Medium-Induced Formation of Indole Alkaloids and Concomitant Changes of Interrelated Enzyme Activities in Cell Suspension Cultures of *Catharanthus roseus*

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Recently medium conditions have been developed which stimulate the formation of the indole alkaloid ajmalicine in cell suspension cultures of *Catharanthus roseus* [6]. When cells were subjected to these conditions the alkaloid accumulation was preceded by a 12-fold increase of the specific activity of tryptophan decarboxylase. The enzyme activity showed a maximum two days after the cell transfer into the induction medium and subsequently declined. In contrast the activity of strictosidine synthase, the enzyme condensing tryptamine and secologanin, was present over the entire measuring period at a constant level. The intracellular content of tryptamine and ajmalicine increased during a period of 6 days after cell transfer and reached a plateau after that time. A possible regulatory function of tryptophan decarboxylase in indole alkaloid biosynthesis is discussed.

Introduction

It has been demonstrated that plant cell cultures have been useful for studying properties and regulation of pathways of secondary metabolism [1–5]. Especially valuable information about regulatory characteristics of a pathway has been gained from inducible systems [1]. Recently we were able to stimulate the biosynthesis of the indole alkaloid ajmalicine in cell suspension cultures of *Catharanthus roseus* by special medium conditions [6]. Tryptophan decarboxylase, the enzyme providing tryptamine, and strictosidine synthase, the key enzyme condensing tryptamine and secologanin (Fig. 1), may play a crucial role in the synthesis of this alkaloid [3, 4]. Therefore we became interested to check for a possible correlation of the enhanced product accumulation with corresponding changes in activities of these two enzymes.

Materials and Methods

Materials

L-[3-^{14}C]Tryptophan and [2-^{14}C]tryptamine bisuccinate were purchased from New England Nuclear (Dreieich). Secologanin was a gift from Prof. Tietze, Göttingen. All other chemicals were reagent grade.

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Cell cultures

Catharanthus cells were routinely grown in MS-medium [7] containing 2×10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D), transferred to 8% sucrose medium and harvested as described [6].

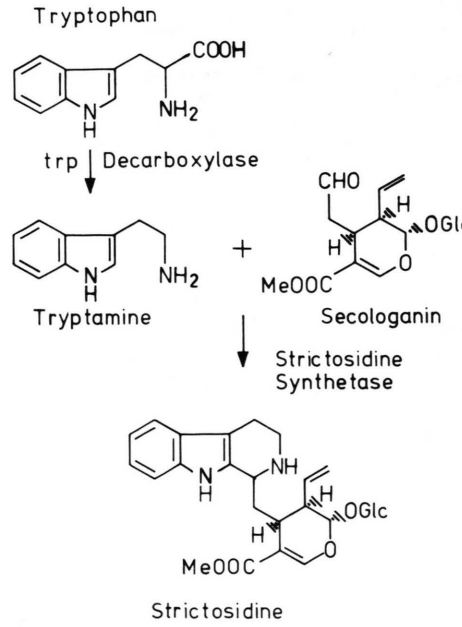

Fig. 1. Enzyme-catalyzed reactions involved in the formation of strictosidine.

Extraction of enzymes

Freshly harvested cells (4 g fresh wt.) were homogenized in a chilled mortar with 2 g quartz sand, 0.4 g polyvinyl pyrrolidone, and 4 ml 0.1 M Tris-HCl buffer, pH 7.5. The homogenate was centrifugated for 15 min at 20000 × *g*. The supernatant was brought to 75% saturation with ammonium sulfate, centrifugated, and the precipitate was dissolved in 2.5 ml 0.05 M Tris-HCl, pH 7.5. The protein solution was subjected to gel filtration on a pre-packed Sephadex G-25 column equilibrated with the same buffer. The protein fraction of this chromatography was used for enzyme assays.

Enzyme assays

The assay for strictosidine synthase activity was done according to Mizukami *et al.* [3], but a higher concentration of δ-D-gluconolactone (0.1 M) and a total volume of 0.2 ml were used. Tryptophan decarboxylase was measured according to Gibson *et al.* [8] with the following modifications: temperature 30 °C, incubation time 60 min, concentration of L-trp 10 mM. The assay was stopped by raising the pH to about 10 with 10% Na_2CO_3. Subsequently tryptamine was extracted with ethyl acetate. After evaporation the extract was analyzed by TLC on silica gel using acetone:methanol:diethylamine (7:2:1) for development. Radioactive bands corresponding to tryptamine were cut out and counted by liquid scintillation counting.

The enzymatic synthesis of cathenamine for the analysis by mass spectroscopy was performed according to Stöckigt [9] with a reduced assay volume of 13 ml.

Protein determination

Protein was determined in the eluates from gel filtration by the biuret method [10].

Determination of products

Determination of ajmalicine and serpentine was done as previously described [6]. Quantitative assay of tryptamine was performed according to Courtois *et al.* [11]. Absorption at 280 nm was measured on TLC-plates by means of a TLC-scanner (Shimadzu; Kyoto, Japan).

Results and Discussion

Cell free extracts from cell cultures of *Catharanthus roseus* have been shown to catalyze the formation of strictosidine and cathenamine [9]. Enzymes involved in this reaction, strictosidine synthase [3, 4] and specific glucosidases [5] have been purified and characterized. In our experiments, crude extracts of *Catharanthus roseus* were incubated after ammonium sulfate precipitation and gel filtration with [2-^{14}C]tryptamine and secologanin according to the enzymatic test for strictosidine synthase described in the literature [3]. It has been shown, that under these conditions strictosidine and cathenamine are formed [9]. We obtained both products, which were formed from labelled tryptamine only in the presence of secologanin and native enzyme (Table I). They could be extracted from basified incubation mixtures with ethyl acetate and separated by TLC according to Mizukami *et al.* [3]. When the glucosidase inhibitor δ-D-gluconolactone was omitted from the incubation mixture (Table I), lower amounts of the polar strictosidine ($R_f = 0.4$) and higher amounts of the unpolar product cathenamine ($R_f = 0.8$) were formed, as described in ref. [9].

The identity of the product cathenamine was confirmed by purification on TLC [9] and subsequent mass spectroscopy of the isolated compound. The spectrum agreed with data given in the literature [9].

When cell suspension cultures of *Catharanthus roseus* were grown in MS-medium supplemented with 2×10^{-6} M 2,4-D, they contained only low levels of indole alkaloids [6]. When these cells were transferred after two weeks of growth into a ten-fold volume of a 8% sucrose solution, they started to accumulate high amounts of ajmalicine [6] (Fig. 2). In order to investigate a possible relationship of product accumulation and enzyme activities related to this metabolic pathway, the time course of the

Table I. Enzyme-catalyzed formation of strictosidine and cathenamine under various assay conditions

Assay	Products formed (μ mol · h^{-1})	
	Strictosidine	Cathenamine
Complete assay	105	12
Denatured enzyme	~ 0	~ 0
Secologanin omitted	~ 0	~ 0
δ-D-Gluconolactone omitted	74	71

activities of tryptophan decarboxylase and strictosidine synthase was followed over a period of ten days after transfer of cells into 8% sucrose medium (Fig. 2).

After the cell transfer a large increase of tryptophan decarboxylating activity was found reaching a maximum after 2 days. Subsequently the enzyme activity gradually decreased until day 10 after cell transfer. The maximum specific enzyme activity induced by medium transfer was 12-fold higher than in MS-medium.

In contrast to the characteristic changes of tryptophan decarboxylase activity, the enzyme catalyzed formation of strictosidine remained more or less constant over the entire period after the cell transfer. The variations of the specific enzyme activity of strictosidine synthase were relatively small and possibly due to variations among parallel cultures or loss of activity during the preparation of the enzyme extract. Small amounts of cathenamine (about 10% of strictosidine) were formed in the assay of strictosidine synthase but were not taken into account for the calculation of the specific activity of strictosidine synthase.

The peak of tryptophan decarboxylase activity was followed by large increases of the pool sizes of tryptamine and indole alkaloids (sum of ajmalicine and serpentine; Fig. 2). Under our experimental conditions, the cultures accumulated mainly ajmalicine as described in ref. [6]. The values measured for tryptamine levels prior to medium induction were similar to those reported by Schallenberg and Berlin [12] but somewhat lower than from other *Catharanthus* cell lines [11].

From these results tryptophan decarboxylase activity seems to be correlated with the accumulation of the products tryptamine and ajmalicine. It is the first enzyme channelling the primary metabolite L-tryptophan into secondary metabolism. Therefore its function can be compared with the role of phenylalanine ammonia lyase, the first enzyme of phenylpropanoid metabolism [1]. However, in this pathway a coordinated regulation of all enzymes has been observed in cell cultures of parsley [2], whereas the activity of strictosidine synthase was present at a constant level and could not be correlated with the medium-induced accumulation of ajmalicine and serpentine. Thus, the activity of tryptophan decarboxylase seems to be at least one regulating factor of alkaloid biosynthesis in *Catharanthus roseus* cell

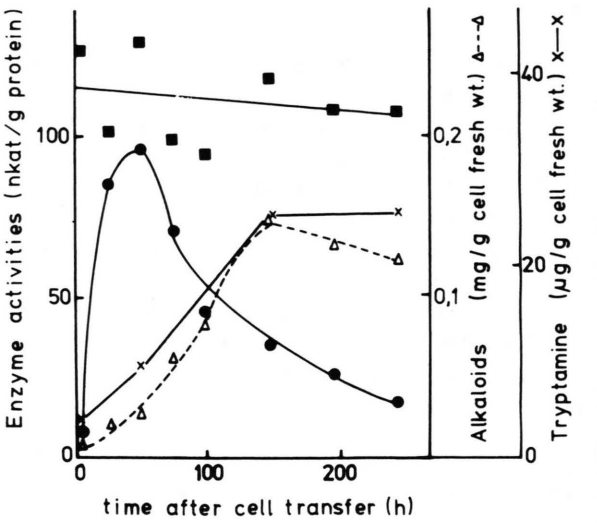

Fig. 2. Time course of specific enzyme activities and product accumulation in cell suspension cultures of *Catharanthus roseus* after transfer into 8% sucrose medium. (●—●) L-tryptophan decarboxylase; (■—■) strictosidine synthase; (×— ×) tryptamine; (△- - -△) sum of ajmalicine and serpentine.

cultures. The findings, that feeding of tryptamine did not enhance the accumulation of ajmalicine and serpentine [13, 14] and the existence of tryptamine pools in very low producing cultures [11] are not contradictory to this conclusion since these pools are located in the vacuole and probably metabolically inactive [11]. Furthermore there is increasing evidence that the biosynthesis of secondary plant compounds is often associated with metabolic channelling (ref. [15] and literature cited herein). Thus intermediates of biosynthetic sequences show a limited diffusion and cannot be substituted by an externally fed compound.

Our results do not rule out the possibility that also other enzyme activities, *e. g.* of monoterpene biosynthesis are involved in the overall regulation of alkaloid biosynthesis and further work on these enzymes is needed to get more information on the regulating factors of indole alkaloid biosynthesis in *Catharanthus roseus* cell cultures.

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[1] K. Hahlbrock and H. Grisebach, Ann. Rev. Plant Physiol. **30,** 105 (1979).
[2] K. Hahlbrock, K.-H. Knobloch, F. Kreuzaler, J. R. M. Potts, and E. Wellmann, Eur. J. Biochem. **61,** 199 (1976).
[3] H. Mizukami, H. Nordlöv, S.-L. Lee, and A. I. Scott, Biochemistry **18,** 3760 (1979).
[4] J. F. Treimer and M. H. Zenk, Eur. J. Biochem. **101,** 225 (1979).
[5] T. Hemscheidt and M. H. Zenk, FEBS Letters **110,** 187 (1980).
[6] K.-H. Knobloch and J. Berlin, Z. Naturforsch. **35 c,** 551 (1980).
[7] T. Murashige and F. Skoog, Physiol. Plant. **15,** 473 (1962).
[8] R. A. Gibson, G. Barret, and F. Wightman, J. Exp. Botany **23,** 775 (1972).
[9] J. Stöckigt, Phytochemistry **18,** 965 (1979).
[10] E. Layne, Methods Enzymol. **3,** 447 (1957).
[11] D. Courtis, A. Kurkdjian, and J. Guern, Plant Sci. Lett. **18,** 85 (1980).
[12] J. Schallenberg and J. Berlin, Z. Naturforsch. **34 c,** 541 (1979).
[13] M. H. Zenk, H. El-Shagi, H. Arens, J. Stöckigt, E. W. Weiler, and B. Deus, Plant Tissue Culture and Its Biotechnological Applications, (W. Barz, E. Reinhard and M. H. Zenk, eds.), p. 27, Springer-Verlag, Berlin 1977.
[14] G. Döller, Production of Natural Compounds by Cell Culture Methods, (A. W. Alfermann and E. Reinhard, eds.), p. 109, Gesellschaft für Strahlen- und Umweltforschung m. b. H., München 1978.
[15] E. E. Conn, Naturwissenschaften **66,** 28 (1979).