*Phytochemistry*, Vol. 21, No. 7, pp. 1585–1588, 1982.
Printed in Great Britain.

0031-9422/82/071585-04$03.00/0


# PARTIAL PURIFICATION AND CHARACTERIZATION OF GEISSOSCHIZINE DEHYDROGENASE FROM SUSPENSION CULTURES OF *CATHARANTHUS ROSEUS*

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(*Received* 13 *July* 1981)

**Key Word Index**—*Catharanthus roseus*; Apocynaceae; geissoschizine dehydrogenase; stereospecificity; biosynthesis; heteroyohimbine alkaloids; cell suspension culture.

**Abstract**—The characterization and partial purification of geissoschizine dehydrogenase from *Catharanthus roseus* cell suspension cultures are described. The 35-fold purified enzyme removes the 21$\alpha$-hydrogen of geissoschizine in a $NADP^+$-dependent reaction. $NAD^+$, FAD or FMN cannot act as cofactors for the dehydrogenation. Structurally related indole alkaloids are not dehydrogenated. In comparison to enzymes of the ajmalicine pathway, geissoschizine dehydrogenase shows an extremely low specific activity.

## INTRODUCTION

As a result of tracer feeding experiments, the alkaloid geissoschizine was assumed to occupy a central role in the biosynthesis of the diverse classes of indole alkaloids in *Catharanthus roseus*, e.g. the *Corynanthé-*, *Strychnos-*, *Iboga-*, *Aspidosperma*-type [1, 2]. More recent studies with cell-free systems confirmed that geissoschizine is converted to heteroyohimbine alkaloids (*Corynanthé*-type) [3, 4] and led to a more detailed understanding of the involved biochemical reactions. Thus, investigations on the cofactor dependence as well as mechanistic studies of the biogenetic sequence geissoschizine→heteroyohimbine alkaloids indicated that the former is only indirectly involved in the biosynthesis of the heteroyohimbine system [4, 5]. We now report on the purification and characterization of the enzyme geissoschizine dehydrogenase which controls the first step in this sequence. This enzyme catalyses the dehydrogenation of geissoschizine at the C-21$\alpha$ position and thus gives rise to 4,21-dehydrogeissoschizine, a central intermediate in the biosynthesis of heteroyohimbine alkaloids [6].

## RESULTS AND DISCUSSION

### *Enzyme assay*

To monitor the purification of the enzyme use was made of the fact that under basic conditions the expected dehydrogenation product 4,21-dehydrogeissoschizine spontaneously cyclizes to the heteroyohimbine system (cathenamine) which is easily extracted with toluene. The starting material geissoschizine, however, remains in the aqueous incubation mixture. Quantitation was achieved by using [aryl-$^3$H]geissoschizine.

### *Isolation, purification and properties of geissoschizine dehydrogenase*

The enzyme was extracted from cell suspensions of *C. roseus* with 0.1 M borate buffer in the presence of 20 mM 2-mercaptoethanol and was purified as shown in Table 1. The 35-fold purified enzyme (yield 41%) did not show any strictosidine synthase activity [7] and was free of $\beta$-glucosidases [8]. Chromatography on CM-cellulose, CM-Sephadex or hydroxylapatite did not result in a further purification of the enzyme. After AcA 54 chromatography the 35-fold purified fractions were used for the determination of the catalytic properties of the enzyme. Under standard conditions, the dehydrogenation of geissoschizine was absolutely dependent on protein between pH 5 and 8.5 and showed a typical saturation curve. In the standard assay, the reaction rate was linear for about 30 min and maximum conversion (~70%) of geissoschizine was reached after 100 min. The pH optimum of the enzymatic reaction was at pH 7.6. The partly purified dehydrogenase was stable at 0° and could be stored without loss of activity for periods of up to 14 days. The remarkable stability of the enzyme could be further demonstrated by its purification by HPLC. Thus, using 17.5-fold purified enzyme, HPLC resulted in a further 5-fold enrichment of the enzyme retaining about 70% activity. At the final stage of partial purification the dehydrogenase was free of the following enzymes characteristic of the ajmalicine pathway: strictosidine synthase and $\beta$-glucosidases I and II, but still contained cathenamine reductase activity.

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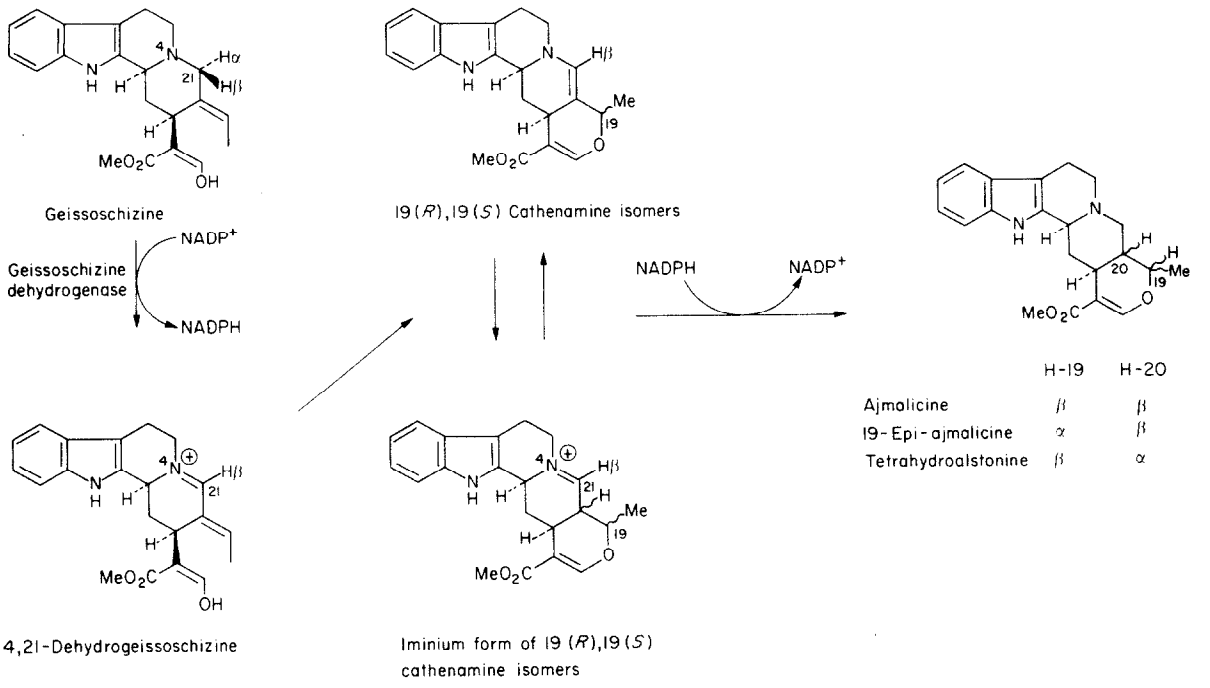


Table 1. Purification of geissoschizine dehydrogenase

| Purification steps | Total protein (mg) | Total activity (pkat) | Sp. activity (pkat/mg) | Recovery (%) | Purification (-fold) |
|---|---|---|---|---|---|
| 1. Centrifuged crude extract | 231 | 18.5 | 0.08 | 100 | 0 |
| 2. DEAE-cellulose chromatography | 7.5 | 10.5 | 1.4 | 58 | 17.5 |
| 3. Ultrogel AcA 54 chromatography | 2.6 | 7.5 | 2.8 | 41 | 35 |

*Dehydrogenation of geissoschizine*

The apparent $K_m$ value for geissoschizine was 83 $\mu$M under standard conditions, $V_{max}$ = 23 pmol/min. A double-reciprocal plot of the dehydrogenase activity with respect to cofactor concentration was linear and the $K_m$ value for $NADP^+$ was 77 nM ($V_{max}$ = 6.8 pmol/min). The dehydrogenation of geissoschizine showed a strict dependence and absolute specificity toward $NADP^+$. Other cofactors were ineffective as hydrogen acceptors, e.g. $NAD^+$, FAD, FMN.

Of the different substrates tested only geissoschizine was dehydrogenated by the enzyme. The structurally related alkaloids [21$\alpha$-$^3$H]tetrahydroalstonine and [3$\alpha$-$^3$H]ajmalicine were not enzymatically dehydrogenated at the 21$\alpha$- or 3$\alpha$-position. This result indicates a high substrate specificity of the dehydrogenase. The [21$\alpha$-$^3$H]tetrahydroalstonine used for these experiments was obtained by reduction of cathenamine with $NaB^3H_4$. The position of the label was determined by MS and $^1$H NMR analysis of $^2$H-labelled tetrahydroalstonine [9]. Due to the exchange of hydrogen with deuterium the corresponding fragment at 184 $m/z$ in the mass spectrum was shifted to 185 $m/z$ and the signal for H-21$\alpha$ in the $^1$H NMR spectrum at $\delta$2.72 disappeared, whereas the doublet of H-21$\beta$ collapsed to a singlet. Both results are clearly consistent with a stereoselective reduction of cathenamine.

To investigate the stereospecificity of the enzymatic dehydrogenation reaction [5-$^{14}$C, 21$\alpha$-$^3$H]geissoschizine was synthesized by $NaB^3H_4$ reduction of 4,21-dehydro[5-$^{14}$C]gleissoschizine at low temperature. The $^{14}$C-labelled 4,21-dehydro compound was prepared by treatment of enzymatically formed [5-$^{14}$C]cathenamine with 2% HCl [10]. The position of

the $^3H$ label was confirmed by the demonstration that the corresponding deuterated form of geissoschizine ($NaB^2H_4$ reduction) gave no $^1H$ NMR signal for H-21$\alpha$. Using this labelled geissoschizine ($^3H/^{14}C$ = 7.5/1) a pre-purified enzyme extract of *C. roseus* cells forms in the presence of $NADP^+$/NADPH heteroyohimbine alkaloids (ajmalicine, tetrahydroalstonine, 19-epi-ajmalicine) with a drastically changed $^3H/^{14}C$ ratio of about 0.5/1. The same result was obtained for ajmalicine by feeding the $^3H/^{14}C$ labelled precursor to seedlings of *C. roseus*. The results clearly demonstrate that the dehydrogenase removes *in vitro* and *in vivo* the 21$\alpha$-hydrogen of geissoschizine in a stereoselective way. Removal of the hydrogen takes place from the less hindered site of the substrate. In a crude cell-free extract, obtained from tissue at the stage of optimum cell yield and heteroyohimbine alkaloid production, the dehydrogenase was found to have an extremely low specific activity of 0.08 pkat/mg protein. In contrast enzymes of the ajmalicine pathway were highly active: strictosidine synthase 360 pkat/mg, $\beta$-glucosidases 108 pkat/mg [7, 8].

In summary, the isolated enzyme catalyses the conversion of geissoschizine into dehydrogeissoschizine, which is a biogenetic intermediate of heteroyohimbine alkaloids. The enzyme, only channels geissoschizine by a side reaction into the main pathway. This channelling process and the fact that geissoschizine is formed by enzymatic reduction of its dehydroform [6] indicates that geissoschizine dehydrogenase does not play a crucial role in the biosynthesis of heteroyohimbine-type alkaloids.

## EXPERIMENTAL

*Plant sources.* Plant cell suspension cultures of *C. roseus* were provided by the cell culture laboratory of our Institute. Cells were routinely grown in 1-l. conical flasks using 250 ml production medium [11] for 7 days at 26°. After filtration by suction the tissue was immediately frozen with liquid $N_2$, stored at −20° and used as an enzyme source.

*Bio- and radiochemicals.* All biochemicals were obtained from Boehringer, Mannheim. $NaB^3H_4$ was obtained from Amersham, Braunschweig. Material for chromatography was purchased from Whatman (DEAE-cellulose) and LKB (AcA 54). [Aryl-$^3$H]geissoschizine was available as described [12]. [21$\alpha$-$^3$H]- and [21$\alpha$-$^2$H]-geissoschizine were synthesized as follows: a four-fold excess of 4, 21-dehydrogeissoschizine was solubilized in MeOH, the soln cooled to −15° and reduced with $NaB^3H_4$ or $NaB^2H_4$ for 1 hr. After evaporation of the solvent the residue was purified by TLC (Polygram Sil G/UV$_{254}$; Machery–Nagel) using $CHCl_3$–MeOH–$NH_4OH$ (90:10:0.1) as the solvent system. The average yield of the purified compound was about 12%. [5-$^{14}$C, 21$\alpha$-$^3$H]Geissoschizine was obtained in an overall yield of 10% by enzymatic synthesis of [5-$^{14}$C]cathenamine followed by reduction with $NaB^3H_4$ as described above. [21$\alpha$-$^3$H]- and [21$\alpha$-$^2$H]-tetrahydroalstonine were synthesized from cathenamine as indicated for [21$\alpha$-$^3$H]geissoschizine except that reduction was carried out at room temperature with a slight excess of the reducing agent. Ajmalicine-[3-$^3$H] was a gift from Dr Rüffer of our Department.

*Enzyme preparation and purification.* Frozen cells (316 g) were stirred with 0.1 M borate buffer (500 ml), pH 7.6, containing 20 mM 2-mercaptoethanol. After filtration through cheese-cloth the filtrate was centrifuged at 48 000 *g* for 15 min, the supernatant was concentrated (to 266 ml) using a Berghoff cell and then dialysed against 0.01 M K-Pi buffer (20 mM 2-mercaptoethanol, pH 7.0) for 1 day. The enzyme soln was applied to a DEAE-cellulose column (25 cm × 7.5 cm, flow rate: 30 ml/hr) and eluted with a linear NaCl gradient (0–250 mM) using the same buffer (pH 7). The enzyme activity was recovered at 50 mM NaCl. The purified fractions were pooled and chromatographed on an Ultrogel AcA 54 column (2.5 cm × 86 cm; flow rate: 24 ml/hr).

The eluted fractions were assayed for protein [13] and then tested for the dehydrogenation of geissoschizine. The enzyme preparation obtained on chromatography on DEAE-cellulose could be further purified (×5) on HPLC using two Waters protein columns I-125, 10 mM K-Pi buffer pH 7.4, 20 mM 2-mercaptoethanol, 150 psi, 0.5 ml/min.

*Enzyme assay.* 185 pmol ($1.9 \times 10^4$ cpm) [aryl-$^3$H]geissoschizine (sp. act. 92.3 $\mu$Ci/$\mu$mol) was incubated with enzyme in a total vol. of 200 $\mu$l 0.1 M K-Pi buffer (pH 7.6) containing 12.5 nmol $NADP^+$ for 60 min at 30°. The enzyme reaction was then stopped by adding 100 $\mu$l 5 M KOH and the mixture extracted with 0.5 ml toluene. 0.2 ml of the organic phase was then assayed for radioactivity.

In vitro *and* in vivo *conversion of* [5-$^{14}$C; 21$\alpha$-$^3$H]*geissoschizine to heteroyohimbine alkaloids.* 63 nmol [5-$^{14}$C, 21$\alpha$-$^3$H]geissoschizine ($^3H/^{14}C$ = 7.5/1) was incubated with 4 mg crude enzyme in 0.5 ml (0.1 M K-Pi buffer, pH 7.6) in the presence of 2.5 $\mu$mol $NADP^+$ and 2.5 $\mu$mol NADPH for 120 min at 30°. The formed alkaloids ajmalicine, tetrahydroalstonine and 19-epi-ajmalicine were purified to constant sp. act. by TLC and the $^3H/^{14}C$ ratio determined; ajmalicine $^3H/^{14}C$ = 0.35/1; tetrahydroalstonine 0.54/1; 19-epi-ajmalicine 0.47/1. For the feeding expt an aq. soln (0.2 ml) of 330 nmol [5-$^{14}$C, 21$\alpha$-$^3$H]geissoschizine ($^3H/^{14}C$ = 5/1) was applied to 6-week-old seedlings of *C. roseus* for 24 hr. The plant material was extracted with MeOH, the isolated ajmalicine purified by TLC, diluted with unlabelled ajmalicine (3 mg) and 3× recrystallized from MeOH. The isolated alkaloid finally showed a $^3H/^{14}C$ of 0.64/1.

*Acknowledgements*—We wish to thank Dr B. Deus and Dr M. Rüffer for the selected strain of *C. roseus* cells and for [3$\alpha$-$^3$H]ajmalicine, Dr H.-P. Husson and C. Kan-Fan, Gif-sur-Yvette, for samples of 4, 21-dehydrogeissoschizine, Prof. Dr E. Winterfeldt, Hannover, for a generous gift of geissoschizine, Dr G. Höfle, Braunschweig, for NMR spectra, and Dr Kern, Waters GmbH, Leverkusen, for performing the HPLC analyses. This research was financially supported by a grant to Prof. M. H. Zenk from the Bundesminister für Forschung und Technologie, Bonn.

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