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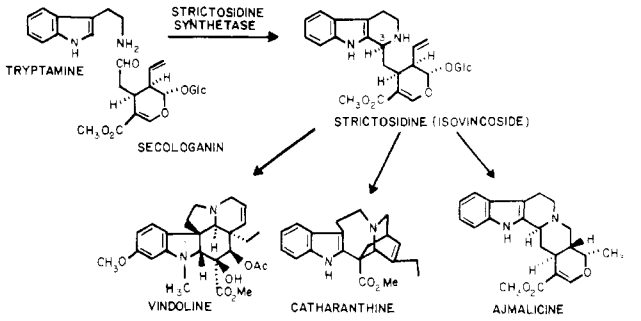
Purification and Properties of Strictosidine Synthetase (an Enzyme Condensing Tryptamine and Secologanin) from *Catharanthus roseus* Cultured Cells†

Hajime Mizukami, Hans Nordlöv, Siu-Leung Lee, and A. Ian Scott*

ABSTRACT: Strictosidine synthetase, which catalyzes the condensation of tryptamine with secologanin to form strictosidine (isovincoside), was purified 740-fold to homogeneity from cultured cells of *Catharanthus roseus* in 10% yield. The specific activity is 5.85 nkat/mg. The molecular weight as estimated by gel filtration is 38 000. The isoelectric point is 4.6. Apparent K_m values for tryptamine and secologanin are 0.83 and 0.46 mM, respectively. The enzyme shows a broad pH optimum between 5.0 and 7.5. The product of the enzymic reaction is exclusively strictosidine, while no trace of its epimer vincoside can be detected. Sulfhydryl inhibitors have no effect on the enzyme. End products in the biosynthetic pathway of indole alkaloids such as ajmalicine, vindoline, and catharanthine do not inhibit the activity of strictosidine synthetase.

Catharanthus roseus (periwinkle) produces more than 100 indole alkaloids (Scott, 1970), many of which are pharmacologically active, the most notable of these being vinblastine and vincristine, the antitumor alkaloids. As the contents of these alkaloids in plant cells are extremely low, various, but so far unsuccessful, attempts have been made to produce these compounds from plant tissue culture. It has therefore become important to understand the regulatory mechanism of the biosynthesis of indole alkaloids in higher plants. There were few enzymological studies of the biosynthesis of the indole alkaloids until the discovery of a cell-free system from *C. roseus*, which yielded some important information concerning the formation of indole alkaloids, at both early (Scott & Lee, 1975; Scott et al., 1977a; Stöckigt et al., 1976; Stöckigt & Zenk, 1977a,b) and final stages (Baxter et al., 1979; Stuart et al., 1978). The first step in the biosynthesis of indole alkaloids in *C. roseus* is a stereospecific enzymic condensation of tryptamine and secologanin to form strictosidine with H-3α(*S*) configuration (Scheme I) (Blackstock et al., 1971; DeSilva et al., 1971). Earlier assignments (Battersby et al., 1968, 1969) of vincoside, the H-3β(*R*) isomer, as the precursor for indole alkaloids have been proven (Scott et al., 1977a; Stöckigt & Zenk, 1977a,b; Battersby et al., 1978) to be incorrect. Later, it was found that this compound is a common precursor for monoterpenoid indole alkaloids with both H-3α(*S*) and H-3β(*R*) configuration (Rueffer et al., 1978). The first enzyme in the biosynthetic pathway normally serves as a site of control (Umbarger, 1956; Yates & Pardee, 1956) including secondary metabolic pathways such as the biosynthesis of ergot alkaloids (Heinstein et al., 1971; Lee et al., 1976). In this paper, we describe the purification of strictosidine synthetase from cultured cells of *C. roseus* and some properties of this enzyme, which, as the first committed synthetase enzyme on the pathway, forges the link between the amino acid and mevalonoid derived segments of the alkaloids of *C. roseus*.

Scheme I



Experimental Procedures

Cell Culture. Cultured cells induced from seedlings of *Catharanthus roseus* G. Don (Apocynaceae) were maintained in SH liquid medium (Schenk & Hildebrandt, 1972) at about 28 °C under dim light with transfer intervals of 2 weeks.

Assay of Strictosidine Synthetase. The activity of strictosidine synthetase was determined by the formation of

† From the Department of Chemistry, Texas A&M University, College Station, Texas 77843. *Received February 28, 1979.* This work was supported by National Institutes of Health Grant CA 22436.

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strictosidine after incubating [*side chain*-2-^{14}C]tryptamine and secologanin at 30 °C for 1 h. The reaction mixture contained 2.5 mM [*side chain*-2-^{14}C]tryptamine (0.4 Ci/mol), 10 mM secologanin, 10 mM 2-mercaptoethanol, 50 mM Tris-HCl (pH 7.5), and enzyme in a final volume of 1.0 mL. In the earlier steps of the purification (before DEAE-cellulose chromatography), 25 mM δ-D-gluconolactone was supplied in order to inhibit endogenous β-glucosidase. The reaction was stopped by basifying to pH 10 with 10% K_2CO_3. Strictosidine was extracted with 2 mL of EtOAc three times. After evaporation, the EtOAc extract was analyzed by thin-layer chromatography on silica gel G by using acetone:MeOH:diethylamine (7:2:1) for development. The band corresponding to strictosidine was scraped off and counted in a Packard Prias scintillation counter. A parallel extraction of standard strictosidine with EtOAc and assay by the TLC method showed a recovery of 93%.

LC[1] *Analysis of the Reaction Products.* The EtOAc extract from the incubation was analyzed with LC (Waters equipment) by using a μC_{18}-Bondapak column eluted with MeOH–10 mM sodium phosphate, pH 7.2 (60:40), at a flow rate of 2.0 mL/min. The eluate was monitored at 254 nm.

Preparative Isoelectric Focusing. Preparative flat-bed isoelectric focusing (Vesterberg & Svensson, 1966) was performed in a LKB Multiphor apparatus by using Sephadex G-75 as a stabilizer. The bed was formed of 1 mL of ampholines (pH 3.5–10), 4 mL of ampholines (pH 4–6), 65 mL of water, and 5 g of Sephadex G-75. The electrofocusing was performed at 10 W and a maximum of 1200 V for 12 h. After focusing, the gel was divided into 30 fractions, each of which was eluted with 3 mL of 20 mM Tris-HCl, pH 7.5.

Analytical Isoelectric Focusing. Analytical isoelectric focusing was carrtied out in a LKB Multiphor apparatus by using LKB PAG plates (pH 3.5–9.5). Staining for proteins was done with Coomassie Brilliant Blue.

Analytical Gel Electrophoresis. Analytical gel electrophoresis modified after Davies (1964) was performed without stacking gel by using 7.5% acrylamide in 0.3 M Tris-HCl, pH 8.9. The gel was stained for proteins with Coomassie Brilliant Blue.

Determination of Molecular Weight by Gel Filtration. Molecular weight was determined by gel filtration according to Andrews (1965) by using a 2.6 × 62 cm Sephadex G-75 column eluted with 20 mM Tris-HCl, pH 7.5, at a flow rate of 25 mL/h. The following proteins were used as standard markers: cytochrome *c* from horse heart; chymotrypsinogen from bovine pancreas; hen's egg albumin; and bovine serum albumin.

Protein Determination. Protein was analyzed by the method of Bradford (1976) with bovine γ-globulin as standard.

Materials. DEAE-cellulose DE-52 was purchased from Whatman. Sephadex G-75 was supplied by Pharmacia. Hydroxylapatite, electrophoresis reagents, protein assay reagent, and bovine γ-globulin were obtained from Bio-Rad. Ampholines were purchased from LKB. Molecular weight standard marker proteins were obtained from Boehringer-Mannheim. Ajmalicine, vindoline, and catharanthine were generous gifts from Eli-Lily Co. [*side chain*-2-^{14}C]Tryptamine bisuccinate was purchased from New England Nuclear. Secologanin was isolated from *Lonicera japonica* according to Kinast & Tietze (1976) with slight modifications. All other chemicals used were of reagent grade.

[1] Abbreviations used: LC, high-pressure liquid chromatography; nkat, nanokatal.

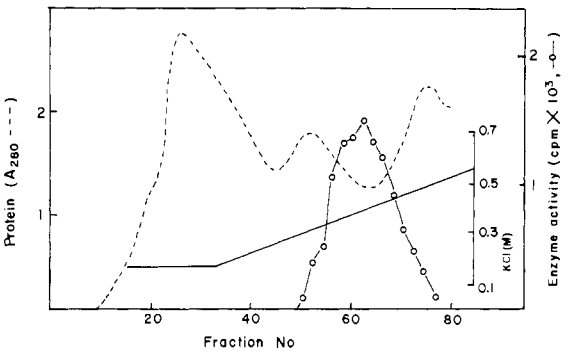

FIGURE 1: Purification of strictosidine synthetase by DEAE-cellulose chromatography. Strictosidine synthetase (5.3 nkat) after the ammonium sulfate precipitation step containing 466 mg of protein was applied. Strictosidine synthetase activity (O); absorbancy at 280 nm (---); KCl concentration (—).

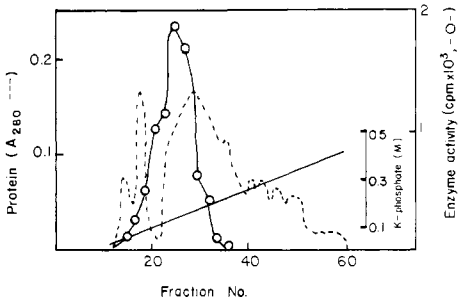

FIGURE 2: Purification of strictosidine synthetase by hydroxylapatite chromatography. Dialyzed strictosidine synthetase (3.9 nkat) after the DEAE-cellulose chromatography step containing 39 mg of protein was applied. Strictosidine synthetase activity (O); absorbance at 280 nm (---); potassium phosphate concentration (—).

Results

Enzyme Purification. All purification steps were carried out at 4 °C and the buffers used contained 10 mM 2-mercaptoethanol.

Step 1: Preparation of Crude Extract. Wet cells (116 g) were homogenized with 120 mL of 50 mM Tris-HCl, pH 7.5, in a Sorvall Omni-Mixer at the highest speed for 2 min. The homogenate was centrifuged at 25000*g* for 15 min and the supernatant collected.

Step 2: Ammonium Sulfate Precipitation. Solid ammonium sulfate corresponding to 70% saturation was added to the crude extract. The resulting precipitate was collected by centrifugation at 10000*g* for 15 min, dissolved in 20 mM Tris-HCl, pH 7.5, and dialyzed for 7 h against two changes of 3.5 L of the same buffer.

Step 3: DEAE-cellulose Chromatography. The dialysate from the preceding step was applied to a 5 × 6 cm DEAE-cellulose column equilibrated with 20 mM Tris-HCl, pH 7.5. The column was washed with 100 mL of 0.15 M KCl in 20 mM Tris-HCl, pH 7.5, and then the enzyme was eluted with a linear gradient formed from 150 mL of 0.15 M KCl in 20 mM Tris-HCl, pH 7.5, and 150 mL of 0.60 M KCl in the same buffer. The flow rate was 30 mL/h, and 5.6-mL fractions were collected. The enzyme was eluted at a KCl concentration of about 0.35 M (Figure 1). Active fractions were pooled, concentrated by Amicon PM-10 ultrafiltration, and dialyzed against 10 mM potassium phosphate, pH 6.8.

Step 4: Hydroxylapatite Chromatography. The solution from the preceeding step was appplied to a 5 × 6 cm hydroxylapatite column equilibrated with 10 mM potassium phosphate, pH 6.8. The elution was made with a linear gradient formed from 200 mL of 10 mM potassium phosphate, pH 6.8, and 200 mL of 500 mM potassium phosphate, pH 6.8.

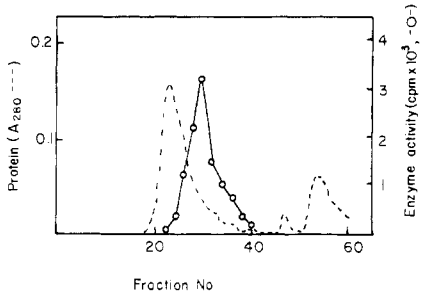

FIGURE 3: Purification of strictosidine synthetase by gel filtration on Sephadex G-75. Strictosidine synthetase (3.6 nkat) after the hydroxylapatite chromatography containing 5.9 mg of protein was applied. Strictosidine synthetase activity (O); absorbancy at 280 nm (---).

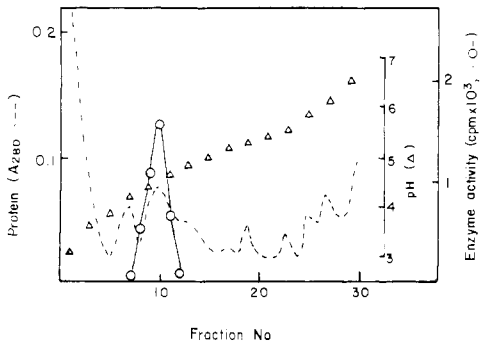

FIGURE 4: Purification of strictosidine synthetase by isoelectric focusing. Strictosidine synthetase (2.0 nkat) after the gel filtration step containing 0.72 mg of protein was applied. Strictosidine synthetase (O); absorbancy at 280 nm (---); pH (Δ). For details, see Experimental Procedures.

The flow rate was 30 mL/h, and 7.0-mL fractions were collected. Strictosidine synthetase was eluted at a potassium phosphate concentration of about 120 mM (Figure 2). Active fractions were pooled and concentrated on a PM-10 filter.

Step 5: *Gel Filtration on Sephadex G-75.* The concentrate from the hydroxylapatite chromatography was applied to a 2.6 × 65 cm Sephadex G-75 column and eluted in 5.2-mL fractions with 20 mM Tris-HCl, pH 7.5, at a flow rate of 20 mL/h (Figure 3). Active fractions were pooled and concentrated by ultrafiltration.

Step 6: *Preparative Isoelectric Focusing.* The concentrated enzyme solution from step 5 was finally purified by flat-bed isoelectric focusing as shown in Figure 4. The isoelectric point of the enzyme was 4.6. The overall purification was 740-fold at a yield of 10% (Table I).

Homogeneity of Purified Strictosidine Synthetase. The homogeneity of purified strictosidine synthetase was tested by analytical polyacrylamide gel electrophoresis and analytical isoelectric focusing showing only one protein band.

Stability of Strictosidine Synthetase. After being partially purified through the Sephadex G-75 chromatography step, the enzyme was stable at 4 °C for more than a week, while the purified enzyme was less stable. Upon freezing, the enzyme lost about half of its activity within a week.

Product of the Enzyme. Purified strictosidine synthetase was incubated with tryptamine and secologanin, and the EtOAc extract was analyzed by TLC and LC. As is shown in Figure 5, the only radioactive product found corresponded to strictosidine, while no trace of the 3β-H epimer vincoside could be detected. Furthermore, the product purified by LC was incorporated into ajmalicine with 7% yield when incubated with a crude extract of *C. roseus* cultured cells supplemented with NADPH.

Table I: Summary of Purification[a]

step	total protein (mg)	enzyme act. nkat/mg of protein	enzyme act. total (nkat)	yield (%)
(I) crude extract	654	0.008	5.2	100
(II) $(NH_4)_2SO_4$ ppt	466	0.011	5.1	98
(III) DEAE-cellulose	38.7	0.10	3.9	75
(IV) hydroxylapatite	5.9	0.64	3.8	73
(V) Sephadex G-75	0.72	2.8	2.0	38
(VI) isoelectric focusing	0.08	5.9	0.5	10

[a] Wet cells (116 g) were used.

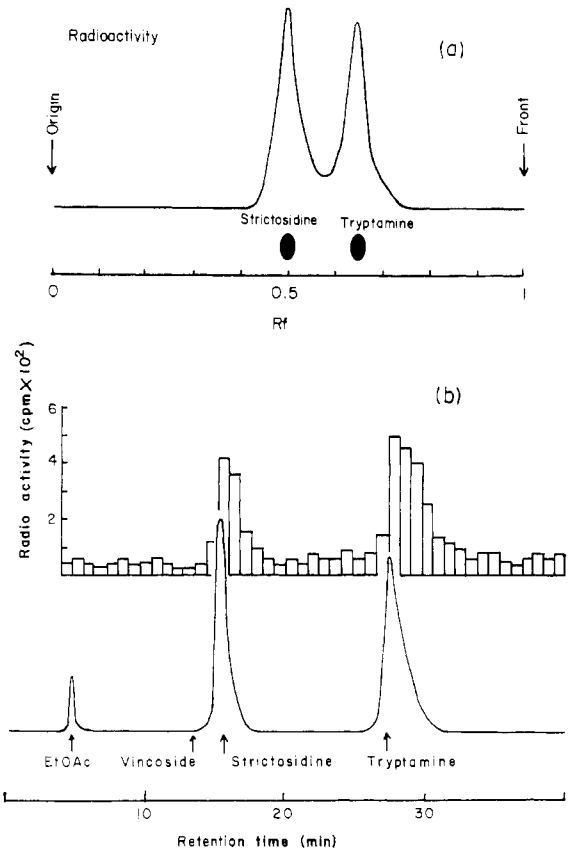

FIGURE 5: Product of strictosidine synthetase. (a) TLC chromatogram of EtOAc extract from reaction mixture incubated with purified strictosidine synthetase. Development with the solvent system acetone/MeOH/diethylamine (7:2:1). Radioactivity was recorded with a Packard radiochromatoscanner. (b) LC of EtOAc extract from reaction mixtures incubated with purified strictosidine synthetase. Fractions were collected in every 30 s and counted for radioactivity. See Experimental Procedures for details.

Molecular Weight Determination by Gel Filtration. Purified strictosidine synthetase was analyzed on a 2.6 × 65 cm Sephadex G-75 column eluted with 20 mM Tris-HCl, pH 7.5, and compared with standard marker proteins: bovine serum albumin (68 000), hen's egg albumin (45 000), chymotrypsinogen (25 000), cytochrome *c* (12 500). The molecular weight was found to be 38 000.

pH Dependence of the Enzyme Activity. Strictosidine synthetase was incubated at different pH in 0.1 M citrate–phosphate buffer (pH 3.7–7.4) and 0.1 M Tris-HCl buffer (pH 7.0–8.9). The enzyme has a broad pH optimum between 5.0 and 7.5.

Kinetic Analysis. Strictosidine synthetase demonstrated Michaelis–Menten kinetics for both of its substrates. Apparent K_m values were determined in 50 mM Tris-HCl buffer, pH

7.5, at 30 °C in the presence of 2.5 mM tryptamine or 10 mM secologanin, respectively. The apparent K_m for secologanin was found to be 0.46 mM and for tryptamine 0.83 mM.

Effect of Sulfhydryl Binding Inhibitors on the Enzyme Activity. Strictosidine synthetase incubated in the presence of *p*-hydroxymercuribenzoate, *N*-ethylmaleimide, and iodoacetic acid up to concentrations of 2 mM did not show significant inhibition, suggesting that a sulfhydryl group is not essential for enzyme activity.

Effect of Some Indole Alkaloids on the Enzyme Activity. The effect of some alkaloids originating from strictosidine on the activity of strictosidine synthetase was tested. The enzyme was preincubated with the alkaloid for 10 min at 30 °C before substrates were added. The enzyme retained 90% of its activity in 2 mM ajmalicine or catharanthine. Vindoline had no effect at this concentration.

Discussion

The enzyme system capable of synthesizing ajmalicine from tryptamine and secologanin was partially purified by gel filtration, showing a β-glucosidase might be involved in this enzymic complex (Scott et al., 1977b). Recently, this ajmalicine-synthesizing enzyme complex was further characterized by Treimer & Zenk (1978). However, no enzyme involved in this system has been purified so far. This report presents the purification of the first enzyme specific for indole alkaloid biosynthesis from higher plant cells.

Recent investigations which used a cell-free system of *C. roseus* have revealed that strictosidine (isovincoside) with 3α(*S*) configuration is the key intermediate leading to all the *Catharanthus* alkaloids bearing a 3α-H (Scott et al., 1977a; Stöckigt & Zenk, 1977b) as well as other known indole alkaloids with 3β-H (Rueffer et al., 1978). The purified strictosidine synthetase gave strictosidine as the only coupling product of tryptamine and secologanin. The molecular weight of strictosidine synthetase is in the range of that of the ajmalicine-synthesizing system (55 000) reported previously (Scott et al., 1977b) and the broad pH optimum of strictosidine synthetase between 5.0 and 7.5 is also consistent with the wide pH tolerance between 6 and 7.5 of the ajmalicine-synthesizing system reported by Treimer & Zenk (1978).

Geraniol 10-hydroxylase involved in the formation of the secologanin moiety was found to be a cytochrome P-450 related enzyme, regulated by feedback inhibition by catharanthine and vindoline (McFarlane et al., 1975). This type of regulation is absent for strictosidine synthetase, although it is the first enzyme leading to the biosynthesis of all the *Catharanthus* alkaloids. Furthermore, a 5-methyltryptophan resistant cell line of *C. roseus* retained the capacity of strictosidine synthesis but could not synthesize ajmalicine in any detectable amounts (Scott et al., 1979). These results suggest that indole alkaloid biosynthesis in *C. roseus* may not be regulated in the same way as in primary metabolism. The fact that no post-*Corynanthe* alkaloids are found in the tissue culture of this plant also poses interesting and challenging questions on regulation of secondary metabolism, inviting further investigation.

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