

Geraniol-10-hydroxylase Activity and Its Relation to Monoterpene Indole Alkaloid Accumulation in Cell Suspension Cultures of *Catharanthus roseus*

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The activity of geraniol-10-hydroxylase was greatly increased when *Catharanthus roseus* cells were transferred to a production medium for monoterpene indole alkaloids. Its activity pattern showed a closer relationship than tryptophan decarboxylase to the pattern of indole alkaloid accumulation. The intermediate accumulation of tryptamine and its later incorporation into ajmalicine indicated that the coordination of the two precursor pathways for the monoterpene indole alkaloid formation was not synchronized under the chosen culture conditions. A better tuning of these two pathways may shorten the time period for induction of indole alkaloid biosynthesis.

Introduction

Cell cultures of *Catharanthus roseus* have received considerable attention as they are regarded as a potential new source of medicinally important and rare monoterpene indole alkaloids [1]. Indeed more than 30 monoterpene indole alkaloids have been detected in cell suspension cultures of *C. roseus* [2–4]. However, the presence of the most desirable compounds, the dimeric indole alkaloids vinblastine and vincristine, has not been unequivocally demonstrated in cell suspension cultures [1]. The product levels of commercially interesting monomeric indole alkaloids, such as ajmalicine or catharanthine, remained too low under biotechnologically relevant culture conditions for a technological exploitation at present [5], as analytical screening for highly productive cell lines resulted, unfortunately, only in rather unstable cell lines [6]. Also screening of many independently established *Catharanthus roseus* lines did not provide cell cultures producing the required high levels of the desired compounds in growth or production media [1]. Therefore biotechnological progress may depend upon finding specific elicitors of monoterpene indole alkaloid biosynthesis in cell suspension cultures [7, 8] or in the selection of cell types not present in sufficient number under the usual culture conditions.

Another approach would be to study biochemical, molecular and regulatory aspects of indole alkaloid biosynthesis with the aim of using such information later for altering the controls needed for higher production rates. Indeed, the initial enzymatic steps of the biosynthesis of indole alkaloids as well as several enzymes within this rather branched and complex pathway have recently been identified [9–11]. However, the knowledge concerning regulatory controls of the pathways providing the precursors or of enzymes controlling the flow of intermediates into the various branches of indole alkaloid biosynthesis is rather poor. It was demonstrated that the activity of tryptophan decarboxylase (TDC) was greatly increased when cells were transferred to a production medium [12]. A correlation between TDC and alkaloid accumulation was initially proposed. However, under other culture conditions *C. roseus* cells failed to produce indole alkaloids despite increased TDC activity [13, 14]. Also selected cell lines expressing high TDC activity, when maintained on the growth medium, did not produce more indole alkaloids [15]. Thus it was clear that high activity or induction of TDC may be required for high alkaloid production but was not sufficient. Therefore we looked for a potential regulatory control within the monoterpene branch, providing secologanin for the indole alkaloid formation. The hydroxylation of geraniol to 10-hydroxygeraniol by geraniol-10-hydroxylase (G10H) may be regarded as such a control within the pathway for secologanin biosynthesis [16]. This enzyme

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has been well characterized from intact plants of *C. roseus* [17, 18] and has also been measured in corresponding cell suspension cultures under conditions of low or non-alkaloid biosynthesis [19]. However, the alteration of G10H-activities were rather small in these low productive cells during the growth cycle [19]. Therefore we followed the pattern of G10H-activity in cell cultures when distinct alkaloid biosynthesis was induced, as this should give more information on the regulatory role of this enzyme.

Materials and Methods

Cell cultures

Maintenance on MX-growth medium and some characteristics of cell suspension culture CP-3 of *Catharanthus roseus* have been described previously [12, 13, 15, 20]. For induction of alkaloid biosynthesis 10 ml of 14-day-old CP-3 suspensions were diluted 1:8 with 70 ml production medium IM 2 [21].

Determination of metabolites

Extraction and TLC analyses of tryptamine and ajmalicine in cell extracts of freeze-dried cells has been described previously [15]. Additionally cell extracts were analyzed by HPLC on Lichrosorb RP-18 (solvent $\text{CH}_3\text{CN}/0.01 \text{ M}$ triethylamine formate 1:1, pH 8.5) as described [20]. Tryptamine and ajmalicine were quantified directly by its absorbance at 280 nm with a Shimadzu TLC scanner [15] or by HPLC analysis using ibogain as internal standard. Incorporation of $[3\text{-}^{14}\text{C}]$ tryptophan or $[3\text{-}^{14}\text{C}]$ tryptamine into tryptamine and indole alkaloids was measured directly with a Silena TLC-Linear Analyzer.

Enzyme assays and determinations

Tryptophan decarboxylase was extracted and measured as described [12].

Geraniol-10-hydroxylase: 4 g fresh mass and 12 ml Tris-buffer (0.1 M Tris-HCl, 0.4 M sucrose, 10 mM KCl, 10 mM MgCl_2 , 10 mM EDTA, 10 mM $\text{K}_2\text{S}_2\text{O}_4$, 1 mM DTT, pH 7.6) [17] were homogenized with 1 g quartz sand in a ice-cooled mortar. The suspension was stirred in the presence of buffer saturated dowex 1 for 5 min and centrifuged at $20,000 \times g$ for 20 min. The supernatant was centrifuged at $110,000 \times g$ for 1 h. The resulting pellet (microsomal fraction) was carefully resuspended in K-phosphate buffer (50 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, 1 mM EDTA, 1 mM DTT,

pH 7.5). The assay contained: 150 μl microsomes, 450 μl K-phosphate buffer, 150 μl NADPH (1 μmol), 10 μl geraniol (3 μmol). After 5 min of preincubation the reaction was started with NADPH at 25 °C for 20 min. The reaction was stopped by the addition 150 μl 2 N KOH and 10 μl decandiol and was then extracted twice with 2 ml ethylacetate. The organic phase was dried under a stream of nitrogen and silylated with 100 μl MSTFA at 70 °C for 30 min. The samples were measured by capillary gas chromatography. Conditions: 15 m glass column DB-15n (J&W Scientific California); carrier gas helium 0.75 bar; split ratio 1:30; temperature program 120–200 °C in 6 °C/min, then 30 °C/min to 300 °C; detection by flame ionization detection. R_i of 10-hydroxygeraniol 1690 and of 1,10-decandiol 1735 (Fig. 1).

Protein determination: The protein content of the microsomal fraction was determined by the Lowry method [22] and of the TDC-extracts by the Bradford method [23].

Synthesis and characterization of 10-hydroxygeraniol (17, 24): 7.5 ml geraniol were acetylated with 4.85 ml acetic anhydride/4.15 ml pyridine at room temperature for 12 h. After addition of H_2O geranyl acetate was extracted into ethyl acetate which was then washed with 1 M HCl followed by 6.6% NaHCO_3 . The dried geranyl acetate was oxidized with a 1.2-fold surplus of SeO_2 in boiling 95% EtOH under reflux for 5 h. 10-Oxo-geranyl acetate was purified on silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 8/2). Ethereal solutions of the acetates were reduced with a 1.4-fold surplus of LiAlH_4 with ice cooling. After removal of excess LiAlH_4 , by the addition of H_2O , 10-hydroxygeraniol was extracted in the ether phase from a 10% NH_4Cl solution. The product was purified on a silica column with toluene/ethyl acetate/acetone 2/2/1. The structure of 10-hydroxygeraniol was confirmed by ^1H NMR-spectroscopy [17] and by GC/MS. m/e m^+ 170 n.d., 152 (m- H_2O) 3%, 134 (m-2 H_2O) 17%, 121/16%, 94/22%, 84/42%, 68/100%, 43/46%, 41/45%. The R_i of the product was 1475 and thus identical with the literature [25].

Results

Assay for geraniol-10-hydroxylase activity: The previously used test for the enzyme activity hydroxylating geraniol to 10-hydroxygeraniol was based on the availability or synthesis of $[1\text{-}^3\text{H}]$ geraniol [17].

We developed a new GC-based test which avoids the need for labelled substrate. Without derivatization 10-hydroxygeraniol gave a rather broad GC-signal which may complicate quantitative measurements. Thus the extracted products compounds were silylated with MSTFA. Among all tested dihydroxy-monoterpenes 1,10-decandiol proved to be the most suitable internal standard, as it gave only one signal after silylation. Fig. 1 shows the area of interest of the GC-chromatograms (between C_{16} – C_{18}). 10-Hydroxygeraniol was only found in chromatograms of complete enzyme assays and no interfering compounds were present in the microsomal extracts. Analysis of parallel samples and repeated injections indicated a reproducibility of $\pm 5\%$. The enzyme assay was linear for 30 min with more than 200 μ l microsomes (data not shown).

Activity pattern of geraniol-10-hydroxylase in CP-3 cells: Cells from the same preculture were transferred to growth medium and to the production medium IM 2 and G10H-activity and ajmalicine formation was followed for 13 days. In both media a rapid initial increase of G10H activity was observed (Fig. 2). While this activity declined steadily for cells in the growth medium until they had reached the stationary

phase, the activity increased greatly for cells in the production medium (Fig. 2). The highest specific activities were measured in cells on the production medium between day 5 and 7. After this a steep decline of G10H activity was observed. The specific content of ajmalicine of these cells increased from day 5 to day 10 from 0.15 mg to 1.1 mg/g dry mass and reached a final value of 1.3 mg/g at day 13. It is a well known fact that addition of more phosphate to the production decreases indole alkaloid accumulation [13]. When 1 mM phosphate was added to the production at day 3 the high increase of G10H-activity was prevented (Fig. 2). However, the remaining activity was sufficient to let the cells produce a specific ajmalicine content of 0.75 mg/g. Addition of mevinolate, a specific inhibitor of HMG-CoA reductase [26] did not effect the development of G10H-activity in the production medium. Thus lack of substrate did not influence the expression of this enzyme. Due to the depletion of precursor supply the ajmalicine content was, however, zero at day 10 and only increased to 0.2 mg/g at day 13. The results indicated a close relationship of G10H to the alkaloid formation pattern provided the flow of precursor for geraniol biosynthesis was guaranteed.

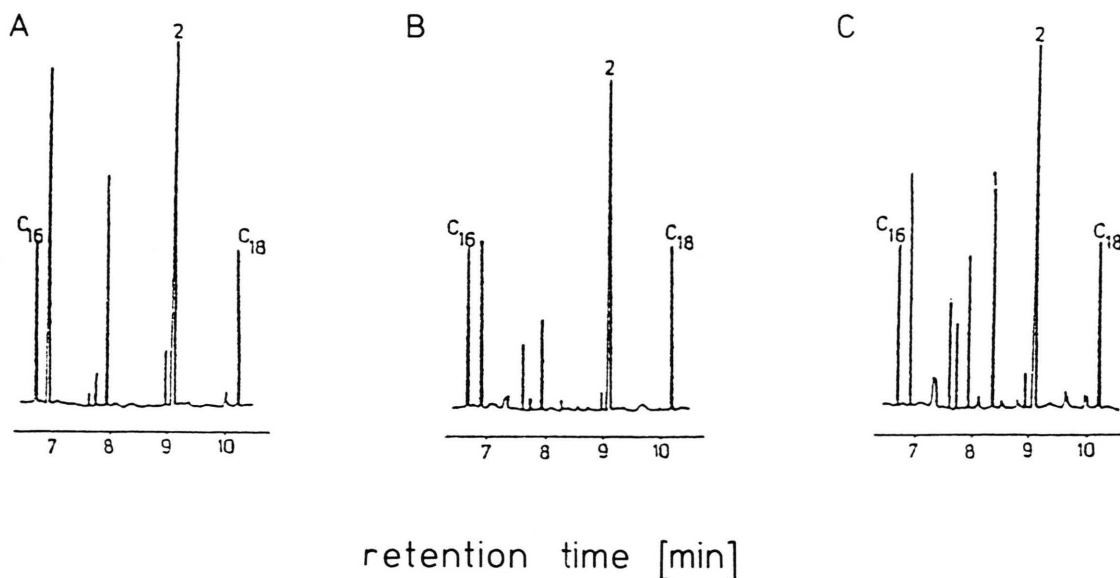


Fig. 1. Gas chromatograms of the ethyl acetate extracts of microsomal enzyme assays for geraniol-10-hydroxylase. A) microsomal fraction of cells grown for 5 days on IM 2, without geraniol and NADPH; B) complete enzyme assay, immediately stopped by KOH; C) complete enzyme assay after 20 min of incubation. 1 = 10-hydroxygeraniol, 2 = 1,10-decandiol.

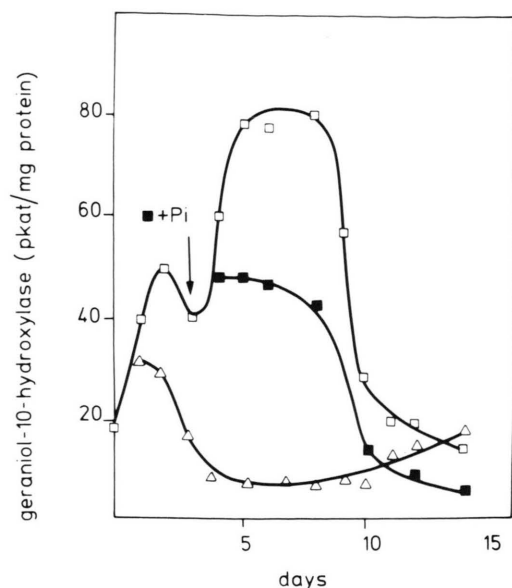


Fig. 2. Activity pattern of geraniol-10-hydroxylase in the production medium IM 2 (□), in the production medium + 1 mM P_i (■), and in the growth medium (△).

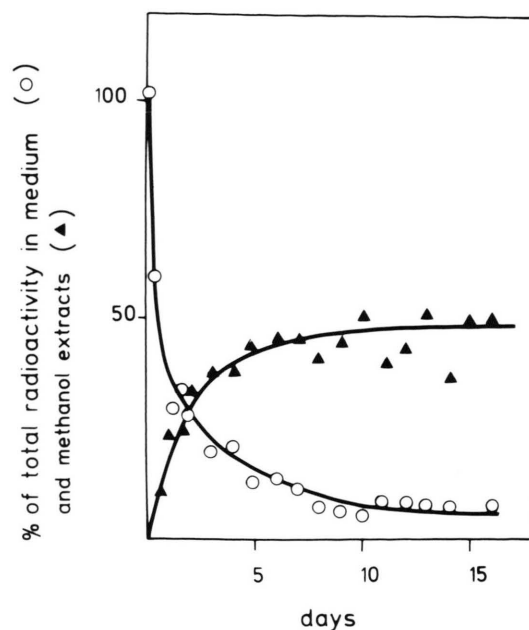


Fig. 3. Distribution of radioactivity in the medium (○) and the methanolic cell extract (▲) of culture grown in the presence of 2 μCi [3-¹⁴C]tryptophan.

Comparison of TDC and G10H-activity in relation to tryptamine and ajmalicine accumulation: Both enzyme activities and the accumulation pattern of the precursor tryptamine and the major indole alkaloid were followed in the production medium. Additionally, the cells were labelled with 2 μCi [3-¹⁴C]tryptophan or [2-¹⁴C]tryptamine/flask to measure the flow of tryptamine into ajmalicine during this period. Uptake of radioactive tryptophan from the medium and recovery of radioactivity by methanol extraction of freeze dried cells is shown in Fig. 3. TDC activity increased greatly and reached highest specific activity just 36 h after the transfer (Fig. 4). From then on TDC activity declined steadily. However, the remaining activity was still distinctly higher than in cells on the growth medium [27]. The pattern of G10H (Fig. 4) was similar to the previously measured one (Fig. 2). However, the highest specific activity was distinctly lower but was maintained over 5–7 days (Fig. 4). Consequently the alkaloid accumulation was lower and delayed (Fig. 5). This type of variation depends upon the state of the cells when transferred to the production medium. Tryptamine accumulated in the cells initially but at the time of indole alkaloid formation this pool was nearly de-

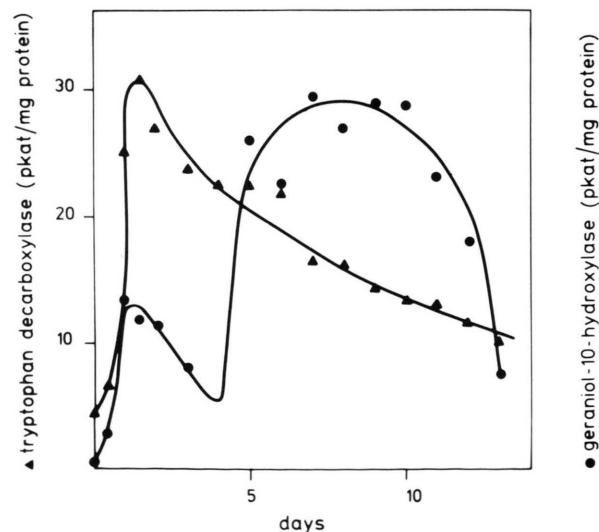


Fig. 4. Activity patterns of tryptophan decarboxylase (▲) and geraniol-10-hydroxylase (●) of cells grown on the production medium.

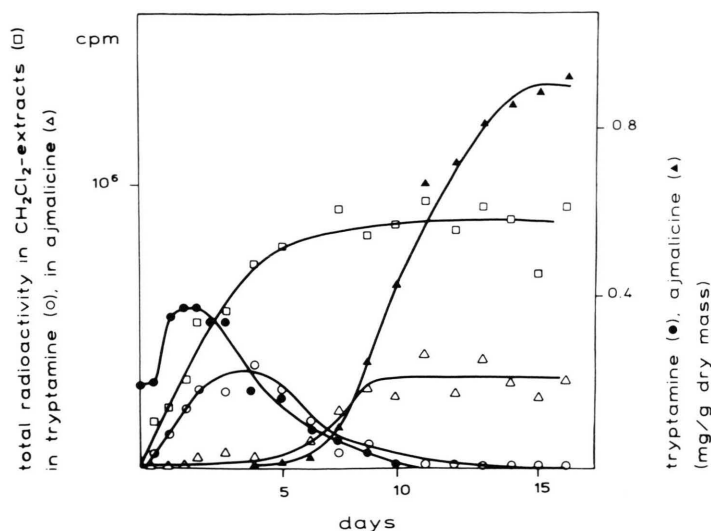


Fig. 5. Accumulation pattern of tryptamine (●) and ajmalicine (▲) in the growth medium. Distribution of radioactivity of the CH_2Cl_2 -cell extract (□) of tryptamine (○) and ajmalicine (Δ).

pleted (Fig. 5). The pattern of tryptamine decrease and ajmalicine accumulation (Fig. 5) did not indicate a direct conversion of tryptamine into the indole alkaloids, even if ajmalicine represents only 50–80% of all monoterpene indole alkaloids synthesized and accumulated by CP-3 cells. The pattern shows clearly that in later stages of the culture period new tryptamine was formed and immediately used for alkaloid formation (Fig. 5).

Therefore the question arose whether the tryptamine produced by the initial high activity of the TDC was of any use for indole alkaloid formation. Around 80–90% of the added radioactivity was taken up by the cells and ca. 60% of the radioactivity taken up was extractable with methanol (Fig. 3). Initially ca. 40–50%, later up to 80% of the radioactivity of the methanol extracts was extracted into CH_2Cl_2 (Fig. 5). Evidently most of the soluble radioactivity was decarboxylated and thus available for indole alkaloid formation. During the first 5 days up to 50% of the radioactivity of the CH_2Cl_2 -extracts was related to tryptamine while the ajmalicine zone had only traces of label (Fig. 5). The total extractable radioactivity remained rather unchanged from day 5 on. Distinct labelling of the ajmalicine pool was measured from day 6 to 9. While the total radioactivity of the ajmalicine stayed stable for the rest of the culture period, de-novo synthesis of ajmalicine continued at least until day 15 (Fig. 5). Radioactivity in ajmalicine accounted for 30–40% of the radioactivity of the CH_2Cl_2 -phase. Several other ceric am-

monium phosphate positive spots also showed distinctly increased incorporation of label with 7–15%, while the tryptamine pool remained depleted. Thus it can be concluded that the initially formed and stored tryptamine was used for indole alkaloid biosynthesis.

When tryptamine and indole alkaloids are labelled by feeding of radioactive tryptophan, one can assume that this portion of the added precursor has entered the correct biosynthetic sequences. Indeed when labelled tryptamine was added (data not shown), a large portion of the radioactivity in the CH_2Cl_2 -extracts (50%) could not be related to peaks which were also labelled by tryptophan. Thus some of the added tryptamine seems to undergo other “artificial” reactions. But in general the same pattern of incorporation into indole alkaloids was observed. After 7 days the tryptamine pool was no longer labelled and radioactivity in ajmalicine accounted for 20–25% of total radioactivity in CH_2Cl_2 -extracts.

Discussion

Rapidly growing cell suspension cultures of *C. roseus* maintained on 2,4-D containing growth medium accumulate no, or only low levels, of indole alkaloids during the growth cycle [8, 20, 28, 29]. When they are transferred to various growth limiting production media, alkaloid accumulation resumes normally after 5–8 days [8, 20, 28]. This rather delayed response of the cells is indeed a problem, espe-

cially if one wants to optimize the productivity of the cells. The rapid induction of tryptophan decarboxylase in the production is not a good marker. This activity is induced by so many non-productive culture conditions [13, 14] that a close correlation between TDC activity and indole alkaloid accumulation should no longer be claimed. On the other hand, it is evident that without sufficient TDC activity alkaloid levels will also remain low. In CP-3 cells TDC activity is always distinctly higher in the production than in the growth medium. The peak activity of TDC during the first 2 days (Fig. 3) is evidently not needed to maintain reasonable de-novo synthesis of indole alkaloids. Fig. 4 shows that highest alkaloid and thus tryptamine formation occurs when TDC activity is declining and the tryptamine pool is depleted. This would be even more evident when one looks at the total and not at specific pool sizes. At day 3–4 the cells contained ca. 150 µg tryptamine/flask while at day 10 ca. 1.3 mg ajmalicine/flask are found. Ajmalicine accounts for only 50–70% of all indole alkaloids (*e.g.* catharanthine, serpentine). Nevertheless the tryptamine produced and stored during the first days is later used for alkaloid biosynthesis.

Thus induction of geraniol-10-hydroxylase seems to be required before indole alkaloid synthesis can occur in CP-3 cells. Its pattern is closely related to the ajmalicine alkaloid accumulation pattern. However, it is questionable whether induction of G10H is the only requirement or the only alteration necessary to make CP-3 cells produce higher levels of indole alkaloids. A distinct increase of G10H-activity is seen also during the first days of culture and together with the high TDC-activity alkaloid formation should be possible. However, during these days no ajmalicine, nor any other ceric ammonium phosphate positive spots, were detected. This may indicate that not only the presence of sufficient enzyme activity but also the substrate supply is required for alkaloid formation. As tryptamine accumulates one may

speculate that secologanin is not produced at that time. Indeed Merillon *et al.* [14] showed that addition of secologanin enhanced ajmalicine and serpentine levels under all culture conditions. The lack of secologanin may not only be a question of poor enzyme activity. Initial substrates may not enter or may not be available for running this pathway or, other unknown regulatory enzyme activities may not be induced at this time. This is indeed the general problem of all such studies aimed at learning more about regulatory controls. As long as all the enzymes of a branch have not been identified and characterized, the interpretation of enzyme activity patterns must remain somewhat dubious. Thus the results presented here can only be regarded as a small step for obtaining further insights into the precursor pathway of monoterpene indole alkaloid formation.

Nevertheless we can assume that the G10H-activity is a better indicator of the potential of CP-3 cells to synthesize indole alkaloids than TDC-activity. Addition of phosphate to the production medium reduced G10H-activity and alkaloid formation (Fig. 2) but had no effect on the induction of TDC [13]. The problem of rather late induction of alkaloid formation on the production medium thus seems to be a problem of late induction of G10H (and correlated biochemical changes!?). Therefore the culture conditions should be changed in such way that earlier G10H induction is possible. At present we are trying to shorten the induction of indole alkaloid formation by elicitor treatment, by alteration of the preculture conditions and by selection of 2,4-D independent cell lines.

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