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Purification and partial amino acid sequences of the enzyme vinorine synthase involved in a crucial step of ajmaline biosynthesis

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Dedicated to Professor Heinz G. Floss on the occasion of his 70th birthday

Abstract—The acetyl-CoA-dependent enzyme vinorine synthase was isolated from hybrid cell suspension cultures of *Rauvolfia serpentina* and *Rhazya stricta*. The sarpagan-type alkaloid gardneral was used as a substrate of the enzyme leading to the ajmalan-type 10-methoxyvinorine. An HPLC-based assay was developed to monitor vinorine synthase activity, which allowed establishing a five step purification procedure combining anion exchange, hydrophobic interaction, hydroxyapatite and gel filtration. Purification resulted in a yield of 0.2% and an approximately 991-fold enrichment of the acetyltransfer activity. SDS-PAGE analysis showed a M_r for the enzyme of ~50 kDa. The four peptide fragments generated by proteolysis of the pure enzyme with endoproteinase LysC and the N-terminal part of the enzyme were sequenced. The enzyme preparation (>875-fold enrichment) delivering the N-terminal sequence was isolated from *R. serpentina* cell suspensions. Sequence alignment of the five peptides showed highest homologies in a range of 30–71% to acetyltransferases from other higher plants involved in natural plant product biosynthesis. Based on the partial sequences vinorine synthase is probably a novel member of the BAHD enzyme super family.

1. Introduction

Acetyl-CoA-dependent acyltransferases play important roles in the secondary metabolism of plant cells. For example, an acetyltransfer takes place in *Catharanthus roseus* (L.) G. Don during the biosynthesis of the monoterpenoid indole alkaloid vindoline,[1–4] a biogenetic precursor of the dimeric alkaloids vinblastine and vincristine used in cancer treatment. Salutaridinol acetyltransferase, again an acetyl-CoA-dependent enzyme, catalyzes a crucial step in the biosynthesis of morphine in *Papaver somniferum*.[5,6] Moreover, in the biosynthesis of the diterpenoid alkaloid taxol in *Taxus cuspidata* several acyltransferases are involved, among are two acetyl-CoA-dependent enzymes.[7,8]

In addition to the biosynthesis of vindoline, an acetyltransferase reaction is also involved in the formation of the alkaloid ajmaline in the Indian medicinal plant *Rauvolfia serpentina* Benth. ex Kurz.[9–11] This alkaloid is of therapeutic interest and is frequently used to treat heart disorders due to its antiarrhythmic activity. During the delineation of ajmaline biosynthesis we have detected nearly all of the enzymes involved in this multistep pathway (Scheme 1). Among these enzymes is the acetyl-CoA-dependent vinorine synthase.[9,11] This acetyltransfer is needed for the biosynthetic formation of the first ajmalan-type alkaloid in the pathway, the indolenine vinorine, generated from a sarpagan structure. Vinorine synthase, therefore, occupies an important position in the metabolism of *Rauvolfia* alkaloids, especially by biosynthetically linking two different

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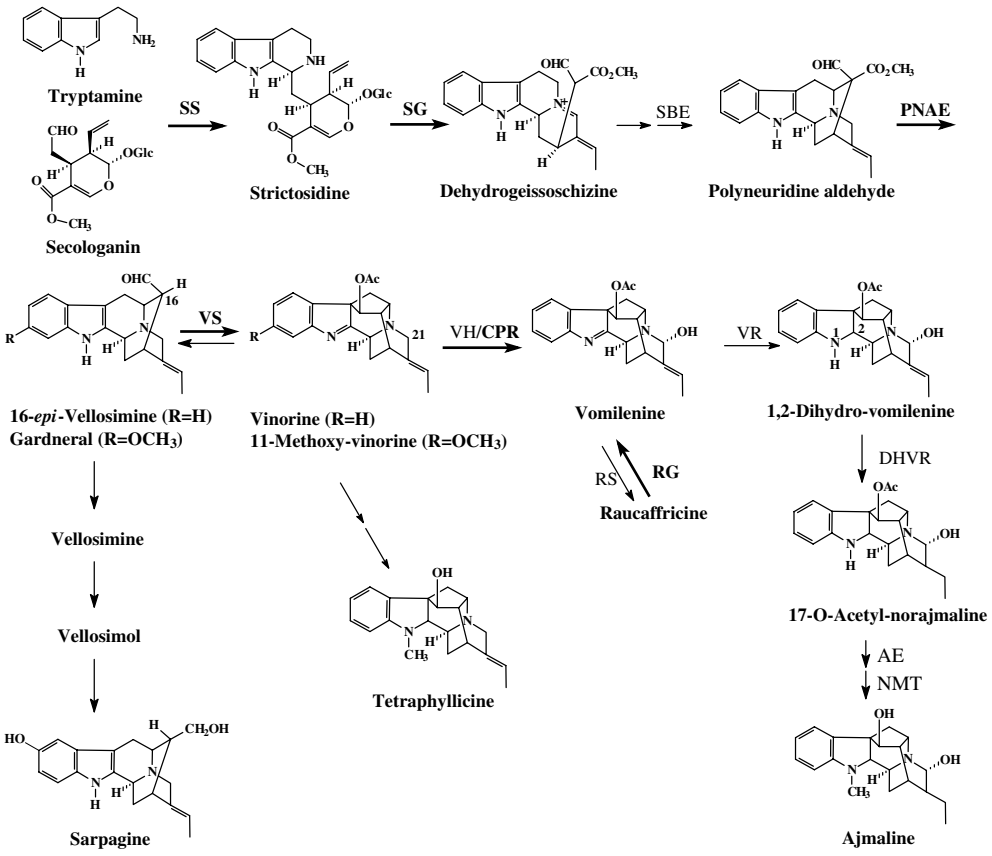

Scheme 1. Biosynthetic pathway leading from strictosidine to the antiarrhythmic monoterpenoid indole alkaloid ajmaline in cell cultures of the medicinal plant *R. serpentina*. The sarpagan structure (16-*epi*-vellosimine) is cyclized to the ajmalan-system (vinorine) combined with an acetyl-CoA-dependent acetylation. This step is catalyzed by vinorine synthase. All reactions catalyzed by enzymes, which have already been expressed heterologously are printed in bold (SS, strictosidine synthase; SG, strictosidine glucosidase; SBE, sarpagan bridge enzyme; PNAE, polyneuridine aldehyde esterase; VS, vinorine synthase; VH, vomilenine hydroxylase; CPR, cytochrome P450 reductase; VR, vomilenine reductase; DHVR, dihydrovomilenine reductase; AE, acetyl esterase; NMT, norajmaline methyltransferase; RG, raucaffricine glucosidase; RS, putative raucaffricine synthase).

groups of alkaloids, the sarpagan and the ajmalan group, respectively (Scheme 1).

Because a previous enrichment of the synthase resulted in only a 160-fold purification, which did not allow sequencing, we developed now more efficient purification procedures for the enzyme from *Rauvolfia*×*Rhazya* hybrid cell line (line *R*×*R*M) and from *Rauvolfia* cell suspensions, respectively. Herein, we report the enrichment, partial amino acid sequences and sequence comparison of vinorine synthase to other acetyltransferases from higher plants. The results of this communication provide for the first time the prerequisite for functional cloning and overexpressing the enzyme heterologously for characterization of its biochemical and molecular properties.

2. Results and discussion

The metabolism of monoterpenoid indole alkaloids in cell suspension cultures of the medicinal plant *Rauvolfia* has been extensively investigated in the past more from the enzymatic than from the genetic point of view. These studies have delineated the complex biosynthetic pathway that leads to the antiarrhythmic alkaloid ajmaline. This alkaloid exhibits a structurally complicated six-membered ring system with a total of nine stereogenic carbon atoms (Scheme 1). According to our present knowledge, the biosynthesis of the intermediate indolenine alkaloid, vinorine, occupies a central role in the overall ajmaline pathway and might function as the most important metabolic branch reaction in the formation of all the sarpagine- and ajmaline-type alkaloids in *Rauvolfia*. On one hand, vinorine is a direct but distant precursor five steps away from the end product ajmaline. On the other hand, after its hydroxylation by a cytochrome P450 enzyme[12,13] it also can be glucosylated leading to raucaffricine, which has been identified as the most prominent alkaloid in *Rauvolfia* cell suspensions, accumulating at levels up to 1.6 g L^{-1} nutrient medium.[14] Obviously vinorine also enters the route to the tetraphyllicines, which are trace alkaloids of *Rauvolfia* and *R*×*R*M hybrid cell cultures.[10,15] This route has not yet been proven at the cell-free level but might resemble that of ajmaline biosynthesis by omitting the 21-hydroxygroup in each intermediate. Moreover, feeding of vinorine to *Rauvolfia* cells indicated its transformation into the alkaloid sarpagine as monitored in vivo by high field (800 MHz) and CryoNMR establishing a further metabolic side route, which vinorine might enter.[16,17]

The biosynthesis of vinorine is catalyzed by the acetyl-CoA-dependent enzyme, vinorine synthase, accepting the sarpagan structure 16-*epi*-vellosimine as the substrate. Acetylation is an essential process in the reaction, because it stabilizes the immediate indolenine 17-deacetyl-vinorine (structure not shown). After removal of the acetyl residue, which in fact acts as a protecting group, the indolenine would spontaneously be re-transformed to the precursor 16-*epi*-vellosimine. However, *epi*-vellosimine is also highly unstable undergoing epimerization at C-16 into vellosimine under basic conditions but also at physiological pH.[18] Since we have no indications to date that this epimerization is reversible (neither spontaneously nor enzyme-catalyzed), vellosimine would not be able to re-enter the ajmaline pathway. In contrast, vellosimine is transformed into the side product of the pathway, sarpagine, by reduction and hydroxylation as we have recently demonstrated.[16] Conclusively, vinorine synthase occupies the crucial function of directing the 'open' 5-ring sarpagan structure to the 'closed' 6-ring ajmalan type finally leading to the target product ajmaline.

Vinorine synthase activity was detected in two plant cell suspension cultures, *R. serpentina* (cell line T30) and somatic $R\times R$M hybrid cell culture. The $R\times R$M cells have an indole alkaloid pattern similar to that of *R. serpentina* cells, whereas the parent cell line *R. stricta* produces alkaloids, which do not belong to the *Rauvolfia*-typical ajmalan type.[15,19] We used both, the $R\times R$M and *R. serpentina* cell lines for isolation and purification of the synthase. Since the *Rauvolfia* cells contained less enzyme activity compared to $R\times R$M cultures, vinorine synthase isolated from this source was applied to only N-terminal sequencing. Crude protein extracts were obtained from 2 kg of $R\times R$M cells. To avoid decrease of enzyme activity, the extraction buffer contained the antioxidant sodium ascorbate and polyvinylpyrrolidone. In addition, mercaptoethanol and glycerol were added to all buffers to maintain sufficient synthase activity throughout the purification procedure. After ammonium sulfate fractionation, the enzyme purification was processed by fast protein liquid chromatography. The final purification protocol included five stages (Fig. 1a–e, Table 1). Separation on anion-exchange column Source 30Q was followed by hydrophobic interaction chromatography using Source phenyl material and hydroxyapatite chromatography resulting in a 36-fold enrichment of enzyme activity. Fractions after the hydroxyapatite column were analyzed by

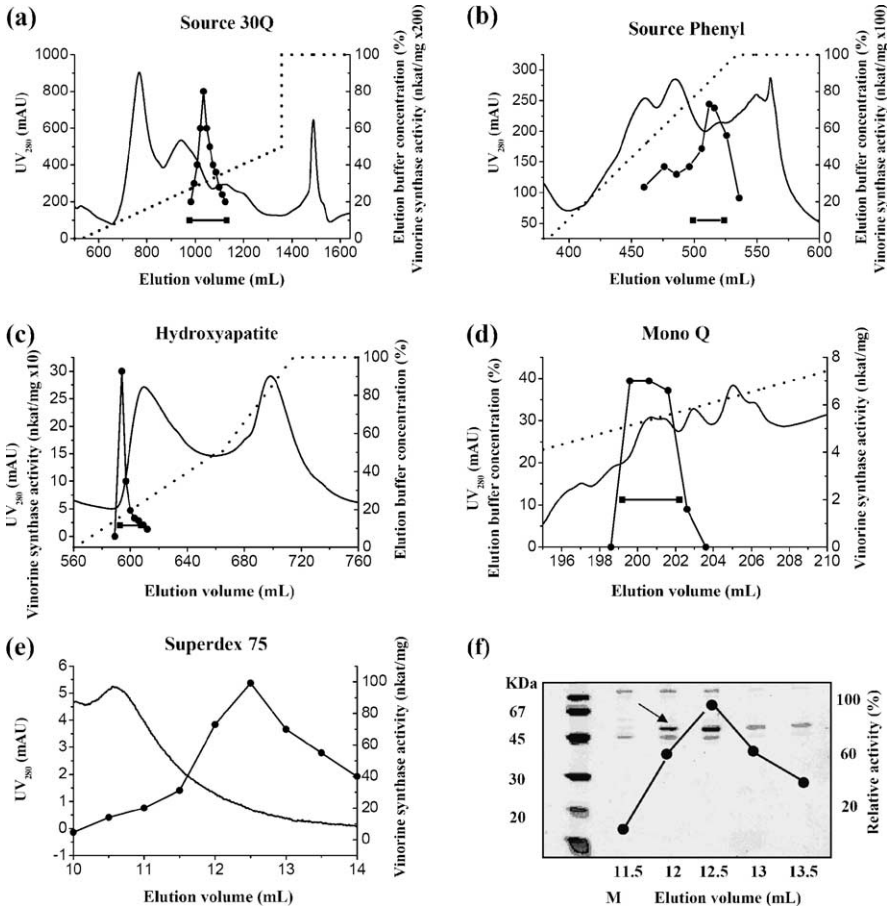

Figure 1. Purification of vinorine synthase from *R. serpentina*×*R. stricta* hybrid cell suspension cultures. Protein elution profiles after chromatography on (a) Source 30Q; (b) Source phenyl; (c) Hydroxyapatite; (d) MonoQ; (e) Superdex 75 chromatography. (— UV_{280}, ··· elution buffer concentration, —•— vinorine synthase activity, —■— combined fractions), (f) Coomassie-blue stained SDS-PAGE of enriched synthase (arrow) after (e). The dotted line represents the relative synthase activity, protein markers are on the left.

Table 1. Summary of the purification of vinorine synthase

Purification step	Volume (mL)	Total protein (mg)	Specific activity (nkat mg^{-1})	Yield (%)	Purification factor (-fold)
Crude extract	2500	6250	0.10	100	1
Source 30Q	144	245	0.20	7.8	2
Source phenyl	30	28	0.55	2.5	5.5
Hydroxyapatite	15	1.7	3.56	1.0	36
MonoQ	3	0.3	5.47	0.3	55
Superdex 75	0.5	0.01	99.1	0.2	991

The synthase was isolated from 2 kg fresh *R. serpentina*×*R. stricta* hybrid cell suspension cultures. Enzyme activities were measured by an HPLC-based assay and protein concentrations were determined by the Bradford method.[24]

SDS-PAGE, which allowed the assignment of a protein band at ~50 kDa to active protein (data not shown). Subsequent chromatographic steps using MonoQ anion-exchange and Superdex 75 size exclusion columns confirmed this assignment (Fig. 1f). The specific enzyme activity in fractions after the final chromatographic stage amounted up to 99.1 nkat mg^{-1}, corresponding to a 991-fold enrichment.

Isolation and enrichment of vinorine synthase from *R. serpentina* cell suspensions was performed in a similar manner (see Experimental). The relative molecular mass (M_r) of the native synthase from *R*×*R*M cells is around 42 ± 5 kDa, as determined by size exclusion chromatography. These results suggested that vinorine synthase is a monomeric protein.

After enzyme purification, the amino acid sequences of four endoproteinase LysC-generated peptides together with the N-terminal amino acid sequence were determined (Table 2). The comparison of these amino acid sequences with those available in Swall database showed homologies of N-terminal sequence and peptide 19 with other acetyltransferases (Table 3), while no significant homologies could be found for the other three peptides. It is interesting to note that peptide fragments of the synthase showed high homologies to salutaridinol 7-*O*-acetyltransferase (SALAT) from *P. somniferum*[6] and minovincinine 19-hydroxy-*O*-acetyltransferase (MAT) from *C. roseus*.[20] Both enzymes are involved in alkaloid biosynthesis and belong to the BAHD enzyme family[21] named after the four enzymes *b*enzylalcohol acetyl-, *a*nthocyanin-*O*-hydroxy-cinnamoyl-, anthranilate-*N*-*h*ydroxy-cinnamoyl/benzoyl- and *d*eacetylvindoline ace-

Table 2. N-Terminal and partial amino acid sequences of vinorine synthase obtained after isolation from *R. serpentina* and *R. serpentina*×*R. stricta* hybrid cell suspension cultures with ~875 and 991-fold enrichment, respectively (X = no clear assignment possible)

Peptide fragment	Amino acid sequence
N-terminal sequence	APQMEKVSEELILPSSXTXQ
Peptide 24	IEVNEDVPLAVK
Peptide 38	VQLVVAYZWK
Peptide 36	GETEIVLPNFD
Peptide 19	FVFDKEK

Table 3. Sequence comparison of vinorine synthase fragments against Swall Database using BLAST

Proteins	Sequences with homologies to N-terminal sequence	Identity (%)	Sequences with homologies to Peptide 19	Identity (%)
PEPTIDE	APQMEKVSEELILPSSXTXQ		FVFDKEK	
MAT	8 ET**E**TL**S**KT**LI**K**PSS**P**T**P**Q 25	45	218 **F**L**F**SP**E**A 224	43
HSR	7 KV**E**TI**S**K**E**I**I**K**PSS**P**T**P 23	40	224 **FVF**EAS**K** 230	57
F21J9.8	5 V**E**IL**S**R**E**IVK**PSS**P**T**P 20	35	221 **FVFD**AS**K** 227	71
AT	15 RVDV**VS**RDI**I**K**PSS**P**T**PN 31	35	230 **FIFD**SSS 236	43
SALAT	9 V**E**VI**S**K**E**T**I**K**P**TTP**T**P 24	30	243 **FVFD**FA**K** 249	71
BAA	5 L**E**VIQR**E**V**I**K**PSS**PAP 20	30	219 **FVF**ESD**K** 225	57

MAT, Minovincinine 19-hydroxy-*O*-acetyltransferase from *C. roseus* (Q8GZU0); HSR, HSR201 like protein from *Arabidopsis thaliana* (O23392); F21J9.8, an ORF from *Arabidopsis thaliana* (Q9FYM1); AT, acetyltransferase-like protein from *Arabidopsis thaliana* (Q9LU88); SALAT, Salutaridinol 7-*O*-acetyltransferase from *P. somniferum* (Q94FT4); BAA, Acetyl-CoA:benzylalcohol acetyltranferase-like protein from *Arabidopsis thaliana* (Q9FI40).

tyltransferase. Based on their similarities in amino acid sequences we conclude that vinorine synthase could be a novel member of the BAHD enzyme super family.

The here reported purification of the synthase together with the sequence information obtained for the first time was the prerequisite for the isolation and expression of the appropriate cDNA. As described in the following paper of this issue,[22] the vinorine synthase cDNA has been functionally overexpressed in *Escherichia coli*, which allowed a detailed molecular analysis of the catalytic properties of the enzyme by inhibitor studies and site-directed mutagenesis.

3. Experimental

3.1. Partial purification of vinorine synthase from *R* × *R*M hybrid cell suspensions and from *R. serpentina* cultured cells

Buffers: Buffer A: 10 mM potassium phosphate (KPi), pH 7.8, 5 mM EDTA, 10 mM 2-mercaptoethanol (MSH) 0.5% (w/v) sodium ascorbate, 2.5% (w/v) polyvinylpyrrolidone, 10% (v/v) glycerol; buffer B: 20 mM Tris–HCl, pH 8.0, 2 mM EDTA, 10 mM MSH, 10% (v/v) glycerol; buffer C: buffer B containing 1 M KCl; buffer D: buffer B containing 1 M $(NH_4)_2SO_4$; buffer E: 10 mM KPi, pH 7.8, 10 mM MSH, 10% (v/v) glycerol; buffer F: buffer E but with 200 mM potassium phosphate.

Somatic hybrid plant cell suspension cultures of *R* × *R*M and cultures of *R. serpentina* were maintained as described.[15] Cell suspensions were harvested after a growth of 6 days (for *R. serpentina* of 8 days) in 1 L Erlenmeyer flasks containing 400 mL Linsmaier and Skoog nutrition medium.[23] Hybrid cells were processed as follows: frozen cells (2 kg) were stirred in 2 L of buffer A, homogenized for 2 min (Ultraturrax) and filtered through cheese cloth. The solution was centrifuged (10,000 g, 10 min, 4 °C) and the supernatant was subjected to ammonium sulfate precipitation. The protein fraction precipitated between 40% and 60% saturation was desalted using buffer B on a Sephadex G-25 column (500 mL, Sigma, Munich, Germany). The resultant crude enzyme solution was applied to a Source 30Q column (XK50/20, Amersham Pharmacia Biotech, Uppsala, Sweden, volume 240 mL), which was equilibrated with buffer B. The column was washed with buffer B and bound enzymes were fractionated with a linear gradient created with buffers B and C (0–0.5 M KCl, 20 mL min^{-1}, 12 mL each fraction). Fractions containing vinorine synthase activity were combined, brought to 40% ammonium sulfate saturation and applied to a Source phenyl column (XK16/20, Amersham Pharmacia, volume 30 mL, equilibrated with buffer D). The column was washed with buffer D and enzymes were eluted with a linear ammonium sulfate gradient made from buffers D and B (1–0 M ammonium sulfate, 5 mL min^{-1}, 5 mL each fraction) leading to 30 mL of enzyme solution, which was desalted on a Sephadex G-25 column (volume 80 mL) using buffer E. The enzyme solution was applied to a Macro-Prep® hydroxyapatite column (C15/30, Amersham Pharmacia, volume 10.6 mL) washed and equilibrated with buffer E. The enzyme was eluted with a linear gradient of potassium phosphate mixed with buffers E and F (see below) (0.01–0.20 M potassium phosphate, 5 mL min^{-1}). Fractions of 3 mL were collected and fractions containing the synthase activity were pooled and applied to a MonoQ column (HR 5/5, Amersham Pharmacia, equilibrated with buffer B). The column was washed with buffer B and bound enzymes were eluted with a KCl gradient generated from buffers B and C (0–0.15 M KCl within 6 min, 0.15–0.45 M within 24 min, 0.45–0.50 M within 5 min, 1 mL min^{-1}, 1 mL each fraction). The combined fractions with highest vinorine synthase activity (total 3 mL) were concentrated to a volume of 100 μL using Microcon 30 concentrators (Amicon, Witten, Germany) and separated on Superdex 75, which was equilibrated with buffer B (column HR 10/30, Amersham Pharmacia, volume 30 mL). The proteins were fractionated with buffer B at a flow rate of 0.4 mL min^{-1} collecting 0.5 mL samples.

The *R. serpentina* cells were processed slightly different: shortly, crude protein (1.58 g) obtained from 1.3 kg cells was first subjected to 0–70% ammonium sulfate precipitation. The precipitated protein (1.34 g with a concentration of 6.1 mg mL^{-1}) was fractionated over DEAE sepharose 'fast flow' anion-exchange chromatography column (5 × 15.3 cm, 2.5 mL min^{-1}) after dialysis overnight. Enzyme activity was eluted by a linear KCl gradient (0–0.5 M) and active fractions (10 mL each) were combined and concentrated. The resulting enzyme solution (5 mL, 0.18 g protein, 7-fold enrichment) was subjected to Ultrogel ACA 54 gel filtration (column 2 × 120 cm, 20 mL h^{-1}). After combining vinorine synthase containing fractions, the protein solution (20 mL) exhibiting <0.7 nkat mg^{-1} activity was concentrated to 1 mL and gave a 48-fold purification with a yield of 55%. In the next step, Mono P chromatofocusing (column 0.5 × 5 cm, 0.25 mL min^{-1}, 0.5 fractions) with 5% polybuffer 74 was performed. The collected enzyme fractions from two chromatofocusing experiments gave an ~300-fold enrichment. A MonoQ anion-exchange chromatography (column 0.5 × 5 cm) with a linear KCl gradient (0–0.4 M, 25 mL, with flow 0.5 mL min^{-1}) resulted in a final 875-fold enrichment of vinorine synthase with a total yield of 3.5%. After SDS-PAGE, the detected protein band (data not shown) was eluted and used for N-terminal sequencing.

3.2. Protein determination and partial amino acid sequence analysis

Protein concentrations were measured as described.[24] The enriched enzyme preparation from *R* × *R*M cells was separated by SDS-PAGE and the Coomassie-stained major band at 46 kDa was digested in situ with endoproteinase LysC as described by Eckerskorn and Lottspeich.[25] The obtained peptide mixture was separated by HPLC and microsequencing of four of the peptides

was performed on an Applied Biosystems model 470 gas-phase sequencer.[26] The N-terminal sequencing was performed as reported.[26]

3.3. Activity assay for vinorine synthase and product identification

To follow the purification of vinorine synthase from the cultured plant cells, the incubation mixture (total volume 25 μL) contained gardneral (final concentration 0.3 mM) acetyl-CoA (final concentration 0.72 mM) and various enzyme activities (1–5 pkat) in 0.1 M potassium phosphate (pH 7.0). The mixture was incubated for 15 min at 30 °C under shaking (1000 rpm). After addition of 2 μL of HCl (0.1 M) and 5 μL of $NaBH_4$ solution (1% in 10 mM NaOH), 200 μL of MeOH were added and the mixture was centrifuged (14,000*g*, 5 min). The supernatant was analyzed by HPLC. 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 Lichrospher 60 RP select B column (125 × 4 mm, 5 mm pre-column, Macherey-Nagel, Düren, Germany). The solvent system was acetonitrile:water (pH 2.3), gradient 28:72 → 35:65 within 6 min → 95:5 within 0.5 min → 95:5 for 2 min → 28:72 within 0.5 min → 28:72 for 5 min, 1.5 mL/min flow rate and detection at 225 nm. Rt values: gardneral—4.1 min; *epi*-gardneral—3.7 min; 11-methoxy-vinorine—7.6 min.

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