all other accepted substrates possess the basic skeleton of strictosidine. The α(S) configuration at C3 is essential for the enzyme reaction. Whereas vincoside, the 3β (R) epimer of strictosidine, is not accepted, the 5α-carboxystrictosidine with 3α (S) configuration has a relative conversion rate of 90%. Changing the structure of strictosidine by acetylation of the β nitrogen leads to more significant decrease of the conversion rate than methylation of the β nitrogen or hydrogenation of the isolated 18,19-double bond. Indole alkaloids possessing a sarpagine or ajmaline ring system were not accepted.

Thus the SG shows a high degree of substrate specificity. Only strictosidine derivatives and ipecoside are accepted. Ipecoside is produced by enzymatic condensation of secologanin and dopamine and has a structure similar to that of

Table 5. Substrate specificity of pure heterologously expressed strictosidine glucosidase.

Glucoside	Structure	Conversion rate, %
Strictosidine	3-Hα, R_1=R_2=H	100
5α-Carboxystrictosidine	3-Hα, R_1= H, R_2=COOH	90.0
18,19-dihydrostrictosidine	3-Hα, R_1=R_2=H	32.7
Nβ-Methylstrictosidine	3-Hα, R_1=CH_3, R_2=H	19.5
Nβ-Acetylstrictosidine	3-Hα, R_1=$COCH_3$, R_2=H	2.5
Strictosidine pentaacetate	3-Hα, R_1=$COCH_3$, R_2= H, Glc-tetraacetyl	0
Vincoside	3-Hβ, R_1=R_2=H	0
Strictosidine lactam	3-Hα	0
Vincoside lactam	3-Hβ	0
Ipecoside		0.8

21-Glucopyranosyl-hydroxysarpagan-17-al	CHO 17 N H H N OGlc 21	0
Raucaffricine 1,2-Dihydroraucaffricine 1-Methyl-1,2-dihydroraucaffricine	OAc 1 N 2 H N OGlc	0 0 0
Secologanin	CHO H OGlc H O CH_3O_2C	0
Loganin	HO H OGlc H O CH_3O_2C	0
   p-Nitrophenylglucoside Arbutin Vanillin-glucoside Vanillylalcohol-phenylglucoside Picein	R_1 R_2 R_3 OGlc R_1=NO_2, R_2=R_3=H R_1=OH, R_2=R_3=H R_1=CHO, R_2=H, R_3=OCH_3 R_1=CH_2OH, R_2=H, R_3=OCH_3 R_1=CH_3-CO, R_2=H, R_3=OCH_3	 0 0 0 0 0
Salicin	OH OGlc	0

Amygdalin		0
Avetiin		0
6-bromo-2-naphtyl-β-D-glucopyranoside		0
Cinnamic acid glucoside		0
Coniferine		0
Esculin		0
Fluorescein-glucoside		0
Isatinoxim-glucoside		0
Prunasin		0
Rhapontin		0
Rutin		0
Sinigrin		0
Zeatin-glucoside		0

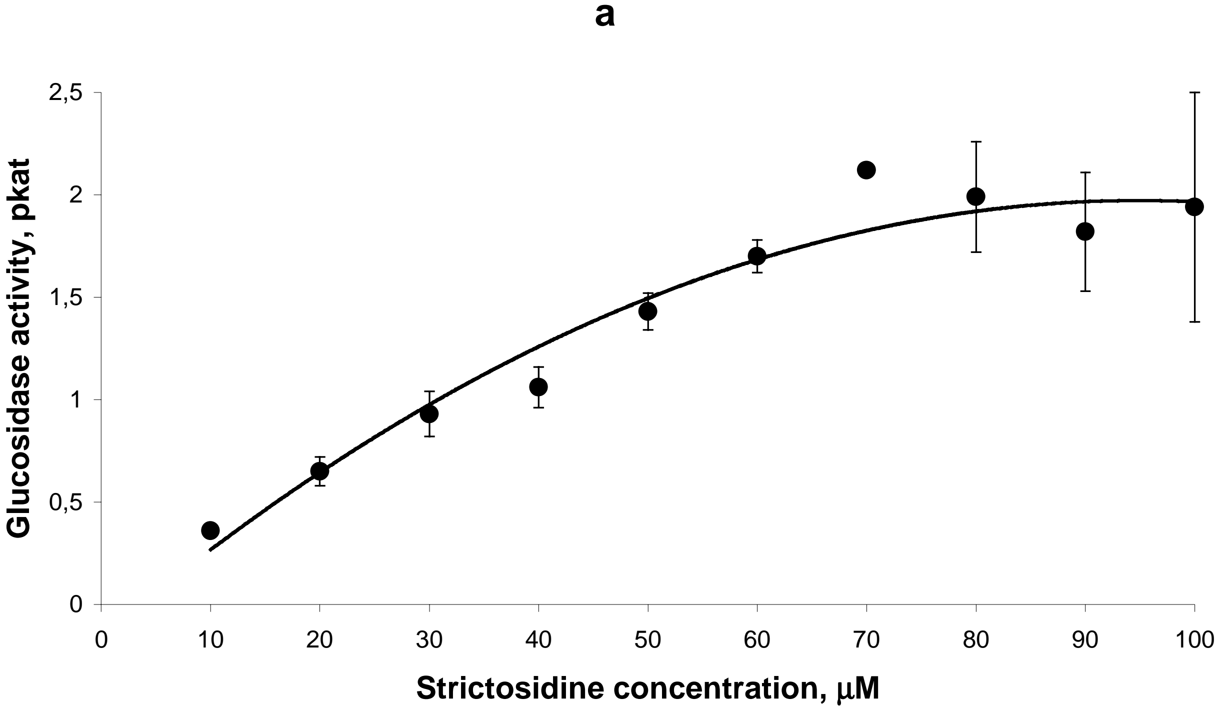
strictosidine. The terpene biogenetic precursors of strictosidine and ipecoside, secologanin and loganin, are not deglucosylated by the SG.

4.8. Enzyme kinetics

Enzyme kinetic parameters were determined in presence of 13 ng (with strictosidine), 26 ng (with 5α-carboxystrictosidine) or 65 ng (with 19,20-dihydro- and Nβ-methylstrictosidine) of SG. The glucosidase activity was tested in substrate concentration range 10 μM - 100 μM for strictosidine (Fig. 37a), 50 μM - 250 μM for 5α-carboxystrictosidine, 100 μM - 250 μM for 19,20-dihydrostrictosidine and 100 μM - 250 μM for Nβ-methylstrictosidine. The K_M and V_{max} values (Table 6) were computed from these data using the method of Lineweaver and Burk (Fig. 37b).

Table 6. Enzyme kinetic parameters of strictosidine glucosidase with different substrates.

Substrate	**K_M, μM**	**V_{max}, pkat**	**V_{max}, pkat/μg**
Strictosidine	121	4.44	342
5α-carboxystrictosidine	278	13.3	512
19,20-dihydrostrictosidine	233	7.58	117
Nβ-methylstrictosidine	435	3.00	46



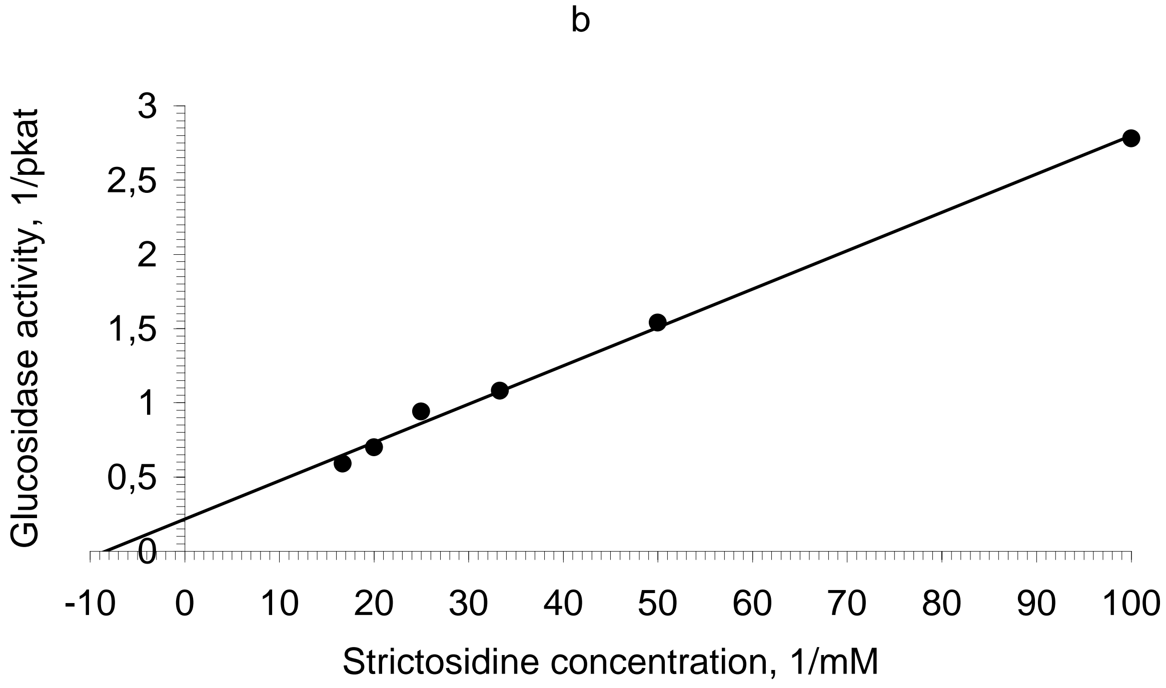


Fig. 37. Determination of enzyme kinetic parameters of strictosidine glucosidase with strictosidine: a) Michaelis-Menten curve ; b) Lineweaver-Burk presentation.

5. Enzymatic deglucosylation of strictosidine

5.1. Formation of cathenamine

Incubation of strictosidine with heterologously expressed SG led to the formation of a pellet. In the control assay containing inactivated boiled enzyme no pellet was detected. The EI-MS investigation indicated that the main component of the pellet is cathenamine. Since the possibility exists, that unstable intermediates formed after strictosidine deglucosylation may change their structure during EI-MS measurement, milder ionization techniques were applied. But the FD-MS spectrum did not display any peaks at *m/z* higher than 350. HR-FAB-MS experiment confirmed that the main deglucosylation product presents cathenamine.

The ^1H NMR spectrum in $CDCl_3$ indicated the presence of N-H as a broad singlet at δ 7.98, H-17 as sharp singlet at δ 7.52, four aromatic protons (two doublets at δ 7.45 and 7.30, H-9 and H-12; two triplets at δ 7.14 and 7.08, H-10 and H-11), H-21 as doublet at δ 6.16, H-19 as quadruplet at δ 4.61, three protons of a methoxy group as a singlet at δ 3.71, and a methyl doublet at δ 1.40. These data are identical with those reported for cathenamine in literature (Stöckigt, 1979). Thus the product of strictosidine deglucosylation catalyzed by the SG from *R. serpentina* is identified as cathenamine.

5.2. Enzymatic strictosidine deglucosylation under reductive conditions

To intercept putative precursors of cathenamine formed immediately after hydrolysis of strictosidine (Fig. 38), the enzymatic reaction was carried out in presence of reducing agents, namely $NaBH_3CN$, KBH_4 and $NaBH_4$, which were expected to reduce aldehyde groups of the intermediates and thus prevent them from further conversion. The reaction mixture was extracted with ethyl acetate and after

SG

Strictosidine

Dialdehyde

- H_2O

Cathenamine

4,21-Dehydrocorynantheine aldehyde

Carbinolamine

$NaBH_3CN$ or KBH_4

$NaBH_3CN$ or KBH_4

Tetrahydroalstonine

Sitsirikine 16 β(R)
Isositsirikine 16 α(S)

21-Hydroxycathenamine

Fig. 38. Conversion of strictosidine by *R. serpentina* strictosidine glucosidase.

evaporation of the solvent the residue was separated by 2-D TLC using the solvent systems SS3 and SS4.

5.2.1. Identification of tetrahydroalstonine

When 2-fold excess of $NaBH_3CN$ was added, only one product, showing yellow colour and blue fluorescence after CAS spraying, was detected. Its chromatographic behavior as well as EI-MS data were identical with those of authentic tetrahydroalstonine. This result supports the identification of cathenamine as the end product of cell free strictosidine deglucosylation, since the reduction of cathenamine leads to tetrahydroalstonine (Fig. 38).

5.2.2. Indirect evidence for 4,21-dehydrocorynantheine aldehyde involvement

When the concentration of $NaBH_3CN$ was increased to 250-fold excess, two products with brown CAS colour and blue fluorescence were detected. They were identified on the basis of their EI-MS data as sitsirikine and isositsirikine. It demonstrates, that 4,21-dehydrocorynantheine aldehyde is involved in the indole alkaloid biosynthesis in *R. serpentina* (Fig. 38).

The increasing of $NaBH_3CN$ concentration up to 4000-fold excess did not change the pattern of deglucosylation products. The experiments using KBH_4 or $NaBH_4$ instead of $NaBH_3CN$ were carried out analogously at pH 8.0 and gave the same results.

5.3. Enzymatic reaction at 4 °C

In order to retard the conversions of intermediates (e.g. the bond rotation necessary for the ring closure), the enzymatic deglucosylation of strictosidine was carried out at low temperature of 4 °C. The reaction mixture was extracted with cold ethyl acetate and after evaporation of the solvent the residue was separated by 2-D TLC using the solvent systems SS3 and SS4. Apart from cathenamine, a colourless product showing blue fluorescence after spraying with CAS reagent was detected. The EI-MS spectrum

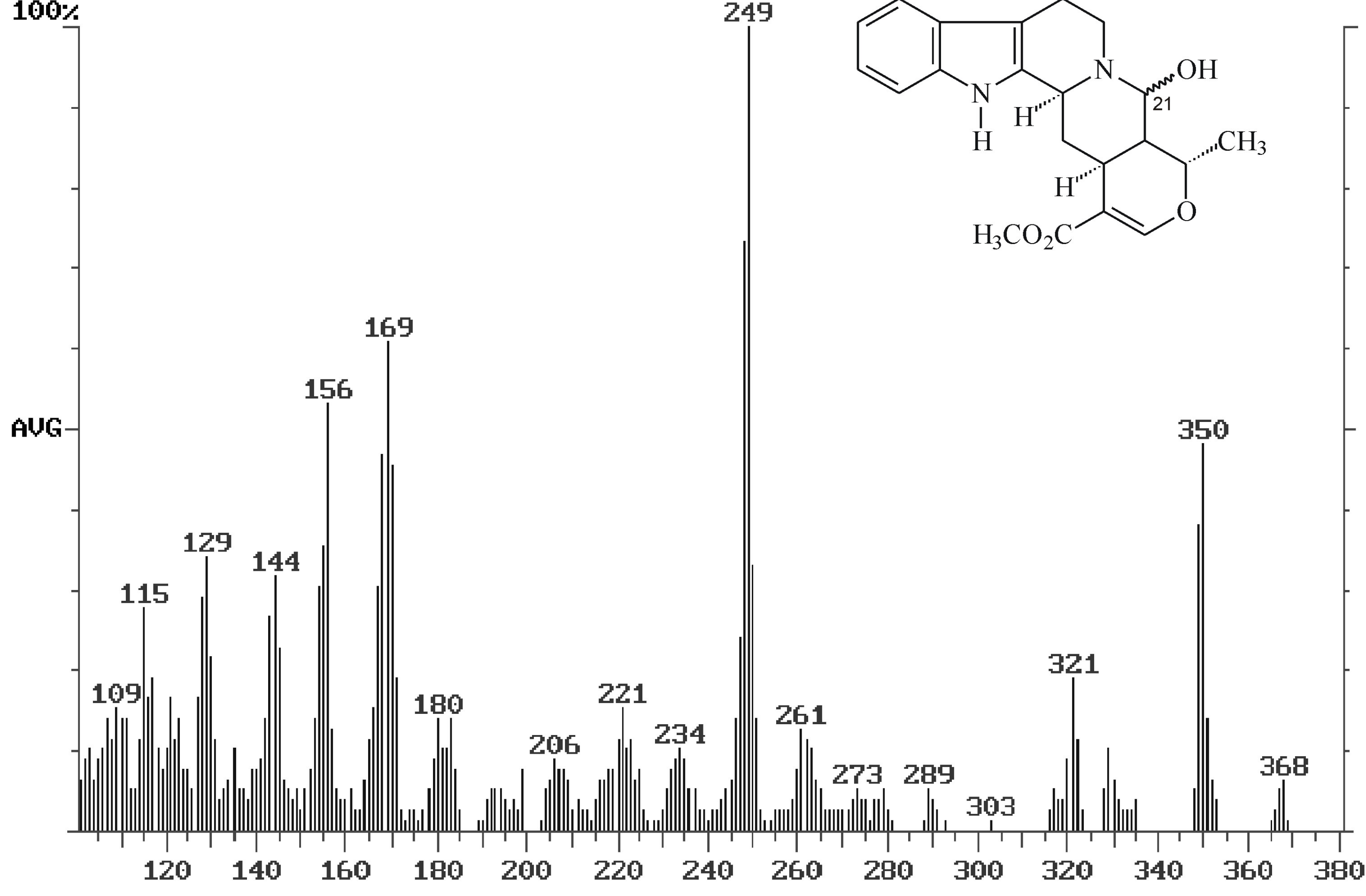


Fig. 39. EI-MS spectrum of a product (putatively 21-hydroxycathenamine) formed after enzymatic deglucosylation of strictosidine at 4^0C.

revealed a molecular weight of 368 (Fig. 39), which indicates that the substance under investigation may present 21-hydroxycathenamine. It has been supposed to be formed spontaneously from a carbinolamine deriving from strictosidine aglycone (Luijendijk et al., 1996) (Fig. 38). But the low yield of the substance precluded its NMR investigation and structure elucidation.

The enzymatic strictosidine deglucosylation under reductive conditions (using 1000-fold excess of $NaBH_3CN$ or KBH_4) at 4 °C resulted in the formation of sitsirikine and isositsirikine.

5.4. Conversion of dolichantoside

A series of experiments was carried out in order to intercept the ring-opened dialdehyde supposed to be formed after strictosidine deglucosylation. The enzymatic reaction was conducted in presence of substances able to react with aldehyde groups, e.g. hydroxylamine and thiols. As all these attempts were unsuccessful, it was decided to modify the structure of strictosidine by methylation of the Nβ amino group in order to retard the intramolecular condensation with the C-21 aldehyde leading to the ring D closure.

5.4.1. Synthesis and purification of dolichantoside

For preparation of dolichantoside (Nβ-methylstrictosidine) strictosidine was methylated using $NaBH_3CN$ and HCHO. In a typical reaction 106 mg strictosidine (200 μmol) were converted into 37 mg (68 μmol, 34% yield) of a product which was identified by EI-MS as methylstrictosidine (Fig. 40a). To prove the Nβ-position of the methyl group the compound was acetylated. The possible acetylation positions in strictosidine are four OH groups of the glucose moiety and the Nβ amino group. Thus to Nβ-methylstrictosidine only four acetates can be added, while Nα-methylstrictosidine should be converted to pentaacetate. The acetylation product was

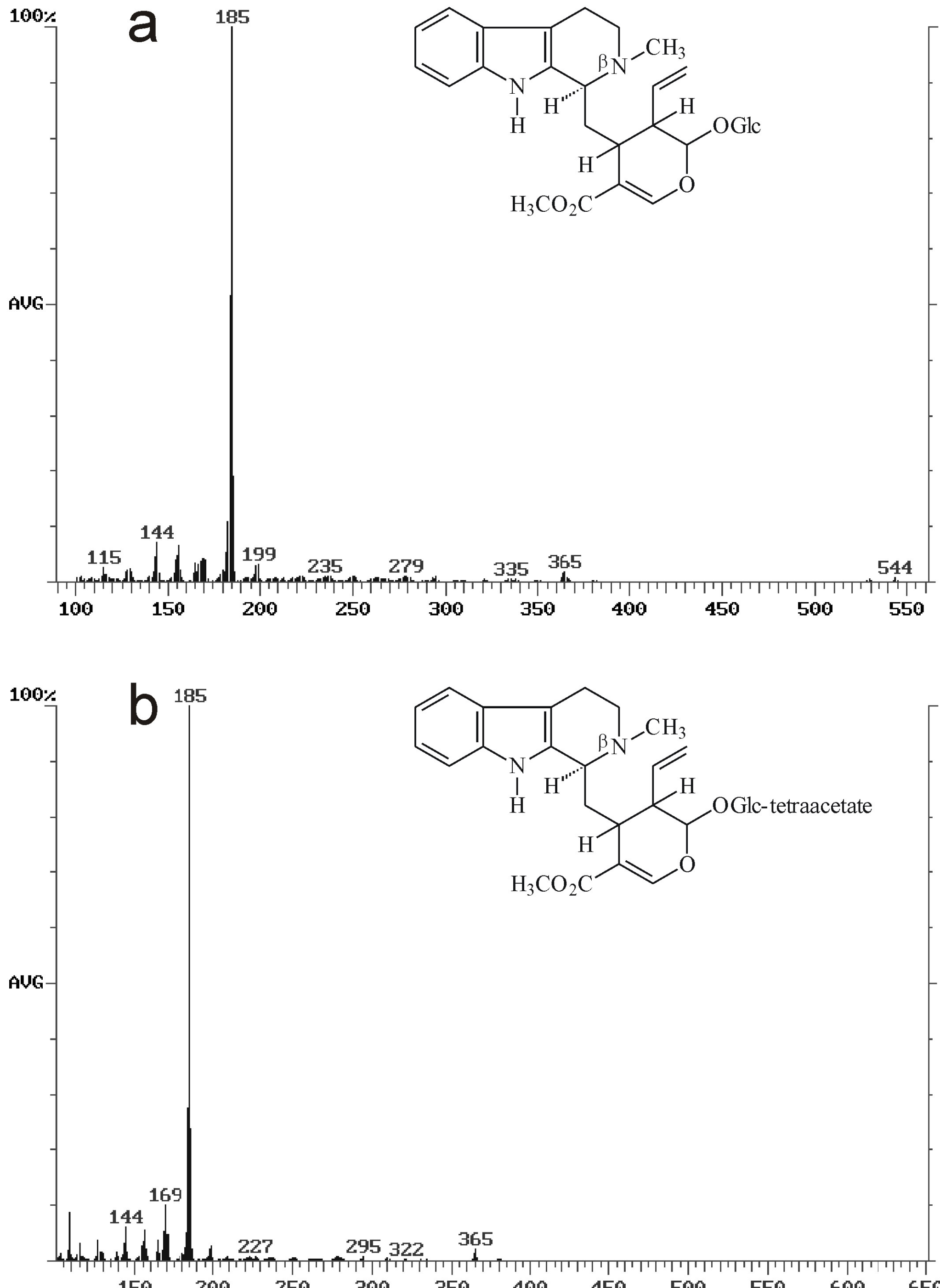


Fig. 40. EI-MS spectra of Nβ-methylstrictosidine (a) and Nβ-methylstrictosidine tetraacetate (the peaks at *m/z* 712 (M^+) and 697 ($[M^+-CH3]$) are not shown) (b).

identified by EI-MS as methylstrictosidine tetraacetate(Fig. 40b), which demonstrates that the obtained product presents Nβ-methylstrictosidine. Its identification was completed by measuring the ^{1}H NMR spectrum which was identical with the literature data reported for dolichantoside (Ohmori et al., 1998).

5.4.2. Enzymatic deglucosylation of dolichantoside

In a preliminary experiment, dolichantoside (1mg) was incubated with SG in 1 ml 0.1 M citrate-phosphate buffer (pH 5.0) over night at 30 °C. The reaction mixture was extracted with EtOAc and after evaporation of the solvent the residue was separated by TLC using solvent system SS3. The control assay containing the inactivated boiled enzyme was processed in the same way. Several products were detected after incubation with the active glucosidase only. EI-MS screening revealed, that the most unpolar of them (Rf 0.64, blue fluorescence at 366 nm after spraying with CAS) had a molecular weight of 382 (Fig. 41), corresponding to the putative Nβ-methyldialdehyde (Fig. 42). HR-EI-MS measurement confirmed the elementar composition $C_{22}H_{26}O_4N_2$: *m/z* 382.1884 ($[M+H]^+$, calc. for $C_{22}H_{26}O_4N_2$, 382.1893), 367.1681 ($[M^+-CH_3]$, calc. for $C_{21}H_{23}O_4N_2$, 367.1658).

To obtain the deglucosylation product for NMR investigation, 30 mg dolichantoside (55 μmol) were incubated with 39 μg homogenous SG which gave 1 mg of the new alkaloid (2.6 μmol, 4.7% yield).

The ^{1}H NMR spectrum (Fig. 43) showed no signals which would correspond to the expected aldehyde protons, as well as to the vinyl side chain. Absence of a signal from Nα-H suggested, that one of the aldehyde groups has reacted with the Nα amino group. In addition, chemical shifts of Nβ methyl protons (δ 2.39), H-3 (δ 3.87) and protons at C-5 (δ 2.60 and 3.52) indicated a tertiary β-nitrogen. Presence of a methyl group at δ 1.57 correlated in the ^{1}H-^{1}H COSY spectrum (Fig. 44) to H19 at δ 4.36

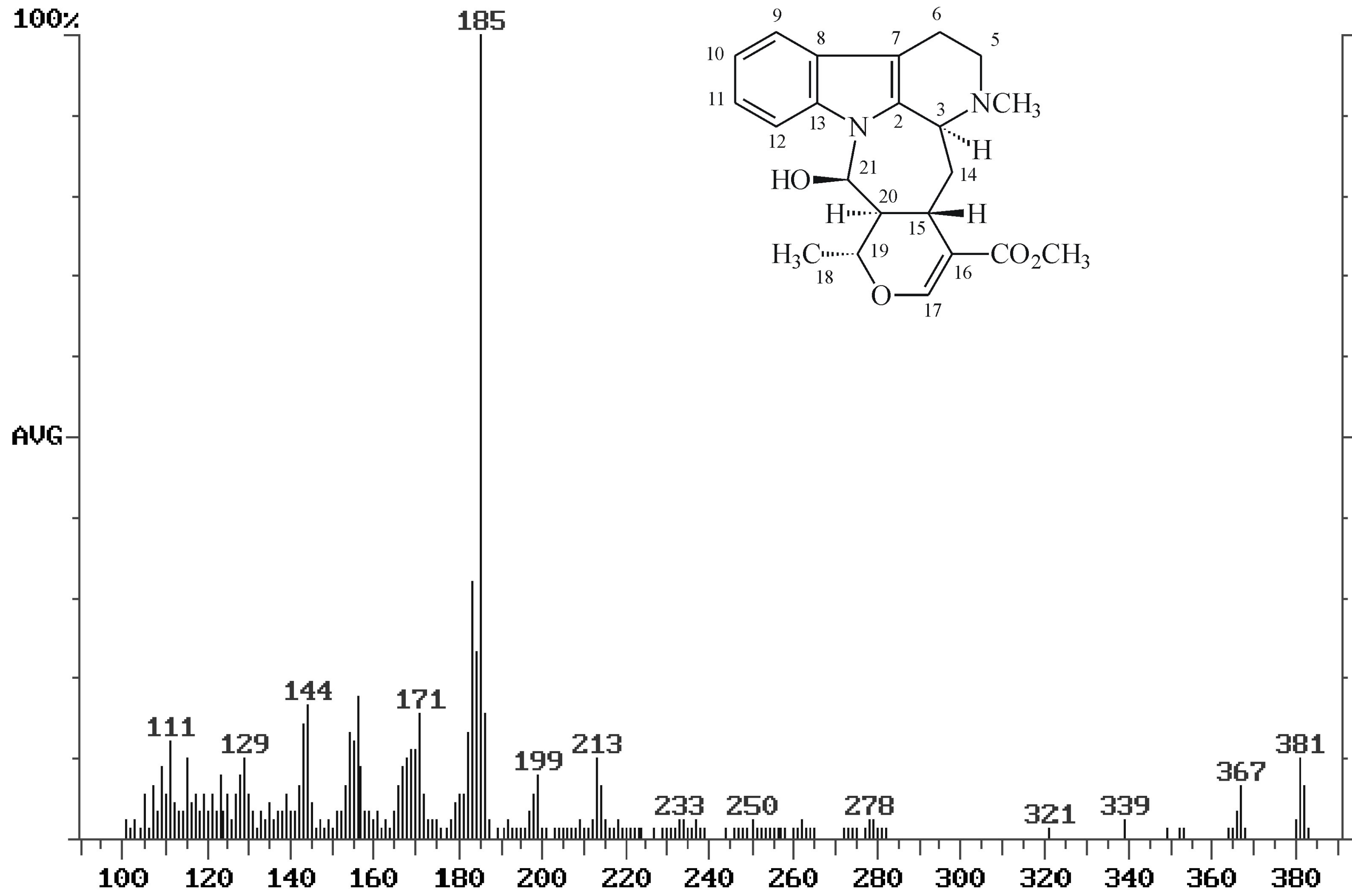


Fig. 41. EI-MS spectrum of 3-iso-correantine A formed after enzymatic dolichantoside deglucosylation.

Nβ-Methylstrictosidine

Aglycone

Dialdehyde

3-Iso-correantine A

Fig. 42. Proposed scheme of 3-iso-correantine A formation after enzymatic hydrolysis of dolichantoside.

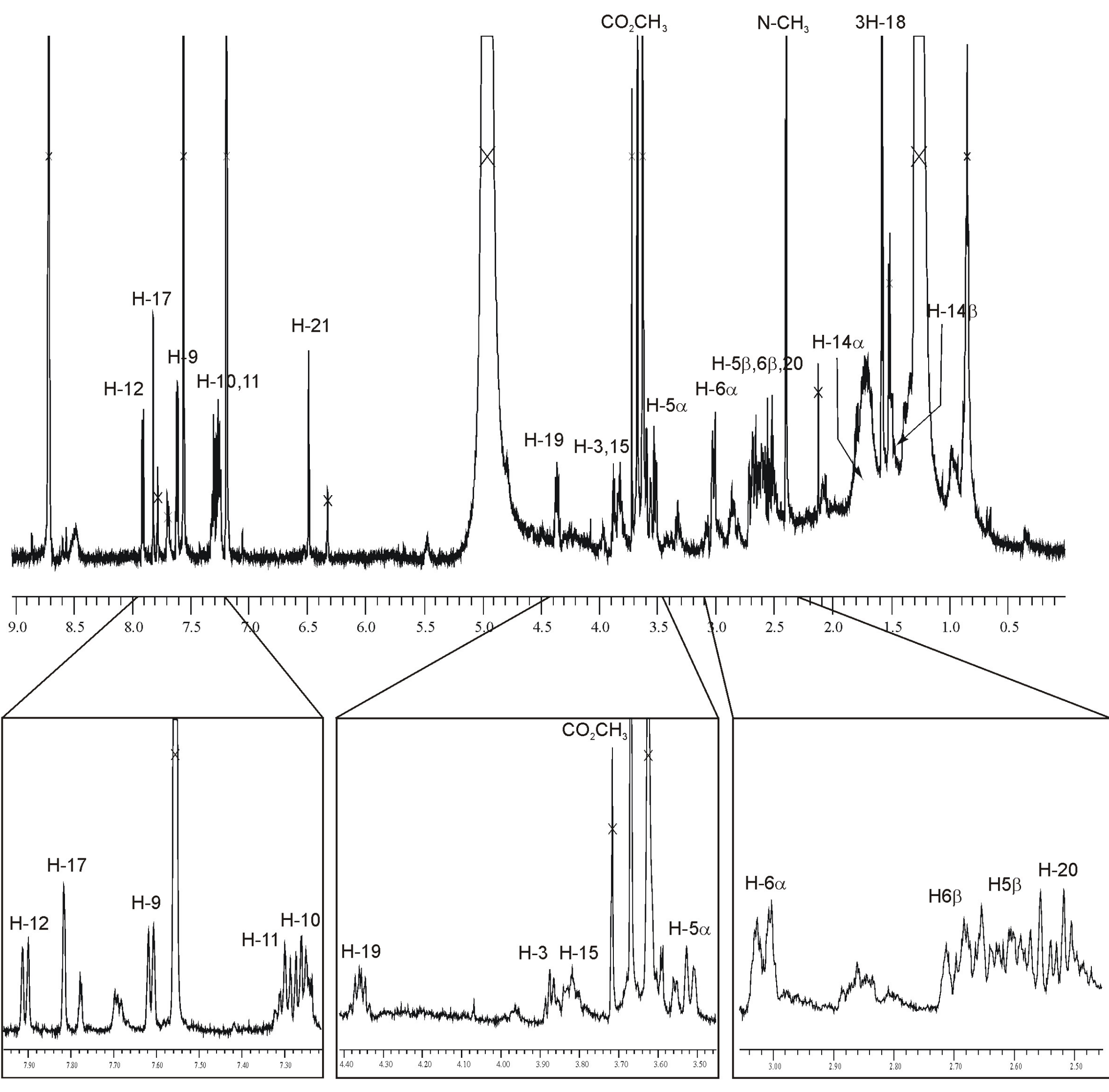


Fig. 43. ^{1}H NMR spectrum of 3-iso-correantine A in pyridine-d_5.

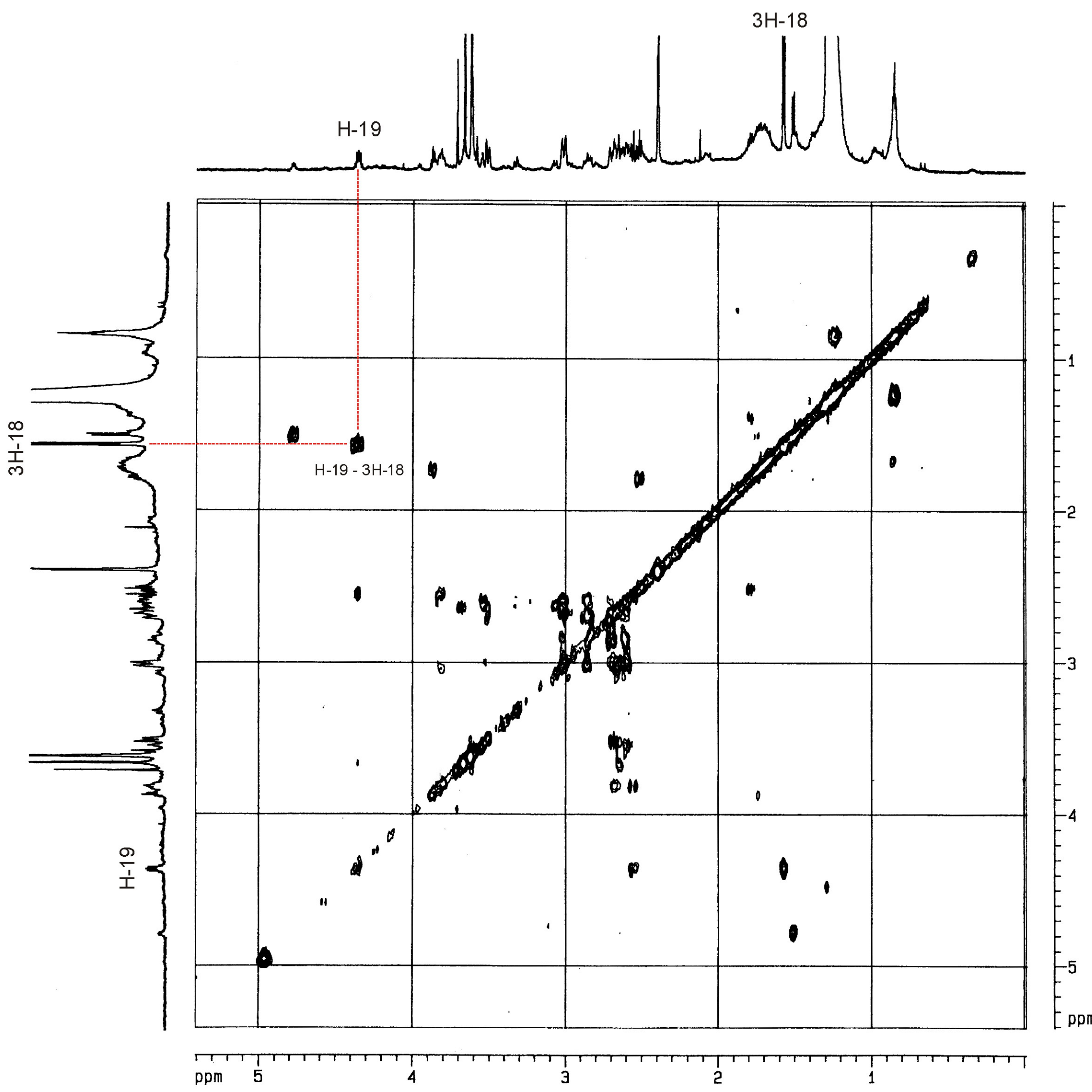


Fig. 44. ^{1}H-^{1}H COSY spectrum of 3-iso-correantine A.

suggested the closure of the ring E, which is confirmed by the shift of H-17 (δ 7.82). H-21 appears as a singlet at δ 6.48 correlated on NOESY spectrum (Fig. 45) to one of the aromatic protons (H-12 at δ 7.91) indicating that C-21 bears an hydroxyl function and is adjacent to Nα. The structure elucidation of the new alkaloid was completed by HSQC and HMBC measurements which allowed to determine the chemical shifts of carbons. The position of H-3 was assumed to be α because of 3(α) configuration of dolichantoside. The relative stereochemical configuration at other chiral carbons was defined by the analysis of NOESY spectrum (Fig. 45). Absence of NOE signal between H-3 and H-15 indicates β position of H-15. The distance between these two protons in identical orientation (2.4 Å as calculated by PCMODEL) is close enough to exhibit a strong NOE signal, while in case of opposite orientation the distance is much longer (3.7 Å) and no significant NOE intensity is expected. Strong NOE interaction between H-15 and H-19 shows β position of H-19 and, consequently, α position of C-18 methyl group. The NOE intensities after irradiation of H-19 suggest the stereochemical configuration at C-20 and C-21. Besides strong interactions with H-15 and 3H-18, two weak signals with H-20 and H-21 are detected, which indicates α conformation of H-20 and H-21. The distance between H-21 in β position and H-19 is 2.4 Å which is closer than between H-19 and H-15 (2.9 Å), thus for such orientation the NOE signals H-19 - H-15 and H-19 – H-21 should have comparable intensities. In case of H-21(α) the distance between H-21 and H-19 suggests lower NOE intensity (3.2 Å). The α position of H-20 and H-21 is confirmed also by strong NOE interactions between themselves and with 3H-18 (Fig. 46). The novel compound is the 3-isomer of correantine A, which has been isolated from *Psychotria correae* (Achenbach et al., 1995). It has been shown, that correantine A and its 21-epimer are

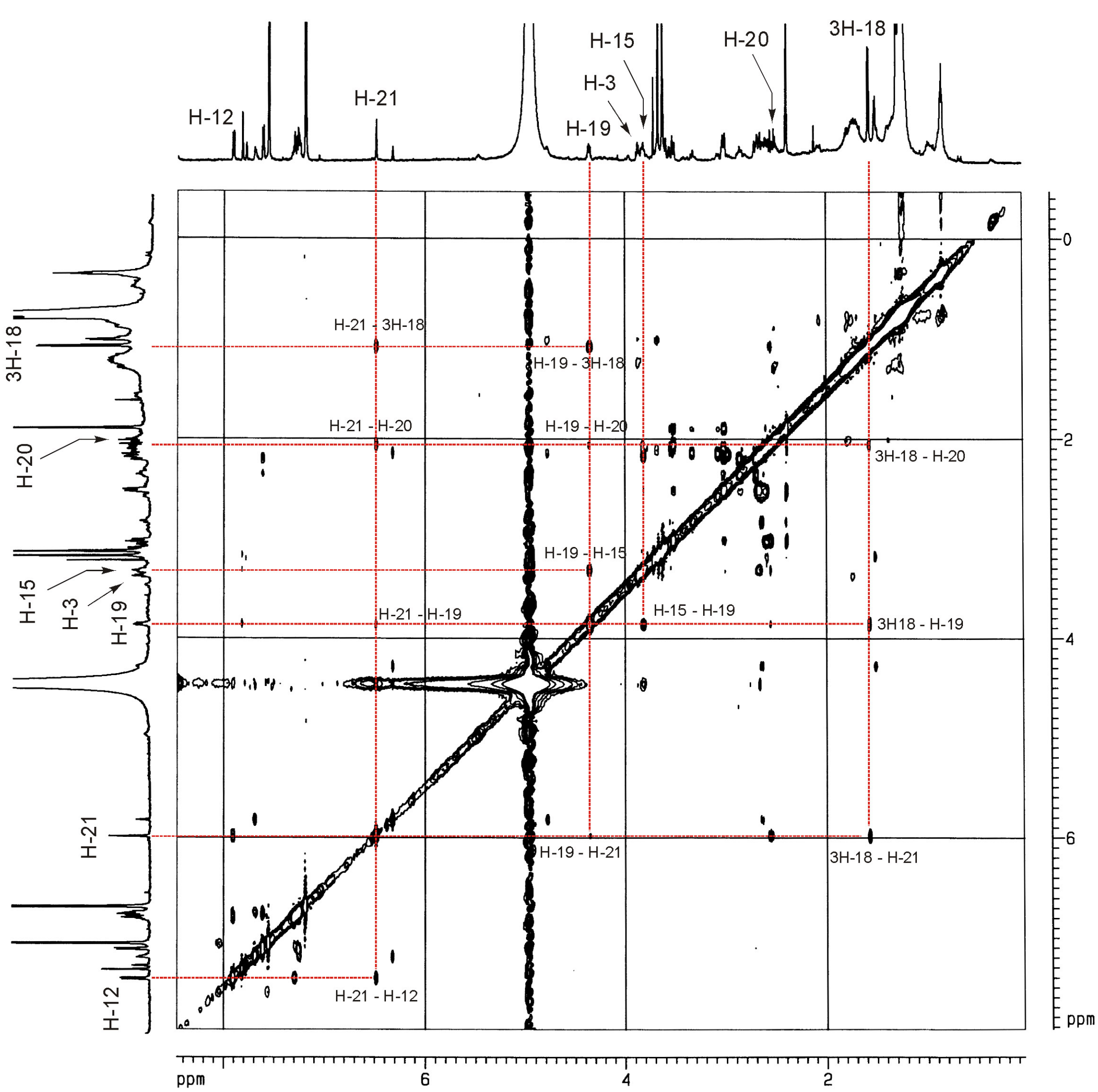


Fig. 45. NOESY spectrum of 3-iso-correantine A.

formed after hydrolysis of isodolichantoside by an unspecific β-glucosidase (Achenbach et al., 1995), which corroborates the structure and stereochemical configuration of the new alkaloid described here.

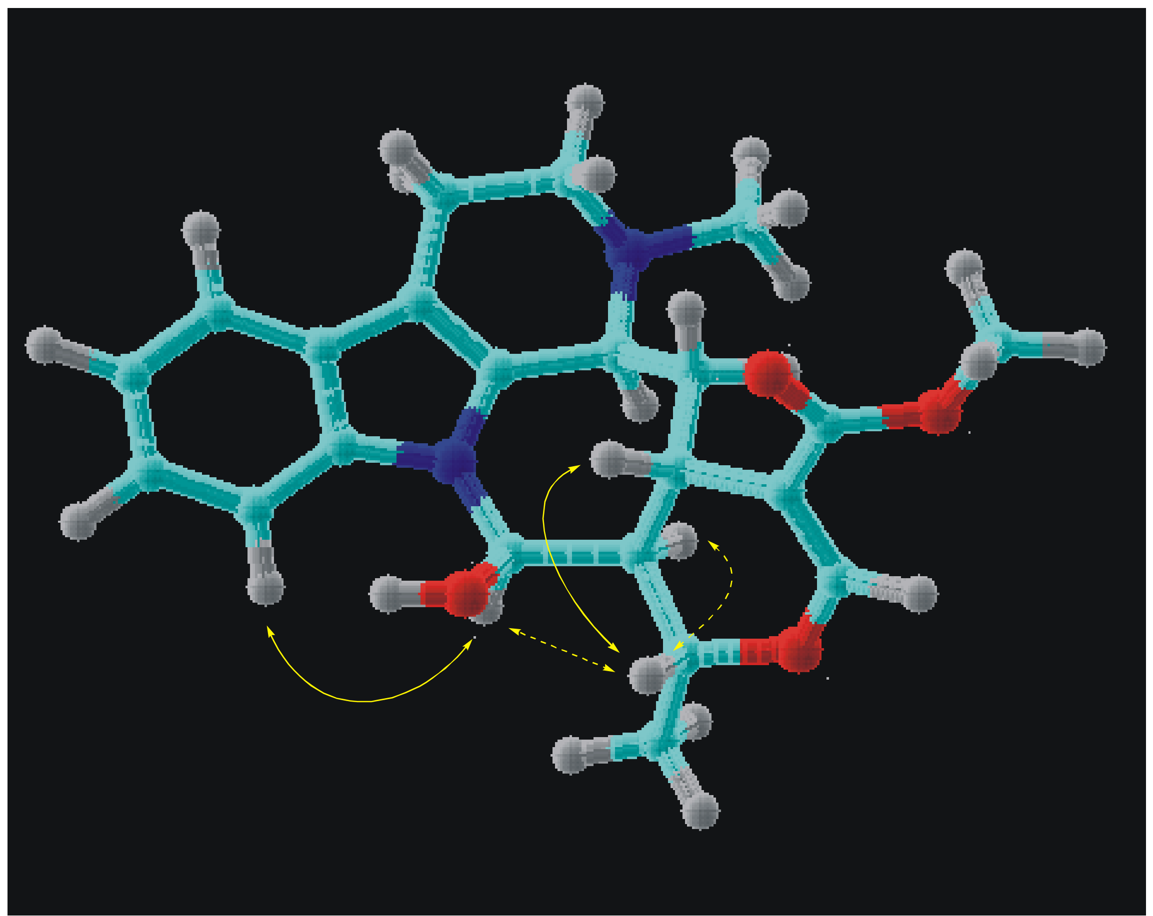


Fig. 46. Important NOE interactions of 3-iso-correantine A. Solid and dotted arrows represent strong and weak NOE signals, respectively.

IV. DISCUSSION

1. Enzymes catalyzing strictosidine deglucosylation in *R. serpentina* cell suspension culture

Deglucosylation of strictosidine is an important step in the biosynthesis of all monoterpenoid indole alkaloids. Up to now more than 2000 of these compounds are known to occur in higher plants, and all this structural diversity derives from strictosidine. At some stage downstream of this glucoalkaloid the biosynthetic pathways diverge to lead to different classes of indole alkaloids. It has been supposed that the enzyme catalyzing strictosidine deglucosylation may play a role in steering the unstable strictosidine aglycone in certain biosynthetic directions (Geerlings et al., 2000).

The cDNA encoding strictosidine glucosidase (SG) was obtained from *R. serpentina* cell suspension culture by the method of homology cloning and heterologously expressed in *E. coli*. Except of this enzyme, another glucosidase found in *R. serpentina* cultivated cells, the raucaffricine glucosidase (RG), was shown to hydrolyze strictosidine, although with considerably lower efficiency. The V_{max} value with strictosidine was determined to be 347 pkat/µg for the SG and 2.56 pkat/µg for the RG, which is ca. 135 times lower. The V_{max} value of the RG with its preferred substrate, raucaffricine, is 199-fold higher (510 pkat/µg) indicating that the hydrolysis of strictosidine is only a side activity of the RG. The K_M value with this glucoalkaloid is 15 times lower for the SG (0.12 mM) than for the RG (1.82 mM), which shows that the former enzyme binds strictosidine significantly better. From these data it can be concluded, that the most part of strictosidine in *R. serpentina* cells is deglucosylated by the SG.

2. Properties of the glucosidases involved in indole alkaloid biosynthesis

The deduced amino acid sequence of the *R. serpentina* SG shows highest homology of 70% to the SG from *C. roseus* followed by the RG from *R. serpentina* (56%) and other plant β-glucosidases. Both SGs as well as RG are assigned to the family 1 of glycosyl hydrolases. The classification of glycosyl hydrolases in families is based on amino acid sequence similarities and is expected to reflect the folding features and catalytic machinery of the enzymes (Henrissat, 1991; Henrissat and Davies, 1997). The family 1 comprises β-O-glucosidases with retaining mechanism of glycosidic bond hydrolysis. As a result of enzymatic reaction the anomeric configuration at C-1 of the glucose is retained. Two amino acid residues are involved in the catalysis: a proton donor and a nucleophile (base). The acid catalyst protonates the glycosidic oxygen and the nucleophile assists the aglycone departure. The resulting glycosylated enzyme is hydrolyzed by a water molecule and a product with the same stereochemistry as a substrate is generated (Fig. 47) (Davies and Henrissat, 1995).

Fig. 47. Retaining mechanism of enzyme catalyzed hydrolysis of a glycosidic bond . AH – proton donor (acid) catalyst, B – nucleophile (base) catalyst, R – aglycone.

The catalytic amino acids are highly conserved throughout the enzyme family. Once they are identified in some family members, the alignment of amino acid sequences might suggest the catalytic residues in new enzymes within the homology group. In

family 1 of glycosyl hydrolases two glutamate residues were shown to participate directly in glycosidic bond cleavage (Withers et al., 1990; Trimbur et al., 1992; Baird et al, 1990; Keresztessy et al., 1994). The analysis of deduced amino acid sequences of three glucosidases involved in indole alkaloid biosynthesis indicates the putative catalytic glutamic acid residues (Fig. 48). The proton donor is preceded by asparagine and followed by proline. This motif is conserved in all three enzymes under investigation. The nucleophile catalyst is suggested to be located within the [LIVMFSTC]-[LIVFYS]-[LIV]-[LIVMST]-E-N-G-[LIVMFAR] pattern, which was found up to now in all members of family 1 of glycosyl hydrolases and is used as a signature of this enzyme family (Hofmann et al., 1999). However, the ENG tripeptide is present only in the amino acid sequence of the RG, while in both SGs asparagine is exchanged, against serine in *R. serpentina* SG and against cysteine in *C. roseus* enzyme. Region-directed mutagenesis of β-glucosidase from *Agrobacterium faecalis* indicated that this asparagine residue does not play a critical role in catalysis (Trimbur et al., 1992). The SGs are the first two members of the glycosyl hydrolases family 1 in which this residue is not conserved, which supports the finding mentioned above. On the contrary, the next glycine proved to be essential for enzyme activity probably due to its small size necessary for the right conformation of the active site (Trimbur et al., 1992). This residue is indeed conserved in both SGs and RG. The sequence DxxRxxY near the C-terminus is also found in most members of the family 1 of glycosyl hydrolases, although it was shown that only asparaginic acid plays an important, but not crucial role in the catalysis (Trimbur et al., 1992). The exact function of this residue is not defined yet, as well as the role of the N-terminal signature of the family 1 of glycosyl hydrolases (Hofmann et al., 1999), which is present near the N-terminus of all the family members.

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SG_Rs   (1) MDNTQAEPLVVAIVPKPNASTEHTNSHLIPVTRSKI----------VVHRRDFPQDFIFG
SG_Cr   (1) MGSKDDQSLVVAI--SPAAEPNGNHSVPIPFAYPSIPIQPRKHNKPIVHRRDFPSDFILG
RG_Rs   (1) M----------AT--QSSAVIDSNDA-------TRI------------SRSDFPADFIMG

SG_Rs  (51) AGGSAYQCEGAYNEGNRGPSIWDTFTQRSPAKISDGSNGNQAINCYHMYKEDIKIMKQTG
SG_Cr  (59) AGGSAYQCEGAYNEGNRGPSIWDTFTNRYPAKIADGSNGNQAINSYNLYKEDIKIMKQTG
RG_Rs  (30) TGSSAYQIEGGARDGGRGPSIWDTFTHRRPDMIRGGTNGDVAVDSYHLYKEDVNILKNLG

SG_Rs (111) LESYRFSISWSRVLPGGRLAAGVNKDGVKFYHDFIDELLANGIKPSVTLFHWDLPQALED
SG_Cr (119) LESYRFSISWSRVLPGGNLSGGVNKDGVKFYHDFIDELLANGIKPFATLFHWDLPQALED
RG_Rs ( 90) LDAYRFSISWSRVLPGGRLSGGVNKEGINYYNNLIDGLLANGIKPFVTLFHWDVPQALED
                                                  A
SG_Rs (171) EYGGFLSHRIVDDFCEYAEFCFWEFGDKIKYWTTFNEPHTFAVNGYALGEFAPGRGGKGD
SG_Cr (179) EYGGFLSDRIVEDFTEYAEFCFWEFGDKVKFWTTFNEPHTYVASGYATGEFAPGRGGADG
RG_Rs (150) EYGGFLSPRIVDDFCEYAELCFWEFGDRVKHWMTLNEPWTFSVHGYATGLYAPGRGRTSP

SG_Rs (231) E----------------------GDPAIEPYVVTHNILLAHKAAVEEYRNKFQKCQEGEI
SG_Cr (239) K----------------------GEPGKEPYIATHNLLLSHKAAVEVYRKNFQKCQGGEI
RG_Rs (210) EHVNHPTVQHRCSTVAPQCICSTGNPGTEPYWVTHHLLLAHAAAVELYKNKFQRGQEGQI

SG_Rs (269) GIVLNSMWMEPLSDVQA-DIDAQKRALDFMLGWFLEPLTTGDYPKSMRELVKGRLPKFSA
SG_Cr (277) GIVLNSMWMEPLNETKE-DIDARERGLDFMLGWFIEPLTTGEYPKSMRALVGSRLPEFST
RG_Rs (270) GISHATQWMEPWDENSASDVEAAARALDFMLGWFMEPITSGDYPKSMKKFVGSRLPKFSP

SG_Rs (328) DDSEKLKGCYDFIGMNYYTATYVTNA--VKSNSEKLSYETDDQV-----TKTFERNQKPI
SG_Cr (336) EVSEKLTGCYDFIGMNYYTTTYVSNA---DKIPDTPGYETDARINKNIFVKKVDGKEVRI
RG_Rs (330) EQSKMLKGSYDFVGLNYYTASYVTNASTNSSGSNNFSYNTDIHV-----TYETDRNGVPI
                                                 B
SG_Rs (381) GHALYGGWQHVVPWGLYKLLVYTKETYHVPVLYVTESGMVEE---------NKTKILLSE
SG_Cr (393) GEPCYGGWQHVVPSGLYNLLVYTKEKYHVPVIYVSECGVVEENRTNILLTEGKTNILLTE
RG_Rs (385) GPQSGSDWLLIYPEGIRKILVYTKKTYNVPLIYVTENGV-DD--------VKNTNLTLSE

SG_Rs (432) ARRDAERTDYHQKHLASVRDAIDDGVNVKGYFVWSFFDNFEWNLGYICRYGIIHVDYK-S
SG_Cr (453) ARHDKLRVDFLQSHLASVRDAIDDGVNVKGFFVWSFFDNFEWNLGYICRYGIIHVDYK-T
RG_Rs (436) ARKDSMRLKYLQDHIFNVRQAMNDGVNVKGYFAWSLLDNFEWGEGYGVRFGIIHIDYNDN

SG_Rs (491) FERYPKESAIWYKNFIAGKSTTSPA-KRR-REEAQ-VELV--KR-QKT
SG_Cr (512) FQRYPKDSAIWYKNFISEGFVTNTA-KKRFREEDKlVELV--KK-QKY
RG_Rs (496) FARYPKDSAVWLMNSFHKNISKLPAVKRSIREDDE--EQVSSKRlRK-
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Fig. 48. Alignment of deduced amino acid sequences of three glucosidases involved in indole alkaloid biosynthesis. SG_Rs: SG from *R. serpentina*, SG_Cr: SG from *C. roseus*, RG_Rs: RG from *R. serpentina*. Identical amino acids are shaded. Motifs conserved in members of glycosyl hydrolases family 1 are highlighted black, the putative catalytic glutamate residues are marked: A – proton donor, B – nucleophile; lysine residues at the C-terminus are in boxes; possible peroxisomal targeting signals are underlined.

The 3D structures of several glycosyl hydrolases belonging to the family 1 were determined to present a $(\beta/\alpha)_8$ barrel, with the proton donor and nucleophile catalysts found on strands 4 and 7, respectively (Jenkins et al., 1995).

The SG from *R. serpentina* cell suspension culture displays under native conditions a considerably higher molecular weight than is calculated for a monomeric protein. The same was shown also for the enzyme from *C. roseus* (Hemscheidt and Zenk, 1980; Luijendijk et al., 1998; Geerlings et al., 2000) (Table 7). It should be noted, that the SGs appear as high molecular weight aggregates also after heterologous expression in yeast (*C. roseus* enzyme) or in *E. coli* (*R. serpentina* SG). It has been supposed, that the *C. roseus* SG presents a membrane bound enzyme which accounts for its high molecular weight (Geerlings et al., 2000). However, the analysis of its deduced amino acid sequence with several programs based on a hidden Markov model (Sonnhammer et al., 1998; Tusnady and Simon, 1998) did not reveal any regions that would probably form transmembrane helices. On the other hand, the *C. roseus* SG is predicted to have an uncleavable N-terminal signal sequence (Nakai and Kanehisa, 1992). Signal sequences play a key role in targeting and membrane translocation of both soluble and membrane bound proteins. These peptides consist of a hydrophobic core flanked on its N- and C-terminal sides by polar regions and show great variation in length and amino acid sequence. Usually they are found at the N-terminal end of a protein, but may be located also within its sequence or at the C-terminus (Martoglio and Dobberstein, 1998). Signal sequences can be cleaved off by the enzyme signal peptidase after membrane translocation or remain in the protein and anchor it in the membrane. The uncleavable signal-anchor sequences can insert the protein into the membrane in either orientation. Depending on whether the N-terminus is kept on the cytoplasmic side or transferred across the membrane, the protein falls into the type II

Table 7. Properties of SGs from *R. serpentina* and *C. roseus*. n.d. – not determined.

	SG from *C. roseus* cell suspension cultures (Hemscheidt and Zenk, 1980)	SG from *C. roseus* cell suspension cultures (Luijendijk et al., 1998)	SG from *C. roseus* expressed in yeast (Geerlings et al., 2000)	SG from *R. serpentina* expressed in *E. coli*
MW (calculated)	-	-	63.043 kD	60.881 kD
MW (measured under native conditions)	230 kD (I) >450 kD (II)	>1500 kD	>660 kD	>450 kD
pH optimum	6.0-6.4	6.0-8.5	n.d.	5.0-5.2
Inhibition by 1 mM serpentine	n.d.	50%	n.d.	25.2%
Temperature optimum	30 ^{O}C	n.d.	n.d.	50 ^{O}C
K_M	0.2 mM (I) 0.1 mM (II)	≤20 μM	n.d.	0.12 mM
V_{max}	0.23 nM/min (I) 0.12 nM/min (II)	180-230 pkat/mg	n.d.	342 pkat/μg
Enrichment factor	120	60.2	-	250

or Ib of transmembrane proteins, respectively (Singer, 1990). The type of orientation is determined by the features of signal-anchor sequences (Martoglio and Dobberstein, 1998) and can be predicted by the net charge difference of polar regions on both sides of the hydrophobic core (Hartmann et al., 1989). The membrane topology seems to be relevant for the subcellular localization site of a protein, e.g. the proteins of type Ib, where the SG from *C. roseus* is predicted to belong, are favored at the endoplasmic reticulum (ER) (Nakai and Kanehisa, 1992). Thus the high molecular weight of the *C. roseus* SG isolated from the plant cells or expressed in yeast is likely to be due to its association with the membranes of the ER.

The analysis of amino acid sequence of the *R. serpentina* SG does not give any cue to account for the high molecular weight of the enzyme. On the contrary to the *C. roseus* SG, the enzyme from *R. serpentina* is predicted to lack both transmembrane helices and uncleavable signal sequence. The possible explanation is the aggregation of the proteins and formation of large complexes. As the protein-protein interactions are difficult to predict (Jones and Thornton, 1997), further experiments (e.g. crystallization of the SG and X-ray analysis of its 3-D structure) are necessary to examine this hypothesis. It is tempting to suppose, that the SG in the plant cell may be associated with other enzymes of indole alkaloid biosynthesis which intercept and convert the unstable intermediates formed after strictosidine deglucosylation. Putative ability of SG units to interact with other proteins and between themselves could explain also the high molecular weight of the heterologously expressed enzyme. On the other hand, the formation of large complexes after the intein-mediated purification of the heterologously expressed SG may be due to the aggregation of several identical SG units failing to fold correctly after intein cleavage. A high concentration of

unfolded or malfolded proteins can lead to abnormal protein-protein interactions and aggregation (Okita and Rogers, 1996).

Anchoring in the ER membrane does not necessary mean that the protein is retained in this cell compartment, it can be further sorted included in the vesicles. The proteins resident to the ER often show C-terminal retention signals. The tetrapeptide KDEL and some related sequences (HDEL, RDEL) were demonstrated to act as ER retention signals in soluble luminal proteins (Vitale et al., 1993). Two lysines positioned three and four or five residues from the C-terminus were shown to confer the ER retention on the type I transmembrane proteins (Jackson et al., 1990). But there exist many examples of the ER associated proteins without any distinct (or identified) retention signals (Okita and Rogers, 1996). Thus the question about the further fate of a protein targeted to the ER often cannot be solved by the primary structure analysis. Three lysine residues are located at the C-terminus of the SG from *C. roseus*, but none of them occupies the –3 position (Fig. 48). Moreover, two lysines are found also at the C-terminal end of the SG from *R. serpentina* and the RG, which are not predicted to be directed to the ER. Whether the lysines at the C-terminus of the *C. roseus* SG function as the ER retention signal, remains unclear. The subcellular localization of this enzyme in the ER was established experimentally (Geerlings et al., 2000), although the evidence exists that at least some part of the SG in *C. roseus* cells is associated with the tonoplast (Stevens et al., 1993).

The availability of pure functionally expressed *R. serpentina* SG would allow to prepare antibodies to this enzyme for future investigation of its subcellular localization. Some features of this glucosidase, e.g. the lower pH optimum and weaker inhibition by serpentine compared with the *C. roseus* SG (Table 7), indicate that the vacuolar compartmentation is not excluded. Serpentine was demonstrated to

be accumulated and stored in the vacuoles of the *C. roseus* protoplasts (Deus-Neumann and Zenk, 1984), so the resistance to high concentrations of this alkaloid is necessary for the vacuolar enzymes (Luijendijk et al., 1998). The targeting signals for peroxisomes (SKL-like tripeptides) (Olsen and Harada, 1995) are absent in the amino acid sequence of the *R. serpentina* SG. Moreover, the internal SKL and SRL motifs found within the sequences of *C. roseus* SG and the RG (Fig. 48) are not likely to act as peroxisomal targeting signals, since the C-terminal position was demonstrated to be important for the function of these peptides (Olsen and Harada, 1995).

An interesting property of *R. serpentina* SG is its high temperature optimum at 50 °C. The temperature optimum for SG from *C. roseus* cell suspensions was reported to be 30 °C (Hemscheidt and Zenk, 1980), although the enzyme was highly stable up to 50 °C (Luijendijk et al., 1998). Whether the high temperature optimum of *R. serpentina* SG may be attributed to the source plant is uncertain. But several other enzymes isolated from the same cell suspension culture, e. g. the vomilenine glycosyl transferase (Ruyter and Stöckigt, 1991) and the arbutin synthase (Arend et al., 2001), also displayed an optimum catalytic activity at 50 °C.

The K_M value with strictosidine of the *R. serpentina* SG corresponds well to the data of two SG enzymes characterized from *C. roseus* cell cultures (Hemscheidt and Zenk, 1980). However, the K_M value determined for *C. roseus* SG by other authors (Luijendijk et al., 1998) is much lower (Table 7). The higher V_{max} value of the heterologously expressed *R. serpentina* SG may be explained by the higher specific activity due to the better purification of the enzyme (Table 7).

Both SGs from *C. roseus* and *R. serpentina* have a high degree of substrate specificity. All the accepted substrates possess a basic skeleton of strictosidine with only one exception, ipecoside, which is deglucosylated by the *R. serpentina* enzyme

Fig. 49. Biogenetic formation of strictosidine and ipecoside. SS –strictosidine synthase, DIS - deacetylipecoside synthase.

at a low rate of 0.8% compared with strictosidine. The SG isolated from *C. roseus* cell suspension cultures was shown not to convert deacetyl-ipecoside and deacetyl-isoipecoside (Hemscheidt and Zenk, 1980), which is difficult to explain because the structure of strictosidine resembles more that of deacetyl-ipecoside than of ipecoside (Fig. 49). Moreover, the acetyl group at Nβ of strictosidine decreased the conversion rate significantly (to 2.5%). It can be supposed, that the deglucosylation of deacetyl-

ipecoside by the SG from *C. roseus* cells was not detected because of lower sensitivity of glucosidase activity assay or not sufficient enzyme amount used for testing. Similar situation was observed during the substrate specificity studies of the RG. The ability of this glucosidase to hydrolyze strictosidine at a low rate of 7.1% compared with raucaffricine was demonstrated using the purified heterologously expressed enzyme, while in the experiments with the RG enriched from the *R. serpentina* cells no conversion of strictosidine was detected (Schübel and Stöckigt, 1986). Thus the possibility to obtain high amounts of pure plant protein *via* heterologous expression can allow to investigate the properties of the enzyme in more detail.

3. Mechanism of enzymatic strictosidine deglucosylation

The glucoalkaloid strictosidine can undergo easy conversions, e.g. lactamization under slightly alkaline conditions yielding strictosidine lactam (Fig. 50). The removal of glucose moiety from strictosidine molecule triggers a sequence of reactions leading to the rearrangements of the carbon skeleton. Vallesiachotamine isomers and the alkaloid nacycline were described as the products of alkaline and acid hydrolysis of strictosidine, respectively (De Silva et al., 1971; Schübel, 1986), while the incubation of this glucoalkaloid with the crude enzyme preparation from *C. roseus* resulted in the formation of cathenamine (Stöckigt, 1979). Vallesiachotamine isomers were also reported to be produced from strictosidine by biotransformation using different bacterial strains (Shen et al., 1998). It has been postulated that after glucose cleavage the unstable strictosidine aglycone converts spontaneously to the dialdehyde (Brown and Chapple, 1974), in which the intramolecular reactions involving the aldehyde group(s) occur, e.g. the condensation with one of the amino groups or the addition (in the enol form) to the ethylidene side chain.

Fig. 50. Products of strictosidine conversion under different conditions. **[OH$^-$]** – alkaline hydrolysis, **[H$^+$]** – acid hydrolysis, **[SG]** – enzymatic deglucosylation.

The enzyme catalyzing the strictosidine deglucosylation may theoretically define further fate of the formed aglycone by influencing the conformation of the dialdehyde to favor certain intramolecular reactions. If this hypothesis is correct, the enzymatic hydrolysis of strictosidine by the SGs from plants producing different types of indole alkaloids would result in the formation of different products. Moreover, the ring-opened dialdehyde would not be released from the enzyme until the definite rearrangements have taken place.

The stable end product of strictosidine deglucosylation by heterologously expressed SG from *R. serpentina* cell suspension cultures was identified as cathenamine. The same alkaloid was produced from strictosidine by the crude enzyme preparation from *C. roseus* cultured cells (Stöckigt, 1979). In order to intercept the precursors of cathenamine formed after removal of the glucose moiety from strictosidine, the enzymatic reaction was carried out in presence of reducing agents which were expected to reduce aldehyde groups of the intermediates and thus prevent them from further conversion. As a result, two products sitsirikine and isositsirikine were identified, which demonstrates that 4,21-dehydrocorynantheine aldehyde is involved in the indole alkaloid biosynthesis in *R. serpentina* as well as it has been shown earlier for *C. roseus* (Stöckigt et al., 1978). Thus the conversion of strictosidine by SGs from two different plants, *C. roseus* and *R. serpentina*, occurs by the same mechanism. The biosynthetic pathways leading to ajmaline and ajmalicine types of indole alkaloids diverge at some later stage, e.g. after formation of 4,21-dehydrogeissoschizine, which has been shown to be converted into ajmalicine type alkaloids or geissoschizine by the enzyme preparations from *C. roseus* (Rueffer et al., 1979) (Fig. 51). Anyhow, the ring-opened dialdehyde could not be intercepted, which did not allow to make the final conclusion about the influence of the SG on the spontaneous conversions of the strictosidine aglycone . On the basis of the results described above the possibility cannot be excluded, that the condensation of C-21 aldehyde and Nβ-amino groups leading to the ring D formation occurs before the product is released from the enzyme.

Therefore, to retard the intramolecular reaction leading to the ring closure the structure of strictosidine was modified by methylation of the Nβ-amino group. The incubation of the obtained dolichantoside with the *R. serpentina* SG led to the

Strictosidine — SG → Aglycone → Dialdehyde

Cathenamine ⇌ 4,21-Dehydrogeissoschizine ← 4,21-Dehydrocorynantheine aldehyde

R I (NADPH → NADP+)

R II (NADPH → NADP+)

NaBH3CN or KBH4

Ajmalicine 19 β, 20β
19-Epi-ajmalicine 19 α, 20β
Tetrahydroalstonine 19 β, 20α

Geissoschizine

Sitsirikine 16 β(R)
Isositsirikine 16 α(S)

Ajmaline

Fig. 51. Scheme of enzymatic conversion of strictosidine into indole alkaloids of ajmaline and ajmalicine types. SG – strictosidine glucosidase, R I and R II – different NADPH-dependent reductases.

SG

Strictosidine R=H
Dolichantoside R=CH_3

Aglycone

Dialdehyde

bond rotation

Dialdehyde

4,21-Dehydrocorynantheine aldehyde

3-iso-correantine A

Cathenamine

Fig. 52. Scheme of enzymatic deglucosylation of strictosidine and its Nβ-methylated analog, dolichantoside, using the heterologously expressed strictosidine glucosidase from *R. serpentina* cell suspension cultures.

formation of 3-iso-correantine A, an alkaloid exhibiting the bond between C-21 and Nα. The detection of such structure suggests that the dialdehyde is released from the enzyme before the ring D is closed and converts spontaneously to either cathenamine or 3-iso-correantine A (Fig. 52). Bearing in mind, that the reduction of the 18,19-double bond in strictosidine can influence its binding to the SG (which is demonstrated by a higher K_M value, Table 6), the bond rotation necessary for the reaction between C-21 and Nα is not likely to occur in the enzyme-substrate complex. It has been reported (Brandt et al., 2001), that the enzymatic deglucosylation of dolichantoside by crude enzyme preparations from *Strychnos mellodora* or *C. roseus* resulted in the formation of a quaternary alkaloid, Nβ-methyl-21-hydroxy-mayumbine, in which the condensation of C-21 aldehyde and Nβ amino groups took place (Fig. 53). However, the participation of several enzymes in the product formation cannot be excluded when a crude preparation is used. The application of homogenous SG is necessary to draw final conclusions about the reaction mechanism.

Dolichantoside Dialdehyde Nβ-Methyl-21-hydroxymayumbine

Fig. 53. Scheme of enzymatic deglucosylation of dolichantoside by a crude enzyme preparation from *Strychnos mellodora* (Brandt et al., 2001).

Although it is concluded, that the SG itself does not determine the further conversions of strictosidine aglycone, this enzyme may play a role in creation of the structural diversity of indole alkaloids. The instability of products formed after strictosidine hydrolysis together with the ability of SG to form large aggregates support the hypothesis that the SG may be associated with other enzymes intercepting and transforming the reactive intermediates. Thus the knowledge of the SG cDNA structure may help to identify further enzyme(s) of ajmaline biosynthesis e.g. using a two-hybrid system for detecting protein-protein interactions (Fields and Sternglanz, 1994). The possibility to obtain high amounts of pure active SG is also a necessary prerequisite for future characterization of enzymes converting unstable products of strictosidine deglucosylation.

V. SUMMARY

The aim of the present work was identification and characterization of enzyme(s) involved in strictosidine deglucosylation in *R. serpentina* cell suspension cultures and a detailed investigation of the catalyzed reaction.

As a prerequisite for studies of strictosidine deglucosylating enzymes, a protocol was developed for isolation and purification of this glucoalkaloid from plant cell suspension cultures allowing to prepare 37.7±2.6 mg of strictosidine from 150 g of dried cells.

Indole alkaloid patterns of two somatic hybrid cell lines between *R. serpentina* and *Rhazya stricta* were studied. Treatment with 100 µM methyl jasmonate was employed to induce the secondary metabolism of cultured plant cells in order to reveal their full biosynthetic potential. In total 15 compounds were identified, including a novel indole alkaloid 3-oxorhazinilam. The changes in content of 7 indole alkaloids under methyl jasmonate treatment were determined. The biosynthesis of different alkaloids was induced to varying extents. The concentrations of strictosidine and 17-O-acetylnorajmaline in methyl jasmonate treated cells were 46- and 43-fold higher, respectively, than in the control cultures. The content of 17-O-acetylajmaline and raucaffricine surpassed the value of the control cultures by 5.4 and 4.5 times, respectively. For ajmaline, ajmalicine and reserpine only a slight increase of ~ 2-3 fold was observed.

The ability of raucaffricine glucosidase to deglucosylate strictosidine was detected for the first time using pure heterologously expressed enzyme.

The cDNA encoding strictosidine glucosidase was cloned from *R. serpentina* cell suspension cultures and heterologously expressed in *E. coli*. The enzyme was purified to homogeneity using intein tag system and its properties were determined, e.g. the

temperature optimum of 50 °C, the pH optimum of 5.0-5.2, the K_M value with strictosidine of 121 μM. The substrate specificity of strictosidine glucosidase was studied using 34 β-glucosides, most of them being plant natural products of different structural types. The enzyme has a high degree of substrate specificity restricted to strictosidine derivatives. The only exception, ipecoside, which is structurally related to strictosidine, is deglucosylated at a very low rate of 0.8%.

The product of strictosidine deglucosylation was identified as cathenamine by its EI-, FD-, HR-FAB-MS and ^1H NMR data. The strictosidine deglucosylation under reductive conditions resulted in the formation of sitsirikine and isositsirikine indicating the involvement of 4,21-dehydrocorynantheine aldehyde as an intermediate in this process.

Enzymatic deglucosylation of dolichantoside (Nβ-methylstrictosidine) led to the formation of a novel indole alkaloid 3-iso-correantine A, which suggests that the dialdehyde formed from the unstable strictosidine aglycone is released from the glucosidase before the ring closure occurs.

The strictosidine glucosidase is the fifth enzyme involved in the ajmaline biosynthesis for which the cDNA cloning and functional heterologous expression has been achieved. These results are an important step towards the heterologous expression of the entire biosynthetic pathway leading from strictosidine to ajmaline.

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APPENDIX. Data of compounds mentioned in text

- **17-O-ACETYLAJMALINE**: CAS red (blue fluorescence); EI-MS *m/z* (rel. int.%) 368(M^+,63); 353(26); 325(10); 224(11); 194(15); 182(100); 157(29); 144(76) (Stöckigt et al., 1981). Acetylation of 17-O-acetylajmaline gave diacetylajmaline (Sheludko et al., 1999).
- **17-O-ACETYLNORAJMALINE**: CAS orange (orange fluorescence); EI-MS *m/z* (rel. int.%) 350(M^+,31); 339(7); 325(4); 265(4); 224(15); 196(13); 182(20); 180(22); 169(54); 168(100); 143(26); 130(50) (Stöckigt et al., 1981; Sheludko et al., 1999).
- **17-O-ACETYLRAUGLUCINE**: CAS red (blue fluorescence); EI-MS *m/z* (rel. int.%) 530(M^+,17); 397(30); 368(100); 353(33); 237(6); 211(4); 194(16); 182(67); 170(12); 158(15); 157(19); 144(55) (Ruyter et al., 1988; Sheludko et al., 1999).
- **AJMALICINE**: CAS brown (blue fluorescence); EI-MS *m/z* (rel. int.%) 352(M^+,100); 351(84); 337(6); 225(8); 223(5); 209(15); 184(50); 170(14); 169(26); 156(83). The stereochemical structure was confirmed by co-TLC with authentic sample in solvent system SS7 reported to separate eight ajmalicine isomers (Phillipson and Hemingway, 1975; Sheludko et al., 1999).
- **AJMALINE**: CAS red; EI-MS *m/z* (rel. int.%) 326(M^+,96); 311(42); 200(24); 199(36); 183(60); 182(82); 158(33); 157(39); 145(64); 144(100) (Falkenhagen et al., 1993; Sheludko et al., 1999). Acetylation of ajmaline gave diacetylajmaline.
- **CATHENAMINE**: EI-MS *m/z* (rel. int.%) 350(M^+,51); 349 (31); 321(16); 249(100); 248(57); 169(34); 156(36); 145(22); HR-FAB-MS *m/z* 351.1720 ($[M+H]^+$, calc. for $C_{21}H_{23}O_3N_2$, 351.1709), 503.2037 ($[M+NBA]^+$, calc. for $C_{28}H_{29}O_6N_3$, 503.2056); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.98 (1H, s, N-H), 7.52 (1H, s, H-17), 7.45 (1H, d J=7.0 Hz, H-9^1), 7.30 (1H, d J=7.0 Hz, H-12^1), 7.14

(1H, ddd J=7.0, 7.0, 0.9 Hz, H-11[2]), 7.08 (1H, ddd J=7.0, 7.0, 0.9 Hz, H-10[2]), 6.16 (1H, s, H-21), 4.61 (1H. q J=6.7 Hz, H-19), 3.71 (3H, s, $COCH_3$), 1.40 (3H, d J=6.7 Hz, H_3-18) (Stöckigt, 1979).

[1] and [2]: the assignment may be interchanged.

- **DIACETYLAJMALINE**: CAS red (blue fluorescence); EI-MS *m/z* (rel. int.%) 410(M^+,100); 409(54); 368(17); 353(19); 325(8); 307(16); 291(8); 237(9); 194(11); 183(36); 182(55); 181(25); 167(12); 157(16); 144(47).
- **1,2α(S)-DIHYDRORAUCAFFRICINE**: CAS orange (orange fluorescence); EI-MS *m/z* (rel. int.%) 514(M^+,4); 381(14); 353(100); 352(74); 335(13); 319(6); 293(9); 275(41); 222(22); 185(56); 168(75); 156(21); 143(37); 130(69) (Schübel, 1986).
- **DOLICHANTOSIDE**: CAS brown-green; EI-MS *m/z* (rel. int.%) 544(M^+,0.6); 529(0.4); 365(1.7); 199(3.1); 198(2.7); 186(18.5); 185(100); 184(51.5); 156(6.3); 144(6.9); ^{1}H NMR (CD_3OD, 400 MHz) δ 1.96 (1H, m, H-14), 2.11 (1H, m, H-14), 2.69 (3H, s, Nβ-CH_3), 2.76 (1H, m, H-6), 2.82 (1H, m, H-6), 2.97(1H, m, H-15), 3.22-3.30 (5H, m, H-5, H-2', H-4', H-5, H-5'), 3.35 (1H, m, H-3'), 3.65 (1H, dd J=6.7, 12.1 Hz, H-6'), 3.77 (3H, s, CO_2CH_3), 3.92 (1H, dd J=2.0, 12.1 Hz, H-6'), 4.76 (1H, d J=7.8 Hz, H-1'), 5.32 (1H, d J=10.2 Hz, H-18), 5.37 (1H, d J=17.2 Hz, H-18), 5.68 (1H, d J=6.7 Hz, H-21), 5.88 (1H, ddd J=10.2, 10.2, 17.2 Hz, H-19), 7.00 (1H, ddd J=7.8, 7.8, 1.2 Hz, H-10), 7.12 (1H, ddd J=7.8, 7.8, 1.2 Hz, H-11), 7.33 (1H, d J=7.8 Hz, H-9), 7.45 (1H, d J=7.8 Hz, H-12), 7.66 (1H, s, H-17) (Ohmori et al., 1998). Acetylation of dolichantoside gave dolichantoside tetraacetate**.**

- **DOLICHANTOSIDE TETRAACETATE**: EI-MS *m/z* (rel. int.%) 712(M^+,1.5); 697(1.3); 365(1.9); 199(2.4); 186(23.8); 185(100); 184(27.5); 169(10.0); 156(5.5); 144(5.9).
- **21-GLUCOHYDROXYSARPAGAN-17-AL**: CAS grey; EI-MS *m/z* (rel. int.%) 470(M^+,4); 441(2); 331(5); 307(21); 291(20); 279(13); 263(12); 183(12); 182(14); 170(36); 169(100); 168(66); 156(19) (Ruyter et al., 1988).
- **21-GLUCOHYDROXYSARPAGAN-17-OL**: CAS grey; EI-MS *m/z* (rel. int.%) 472(M^+,5); 471(5); 331(5); 311(23); 309(36); 293(24); 279(4); 263(5); 183(7); 182(10); 170(43); 169(100); 168(48); 156(19).
- **3-ISO-CORREANTINE A**: CAS colourless (blue fluorescence); EI-MS *m/z* (rel. int. %) 382 (7, M^+), 381 (10), 367 (7), 213 (10), 199 (8), 185 (100), 171 (15), 156 (18), 144 (17); HR-EI-MS: *m/z* 382.1884 (M^+, calc. for $C_{22}H_{26}O_4N_2$, 382.1893), 367.1681 (M^+-CH_3, calc. for $C_{21}H_{23}O_4N_2$, 367.1658); ^{1}H NMR (pyridine-d_5, 600 MHz): δ 1.50 (1H, m, H-14β), 1.57 (3H, d J=6.5 Hz, H_3-18), 1.73 (1H, m, H-14α), 2.39 (3H, s, Nβ-CH_3), 2.56 (1H, m, H-20), 2.60 (1H, m, H-5β), 2.68 (1H, m, H-6β), 3.02 (1H, dd J=14.2, 2.7 Hz, H-6α), 3.52 (1H, d J=11.5 Hz, H-5α), 3.67 (3H, s, CO_2CH_3), 3.82 (1H, m, H-15), 3.87 (1H, dd J=13.7, 6.4 Hz, H-3), 4.36 (1H, dq J=9.3, 6.5 Hz, H-19), 6.48 (1H, s, H-21), 7.26 (1H, dd J=7.7, 7.7 Hz, H-10), 7.30 (1H, dd J=7.7, 7.7 Hz, H-11), 7.61 (1H, d J=7.7 Hz, H-9), 7.82 (1H, s, H-17), 7.91 (1H, d J=7.7 Hz, H-12); ^{13}C NMR (determined from HSQC and HMBC spectra, pyridine-d_5, 600 MHz) δ 18.3 (q, C-18), 24.9 (t, C-14), 29.1(d, C-15), 32.4 (t, C-6), 41.8 (q, Nβ-CH_3), 46.0 (d, C-20), 50.4 (q, $CO_2\mathbf{C}H_3$), 52.9 (t, C-5), 60.8 (d, C-3), 76.7 (d, C-19), 78.2 (d, C-21), 108.7 (s, C-7), 111.6 (d, C-12), 112.1 (s, C-16), 118.3 (d, C-9), 119.9 (d, C-10), 122.0 (d, C-11), 128.0 (s, C-8), 137.6 (s, C-13), 138.3 (s, C-2), 154.7 (d, C-17), 168.1 (s, $\mathbf{C}O_2CH_3$).

- **ISOSITSIRIKINE**: CAS brown (blue fluorescence); EI-MS *m/z* (rel. int.%) 354(M^+,92); 353(78); 323(8); 252(45); 251(74); 250(39); 223(36); 184(32); 170(100); 169(83); 156(65); 144(21) (Stöckigt et al., 1978).
- **1-Methyl-2(α)S-DIHYDRORAUCAFFRICINE**: CAS red (blue fluorescence); EI-MS *m/z* (rel. int.%) 528(M^+,12); 527(6); 395(6); 367(47); 349(20); 333(6); 307(7); 289(32); 222(13); 199(30); 182(64); 170(27); 157(45); 144(100) (Schübel, 1986).
- **3-OXO-RHAZINILAM**: white powder; mp 201-204^{0}C; CAS yellow; $[\alpha]_D$ – 247.2^0 (c 0.3; $CHCl_3$); UV (MeOH) λ_{max} (log ε) 241 (3.63); EI-MS *m/z* (rel. int.%) 308 $[M]^+$ (17), 279 $[M - CH_2CH_3]^+$ (100), 251 (45), 237 (15), 223 (25), 195 (15); HR-EI-MS *m/z* 308.1535 (calcd for $C_{19}H_{20}N_2O_2$, 308.1524); 279.1141 (calcd for $C_{17}H_{15}N_2O_2$, 279.1133), $[M - CH_2CH_3]^+$; ^1H NMR (pyridine-d_5, 400 MHz) δ 10.00 (1H, s, N-H), 7.55 (1H, d J=3.2 Hz, H-5), 7.50 (1H, d J=7.3 Hz, H-9), 7.33-7.47 (3H, m, H10-12), 5.94 (1H, d J= 3.2 Hz, H-6), 2.88 (1H, ddd J=18.2, 13.5, 5.3 Hz, H-14α), 2.65 (1H, dd J=13.2, 12.3 Hz, H-17'), 2.60 (1H, ddd J=18.2, 4.7, 3.2 Hz, H-14β), 2.51 (1H, dd J=14.1, 12.3 Hz, H-16'), 2.19 (1H, dd J=14.1, 7.9 Hz, H-16), 1.91 (1H, ddd J=14.1, 13.5, 4.7 Hz, H-15α), 1.50 (1H, ddd J=14.1, 5.3, 3.2 Hz, H-15β), 1.47 (1H, m, H-19'), 1.46 (1H, dd J=13.2, 7.9 Hz, H-17), 1.29 (1H, m, H-19), 0.62 (3H, t J=7.3 Hz, H-18); ^1H NMR (CD_2Cl_2, 400 MHz) δ 7.27-7.45 (4H, m, H9-12), 7.39 (1H, d J=3.2 Hz, H-5), 6.68 (1H, s, N-H), 5.95 (1H, d J= 3.2 Hz, H-6), 2.91 (1H, ddd J=17.9, 13.5, 5.3 Hz, H-14α), 2.66 (1H, ddd J= 17.9, 4.7, 3.2 Hz, H-14β), 2.38 (2H, m, H-16', H-17'), 2.11 (1H, ddd J=14.1, 13.5, 4.7 Hz, H-15α), 2.03 (1H, dd J=12.3, 7.9 Hz, H-16), 1.72 (1H, ddd J=14.1, 5.3, 3.2 Hz, H-15β), 1.56 (1H, dd J=12.3, 7.9 Hz, H-17), 1.46 (1H, m, H-19'), 1.31 (1H, m, H-

19), 0.72 (3H, t J=7.3 Hz, H-18); ^{13}C NMR (CD_2Cl_2, 100 MHz) δ 176.4 (s, C-2), 168.3 (s, C-3) 138.0 (s, C-13), 137.7 (s, C-21), 134.1 (s, C-8), 130.8 (d, C-11), 129.3 (d, C-9), 128,1 (d, C-10), 128,1 (d, C-12), 122.5 (s, C-7), 116.5 (d, C-5), 115.1 (d, C-6), 38.8 (s, C-20), 34.0 (t, C-17), 32.2 (t, C-15), 30.1 (t, C-19), 29.5 (t, C-14), 28.7 (t, C-16), 8.1 (q, C-18) (Gerasimenko et al., 2001b).

- **RAUCAFFRICINE**: CAS colourless (blue fliorescence); EI-MS *m/z* (rel. int.%) 512(M^+,2); 379(7); 351(52); 334(11); 307(17); 291(24); 273(18); 263(9); 246(5); 206(5); 183(13); 169(100); 156(21); 109(8) (Schübel et al., 1984; Sheludko et al., 1999).
- **RESERPINE**: CAS colourless (blue fliorescence); EI-MS *m/z* (rel. int.%) 608(M^+,100); 607(96); 593(12); 577(7); 450(10); 413(5); 397(31); 395(52); 381(27); 365(13); 265(11); 251(22); 214(19); 200(25); 199(27); 195(63); 186(20); 174(13)) (Hesse, 1974; Sheludko et al., 1999).
- **SITSIRIKINE**: CAS brown (blue fluorescence); EI-MS *m/z* (rel. int.%) 354(M^+,98); 353(100); 323(9); 252(36); 251(75); 250(43); 223(35); 184(30); 170(90); 169(80); 156(62); 143(18) (Stöckigt et al., 1978).
- **STEMMADENINE**: CAS violet; $[\alpha]_D$ +186^0 (c 0.05 in pyridine); EI-MS *m/z* (rel. int.%) 354(M^+,7); 322(2); 224(6); 194(4); 180(3); 167(5); 154(6); 144(5); 123(100); 122(31) (Kostenyuk et al., 1995; Sheludko et al., 2000b).
- **STRICTOSIDINE**: CAS brown-green (blue fluorescence); $[\alpha]_D$ -204^0 (c 0.18 in MeOH); ^{1}H NMR (CD_3OD, 400 MHz) δ 7.73 (1H, s, H-17), 7.41 (1H, d J=7.8 Hz, H-9), 7.29 (1H, d J=8.2 Hz, H-12), 7.07 (1H, ddd J=1.2, 7.0, 8.2 Hz, H-11), 6.99 (1H, ddd J=1.2, 7.0, 7.8 Hz, H-10), 5.87 (1H, m, H-19), 5.87 (1H, d J=8.6 Hz, H-21), 5.36 (1H, d J=17.2 Hz, H-18*Z*), 5.26 (1H, d J=10.6 Hz, H-18*E*), 4.82 (1H, d J=7.8 Hz, H-1'), 3.99 (1H, m, H-6'), 3.80 (3H, s, CO_2CH_3), 3.71-3.65 (2H,

m, H-6', H-5β), 3.46-3.24 (4H, m, H-2'- H-5'), 3.07 (1H, m, H-15), 2.86 (1H, m, H-6α), 2.72 (1H, m, H-20), 2.13-2.04 (2H, m, 2H-14) (Patthy-Lukats et al., 1997). Acetylation of strictosidine gave strictosidine pentaacetate (Sheludko et al., 1999; Sheludko et al., 2000b).

- **STRICTOSIDINE PENTAACETATE**: CAS yellow (yellow fluorescence); EI-MS *m/z* (rel. int.%) 740(M^+,3); 697(28); 409(3); 393(5); 392(7); 349(7); 331(4); 323(7); 322(7); 265(8); 264(8); 214(35); 213(100); 171(59); 169(76); 127(29); 115(26); 109(77) (Stöckigt, 1979).
- **STRICTOSIDINE LACTAM**: CAS yellow (yellow fluorescence); EI-MS *m/z* (rel. int.%) 498(M^+,6); 336(8); 335(7); 319(5); 289(6); 266(61); 265(67); 249(15); 236(62); 235(100); 221(11); 207(21); 206(26); 171(41); 169(36); 156(15); 154(15); 144(30); 143(29) (Stöckigt, 1979; Sheludko et al., 1999). Acetylation of strictosidine lactam gave strictosidine lactam tetraacetate (Sheludko et al., 2000b).
- **STRICTOSIDINE LACTAM TETRAACETATE**: $[\alpha]_D$ –80^0 (c 0.27 in $CHCl_3$) (Sheludko et al., 2000b).
- **TETRAHYDROALSTONINE**: CAS yellow (blue fluorescence); EI-MS *m/z* (rel. int.%) 352(M^+,78); 351(100); 337(23); 251(17); 225(6); 223(28); 209(12); 197(16); 183(23); 169(49); 168(38); 156(71) (Hesse, 1974).
- **TRYPTAMINE**: CAS yellow, EI-MS *m/z* (rel. int.%) 160(M^+, 13); 131(61); 130(100); 115(2); 103(13) (Sheludko et al., 2000b).
- **TUBOTAIWINE**: CAS blue; $[\alpha]_D$ +598^0 (c 0.13 in $CDCl_3$); EI-MS *m/z* (rel. int.%) 324(M^+,29); 267(31); 253(19); 229(100); 208(29); 197(41); 194(51); 182(63); 181(67); 180(97); 167(79); 154(25) (Kostenyuk et al., 1995; Sheludko et al., 2000b).

- **VALLESIACHOTAMINE ISOMERS**: CAS yellow (yellow fluorescence); EI-MS *m/z* (rel. int.%) 350(M^+,56); 335(10); 322(47); 318(17); 307(32); 291(50); 279(92); 265(39); 264(41); 263(100); 249(23); 247(18); 236(7); 221(83); 209(36); 184(19); 170(28); 169(31); 156(21); 154(27); 144(28) (Kostenyuk et al., 1995; Sheludko et al., 1999; Sheludko et al., 2000b; Sheludko et al., 2000b).
- **VOMILENINE ISOMERS**: CAS colourless (blue fluorescence); EI-MS *m/z* (rel. int.%) 350(M^+,18); 307(7); 291(6); 183(10); 169(100); 168(30); 156(6) (Kostenyuk et al., 1995; Sheludko et al., 1999).
- **ISOMER OF YOHIMBINE**: CAS brown (blue fluorescence); EI-MS *m/z* (rel. int.%) 354(M^+,65); 353(100); 295(4); 223(4); 221(4); 197(4); 184(18); 169(35); 170(15); 156(18); 144(15); 143(17) (Hesse, 1974; Sheludko et al., 1999).