Characterization of 2β(*R*)-17-O-Acetylajmalan: Acetylesterase – a Specific Enzyme Involved in the Biosynthesis of the *Rauwolfia* Alkaloid Ajmaline

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Dedicated to Professor Helmut Simon on the occasion of his 60th birthday

Rauwolfia serpentina Benth., Cell Suspension Culture, 2β(*R*)-17-O-Acetylajmalan: Acetylesterase, Ajmaline Biosynthesis

A novel enzyme was isolated, partially purified (217-fold) and characterized from cell suspension cultures of *Rauwolfia serpentina* Benth. The enzyme catalyzes one of the late biochemical reactions in the biosynthesis of ajmaline by hydrolysis of 17-O-acetylated alkaloids of the ajmalan group forming the appropriate deacetylated compounds. This esterase exhibits an unusually high substrate selectivity and exclusively accepts acetylated ajmaline derivatives with the naturally occurring 2β(*R*)-configuration. The properties of the enzyme were determined showing an optimum pH at 7.5, an isoelectric point of pH 4.9 and a relative molecular weight of 33 ± 2 kDa. Inhibition studies of enzyme activity point to the necessity of SH-groups. The esterase seems not to be inhibited by ajmaline, the end product of the pathway. The highest enzyme activities were observed in leaves and cell suspension tissues of the tribe *Rauwolfieae* which are known to synthesize ajmaline and its congeners. The specific function of the esterase in the biosynthesis of the later alkaloids was established.

Introduction

Ajmaline, a major plant constituent of *Rauwolfia serpentina* Benth., is a monoterpenoid indole alkaloid which is used for the treatment of cardiac arrhythmias. The molecular structure of this compound, as well as a number of other alkaloids belonging to this group, is very complex. Ajmaline, for example, consists of a hexacyclic ring system bearing 9 centers of chirality. Because of the complex nature of this structure, the biosynthesis of ajmaline and related compounds would be expected to be a complicated process involving many different biochemical reactions.

Studies designed to determine the biosynthetic pathway of ajmaline in the past have been limited due to the difficulty in cultivating *R. serpentina* plants. Because of this problem, only a very few *in vivo* feeding experiments with labelled precursors have been described in the literature (*e.g.* [1–5]). None of these studies, however, provided a detailed description of any biosynthetic step. Investigations using plant cell cultures and the cell-free systems, on the other hand, prove to be very useful in providing a means of obtaining experimental data which eventually lead to the elucidation of the ajmaline biosynthetic route.

During the last few years we discovered and characterized several of the enzymes catalyzing the formation of *Rauwolfia* alkaloids, especially those of the sarpagan/ajmalan group [6, 7]. Here we report the isolation and the properties of a "late" enzyme of the ajmaline pathway, named 2β(*R*)-17-O-acetylajmalan: acetylesterase which catalyzes the deacetylation of alkaloids of the ajmaline class as illustrated in Scheme 1.

Results

Isolation and enrichment of 2β(R)-17-O-acetylajmalan: acetylesterase

When cultivated *Rauwolfia serpentina* cells were extracted with 0.1 M potassium phosphate buffer, the crude protein mixture obtained catalyzed the hydrolysis of acetylated alkaloids of the ajmaline group. Using [^{3}H-acetyl]acetyltetraphyllicine as a test compound, the hydrolytic activity of this protein fraction was monitored by measuring the liberated [^{3}H]acetic acid. This alkaloid was selected not only for its ease in hydrolysis by crude protein extracts, but also be-

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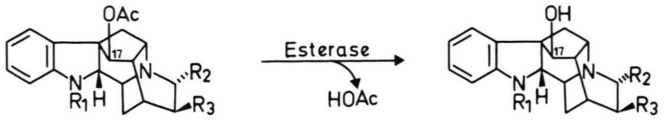


Scheme 1. The reaction catalyzed by 2β(*R*)acetylajmalan: acetylesterase from *R. serpentina* cell suspension cultures.

17-O-Acetylnorajmaline
(R_1 = H, R_2 = OH, R_3 = ethyl)
17-O-Acetylajmaline
(R_1 = CH_3, R_2 = OH, R_3 = ethyl)
17-O-Acetyltetraphyllicine
(R_1 = CH_3, R_2 = H, R_3 = ethylidene)

Norajmaline
(R_1 = H, R_2 = OH, R_3 = ethyl)
Ajmaline
(R_1 = CH_3, R_2 = OH, R_3 = ethyl)
Tetraphyllicine
(R_1 = CH_3, R_2 = H, R_3 = ethylidene)

cause it is not difficult to label. In addition, the compound closely resembles the structures of those intermediates which eventually lead to ajmaline. Based on the hydrolytic reaction an enzyme assay was developed to follow the enzyme activity at different stages of purification. The minimum activity of the esterase that could be measured was 0.1 pkat per ml incubation mixture. A time course experiment was carried out for a period of 22 days and showed that maximum enzyme activity per l nutrition medium could be extracted from the *Rauwolfia* cells around day 9 or 11 of the growth period depending on the applied medium. Using a growth medium published by Linsmaier and Skoog (LS-medium) [8], 1 nkat/l medium of enzyme activity was recovered after 9 days (Fig. 1). Cells cultivated in an alkaloid production medium (AP) [9] provided about 1.8 nkat/l of activity. Determination of cell growth under the same conditions showed the production of 27 g dry weight/l in LS medium and 34 g/l in AP-medium, respectively (Fig. 2). From 0.75 kg of cells grown for 11 days in AP-medium 650 mg of protein were extracted with 0.1 M potassium phosphate buffer. Employing G-25 gel chromatography, protamine sulfate precipitation, a combined set of columns consisting of DEAE-, CM- and hydroxylapatite-chromatography and additional purification by hydrophobic chromatography on phenyl-TSK an esterase enrichment of about 220-fold was achieved with an overall yield of 58%. The enzyme enrichment data are illustrated in Table I. Further studies showed that this esterase fraction, concentrated with polyethylene glycol (1.5 mg protein, specific activity 437 pkat/mg protein), did not contain other known enzymes of the ajmaline/sarpagine biosynthesis (strictosidine synthase, strictosidine glucosidase, polyneuridine aldehyde esterase, vinorine synthase, norajmaline-N-methyltransferase, raucaffricine glucosidase, vellosimine reductase) or the very recently discovered enzymes preliminarily named vomilenine isomerase and perakine reductase.

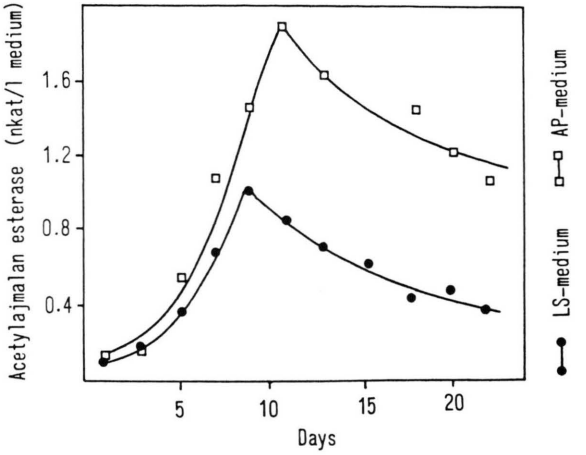


Fig. 1. Time course of acetylajmalan esterase activity in a cell suspension culture of *R. serpentina* grown in LS- and AP-medium.

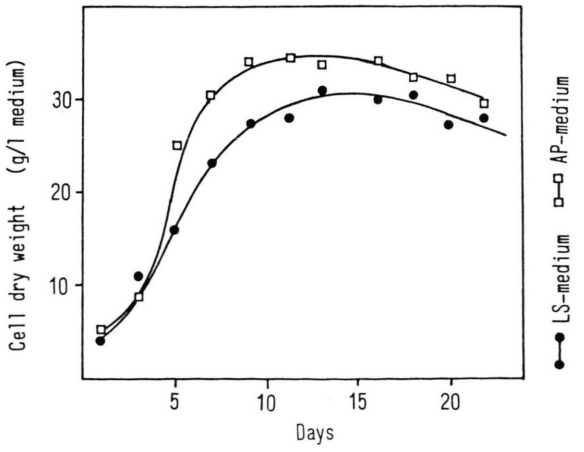


Fig. 2. Time course of cell dry weight in a cell suspension culture of *R. serpentina* grown in LS- and AP-medium.

Table I. Partial purification of 2β(*R*)acetylajmalan: acetylesterase from 0.75 kg *R. serpentina* cells.

Purification step	Total volume [ml]	Total protein [mg]	Total activity [pkat]	Specific activity [pkat/mg]	Yield [%]	Purification (-fold)
1. Supernatant of the crude extract	1300	650	1820	2.8	100	1.0
2. Ammonium sulfate fractionation	110	430	1547	3.6	85	1.3
3. Protamine sulfate fractionation	115	378	1587	4.2	87	1.5
4. TSK–DEAE, TSK–CM and hydroxylapatite chromatography	230	13.82	1564	113.2	86	40.4
5. Phenyl-TSK chromatography	58	1.74	1056	607	58	217

Characteristics of the esterase

As depicted in Fig. 3, the esterase reaction was clearly dependent on protein and showed a typical saturation curve and normal Michaelis-Menten kinetics. For the substrate acetyltetraphyllicine an apparent K_m-value of 30 μM (V_{max} = 5 pkat) was determined. The optimum pH for the enzyme activity, as determined under conditions described in the experimental was found to be pH 7.5 with half maximum activity at pH 6.3 and 8.5. The optimum temperature and the pH of the isoelectric point was found to be 37 °C and 4.9 ± 0.2, resp. (Fig. 4). An 11 ml elution volume containing the esterase corresponded to a relative molecular weight of 33 ± 2 kDa on a calibrated TSK G 3000 column. When different enzyme inhibitors were tested only 2,2'-dithiodipyridine, iodoacetamide and 5,5'-dithiobis(2-nitrobenzoic acid) exhibited a significant effect ranging between 30 and 40% at 0.5 mM concentration of inhibitor. The deacetylated alkaloids ajmaline and norajmaline did not show any inhibition of the ester hydrolysis up to a concentration of 2.9 mM, as well as acetate, which had no effect up to 75 mM. The hydrolase was relatively stable at the stage of a 150-fold enrichment in 0.1 M phosphate buffer (pH 7.0, 1 mM dithiothreitol) at 4 °C. The half-life of the enzyme was found to be 21 days under these conditions.

Substrate specificity of the enzyme

Of 14 acetylated compounds tested only 3 alkaloids were found to be hydrolyzed by the esterase. The accepted substances were exclusively of the ajmaline class with 2β(*R*)-configuration (Table II). The K_m-values of these substrates did not differ very much. However, the enzyme exhibited a slight preference for 17-O-acetylnorajmaline (K_m = 22 μM, Fig. 3). The K_m-values for 17-O-acetylajmaline and 17-O-acetyltetraphyllicine were about 27 and 30 μM. Acetylated alkaloids of this particular type, but with 2α(*S*)-configuration were not hydrolyzed. Compounds of the indolenine group, of the *Sarpagine, Yohimbine, Aspidosperma* type and *p*-nitrophenyl acetate or β-naphthyl acetate were also not reacted by this enzyme.

Taxonomic distribution of acetylajmalan esterase activity

Plant materials (cell suspension cultures and leaves) of different origin were investigated for the presence of acetyltetraphyllicine hydrolyzing enzyme activity. Crude enzyme preparations, dialyzed 10 h against potassium phosphate buffer, were used. The highest specific activities of the esterase were discovered in all the species of *Rauwolfia* tested and ranged from 2 pkat/mg protein for *R. chinensis* to 5 pkat/mg protein in *R. verticillata* leaves. An intensive phytochemical screening of plants belonging to the tribe *Rauwolfieae* demonstrated that all of the above species tested contained ajmaline [10, 11]. Plant leaves or cultivated cells of other indole alkaloid producing species or their cell cultures (*Catharanthus, Vinca* species) exhibited lower specific activities of the esterase and ranged between 0.4 and 1.2 pkat/mg protein. The enzyme activity found in cell material free of indole alkaloids *(Malus sylvestris, Nicotiana sylvestris, Daucus carota)* was between 0.2 and 0.4 pkat/mg protein (Table III).

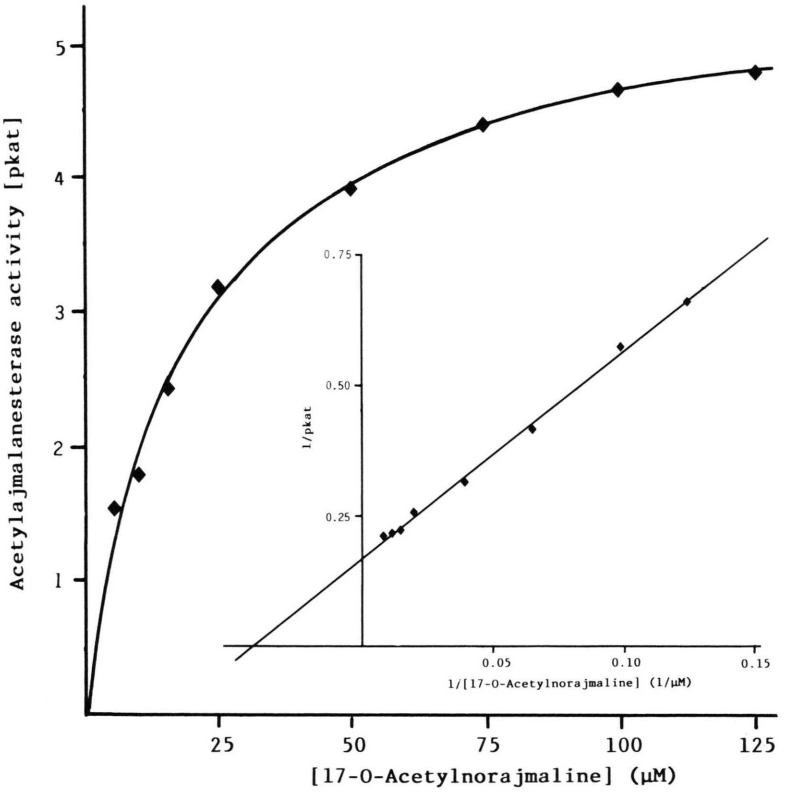


Fig. 3. The effect of the concentration of 17-O-acetylnorajmaline on the reaction rate of acetylajmalan esterase (standard incubation). K_m = 22 μM; V_{max} = 5.7 pkat.

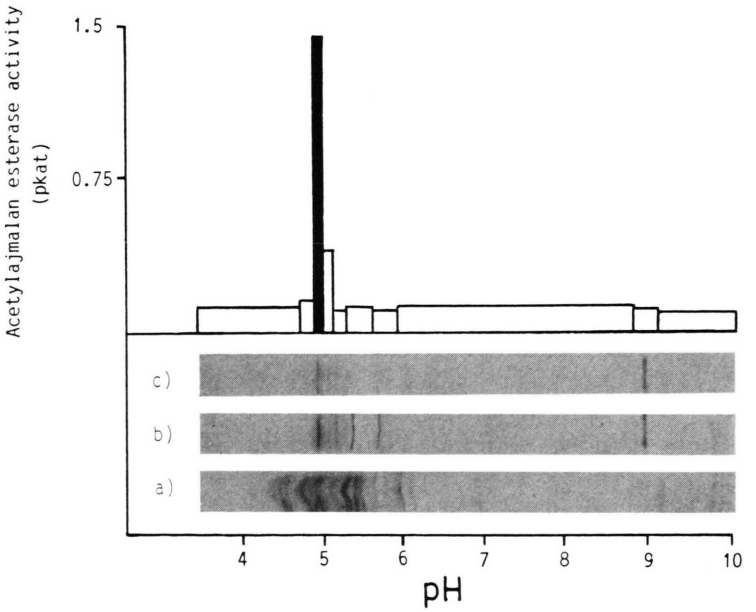
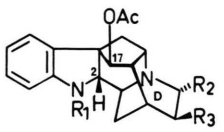
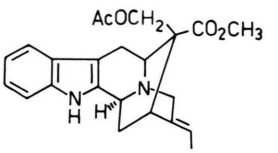
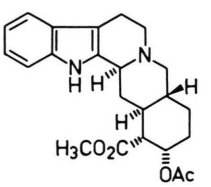
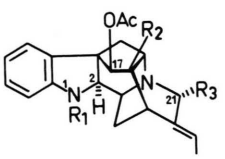
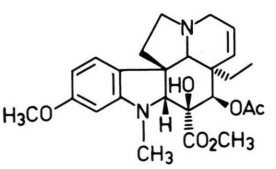
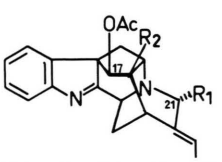
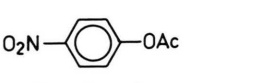
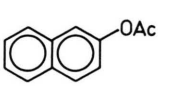


Fig. 4. Isoelectric focussing of acetylajmalan esterase at different stages of enrichment: a) ammonium sulfate precipitation, b) DEAE- CM- and hydroxylapatite chromatography, c) phenyl-TSK chromatography.

Table II. Substrate specificity of acetylajmalan esterase.

Substrates	Km [μM]	Rel. enzyme activity [%]	Substrates	Rel. enzyme activity [%]
2 β-(*R*)-17-O-Acetyl ajmalan alkaloids				
				
17-O-Acetylnorajmaline (R_1 = H, R_2 = OH, R_3 = ethyl)	22	100	Acetylpolyneuridine	< 1
17-O-Acetylajmaline (R_1 = CH_3, R_2 = OH, R_3 = ethyl)	27	84		
17-O-Acetyltetraphyllicine (R_1 = CH_3, R_2 = H, R_3 = ethylidene)	30	86		
2 α-(*S*)-17-O-Acetyl ajmalan alkaloids				
			Acetylyohimbine	< 1
1,2-Dihydrovomilenine (R_1 = R_2 = H, R_3 = OH)	–	< 1		
1,2-Dihydroraucaffricine (R_1 = R_2 = H, R_3 = Oglu)	–	< 1		
Nα,17-O-Diacetylquebrachidine (R_1 = OAc, R_2 = CO_2CH_3, R_3 = H)		< 1	Vindoline	< 1
Indolenine-17-O-acetyl ajmalan alkaloids				
				
Vinorine (R_1 = R_2 = H)	–	< 1	p-Nitrophenyl acetate	< 1
Vomilenine (R_1 = OH, R_2 = H)	–	< 1		
Raucaffricine (R_1 = Oglu, R_2 = H)	–	< 1	β-Naphthyl acetate	< 1

Discussion

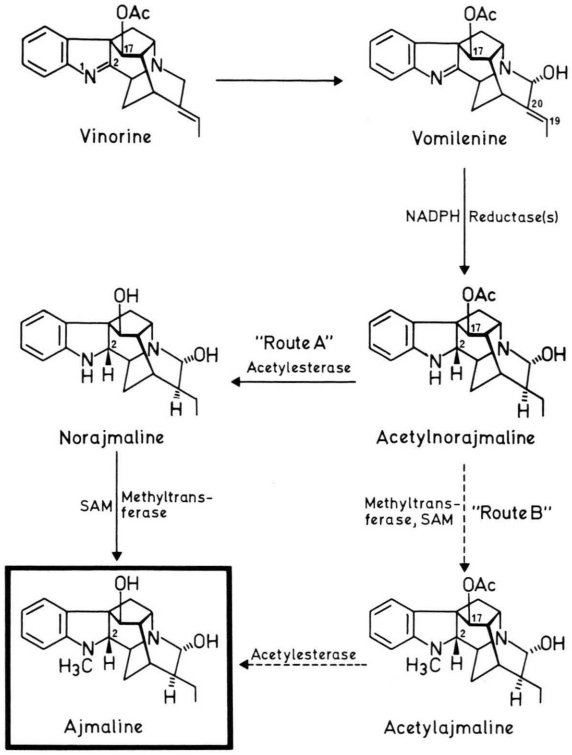
An O-acetylesterase has been identified and partially purified from cell suspension cultures of *Rauwolfia serpentina* Benth., which produces a whole range of typical *Rauwolfia* alkaloids, especially ajmaline and its derivatives [12]. The enzyme converts by hydrolysis 17-O-acetylated alkaloids of the ajmaline group to the appropriate deacetylated compounds (Scheme 1). A simple assay to monitor the enzyme activity was developed by labelling of tetraphyllicine with [^{3}H]aceticanhydride to give [^{3}H-acetyl]acetyltetraphyllicine. Enzymatic hydrolysis of this radioactive alkaloid releases the label in the ambient water and enzyme activity can be easily determined by scintillation counting of the incubation mixture

after the alkaloid has been adsorbed on charcoal. For evaluation of the optimum conditions of enzyme isolation we followed the change of enzyme activity in cultivated cells over a growth period of 22 days. As depicted in Fig. 1 and 2, the increase of total esterase activity is closely correlated with cell growth. Maximum enzyme yield (1 nkat/l medium) was extracted from cells grown in Linsmaier-Skoog medium 9 days after inoculation. At this time cell growth is at the end of the logarithmic phase. Using an alkaloid production medium, highest enzyme activity could be isolated at the beginning of the stationary phase 11 days after inoculation. About twice of the esterase activity (1.8 nkat/l medium) compared to the results for the LS-medium could be isolated under these conditions.

From 0.75 kg of fresh *Rauwolfia* cells cultivated in AP-medium, 650 mg of a crude protein mixture were obtained and partially purified. G-25 gel filtration, protamine sulfate precipitation, purification on a combined set of columns of ion exchange (TSK-DEAE, TSK-CM) and hydroxylapatite chromatography removed all the other known *Rauwolfia* enzymes and gave a 40-fold enrichment of the esterase in a good yield of 86%. Then, following the use of hydrophobic chromatography (phenyl-TSK) an overall yield of protein recovery of 58% with a 217-fold enrichment was achieved. Comparison of the protein pattern after isoelectric focussing between pH 3 and 10 shows that most of the proteins were removed when the stage of a 40-fold enrichment was reached. After the final purification step the esterase preparation still consisted of at least seven proteins (Fig. 4). From these results we expect that preparative isoelectric focussing can be applied for the isolation of homogenous acetylajmalan esterase in future experiments. The properties of the enzyme were determined using a preparation at the stage of a 150-fold enrichment. In comparison to the other enzymes acting in the biosynthesis of ajmaline, the esterase has also a relative low molecular weight of 33 ± 2 kDa assuming a globular shape of the enzyme. The optimum pH of the enzyme was 7.5; however, 50% of its activity was still observed at pH 6.3 and 8.5 pointing to a broad pH tolerance of the esterase. From a selection of enzyme inhibitors, only iodoacetamide, 2,2′-dithiodipyridine and 5,5′-dithiobis(2-nitrobenzoic acid) showed an inhibitory effect (30–40%) at 0.5 mM concentration, which indicates the necessity of SH-groups for full enzyme activity. Therefore, the enzyme is relatively stable in the presence of SH-protecting reagents like dithiothreitol, with a half-life of about 20 days at 4 °C.

The biosynthetic significance of the esterase was established by the determination of its substrate specificity. Esterases are usually believed to exhibit a broad substrate acceptance [13], but few examples from plants have shown high substrate specificity, *e.g.* 5-(4-acetoxy-1-butinyl)-2,2′-bithiophene: acetate esterase from *Tagetes patula* [14] and polyneuridine aldehyde esterase earlier identified from the cell culture investigated here [15]. Because these cells contain about 10 different proteins hydrolyzing β-naphthyl acetate, it was interesting to clarify whether the isolated esterase was one of these enzymes. When the 150-fold purified esterase was incubated with β-naphthyl- or *p*-nitrophenyl acetate no hydrolysis of either compound was observed. In addition, of 12 different O-acetylated alkaloids tested only three, 17-O-acetyltetraphyllicine, 17-O-acetylajmaline and its nor-derivative, were accepted as substrates by the esterase (Table II). These alkaloids differ structurally only by the substituents on the indole nitrogen or the D-ring. The K_m-values or the relative enzyme activities were not significantly influenced by the substituents. In sharp contrast to the above mentioned substrates with the natural 2β(*R*)-configuration synthetic alkaloids having the 2α(*S*)-stereochemistry were not hydrolyzed. Neither related compounds with the indolenine double bond (vinorine or vomilenine), nor acetylated alkaloids of the *Sarpagan, Yohimbine,* or *Aspidosperma* group were enzymatically transformed (Table II). The inability of the esterase to hydrolyze vinorine or vomilenine is of special interest, because these compounds appear as earlier intermediates of the ajmaline pathway. Vinorine, with the basic skeleton of ajmaline is enzymatically synthesized from a sarpagan-type alkaloid in the presence of acetyl-CoA as co-substrate and afterwards it is hydroxylated leading to vomilenine [16]. Both indolenines are only stable in their acetylated form (17-O-acetyl group). Chemical cleavage of this protecting function leads back to the sarpagan-type. NADPH dependent reduction of the indolenine and the C-19 double bond of vomilenine yields, however, 17-O-acetylnorajmaline, the substrate with the highest affinity for the acetylajmalan esterase (K_m = 22 μM). The biosynthetic function of this enzyme is obviously to remove the protecting group which was introduced in

Scheme 2. "Late steps" of the ajmaline biosynthesis in *R. serpentina* cell cultures.



an earlier biosynthetic step by acetylation. The final step in the ajmaline biosynthesis which is the methylation of the indole nitrogen is then carried out by a *S*-adenosylmethionine dependent N-methyltransferase (Scheme 2).

The late reactions in the ajmaline pathway are obviously more complex as just discussed, because 17-O-acetylajmaline is also an excellent substrate for the esterase (K_m = 27 μM). As depicted in Scheme 2, we conclude that both route A and B might finally give rise to the biosynthesis of the end product ajmaline. When ajmaline or its precursor norajmaline with a concentration up to 2.9 mM, or acetate (up to 75 mM) was incubated in the presence of the esterase and [^{3}H-acetyl]acetyltetraphyllicine as substrate, we did not observe any influence on the catalytic activity of the enzyme. Therefore, the activity of the enzyme under investigation seems not to be inhibited by its end product or the final compound of the pathway, ajmaline. The enzymes of that pathway have a distinct taxonomic occurrence. However, when the distribution of acetyltetraphyllicine hydrolyzing enzymes was investigated (as crude enzyme mixture) in cell material of different origin, we found esterase activity in each of the tested plants and cell cultures (Table III). Highest activities were found in those

Table III. Taxonomic distribution of acetyltetraphyllicine hydrolyzing esterase in cell suspension cultures and leaves* of different genera of the Apocynaceae and other families.

Plant material	Family	Tribe	Esterase activity pkat/g cells (fresh weight)	pkat/mg Protein	Occurrence of ajmaline in the plant
Rauwolfia verticillata Chevalier*	Apocynaceae	Rauwolfieae	4.5	5.0	yes
Rauwolfia vomitoria Afz.	Apocynaceae	Rauwolfieae	3.5	4.8	yes
Rauwolfia serpentina Benth.	Apocynaceae	Rauwolfieae	3.2	3.6	yes
Rauwolfia caffra Sond.	Apocynaceae	Rauwolfieae	2.2	2.6	yes
Rauwolfia verticillata Chevalier	Apocynaceae	Rauwolfieae	2.1	2.2	yes
Rauwolfia chinensis Hemsl.	Apocynaceae	Rauwolfieae	1.9	2.0	yes
Catharanthus roseus (L.) G. Don*	Apocynaceae	Alstonieae	0.9	1.4	no
Catharanthus roseus (L.) G. Don	Apocynaceae	Alstonieae	1.3	1.3	no
Vinca minor L.*	Apocynaceae	Alstonieae	0.6	0.6	no
Vinca minor L.	Apocynaceae	Alstonieae	0.3	0.4	no
Vinca major L.	Apocynaceae	Alstonieae	0.2	0.5	no
Vinca major L.*	Apocynaceae	Alstonieae	0.5	0.4	no
Vinca herbacea Waldst. et Kit.	Apocynaceae	Alstonieae	0.2	0.4	no
Rhazya stricta Decaisne	Apocynaceae	Alstonieae	0.8	0.6	no
Alstonia scholaris (L.) R. Br.*	Apocynaceae	Alstonieae	0.6	0.6	no
Alstonia scholaris (L.) R. Br.	Apocynaceae	Alstonieae	0.4	0.4	no
Voacanga africana Stapf.	Apocynaceae	Tabernae-montaneae	0.5	0.6	no
Malus sylvestris (L.) Mill.	Rosaceae	–	0.4	0.3	no
Nicotiana sylvestris Spegazz. et Comes	Solanaceae	–	0.2	0.3	no
Daucus carota L.	Apiaceae	–	0.2	0.2	no

samples of plants or cell cultures which produced ajmaline, all belonging to the tribe *Rauwolfieae*. Much lower, but nevertheless significant hydrolysis was also observed in enzyme preparations from plant leaves and cell suspensions of species of the tribe *Alstonieae* and in tissue samples which do not synthesize indole alkaloids. Because of the high substrate specificity of the enriched esterase from *Rauwolfia*, ester cleavage in crude protein mixtures from different plant origin might be due to the presence of rather unspecific esterases.

Therefore, our earlier observation [16] that unpurified preparations of acetylajmalan esterase have some hydrolytic activity on 17-O-acetylindolenines (like vinorine), is now known to be due to the occurrence of such unspecific esterases.

From the properties of the enzyme described in this paper, it is now obvious that 2β(*R*)-acetylajmalan: acetylesterase fits perfectly into the whole structure of the biochemical pathway of ajmaline formation in *Rauwolfia serpentina* cell cultures as summarized in Scheme 2.

Materials and Methods

Plant cell material

Plant cell suspension cultures of *Rauwolfia serpentina* were cultivated in 1 l Erlenmeyer flasks containing 250 ml Linsmaier-Skoog [8] or alkaloid production medium [9] for 9 and 11 days, resp., as described earlier [16]. Plants were grown under normal greenhouse conditions. The fresh weight of cell suspensions was determined after suction filtration. For measuring the dry weight the cells were dried to constant weight at 110 °C (24 h).

Spectroscopic methods

Structural identification of substrates and products was performed by UV (Perkin-Elmer 551 S spectrophotometer) in methanol as solvent, by MS (Finnigan MAT 44 S quadrupol instrument) in EI-mode, and by ^{1}H NMR (Bruker AM 360 spectrometer) at 360 MHz in pyridine-d_5 as solvent. Chemical shifts (δ) are reported in ppm and coupling constants *(J)* in Hz. Signal multiplicities are abbreviated as: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet (br = broad).

Chromatography

Chromatographic identification and purification of alkaloids were carried out by thin layer chromatography on Sil gel plates (Macherey and Nagel G/UV_{245}, 0.25 mm). The following solvent systems were used: (a) chloroform/methanol/ammonia = 90:10:0.2, (b) petroleum ether (40–60 °C)/acetone/diethylamine = 7:2:1, (c) ethyl acetate/*i*-propanol/ammonia = 16:4:1. Visualization of alkaloids was made by spraying the plates with a 5% (w/w) solution of ceric ammonium sulfate in 85% phosphoric acid. Quantitation of alkaloids was done on a Spectra Physics HPLC instrument coupled with a UV-detector (SP 8440) and a RP 18 column (Merck, Hibar LiChroCART 250–4). The applied HPLC solvent system was acetonitrile/water/methanol containing 0.01% trifluoroacetic acid (gradient 1/8/1→8/1/1 within 30 min). The flow rate was 1 ml/min and alkaloids were detected at 290 nm.

Enzyme assays

Assay A. The standard incubation volume used was 0.3 ml and contained the substrate [^{3}H-acetyl] acetyltetraphyllicine (56 Bq/nmol) at a final concentration of 0.2 mM in 0.1 M potassium phosphate buffer (pH 7.0). Incubations were carried out at 30 °C for 30 min. Then 0.2 ml charcoal suspension (60 mg/ml) was added. After shaking for 10 min the mixture was centrifuged (Eppendorf centrifuge 5414) for 10 min and 0.4 ml of the supernatant was counted for radioactivity with a Berthold BF 5000 scintillation counter using 5 ml rotiszint (Roth company). The limit of detection for this assay was 0.1 pkat per ml incubation volume. This assay was employed for the isolation and purification of the esterase.

Assay B. Using the above conditions, the substrates were incubated in a total volume of 0.3 ml (0.1 M potassium phosphate buffer, pH 7.0) at a final concentration of 0.1 mM in the presence of 0.2 ml of the appropriate enzyme solution. The enzyme reaction was terminated by adding 0.5 ml methanol. Precipitated protein was removed by centrifugation and 0.1 ml of the supernatant was taken for the HPLC analysis. The sensitivity of this test allowed the measurement of 0.6 pkat per ml incubation volume.

Enzyme isolation and enrichment

For the isolation of *Rauwolfia* enzymes, 0.75 kg of fresh cells from AP medium were frozen with liquid nitrogen and stirred with 1.2 l of 0.1 M potassium phosphate buffer (pH 7.0, 10 mM β-mercaptoethanol) for 1 h. After filtration through cheese cloth the protein solution was centrifuged (20,000 × *g*,

30 min). The resulting crude enzyme mixture (supernatant) contained 650 mg protein with a specific activity of 2.8 pkat/mg protein. Ammonium sulfate was then added to the solution (final concentration was 65%) with stirring. After 45 min centrifugation ($20{,}000 \times g$) the precipitated protein was dissolved in 100 ml potassium phosphate buffer (15 mM, pH 7.0, 10 mM β-mercaptoethanol) and again centrifuged at $30{,}000 \times g$ for 30 min. The supernatant was then applied to a Sephadex G-25 chromatography column. The enzyme containing fractions were combined resulting in 430 mg protein (spec. act. 3.6 pkat/mg protein). At this stage a 1.3-fold enzyme purification was measured. To this protein mixture 14 mg protamine sulfate was added with stirring over a period of 45 min. Centrifugation at $30{,}000 \times g$ gave 378 mg protein with a specific activity of 4.2 pkat/mg protein (1.5-fold enrichment). The obtained enzyme preparation was transferred to a three-column system equilibrated with 15 mM potassium phosphate buffer (pH 7.0) consisting of a Fractogel TSK-DEAE 650(S) column (2.4 × 20 cm), a TSK-CM 650(S) column (2.4 × 15 cm) and a hydroxylapatite column (1.5 × 6 cm). The protein was eluted with the same buffer at a flow rate of 8 ml/h. Fractions of 4 ml were collected. Fractions 5–60 were combined and contained 13.8 mg protein (113 pkat/mg protein; 40.4-fold enrichment). β-Mercaptoethanol and ammonium sulfate were added to make a final concentration of 10 mM and 30%, resp. After 45 min the solution was centrifuged ($30{,}000 \times g$, 30 min) and applied to a phenoxylated Fractogel TSK 65(S) column (2 × 5.5 cm; flow rate 20 ml/h), which was equilibrated with 15 mM potassium phosphate buffer pH 7.0 containing 30% ammonium sulfate. Protein was eluted by a decreasing gradient (30–0% ammonium sulfate) for 4 h at a flow rate of 40 ml/h. Fractions of 4 ml were collected. The esterase activity was found in fractions 12–26. These fractions were combined and assayed for protein. The total protein in this solution was 1.74 mg with a specific activity of 607 pkat/mg protein corresponding to a 217-fold enrichment of activity. All the protein concentrations were determined by Bradford's method [17].

Taxonomic distribution of the esterase and determination of enzyme activities depending on the growth of R. serpentina cell cultures

Of the crushed tissue (IKA mill M 20) 25 g were stirred in 0.1 M potassium phosphate buffer (pH 7.0, 10 mM β-mercaptoethanol) for 1 h at 4 °C. After filtration, protein was concentrated by 0–65% ammonium sulfate precipitation. The residue was dissolved in 5 ml of the above buffer and the solution was dialyzed twice against the same buffer (5 l) without mercaptoethanol for 10 h. Enzyme activity was determined using enzyme assay A.

Characteristics of acetylajmalan esterase

The optimum pH of the acetylajmalan esterase was determined in 0.1 M potassium phosphate/citrate buffer (pH 4.5–6.0), 0.1 M potassium phosphate buffer (pH 6.0–8.0) and 0.1 M potassium borate buffer (pH 8.0–9.0) employing enzyme assay A.

For determination of the optimum temperature the enzyme was incubated at different temperatures ranging from 4–60 °C and the activity was measured under the same assay conditions.

The relative molecular weight of the esterase was determined by FPLC-gel filtration chromatography with a FPLC chromatography unit from LKB (Bromma, Sweden; LKB 2150 HPLC pump, LKB 2152 LC controlling unit, LKB 2158 UV-photometer). The enzyme solution (0.1 ml) was applied to a TSK G 3000 SW column (0.75 × 30 cm) which was calibrated with known proteins: Cytochrome C ($M_r = 12.5$ kDa), chymotrypsinogen ($M_r = 25$ kDa), chicken serum albumin ($M_r = 45$ kDa), bovine serum albumin ($M_r = 68$ kDa), catalase ($M_r = 240$ kDa), ferritin ($M_r = 450$ kDa). The elution was carried out with 50 mM potassium phosphate buffer, pH 6.5, containing 0.1 M potassium chloride at a flow rate of 0.3 ml/min. The hydrolyzing enzyme activity was eluted at 11 ml buffer corresponding to a relative molecular weight of 33.0 ± 2 kDa of the acetylajmalan esterase.

Determination of the isoelectric point of the acetylajmalan esterase

Isoelectric focussing was performed on a Servalyt-Precote gel (125 × 125 × 0.15 mm) with 5% Servalyt carrier ampholines generating a linear pH gradient (pH 3–10). The esterase preparation (40 µl containing 8 µg protein, 450 pkat/mg protein) was applied to the gel. After focussing the gel was cut in 0.2 mm slices, then the enzyme activity was eluted with 0.1 M potassium phosphate buffer pH 7.0 and determined by assay A.

Synthesis and structure of substrates

17-O-Acetyltetraphyllicine, acetylpolyneuridine, acetylyohimbine and N_α, 17-O-diacetylquebrachidi-

ne were acetylated by the following standard procedure: 30 μmol of the compound were stirred for 10 h at room temperature in a mixture of 3 ml acetic anhydride/pyridine = 1:1 (v/v). The reaction was stopped by adding 3 ml ethanol and the reaction mixture was evaporated. After dissolving the residue in methanol the alkaloids were purified by thin layer chromatography using solvent system (a), (b) and (c).

Radioactively labelled [^{3}H-acetyl]acetyltetraphyllicine (16.9 MBq/μmol) was prepared from tetraphyllicine and [^{3}H]acetic anhydride by a published procedure [16].

1,2(*S*)-Dihydroraucaffricine was obtained from raucaffricine by reduction with sodium cyanoborohydride as described in [18].

^{1}H NMR (pyridine-d_5): 7.3, 7.2 (d, $J=8$, 2H−C(9, 12)); 6.95, 6.85 (t, $J=8$, 2H−C(10, 11)); 5.85 (q, $J=6.5$, H−C(19)); 5.7 (br s, H−C(21)); 5.5 (s, H−C(17)); 5.35 (d, $J=8$, H−C(1′)); 4.66 (dd, $J=5.5$ and 9, H−C(3)); 4.5 (dd, $J=11.5$ and 2, H−C(6′)); 4.3–3.9 (m, 5H−C(2, 2′, 3′, 4′, 6′)); 3.6 (t, $J=6$, H−C(5)); 3.3 (t, $J=5$, H−C(15)); 2.72 (dd, $J=5$ and 13.5, H−C(14)); 2.68 (dd, $J=4.5$ and 12, H−C(6)); 2.34 (t, $J=6$, H−C(16)); 2.2 (d, $J=12$, H−C(6)); 1.96 (s, $OCOCH_3$); 1.9 (m, H−C(14)); 1.5 (d, $J=6.5$, H−C(18)).

1,2(*S*)-Dihydrovomilenine: 10 mg (19 μmol) 1,2(*S*)-dihydroraucaffricine were incubated with 20 mg lyophilized protein extract from *R. serpentina* cells containing raucaffricine β-D-glucosidase [according to 18] in 15 ml water for 60 min at 30 °C. The aglycone was extracted with ethyl acetate (3 × 20 ml). After evaporation of the organic phase the residue was purified by TLC in solvent system (a) and (c) yielding 4.8 mg 1,2(*S*)-dihydrovomilenine (14 μmol, 73%). EI-MS (rel. int. %): 352 (M^+, 23), 297 (9), 222 (20), 185 (100), 184 (34), 169 (19), 168 (50), 143 (28), 131 (49), 130 (53). ^{1}H NMR (pyridine-d_5): 7.35, 7.3 (d, $J=8$, 2H−C(9, 12)); 6.93, 6.9 (t, $J=8$, 2H−C(10, 11)); 6.1 (q, $J=6.5$, H−C(19)); 5.6, 5.2 (s, 2H−C(17, 21)); 4.07 (dd, $J=5.5$ and 9, H−C(3)); 4.0 (d, $J=5.5$, H−C(2)); 3.75 (t, $J=6$, H−C(5)); 3.25 (t, $J=5$, H−C(15)); 2.7 (dd, $J=5$ and 13.5, H−C(14)); 2.58 (dd, $J=4.5$ and 12, H−C(6)); 2.37 (t, $J=6$, H−C(16)); 1.93 (s, $OCOCH_3$); 1.67 (d, $J=6.5$, H−C(18)).

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