Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 1913–1918

BIOORGANIC & MEDICINAL CHEMISTRY

Vomilenine Reductase — a Novel Enzyme Catalyzing a Crucial Step in the Biosynthesis of the Therapeutically Applied Antiarrhythmic Alkaloid Ajmaline

Gerald von Schumann, Shujuan Gao and Joachim Stöckigt*

Department of Pharmaceutical Biology, Institute of Pharmacy, Johannes Gutenberg-University, Staudinger Weg 5, D-55099 Mainz, Germany

Received 26 November 2001; accepted 18 December 2001

Abstract—Delineation of the biochemical pathway leading to the antiarrhythmic *Rauvolfia* alkaloid ajmaline has been an important target in biosynthetic research for many years. The biosynthetic sequence starting with tryptamine and the monoterpene secologanin consists of about 10 different steps. Most of the participating enzymes have been detected and characterized previously, except those catalyzing the reduction of the intermediate vomilenine. A novel NADPH-dependent enzyme that reduces the intermediate has been isolated from *Rauvolfia serpentina* cell suspension cultures. Vomilenine reductase (M_r 43 kDa, temp opt 30 °C, pH opt 5.7–6.2), saturates the indolenine double bond of vomilenine with stereospecific formation of 2β(*R*)-1,2-dihydrovomilenine. The described detection, enrichment and properties of the reductase not only closes a gap in ajmaline biosynthesis but is also a prerequisite for overexpressing the protein heterologously for final clarification of its molecular properties.

Introduction

Ajmaline is an antiarrhythmic alkaloid introduced on the pharmaceutical market about four decades ago.[1] Its pharmacological activity is similar to that of the well known antiarrhythmic alkaloid quinidine. Based on activity mechanism, ajmaline and the N_β-propylderivative prajmalium belong to the class I of antiarrhythmic drugs,[2] all of which block sodium channels. Therefore, they prolong the length of the action potential time and are indicated for the treatment of heart disorders such as supraventricular and ventricular tachycardia, trial fibrillation and premature beats.[3] Because of the low bioavailability of ajmaline, the quarternary prajmalium compound became the preferred prescription drug, and it is only very recently that the long standing question of why this more polar compound shows much better uptake compared to ajmaline[4] was solved. For its therapeutic application and the synthesis of prajmalium, ajmaline needs to be isolated from the root bark of several years old medicinal plants such as Indian *Rauvolfia serpentina*, American *Rauvolfia tetraphylla*, or African *Rauvolfia vomitoria*, species which have been phytochemically investigated in much detail.[5] None of the developed total chemical syntheses of ajmaline has been exploited commercially so far.

The alkaloid is also a major compound of cell suspension cultures of *Rauvolfia*.[6] The in vitro formation of ajmaline is catalyzed by at least 10 enzymes. Seven of them were well characterized in the past[7,8] and some were recently overexpressed in *Escherichia coli*.[9–11] However, an important reaction, the reduction of the pathway intermediate vomilenine at the end of the total biosynthetic sequence, remained to be investigated.

In this communication, we report on the detection, separation, and properties of a novel NADPH-dependent reductase from *Rauvolfia* cell culture, the enzyme named vomilenine reductase which closes one of the few remaining gaps in the ajmaline biosynthesis.

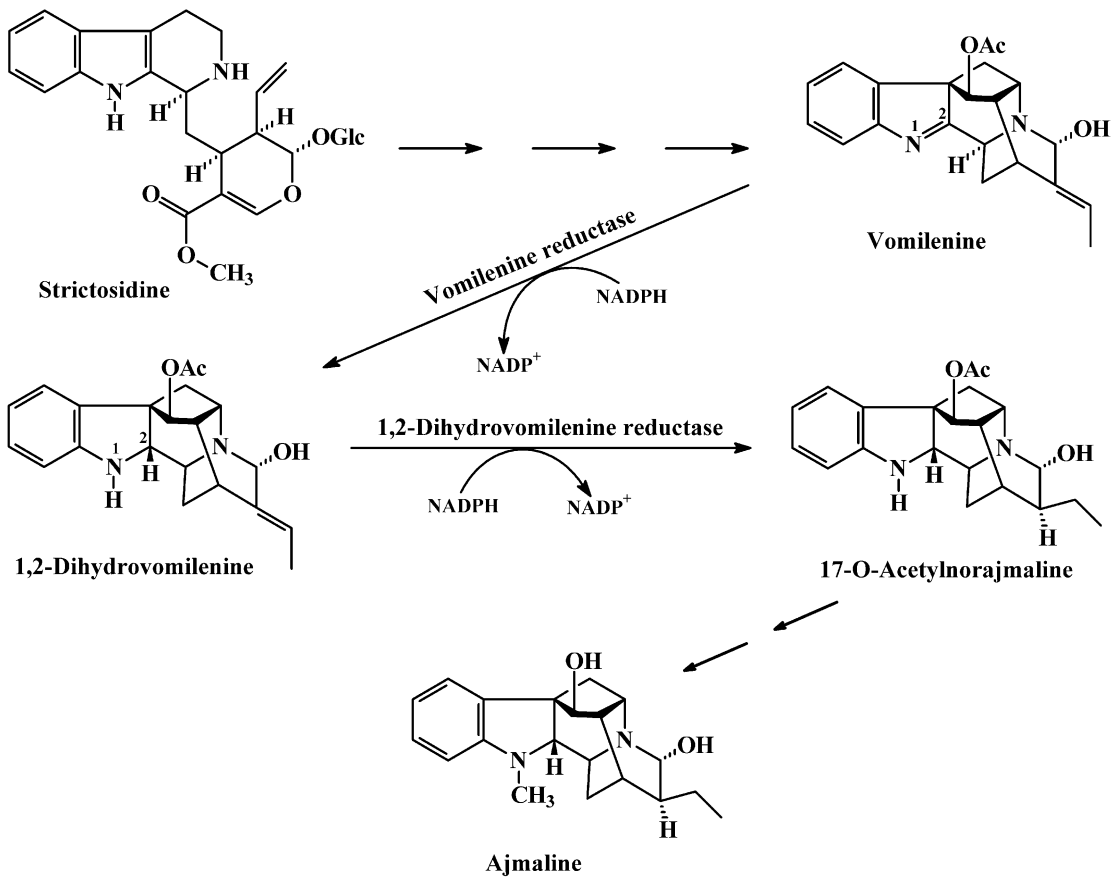
Results and Discussion

Isolation and enrichment of vomilenine reductase

A crude protein extract from cultivated *R. serpentina* cells was tested for enzymatic reductase activity accepting the

*Corresponding author. Tel.: +49-6131-39-25751; fax: +49-6131-39-23752; e-mail: stoeckig@mail.uni-mainz.de

0968-0896/02/$ - see front matter
PII: S0968-0896(01)00435-7



Scheme 1. The reaction catalyzed specifically by the novel NADPH-dependent enzyme, vomilenine reductase, isolated from cell suspension cultures of the Indian medicinal plant *Rauvolfia serpentina*. The depicted conversion is a late reaction in the multistep enzymatic biosynthesis of the monoterpenoid indole alkaloid ajmaline, starting from the precursor strictosidine.

intermediate vomilenine. This alkaloid is produced in the later stages of ajmaline biosynthesis (Scheme 1). Applying the above-mentioned standard enzyme assay, the incubation mixtures could be analyzed by a short HPLC run requiring only 10 min. Both reduction products, 2β(*R*)-1,2-dihydrovomilenine (t_R: 4.60 min) and 17-*O*-acetylnorajmaline (t_R: 5.26 min), were well separated, which allowed quantitation of overall enzyme activity. The reduction was dependent on the presence of active enzyme and NADPH but did not clarify whether two different reductases are involved in the reduction of vomilenine.

Based on these results, the above mentioned enzyme assay was used to follow the reductase activity during different stages of enzyme purification, after a time course experiment had clearly shown that maximum enzyme activity could be obtained from 10-days-old *Rauvolfia* cells (data not shown). After fractionated ammonium sulphate precipitation, vomilenine reductase was subjected to a purification procedure consisting of only three chromatographic steps (Fig. 1). SOURCE 30Q chromatography gave at low KCl concentrations an enzyme preparation reducing the indolenine double bond of vomilenine with formation of 2β(*R*)-1,2-dihydrovomilenine. The enzyme was therefore named vomilenine reductase. At increasing KCl concentrations 2β(*R*)-1,2-dihydrovomilenine formation decreased in favour of 17-*O*-acetylnorajmaline accumulation due to reduction of the 19,20-double bond suggesting that also a second enzyme, preliminary named 1,2-dihydrovomilenine reductase, is present in the *Rauvolfia* cells.

For further enzyme purification and characterization, only vomilenine reductase containing fractions were combined and chromatographed on SOURCE 15Phe material resulting in 18-fold enrichment of this reductase with an excellent yield of 52% (Table 1).

Affinity chromatography on 2′,5′ADP Sepharose resulted in a sharp peak of enzyme activity (Fig. 1) with a final 20-fold enzyme enrichment and good overall reductase recovery of ~23% (Table 1). Because of the presence of 1,2-dihydrovomilenine reductase in the crude enzyme extract and also in the ammonium sulphate precipitation, the vomilenine reductase activity cannot be accurately measured quantitatively at these particular stages of enzyme purification. Therefore, it is obvious that the final enrichment of vomilenine reductase should be higher than 20-fold. To prove the enzyme purity, we performed SDS-PAGE after affinity chromatography (Fig. 2). As the electrogram illustrates, only a few protein bands are present together with one major protein at 43 kDa. Compared with the relative enzyme activity of the single fractions, reductase activity coincides with the major band only, indicating that the 43

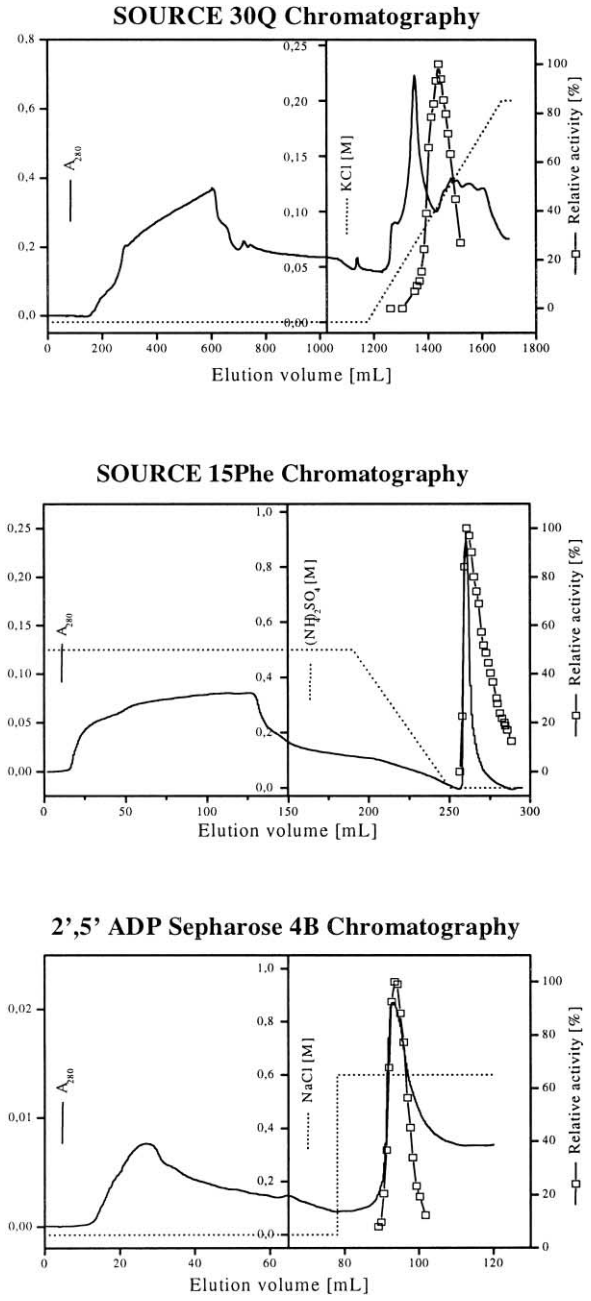


Figure 1. Elution profiles of the enzyme mixture from *Rauvolfia* cell suspension in order to enrich the new enzyme vomilenine reductase by three different columns.

kDa protein should represent the enriched vomilenine reductase. The determined M_r could be verified also by Superdex 75 gel chromatography (Fig. 3) resulting in a molecular mass of 43 ± 5 kDa and suggesting that vomilenine reductase is a monomeric enzyme.

The SDS analysis also indicates that a very short purification procedure leads to a highly enriched enzyme. Such an enzyme preparation could be further investigated by microsequencing and for general properties such as dependence of the catalyzed reaction on temperature and pH. The temperature optimum of vomilenine reductase is around 30 °C with half maximum enzyme activity around 45 °C, pointing to a good thermal stability of the enzyme and, therefore, allowing simple handling. However, it is noteworth that the half life of the enzyme at different stages of purification is only one day (data not shown). At pH 6, the enzyme shows its optimum activity in phosphate buffer with half maximum activity at pH 5.2 and 6.8 whereas its optimum is slightly shifted to pH 6.5 in phosphate/citric acid buffer (data not shown). These values correspond well to those of other enzymes participating in the biosynthesis of the antiarrhythmic ajmaline and might indicate its occurrence in the neutral cytosol of the *Rauvolfia* cell.

The enzyme activity of vomilenine reductase was totally inhibited by a sulfhydryl reagent, *p*-hydroxymercuribenzoate at 200 μM, which suggests that sulfhydryl group(s) of the enzyme play an important role in the catalyzed reaction. EDTA also inhibits the reductase activity. Under standard conditions, the inhibition was about 45% in the presence of 2 mM EDTA. In contrast, pre-incubation of the enzyme with 1 mM EDTA at room temperature for 30 min led to complete loss of enzyme activity indicating the sensitivity of the enzyme against the chelating EDTA. When the EDTA-treated enzyme was incubated with metal ions at room temperature for 30 min, almost the total activity of the enzyme could be restored with Co^{2+}, Ni^{2+}, and Mn^{2+} at 2 mM concentrations. These results demonstrate that vomilenine reductase is most probably a metalloprotein, but the nature of the metal-ion is so far unknown and can only be investigated after purifying the reductase to complete homogeneity, for example, after heterologous expression.

These enzyme parameters are also important for the optimum synthesis of the enzyme product which is

Table 1. Enrichment of vomilenine reductase from plant cell cultures of *Rauvolfia serpentina*

Purification steps	Total protein (mg)	Total activity (pkat)	Specific activity (pkat/mg)	Purification (fold)	Recovery (%)
Crude extract[a]	2347.1	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
Ammonium sulphate (40–60%)[a]	865.2	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
SOURCE 30Q	99.3	248.3	2.5	1.0	100.0
SOURCE 15Phe	2.9	130.0	44.8	18.0	52.4
2′,5′ ADP Sepharose 4B	1.1	56.7	51.5	20.6	22.8

[a]The activity of vomilenine reductase cannot be determined (n.d.) at these steps because of the presence of 1,2-dihydrovomilenine reductase, which transfers directly the product of vomilenine reductase, 1,2-dihydrovomilenine, into 17-*O*-acetylnorajmaline.

essential for its structural confirmation. Enzymatically prepared 2β(*R*)-1,2-dihydrovomilenine was purified by thin layer chromatography. UV analysis of the product showed maximum absorption at 242 nm in contrast to the substrate vomilenine (263 nm), a result, proving the conversion of the indolenine-substrate into the indoline-product. The reduction of the 1,2-double bond is also supported by the fact that ceric ammonium sulphate gives an orange color with the enzyme product, which is characteristic for indoline-type alkaloids.[12] Moreover, MS analysis provided evidence of the reduction of only one double bond because the M^+ was shifted from 350 *m*/*z* for the substrate vomilenine to 352 *m*/*z* for the enzyme product 1,2-dihydrovomilenine. But these results do not allow conclusions on the stereochemistry of the reduction step.

However, the enzymatically prepared alkaloid can be separated by HPLC from synthetic 2α(*S*)-1,2-dihydrovomilenine (t_R: 3.79 min) obtained chemically by sodium borohydride reduction of vomilenine. Conclusively, the enzyme product must exhibit the 2β(*R*) configuration that all the further alkaloids in the biosynthetic sequence to ajmaline show. In addition, MS analysis of the formed 1,2-dihydrovomilenine demonstrated the following fragmentation pattern: EI–MS *m*/*z* (rel int): 352 (46), 323 (3), 309 (4), 293 (8), 264 (4), 250 (4), 232 (4), 222 (25), 209 (6), 194 (8), 180 (23), 169 (100), 156 (14), 143 (23), 130 (31), 111 (17). This pattern with the base peak of 169 *m*/*z* is clearly consistent with the 2β(*R*) configuration of the reduction product. 2α(*S*)-1,2-dihydrovomilenine (base peak: 185 *m*/*z*), on the other hand, shows a different fragmentation pattern as described for the α(*S*)-ajmalan alkaloids.[13] Therefore, vomilenine reductase delivers the next pathway intermediate after vomilenine which exhibits the natural 2β-stereochemistry. It is obvious that the reductase has a high degree of stereospecificity, because the non-natural 2α(*S*)-1,2-dihydrovomilenine was never observed during the enzyme incubations. The high stereospecificity of vomilenine reductase suggests also that the enzyme is a specific member of the set of enzymes operating in the ajmaline biosynthetic pathway. If so, one also would expect a limited taxonomic distribution of the reductase among plants or cell cultures producing ajmaline type alkaloids. We, therefore, tested cell suspensions from plants of five different families (Table 2). Exclusively, cell cultures of the Apocynaceae family showed vomilenine reduction, but only if their members biosynthezise ajmaline alkaloids as in *Rauvolfia* species such as *R. serpentina*, *Rauvolfia mannii*, or *Rauvolfia mombasiana*. *Catharanthus*, which also belongs to the Apocynaceae family is unable to produce ajmaline alkaloids and, therefore, does in fact not express vomilenine reductase. This result is also observed for genera of other families which exhibit alkaloid synthesis (e.g., *Nicotiana* or *Solanum*) but not of the ajmaline-type. The obtained results strongly indicate that vomilenine reductase is in fact a novel and ajmaline pathway-specific enzyme.

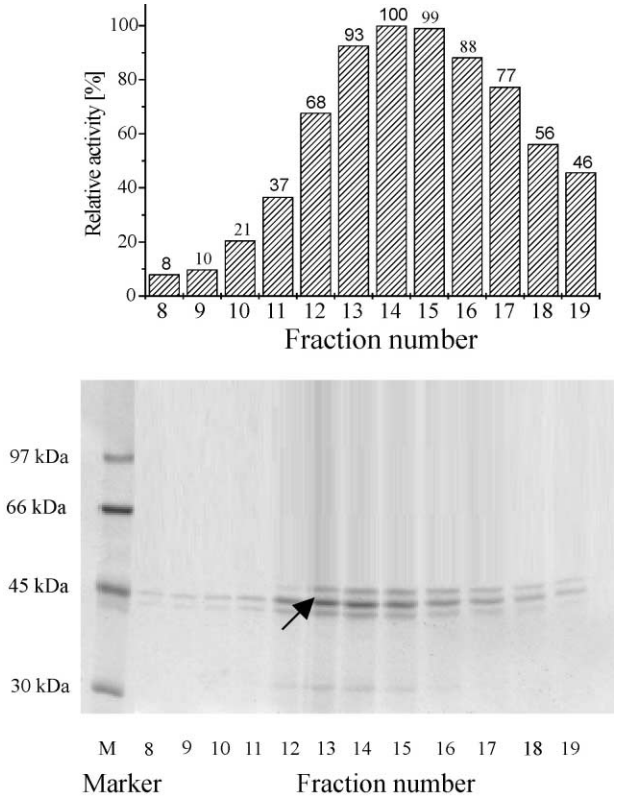


Figure 2. SDS-PAGE of protein fractions from 2′,5′ ADP Sepharose 4B chromatography compared with the vomilenine reductase activity of the single fractions. Vomilenine reductase is indicated by the arrow.

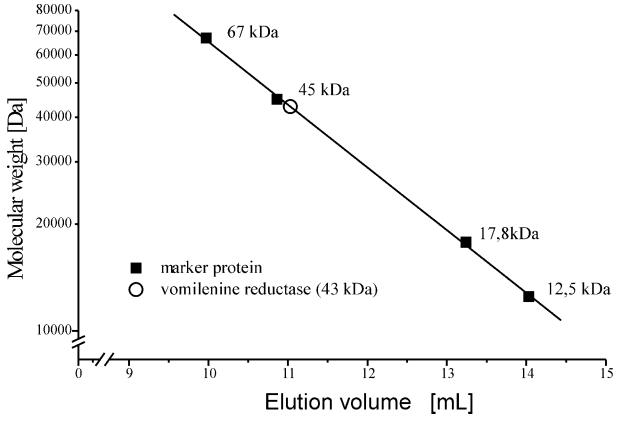


Figure 3. Molecular weight determination of vomilenine reductase by Superdex 75 chromatography.

Table 2. Taxonomic distribution of vomilenine reductase activity in different plant cell suspension cultures

Plant cell cultures	Family	Enzyme activity
Rauvolfia serpentina	Apocynaceae	+
Rauvolfia mannii	Apocynaceae	+
Rauvolfia mombasiana	Apocynaceae	+
Catharanthus roseus	Apocynaceae	–
Gossypium hirsutum	Malvaceae	–
Lonicera morrowii	Caprifoliaceae	–
Nicotiana tabacum	Solanaceae	–
Saponaria officinalis	Caryophyllaceae	–
Solanum lycopersicum	Solanaceae	–

Conclusion

The detection and characterization of vomilenine reductase now closes for the first time a gap between the intermediates vomilenine and acetylnorajmaline at late stages of the biosynthesis of the antiarrhythmic alkaloid ajmaline.

The presented study will allow the expression of this enzyme in other than plant systems such as bacteria or yeast providing much higher and pure enzyme preparation for its detailed investigation at the molecular level.

Experimental

Plant cells

Plant cell suspension cultures of *R. serpentina* and other species were usually grown in 200 mL Linsmaier and Skoog medium[14] for 10 days at 24 °C, with constant diffuse light (~600 lux) and shaking (100 rpm). Cells were harvested by filtration, frozen with liquid nitrogen, and stored at −25 °C.

Used buffers

Buffer A: 10 mM Tris–HCl, pH 7.8; B: 30 mM Tris–HCl, pH 7.3; C: 10 mM Tris–HCl, pH 7.3.

Enzyme assay

Enzyme assay to monitor vomilenine reductase activity. In a total volume of 200 μL (50 mM KPi, pH 7.0) 0.14 mM vomilenine were incubated in the presence of 2 mM NADPH and various enzyme amounts for 1 h at 30 °C with shaking (550 rpm). The reaction was terminated by adding 200 μL methanol. The solution was mixed for 5 s (vortex) and centrifuged at 18,000×*g* for 5 min. The supernatant was analyzed by HPLC.

Protein analyses

Protein concentrations were determined by the method of Bradford[15] using bovine serum albumin as a standard. SDS-PAGE was carried out with a discontinuous system described by Laemmli[16] in gels containing 11% acrylamide. Gels were stained with Coomassie Brilliant Blue solution (0.25% Coomassie Brilliant Blue R 250, 45% MeOH and 9% acetic acid). Molecular weight markers were obtained from Amersham-Pharmacia, (Freiburg).

HPLC analyses

The HPLC analyses were performed with a LiCrospher 60 RP-Select B column (125 × 4 mm, Merck, Darmstadt) equipped with a select B pre-column obtained from the same company. The solvent system used was: A, acetonitrile; B 25 mM K_2HPO_4 containing 0.45% H_3PO_4. The following gradient was applied: A 28% → 30% within 4 min, then to 35% within 2 min; flow rate was 1.68 mL min^{-1}. Retention times were: vomilenine 3.51 min, 2α(*S*)-1,2-dihydrovomilenine 3.79 min, 2β(*R*)-1,2-dihydrovomilenine 4.60 min, 17-*O*-acetylnorajmaline 5.26 min. The test was performed at a wavelength of 250 nm.

Spectroscopic analysis

UV spectra were recorded on an Ultrospec II instrument (LKB-Biochrom) in MeOH as solvent. Mass spectra were measured on a Finnigan Quadrupole instrument (MAT 44S) in EI-mode and direct inlet at 70 eV.

Isolation and enrichment of vomilenine reductase

Frozen plant cells (2 kg) were stirred at 4 °C after adding 2.000 mL Tris–HCl (100 mM, pH 8.0) containing 20 mM β-mercaptoethanol (BME) for about 40 min. The obtained slurry was homogenized with Ultraturrax for 2.5 min followed by centrifugation for 30 min at 10,000×*g*. The supernatant was subjected to ammonium sulphate precipitation. The protein fraction precipitating between 40% and 60% saturation was resuspended in buffer A containing 2.5 mM BME and dialyzed against the same buffer overnight.

After centrifugation (10,000×*g*, 30 min) the supernatant was loaded onto a SOURCE 30Q column (120 × 50 mm) which was equilibrated with buffer A. Elution of the enzyme was carried out with a linear gradient of KCl (0–200 mM) in buffer A (500 mL). The flow rate was 8.0 mL min^{-1} and fractions of 10 mL each were collected. Fractions 35–41 were pooled. Ammonium sulphate to this solution was added (0.5 M final concentration) and centrifuged for 30 min at 18,000×*g*. The supernatant was applied onto a SOURCE 15Phe column (100 × 16 mm) which was equilibrated with buffer B containing 0.5 M ammonium sulphate. The enzyme was eluted with a decreasing linear gradient of ammonium sulphate (0.5 M to 0) in buffer B (60 mL) with a flow rate of 2 mL min^{-1}. The column was eluted further with water until no protein was monitored (UV 280 nm), and fractions of 1.2 mL each were collected. Fractions 18 to 30 were combined and desalted on a Sephadex G 25 column (370 × 26 mm) which was equilibrated with buffer C. Pooled fractions containing vomilenine reductase were loaded onto a 2′,5′ ADP Sepharose 4B column (80 × 16 mm) equilibrated with buffer C. Proteins were eluted by step gradient with the same buffer containing 0.6 M NaCl at a flow rate of 1.0 mL min^{-1}. Fractions were tested for reductase activity by HPLC and separated with SDS-PAGE.

1,2-Dihydrovomilenine production

After SOURCE 30Q column partial purification, vomilenine reductase was incubated with vomilenine; 120 incubations, each with 400 μL total volume, including 310 μL enzyme solution (specific activity: 2.5 pkat/mg), KPi-buffer (50 mM final concentration, pH 7.0), 10 mM NADPH and 0.35 mM vomilenine were shaken (550 rpm) at 30 °C. After 3 h ~60% of the substrate was

converted. The pH of the solution was adjusted with concd ammonia to pH 9. After adding 800 μL ethyl acetate and mixing (30 s vortex), centrifugation at 18,000×*g* followed (5 min). The upper phases containing the alkaloids were combined. The lower phases were extracted again with 400 μL ethyl acetate as above. The organic phases were combined and evaporated. To the yellowish, oily residue 500 μL of the mixture chloroform/methanol (8:2) was added.

Separation of the alkaloids was done by TLC using the solvent system dichloromethane/methanol/ammonia (8.8:1.2:0.1). 1,2-dihydrovomilenine was detected by its orange colour with ammonium cerium(IV)sulphate under daylight[12] and eluted from the TLC-plate with dichloromethane and methanol (7:3). A few μL of the 1,2-dihydrovomilenine solution were taken, evaporated and analyzed by mass spectrometry.

Influence of EDTA and dependence on metal-ions

Influence of EDTA and cations on the reductase activity was tested using the standard assay in the presence of different EDTA concentrations. Recovery of the activity of EDTA-treated enzyme (1.0 mM EDTA, 30 min at 25 °C) was measured after adding metal-ions (2.0 mM final concentration) followed by standard incubation and HPLC analysis.

Taxonomic distribution of vomilenine reductase

For determination of the taxonomic distribution of the reductase, crude enzyme mixtures were isolated from the appropriate cell cultures. About 20 g frozen cell material was ground for 10 s and transferred to a Falcon vial. Addition of the same amount of Tris–HCl (100 mM, pH 7.8, 20 mM BME) followed and after thawing of the mixture (30 min, 700 rpm, 4 °C) the obtained cell slurry was centrifuged (10.000×*g*, 4 °C) for 25 min. The supernatant was taken and precipitated with 75% ammonium sulphate for 1 h at 4 °C. Another centrifugation at 10,000×*g* for 30 min was carried out, followed by resuspension of the pellet in 1 mL 50mM KPi (pH 7.0) containing 0.15 M NaCl. The solution, free of particles, was loaded on a HiTrap desalting column (bed-volume 5 mL) via a syringe. The elution profile recorded at 280 nm showed two major peaks (data not shown). The first one indicated the isolated crude enzyme mixture while the second peak resulted from endogenous alkaloids of the cells. The first fraction was used for standard enzyme incubation followed by HPLC assay.

Acknowledgements

This work was supported by a grant of Deutsche Forschungsgemeinschaft (Bonn, Bad-Godesberg, Germany), by BMBF, DLR Bonn project CHN 96/303 and by the Fonds der Chemischen Industrie (Frankfurt/Main, Germany). We are thankful to Prof. Dr. W. E. Court (Mold, Wales) for linguistic advice.

References and Notes

1. Kleinsorge, H. *Med. Klin* **1959**, *54*, 409.
2. Schütz, W. In *Pharmakologie und Toxikologie*; Forth, W., Henschler, D., Rummel, W., Starke, K., Eds.; Spektrum Akademischer: Heidelberg, Berlin, Oxford (7 Auflage, 1. Nachdruck) 1998, p 371.
3. Mutschler, E. In *Mutschler Arzneimittelwirkungen*; Mutschler, E., Geisslinger, G., Kroemer, H.K., Schäfer-Korting, M., Eds.; Wissenschaftliche: Stuttgart (8 Auflage) 2001, p 545.
4. Hinse, C.; Stöckigt, J. *Pharmazie* **2000**, *55*, 531.
5. Court, W. E. *Planta Med.* **1983**, *48*, 228.
6. Stöckigt, J.; Pfitzner, A.; Firl, J. *Plant Cell Rep*. **1981**, *1*, 36.
7. Stöckigt, J. In *The Alkaloids*; Cordell, G.A., Ed., Academic: New York, 1995; Vol. 47, p 115.
8. Stöckigt, J. In *Natural Product Analysis*; Schreier, P., Herderich, M., Humpf, H.-M., Schwab, W., Eds., Vieweg: Braunschweig-Wiesbaden, 1998; p 313.
9. Kutchan, T. M.; Dittrich, H.; Bracher, D.; Zenk, M. H. *Tetrahedron* **1991**, *47*, 5945.
10. Dogru, E.; Warzecha, H.; Seibel, F.; Haebel, S.; Lottspeich, F.; Stöckigt, J. *Eur. J. Biochem*. **2000**, *267*, 1397.
11. Warzecha, H.; Gerasimenko, I.; Kutchan, T. M.; Stöckigt, J. *Phytochemistry* **2000**, *54*, 657.
12. Court, W. E.; Iwu, M. M. *Chrom*. **1980**, *187*, 199.
13. Gorman, M.; Burlingame, A. L.; Biemann, K. *Tetrahedron Lett*. **1963**, *1*, 39.
14. Linsmaier, E. M.; Skoog, F. *Physiol. Plant*. **1965**, *18*, 100.
15. Bradford, M. M. *Anal. Biochem*. **1976**, *72*, 248.
16. Laemmli, U. K. *Nature* **1970**, *227*, 680.