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Purification of the cytochrome P-450 enzyme geraniol 10-hydroxylase from cell cultures of *Catharanthus roseus*

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ABSTRACT

The cytochrome P-450 enzyme geraniol 10-hydroxylase (G10H) was purified from a suspension culture of *Catharanthus roseus*, grown on an alkaloid production medium. The cholate-solubilixed G10H was purified in a four-step procedure, consisting of chromatography on DEAE-Sephacel, hydroxyapatite Ultrogel, o-aminooctylagarose and TSK Phenyl-5PW. On DEAE-Sephacel a virtually complete separation of the cytochrome P-450 enzyme from NADPH:cytochrome P-450 (cytochrome c) reductase (EC 1.6.2.4) was achieved. The G10H activity of P-450containing fractions was reconstituted by addition of the reductase and a lipid extract. Although a substantial loss of G10H activity occurred on solubilisation and the activity was much lower in the reconstituted system compared with solubilixed preparations, the enzyme was remarkably stable during the later stages of its purification. An efficient separation of G10H from contaminating proteins was achieved by hydrophobic interaction chromatography on a high-performance TSK Phenyl-SPW column that was presaturated with non-ionic detergent. The G10H peak fractions from this column showed a single band on sodium dodecyl sulphate polyacrylamide gel electrophoresis with silver staining, corresponding to an M_r of 56 000. The purified enzyme catalyses the hydroxylation of both geraniol and nerol, and has a specific cytochrome P-450 content of 4.7 nmol/mg protein.

INTRODUCTION

The conversion of geraniol into its 10-hydroxy derivative, catalysed by the enzyme geraniol 10-hydroxylase (G10H, monoterpene hydroxylase), represents one of the initial steps in the biosynthesis of the monoterpenoid indole alkaloids in the plant family of the Apocynaceae. G10H was characterized as a membrane-bound cytochrome P-450 monooxygenase by Meehan and Coscia [1]. The hydroxylation of geraniol, or of its *cis* isomer nerol, was among the first cytochrome P-450-catalysed reactions discovered in higher plants. It has now been demonstrated that plant cytochrome P-450 enzymes are involved in a variety of biosynthetic pathways, e.g., those leading to benzophenanthridine alkaloids [2], lignins and flavonoids, phytoalexins, cutins and suberins, phytohormones and steroids (for reviews, see refs. 3-6). In addition, the metabolism of exogenous compounds by plant cytochrome P-450 enzymes has been described (for reviews, see refs. 7 and 8). The substrate specificity of cytochrome P-450 enzymes and the regulation of their expression have been extensively studied in mammalian systems and in microorganisms, but the knowledge of plant P-450 enzymes is still limited. Only recently was the first cDNA sequence encoding a plant P-450 enzyme reported [9]. This concerned a ripening-related cDNA from avocado fruit (*Persea*

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americana). The polypeptide predicted from the nucleotide sequence shares some common features with animal P-450 proteins and probably the clone represents a P-450 enzyme that was purified to homogeneity from avocado mesocarp by O'Keefe and Leto [10]. Among the few other P-450 proteins that have been purified partially or to homogeneity from a plant source are *trans*-cinnamate 4-hydroxylase (EC 1.14.13.11) from *Helianthus tuberosis* [11], digitoxin 12β-hydroxylase from *Digitalis lanata* [12], a P-450 with unknown enzyme activity from *Tulipa gesneriunu* [13] and 3,9-dihydroxypterocarpan 6a-hydroxylase (EC 1.14.13.28) from *Glycine max* [14]. *The* latter was purified to homogeneity and its separation from *trans*-cinnamate 4-hydroxylase provided the first real evidence for the existence of different types of P-450 proteins in a higher plant.

Madyastha *et al.* [15] described the isolation of G10H from seedlings of *Catharanthus roseus*. By ion-exchange chromatography the P-450 enzyme could be separated from NADPH:cytochrome P-450 (cytochrome c) reductase (EC 1.6.2.4), an enzyme that is essential for any cytochrome P-450-catalysed reaction, as it functions in the transfer of electrons from NADPH to the P-450 protein. The purification of the reductase to homogeneity and its characterization were described [16]. G10H activity could be reconstituted with the reductase, the partially purified cytochrome P-450 and a lipid fraction [15,17]. McFarlane *et al.* [18] proposed that a feedback inhibition at the level of G10H might be one of the regulatory mechanisms in alkaloid biosynthesis, as they observed that this enzyme is inhibited by catharanthine, an end-product of the pathway. G10H was also regarded as a potential site for regulatory control by Schiel *et al.* [19], who observed an increased G10H activity in cultures transferred to an alkaloid production medium and a close relation of G10H activity to the pattern of alkaloid accumulation. A detailed investigation of the expression of G10H and NADPH:cytochrome P-450 reductase on the protein and transcriptional level will help to gain a better insight into the regulation of alkaloid biosynthesis and will attribute to the knowledge of plant cytochrome P-450 systems in general. The purification of G10H is a first step in this direction. Here we describe the purification of this cytochrome P-450 enzyme from cell cultures of *Catharanthus roseus* by an efficient procedure consisting of four chromatographic steps.

EXPERIMENTAL

Materials

Geraniol (96%), nerol (95%) and citral (97%) were obtained from Fluka (Buchs, Switzerland). NaB^3H_4 (>100 Ci/mol) was from NEN Du Pont ('s-Hertogenbosch, Netherlands). [1-^{3}H]Farnesyl pyrophosphate (40 Ci/mol) and [4-^{3}H]squalene (10 Ci/mol) were gifts from Dr. D.R. Threlfall (University of Hull, UK). CHAPS {3-[(3-cholamidopropyl)dimethylammonio]propane -1-sulphonic acid}, CHAPS0 {3-[(3-cholamidopropyl)dimethylammonio]- 2 -hydroxypropane-1-sulphonic acid}, polyvinylpyrrolidone (PVP) (insoluble form), leupeptin, cytochrome c (type III from horse heart) and w-aminooctyl agarose were purchased from Sigma (St. Louis, MO, USA). Sodium cholate was from Merck (Darmstadt, Germany). Renex 690 was a gift from ICI Specialty Chemicals (Rotterdam, Netherlands). Glucose-6-phosphate dehydrogenase (lyophilized, from yeast) was obtained from Boehtinger (Mannheim, Germany). Bovine serum albumin standard was obtained from Bio-Rad Labs. (Veenendaal, Netherlands). The TSK Phenyl-5PW column, DEAE-Sephacel, 2',5'-ADP-Sepharose 4B and low-molecular-mass markers were purchased from Pharmacia LKB Biotechnology (Woerden, Netherlands), hydroxyapatite Ultrogel from IBF (Villeneuve la Garenne, France) and Extracti-gel D from Pierce (Rockford, IL, USA). Organic solvents were of analytical-reagent grade or distilled before use. All other chemicals were of the highest purity commercially available.

Synthesis of 10-hydroxygeraniol and IO-hydroxynerol

10-Hydroxygeraniol was synthesized essentially as described by Madyastha *et al.* [15] and Schiel *et al.* [19]. A 7.5-ml portion of geraniol was acetylated with 4.85 ml acetic anhydride

(95%) and 4.15 ml pyridine for 15 h at room temperature. After addition of water, geranyl acetate was extracted with ethyl acetate. The organic phase was washed with 1 *M* HCl and subsequently with 6.6% (w/v) $NaHCO_3$. After evaporation of the solvent under vacuum, geranyl acetate was subjected to SeO_2 oxidation to yield a mixture of 10-oxogeranyl acetate and 10-hydroxygeranyl acetate. The reaction was performed with 5.5 g of SeO_2 in 100 ml of boiling ethanol (96%) under reflux for 6 h. After evaporation of the solvent under vacuum, the product was applied to a silica gel 60 (Merck) column to remove selenium. The product was eluted with dichloromethane-methanol (8:2, v/v) and subjected to LiAlH, reduction to yield 10-hydroxygeraniol. The dried acetates were dissolved in 50 ml of sodium-dried diethyl ether and added dropwise with stirring to 2.5 g of LiAlH, in 100 ml of sodium-dried diethyl ether at 0°C and under reflux. After 45 min, excess of LiAlH, was removed by the dropwise addition of 25 ml of water. The reaction mixture was extracted with 10% (w/v) NH_4Cl and the aqueous phase was washed twice with diethyl ether. The combined ether phases were dried under vacuum and applied to a silica gel 60 column. After elution of unreduced acetates with chloroform-ethyl acetate (3:1, v/v), 10-hydroxygeraniol was eluted with methanol. 10-Hydroxynerol was synthesized from nerol by the same procedure.

The structures of 10-hydroxygeraniol and 10-hydroxynerol were confirmed by PMR spectroscopy [15] with tetramethylsilane as reference compound. 10-Hydroxygeraniol: δ (ppm) [C^2HCl_3], 1.670, 1.674 (3H, s, H-4; 3H, s, H-9), 2.10 (4H, m, H-5, H-6), 3.98 (2H, s, H-10), 4.14 (2H, d, *J* 6.9 Hz, H-l), 5.38 (2H, m, H-2, H-7). 10-Hydroxynerol: δ (ppm) [C^2HCl_3], 1.65 (3H, d, *J* 1.4 Hz, H-9), 1.75 (3H, d, *J* 1.2 Hz, H-4), 2.14 (4H, m, H-5, H-6), 3.98 (2H, s, H-10), 4.06 (2H, dd, *J* 7.3 and 0.7 Hz, H-l), 5.44 (2H, m, H-2, H-7).

Synthesis of labelled substrates

The synthesis of [1-^{3}H]geraniol and [1-^{3}H]-nerol was modified from Van Aller and Nes [20]. A 300-mg portion of citral [geranial-neral (65:35)] in 1 ml of ethanol was added slowly to 14.5 mg of NaB^3H_4 in 1 ml of ethanol on ice. After 30 min, the reaction mixture was diluted with diethyl ether and washed with 0.5 *M* HCl. The organic phase was concentrated under a stream of nitrogen and the products were purified by TLC on silica gel 60 F_{254}s (0.5 mm thickness, 20 *x 20 cm,* with a 4 cm concentration zone) (Merck). The plates were developed twice with hexane-ethyl acetate (4:1, v/v). By GC analysis less than 3% nerol was detected in the geraniol fractions and *vice versa.*

[1-^{3}H]Farnesol (40 Ci/mol) was obtained from [1-^{3}H]farnesyl pyrophoshate by hydrolysis with calf intestinal alkaline phosphatase.

[1-^{3}H]Geranyl pyrophosphate (0.5 Ci/mol) and [1-^{3}H]geranyl monophosphate (0.5 Ci/mol) were synthesized from [1-^{3}H]geraniol as described by Cornforth and Popják [21]. The products were purified by TLC on silica gel H (0.5 mm thickness, 20 *x* 20 cm) (Merck) with 2-propanol–ammonia solution-water (6:3: 1, v/v/v).

Plant material

Cell suspension cultures were grown in the light at 25°C on gyratory shakers at 120 rpm. Cultures of *Catharanthus roseus* (L.) G. Don were subcultured every 7 days and *Tabernaemontana* cultures every 14 days by a fourfold dilution. C. *roseus* cultures were grown in LS medium [22] containing 3% sucrose, 2 mg/l 1-naphthaleneacetic acid and 0.2 mg/l kinetin. The alkaloid production medium was as described by Berlin *et al.* [23]. Cultures of *Tabernaemontana divuricatu* (L.) R.Br. *ex* Roem. *et* Schult. and Tubernaemontunu *pundacaqui* Poir. were grown in MS medium [24] containing 3% sucrose, 1 mg/l 2,4-dichlorophenoxyacetic acid and 1 mg/l kinetin.

Enzyme preparation

For purification of G10H, 7-day-old C. *roseus* cultures were diluted five-fold in 500 ml of alkaloid production medium and grown in 2-l erlenmeyer flasks for 5-6 days. The cells were harvested by filtration under suction, washed once with water and frozen in liquid nitrogen. Cells were kept at -80°C for storage. Liquid

nitrogen-frozen cells were homogenized in a Waring blender equipped with a stainless-steel beaker at maximum speed for 1 min, rapidly thawed in two parts of homogenization buffer containing 50 mM potassium phosphate (pH 7.6), 0.3 M sucrose, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 5 μg/ml leupeptin, and additionally homogenized with an Ultra Turrax at medium speed for 2 min. Membranes sedimenting between 20 min at 1000 g and 60 min at 20000 g were suspended in an approximately equal volume of 50 mM Tris-HCl buffer (pH 7.8) containing 20% (v/v) glycerol, 1 mM EDTA, 1 mM DTT and 5 μg/ml leupeptin.

In the screening experiment for G10H activity in different cell lines, 4 g of cell material were homogenized with 200 mg of PVP and two volumes of homogenization buffer without leupeptin by means of an Ultra Turrax at medium speed for 2 min at 0°C. A 1000-20 000 g pellet was isolated and suspended in 300 μl of 50 mM potassium phosphate buffer (pH 7.6) containing 1 mM EDTA and 1 mM DTT.

Solubilization and enzyme purification

The 1000-20000 g membrane fraction (S-10 mg/ml of protein) was solubilized at 0°C by the dropwise addition of a 10% (w/v) sodium cholate solution to a final concentration of 1%. After 30 min of gentle stirring the suspension was centrifuged at 100000 g for 90 min.

Solubilized protein isolated from 2.7 kg of cell material was applied on a DEAE-Sephacel column (34 x 2.6 cm I.D.), equilibrated in 50 mM Tris–HCl buffer (pH 7.8) containing 15% (v/v) glycerol, 0.1% (v/v) Renex 690 and 0.1 mM DTT. The column was washed with 400 ml of equilibration buffer and protein was eluted with a KC1 gradient. The cytochrome P-450 was eluted with a linear gradient from 0 to 100 mM KC1 in 1000 ml of equilibration buffer. The column was then washed with 400 ml of 100 mM KC1 in equilibration buffer, Subsequently NADPH:cytochrome P-450 reductase was eluted with 600 ml of 300 mM KC1 in equilibration buffer. The flow-rate was 0.5 ml/min and the fraction size was 10 ml.

G10H (cytochrome P-450)-containing fractions from DEAE-Sephacel were pooled, concentrated by ultrafiltration (Omega 30K membrane, Filtron) and applied to a hydroxyapatite Ultrogel column (24 x 1.6 cm I.D.) equilibrated in 10 mM potassium phosphate buffer (pH 7.7) containing 15% (v/v) glycerol, 0.2% (w/v) sodium cholate and 0.1 mM DTT. The column was washed with 50 ml of equilibration buffer. Elution was with a linear gradient from 10 to 500 mM potassium phosphate in 250 ml of equilibration buffer. The flow-rate was 0.25 ml/min and the fraction size was 7.5 ml.

The G10H pool from hydroxyapatite Ultrogel was concentrated by ultrafiltration (Omega 30K membrane, Filtron). Phosphate and cholate concentrations were reduced by repetitive dilution with aminooctyl equilibration buffer (see below), followed by concentration. The concentrate was applied to an w-aminooctylagarose column (23 × 1.6 cm I.D.) equilibrated in 50 mM potassium phosphate buffer (pH 7.7) containing 15% (v/v) glycerol, 1 mM EDTA and 0.1 mM DTT. The column was washed with 100 ml of equilibration buffer. Elution was with a linear gradient from 0 to 0.2% (v/v) Renex 690 in 300 ml of equilibration buffer. The flow-rate was 0.25 ml/min and the fraction size was 7.5 ml.

The G10H pool from w-amiuooctylagarose was concentrated by ultrafiltration (Omega 30K membrane, Filtron) and applied to a TSK Phenyl-5PW HPLC column (7.5 x 0.75 cm I.D.). The column was equilibrated in 20 mM potassium phosphate buffer (pH 7.7) containing 15% (v/v) glycerol, 0.1% (v/v) Renex 690, 1 mM EDTA and 0.1 mM DTT until the absorbance at 280 nm (absorption of Renex 690) had reached a constant level. About 180 ml of equilibration buffer were required to saturate the column with detergent. After sample application the column was washed with 10 ml of equilibration buffer. Elution was with a linear gradient from 0.1 to 0.6% (v/v) Renex 690 in 35 ml of equilibration buffer. The flow-rate was 0.5 ml/min and the fraction size was 1 ml.

All chromatographic steps were performed at 4–7°C, except for the TSK Phenyl-5PW chromatography, which was performed at room temperature. The absorbance of the effluent was monitored at 280 and 405 nm. Purified enzyme preparations were stored at -80°C.

Determination of cytochrome P-450

Cytochrome P-450 concentrations (ε = 91 l mmol^{-1} cm^{-1} for absorbance at 450-490 nm) were determined from dithionite-reduced CO-difference spectra according to Omura and Sato [25].

Determination of G10H activity

The assay for G10H activity, based on the conversion of [1-^{3}H]geraniol into [1-^{3}H]-10-hydroxygeraniol, was modified from Madyastha *et al.* [15]. The incubation mixture (total volume 550 µl) contained 12.5 nmol of [1-^{3}H]geraniol (127 Ci/mol) in 10 µl of acetone, 1 I.U. of glucose-6-phosphate dehydrogenase, 2.5 µmol of glucose&phosphate, 0.5 µmol of $NADP^+$ and the enzyme preparation (10–50 µg of protein for 1000-20000 g membrane fractions) in 50 m*M* potassium phosphate buffer (pH 7.6) containing 1 m*M* EDTA, 1 m*M* DTT, 10 µ*M* FAD and 10 µ*M* FMN. The geraniol concentration in the assay is approximately five times higher than the apparent K_m reported by Madyastha *et al.* [15]. The incubations were started by the addition of [1-^{3}H]geraniol, after a preincubation for 5 min at 30°C. After 60 min at 30°C, the mixture was extracted with 3 ml of ethyl acetate. The organic phase was dried under a stream of nitrogen, dissolved in 100 µl of acetone and a 10-µl aliquot was subjected to TIC (silica gel 60, 0.5 mm thickness, 20 x 20 cm). The plates were developed with toluene-ethyl acetate-acetone (6:4:1, v/v/v). Reference 10-hydroxygeraniol was rendered visible with anisaldehyde-acetic acid-methanol-sulphuric acid (1:20:170:10, v/v) spray reagent. The radioactivity in 10-hydroxygeraniol was determined by liquid scintillation spectrometry. Emulsifier-Safe scintillation fluid (Packard) was used.

The above-described assay for G10H activity was used during G10H purification and for all other experiments with the exception of the screening experiment for G10H activity in different cell suspension cultures. For this experiment a GC-based assay modified from Schiel *et al.* [19] was used. The incubation mixture (total volume 380 µl) contained 1.5 µmol of geraniol in 5 µl of ethanol, 0.5 µmol of NADPH and 75 µl of enzyme preparation (1000–20 000 g pellet, 5-10 mg/ml of protein), in 50 m*M* potassium phosphate buffer (pH 7.6) containing 1 m*M* EDTA and 1 m*M* DTT. After 30 min at 30°C the reaction was stopped with 75 µl of 2 *M* KOH. As an internal standard 1,10-decanediol (10 nmol in 20 µl of ethanol) was added and the mixture was extracted twice with 1 ml of ethyl acetate. The organic phase was dried under a stream of nitrogen and silylated with 50 µl of N-methyltrimethylsilyltrifluoroacetamide at room temperature for 15 min. The silylated samples were analysed by GC under the following conditions: 10 m x 0.22 mm I.D. WCOT CP-Sil 5cb fused-silica column, film thickness 0.13 µm (Chrompack); carrier gas, nitrogen at 100 kPa; splitting ratio 1:50; detector temperature, 290°C; injector temperature, 275°C; column temperature programme, 120°C for 3 min then increased from 120 to 195°C at 15°C/min and from 195 to 290°C at 30°C/min, then isothermal at 290°C for 10 min; detection, flame ionization.

Reconstitution of G10H activity

The G10H activity of P-450-containing fractions was reconstituted by addition of NADPH: cytochrome P-450 reductase and C. *roseus* lipids. The lipid fraction was extracted from 1000–20000 g membrane fractions with chloroform-methanol (2:1, v/v) and dissolved in incubation buffer (see below) with a Branson microtip sonicator. To cytochrome P-450-containing fractions were added 360 pkat of reductase (from DEAE-Sephacel) and 10 µg of C. *roseus* lipids, in that order. Incubation buffer consisting of 50 m*M* potassium phosphate (pH 7.6), 1 m*M* EDTA, 1 m*M* DTT, 10 µ*M* FAD and 10 µ*M* FMN, was added to make a total volume of 200 µl. This mixture, followed by 300 µl of incubation buffer, was applied to a 250-µl column of Extracti-gel D detergent-removal gel. The eluate was collected and 50 µl of incubation buffer, containing 1 I.U. of glucose&phosphate dehydrogenase, 2.5 µmol of glucose&phosphate and 0.5 µmol of $NADP^+$ was added. After 5 min of preincubation at 30°C the reaction was started with [1-^{3}H]geraniol (12.5 nmol in 10 µl of acetone). After incubation for 60 min the

radioactivity in 10-hydroxygeraniol was determined as in the G10H assay described above.

Determination of NADPH:cytochrome c (P-450) reductase activity

The assay for NADPH:cytochrome c (P-450) reductase was modified from Madyastha *et al.* [15]. The incubation mixture (total volume 1.0 ml) consisted of 20 μl of enzyme solution and 50 nmol of cytochrome c (type III, from horse heart), 0.15 μmol of NADPH, 0.5 μmol of KCN, 5 nmol of flavin mononucleotide and 5 nmol of flavin-adenine dinucleotide (FAD) in buffer containing 0.5 M Tris–HCl buffer (pH 7.5). The reactions were started by the addition of enzyme and the reduction of cytochrome c was monitored for 3-4 min at 550 nm (ε = 21 1 $mmol^{-1}$ cm-') and at 20°C.

Determination of trans-cinnumate *4-hydroxylase activity*

trans-Cinnamate 4-hydroxylase activity was assayed by an HPLC method according to Dr. M. Petersen (University of Dusseldorf, Germany; personal communication). The incubation mixture (total volume 500 μl) consisted of 0.5 μmol of *trans*-cinnamate in 20 μl of 50% ethanol, 0.5 μmol of NADPH and the enzyme preparation (50-200 μg of protein for 1000–20 000 g membrane fractions) in 0.1 *M* Tris–HCl buffer (pH 7.5) containing 20 m*M* DTT. After incubation for 30 min at 30°C, the reaction was stopped by the addition of 100 μl of 6 *M* HCl and the mixture was extracted twice with 1 ml of ethyl acetate. The organic phase was dried under a stream of nitrogen and the residue was dissolved in 150 μl of methanol-water (50:50, v/v), containing 100 μl of H_3PO_4 (85%) per litre. HPLC was carried out at room temperature on a 250 *x* 4.6 mm I.D. Hypersil ODS column (particle size 5 μm) at a flow-rate of 1.5 ml/min. The analytical column was used in combination with a 20 mm *x* 2 mm I.D. precolumn (Upchurch) hand-packed with Perisorb RP-8 (Merck) with a particle size of 30-40 μm. The mobile phase consisted of methanol-water containing 100 μl of H_3PO_4 (85%) per litre and elution was with a linear gradient from 40 to 60% (v/v) methanol in 10 min, followed by 60% methanol for 3 min. The injection volume was 20 μl and detection was at 309 nm.

trans-Cinnamate 4-hydroxylase activity was reconstituted in an analogous manner to the method described for G10H. Incubation times up to 4 h were used in reconstitution experiments.

Gel electrophoresis, isoelectric focusing and N-terminal protein sequence analysis

Gel electrophoresis and isoelectric focusing were performed with a PhastSystem (Pharmacia LKB Biotechnology). Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in PhastGel gradient medium 10–15 or PhastGel homogeneous medium 12.5. Protein staining was done according to the instructions for the PhastGel silver kit. Isoelectric focusing was with PhastGel IEF 3-9. N-Terminal sequence analysis by Edman degradation of protein electroblotted onto a polyvinylidene fluoride membrane was performed by Eurosequence (Groningen, Netherlands).

Alkaloid analysis

A 50-mg portion of freeze-dried cell material, 50 μl of internal standard (1.0 g/l dihydroquinine), 1 ml of buffer (pH 10) consisting of 62.5 m*M* glycine, 62.5 m*M* NaCl and 0.0375 *M* NaOH, 57.5 μl of 1 *M* NaOH and 5 ml of CH_2Cl_2 were mixed thoroughly for 1 min on a vortex apparatus. After centrifugation, the organic phase was evaporated under vacuum. The residue was dissolved in 0.5 ml of eluent consisting of 50 m*M* sodium phosphate (pH 3.9)–acetonitrile–2-methoxyethanol (80:15:5, v/v/v) and 100 μl were injected on to a 300 mm × 3.9 mm I.D. μBondapak Phenyl (Waters, Milford, MA, USA) column [26]. The flow-rate was 2 ml/min and a Waters 990 photodiode-array detector was used.

Protein determination

Protein was determined according to Peterson [27] with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

G10H activity *in* Catharanthus roseus *and* Tabernaemontana *species*

To select a suitable source for G10H purification we determined G10H activity levels in cell suspension cultures of *Catharanthus roseus*, *Tabernaemontana divaricata* and *Tabernaemontana pandacaqui*, three members of the Apocynaceae family that produce related terpenoid indole alkaloids. During this screening experiment we also studied the relationship between the activity of G10H and the accumulation of terpenoid indole alkaloids. G10H activity was measured in a membrane fraction sedimenting between 1000 and 20000 g. In addition to organelles such as mitochondria and plastids, this pellet contains provacuolar membranes, in which, according to Madyastha et al. [28], the G10H from C. *roseus* is localized. In agreement with their results, we found that the bulk of G10H activity in C. *roseus* was present in this fraction. Only about 15% of the total activity was detected in a 100 000 g microsomal fraction. The same distribution pattern was found for G10H from *T. divaricata*. Both in C. *roseus* and in *T. divaricutu we* could also demonstrate the activity of the cytochrome P-450 enzyme *trans*-cinnamate 4-hydroxylase in the 1000-20000 g pellet. In C. *roseus trans*-cinnamate 4-hydroxylase, like G10H, appeared to occur predominantly in this fraction, whereas only about 25% was found to be associated with microsomes.

G10H activity was monitored during growth of several suspension cultures. In a cell line of C. *roseus* that accumulates tryptamine, but no terpenoid indole alkaloids, G10H activity could not be detected. The enzyme, however, became induced when the culture was transferred to an alkaloid production medium. The terpenoid indole alkaloid ajmalicine appeared just after G10H activity had reached a maximum (Fig. 1). An increase in the activity of this enzyme in a C. *roseus* cell line on production medium was also observed by Schiel *et* al. [19].

In a cell line of *T. divaricutu* maximum G10H activity was present during the first week of growth. The enzyme activity could no longer be detected when the stationary phase was reached.

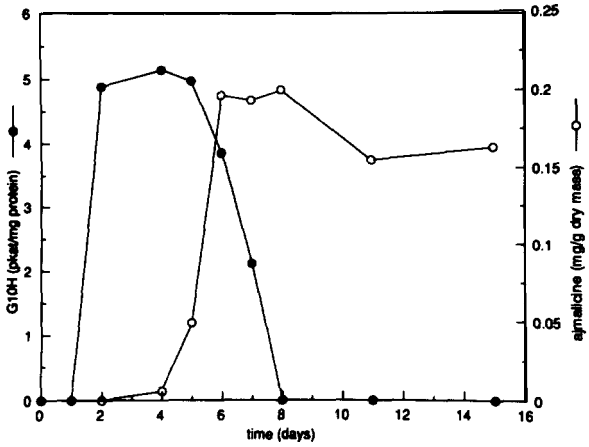

Fig. 1. G10H activity and ajmalicine accumulation in cell cultures of *Catharanthus roseus on* alkaloid production medium. Erlenmeyer flasks (250 ml) containing 50 ml of medium were inoculated with 5 g of cell material, filtered over a sterile glass filter. At each time point one flask was harvested. Cell material was homogenized in two volumes of 50 m*M* potassium phosphate buffer (pH 7.6), containing 0.3 *M* sucrose, 1 m*M* EDTA, 1 m*M* DTT and polyvinylpyrrolidone (50 mg/g cell material). G10H was measured in a 1000–20 000 *g* membrane fraction.

This cell line produced a variety of terpenoid indole alkaloids, with vallesamine and O-acetylvallesamine as major and voaphylline, tubotaiwine, apparicine and several acylindole alkaloids as minor components. Maximum levels were reached after the peak in G10H activity (Fig. 2).

During the growth of a cell suspension culture of *T. pandacaqui* no G10H activity could be detected. This cell line produced no terpenoid indole alkaloids. Only tryptamine (0.03-0.1 mg/g dry mass) and large amounts of a tryptamine derivative, probably 5-hydroxytryptamine, were present (at approximately ten times the level of tryptamine). G10H activity in this cell line was also found to be absent when in a later stage enzyme extractions were performed in the presence of leupeptin, a protease inhibitor which increased substantially the recovery of G10H from C. *roseus* cells.

Parallel to the experiments described here, cell line characterizations were performed, in which the activities of tryptophan decarboxylase and strictosidine synthase were measured during

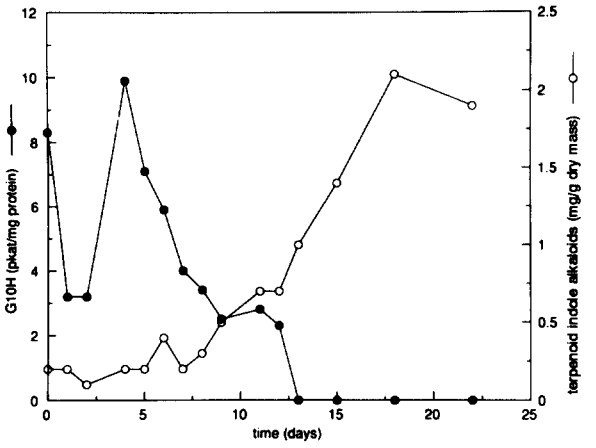

Fig. 2. G10H activity and alkaloid accumulation during growth of cell cultures of *Tabernaemontana divaricata*. The cultures reached stationary phase at day 11. Experimental details as in Fig. 1.

growth [29]. It appeared that the *C. roseus* line on growth medium and the *T. pandacaqui* line, in both of which G10H and terpenoid indole alkaloids were not detected, did possess tryptophan decarboxylase and strictosidine synthase activity. The absence of indole alkaloids in these cell lines seems therefore to be correlated with a shortage of terpenoid precursors. The fact that G10H activity could not be detected supports the arguments of McFarlane *et al.* [18] and Schiel *et al.* [19] to regard this enzyme as a potential site for control in the pathway for secologanin biosynthesis. However, other unknown regulatory enzyme activities in the secologanin pathway may also represent bottlenecks in alkaloid production.

The presence of induced levels of G10H activity in *C. roseus* suspensions rapidly after transfer to alkaloid production medium was found to be reproducible, and this cell material was considered the best source for G10H purification. Madyastha *et al.* [15] had reported partially purified G10H to be highly labile. After selection of the *C. roseus* cell material for G10H purification, we therefore tried to optimize the extraction procedure for this enzyme source in order to obtain a membrane preparation with high G10H specific activity as starting material. In the above-described screening experiments for G10H activity, enzyme extractions were performed in the presence of PVP (insoluble form) to bind phenolics and in the presence of the protease inhibitor EDTA. The addition of PVP (insoluble or soluble form), however, appeared not to be benificial. Several protease inhibitors were tested. With leupeptin (5 μg/ml), a fivefold increase in G10H activity was observed. Phenylmethylsulphonyl fluoride was not effective and benzamidine improved the recovery of G10H slightly. Leupeptin, however, was more effective alone than in combination with the other protease inhibitors. For purification of G10H, the *C. roseus* cell material was homogenized in the presence of only EDTA and leupeptin. In this way membrane preparations were obtained with a ten-fold higher G10H specific activity than the membrane fractions isolated by Madyastha *et al.* [15] from *C. roseus* seedlings.

Solubilization

The ionic detergent sodium cholate is often used for solubilization of cytochrome P-450 systems, since it causes little conversion of cytochrome P-450 to the catalytically inactive cytochrome P-420. Moreover, in contrast to most non-ionic detergents, cholate is only slightly inhibitory to the enzyme activity, even at the high concentrations necessary for solubilization [30]. Solubilization of cytochrome P-450 with the zwitterionic detergent CHAPS has also been successful [31]. We tested cholate, CHAPS, and CHAPSO for solubilization of G10H in the concentration range 0.2–1.2% and at a protein concentration of 5 mg/ml. In all instances most of the activity remained unsolubilized at concentrations below the critical micellar concentration (0.5% for CHAPS and CHAPSO, 0.6% for cholate). The solubilized activity was maximum when concentrations above the critical micellar concentration were used, but under those conditions the total of unsolubilized and solubilized activity was always less than 10% of the original activity. Enzyme solubilized with CHAPS or CHAPSO had to be passed through detergent-removal gel (Extracti-gel D) to measure G10H activity. This treatment had no effect on cholate-solubilized enzyme. Sonication with a microtip sonifier prior to cholate solubilization did not improve the yield of solubilized activity at any of

the detergent concentrations tested. In cholate-solubilixed preparations the activity of *trans*-cinnamate Chydroxylase was also more than 90% lower than in the membrane fraction. With all the detergents tested about 100% of the NADPH:cytochrome c reductase activity could be solubilixed. The loss of monooxygenase activity on solubilixation can be explained by an inactivation or denaturation of the enzyme or by a less efficient interaction between the cytochrome P-450 and the reductase, possibly owing to their distribution over different micelles.

Separation of G10H from NADPH:cytochrome P-450 reductase and reconstitution of G10H activity

The cholate-solubilixed protein was subjected to DEAE-Sephacel chromatography and eluted with a KC1 gradient. Although the use of cholate has advantages for solubilixation, cytochrome P-450 can be separated more completely from NADPH:cytochrome P-450 (cytochrome c) reductase in the presence of a non-ionic detergent [30]. Therefore, ion-exchange chromatography was performed in the presence of 0.1% Renex 690, a non-ionic detergent of the polyoxyethylene nonylphenol type, which is non-denaturing with respect to cytochrome P-450 [30]. A good separation of the two proteins was achieved. The cytochrome P-450 eluted between 25 and 35 m*M* KC1 and the reductase could be eluted after a stepwise increase in the KC1 concentration from 100 to 300 m*M*. No NADPH:cytochrome c reductase activity was associated with the P-450 peak. The reductase fractions contained only traces of cytochrome P-450.

After the separation of the P-450 component and the reductase, G10H activity can only be measured in a reconstituted system, in which the protein components are integrated into liposomes. Reconstitution of G10H activity could be achieved by the addition of partially purified reductase (DEAE-Sephacel fraction) and a crude extract of C. *roseus* lipids to the P-450-containing fraction. To avoid the sensitivity of the G10H assay becoming limiting during subsequent purification steps, it was essential to optimize the conditions of the reconstitution method. We found that the efficiency of reconstitution could be improved when the mixture of cytochrome P-450, reductase and lipid was passed through a small column of Extracti-gel D for detergent removal. When the Extracti-gel D step was omitted, more than 80% reduced G10H activities were observed. A similar decrease in activity was found on addition of 0.1% Renex 690 to the reconstituted system after the Extracti-gel treatment. The dependence of G10H activity on the concentrations of cytochrome P-450 and reductase was investigated. Omitting either of the protein components resulted in a complete loss of G10H activity. To allow us to determine the recovery of G10H activity during purification, a saturating reductase concentration was used in the reconstitution assay, which made G10H activity linearly dependent on the concentration of (geraniol-specific) cytochrome P-450. The reconstituted system was optimized with respect to lipid composition and concentration. Reconstitution with a crude extract of C. *roseus* lipids (10–250 μg) stimulated G10H activity. The highest (two-fold) increase was with 10 μg, and this amount was chosen for the routine procedure. A stimulation (1.5–2-fold) could also be effected with 10 μg of purified phospholipids [dilauroylphosphatidylcholine or a mixture of phosphatidylcholine and phosphatidylinositol (9:1)], but these were inhibitory at higher concentrations.

The highest G10H activity achieved in the reconstituted system was always less than 10% of the activity of the solubilixed membrane fraction. A low G10H activity after reconstitution was also reported by Madyastha *et al.* [15]. Similar results were obtained with reconstitution of digitoxin 12β-hydroxylase [12] and *trans*-cinnamate Chydroxylase [32], but 3,9-dihydroxypterocarpan 6a-hydroxylase [14] could be reconstituted with a much higher yield of activity. Madyastha *et al.* [15] assumed that the low activity after reconstitution was probably due to the highly labile nature of cytochrome P-450. In contradiction to this, we found that the recovery of G10H activity was remarkably high during all subsequent chromatographic steps in our purification procedure. Moreover, we did not observe conversion of cytochrome P-450 to the P-420 form during these steps. Loss or denaturation of

protein may partly explain the initial decrease in activity. However, we consider it more likely that this is mainly caused by other effects, such as an incomplete removal of inhibitory detergent and a less efficient interaction between reductase and P-450 in the reconstituted system. Such differences in the efficiency of catalysis of the hydroxylation complicate the interpretation of the purification scheme (Table I). We believe that DEAE-Sephacel forms an efficient purification step, because there was virtually complete separation of P-450 and reductase, and the total P-450 was enriched seven-fold. A less efficient catalysis in the reconstituted system may explain the decrease in specific G10H activity.

Purification of G10H

The G10H pool from DEAE-Sephacel was subjected to hydroxyapatite Ultrogel chromatography. G10H and cytochrome P-450 eluted in a broad peak between 150 and 450 m*M* potassium phosphate, with the highest amounts at 250 m*M*. A three-fold increase in the specific activity of G10H was achieved. On this column the non-ionic detergent Renex 690 was exchanged for cholate, because Renex interferes with the binding of G10H to w-aminooctyl agarose, which was planned as the next purification step, as aminooctyl has been described as an efficient ligand for the iron(III) form of cytochrome P-450 [33]. The G10H pool from the hydroxyapatite column was applied to an o-aminooctylagarose column and G10H was eluted with a gradient from 0–0.2% Renex 690.

The G10H pool from o-aminooctylagarose was further purified by hydrophobic interaction chromatography on a high-performance TSK Phenyl-5PW column. Protein purification by hydrophobic interaction chromatography usually involves binding of protein to the matrix under high-salt conditions and elution with a decreasing salt gradient. To elute strongly bound proteins, addition of a (poly)alcohol or detergent may be necessary. G10H, however, appeared to have such a high hydrophobicity that it could even be bound in the presence of detergent. Before the application of protein, the TSK Phenyl-5PW column was washed with buffer, containing 0.1% of the non-ionic detergent Renex 690, until the absorbance of the effluent at 280 nm reached a constant level. This presaturation of the column with detergent made the TSK Phenyl-5PW chromatography a very efficient purification step, as it prevented most of the protein in the sample from binding, while G10H was still retained on the column. G10H could be eluted by further increasing the detergent concentration with a linear gradient of Renex 690 from 0.1 to 0.6% (Fig. 3a). Four of the peak fractions showed an electrophoretically homogeneous protein with an M_r of 56000 on SDS-PAGE with silver staining (Fig. 3b). The p*I* of this protein was 8.3. In earlier fractions of the peak a second band with a slightly lower molecular mass was present, and the later fractions contained minor impurities.

TABLE I

PURIFICATION OF THE CYTOCHROME P-450 ENZYME G10H FROM *CATHARANTHUS ROSEUS*

***The* table is based on purification from 2.7 kg (fresh mass) of C. *roseus* cell material, harvested 5-6 days after transfer of suspension cultures to alkaloid production medium.**

Purification step	Protein (mg)	Cytochrome P-450 (nmol)	Specific cytochrome P-450 content (nmol/mg)	G10H activity (pkat)	Specific activity (pkat/mg)	Purification (-fold)	Recovery (%)
20 000*g* Membrane fraction	1891.2	483.5	0.26	154 668.6	81.8	1.00	100.00
Cholate solubiition	600.6	61.1	0.10	5978.0	10.0	0.12	3.87
DEAE-Sephacel	55.1	38.8	0.70	358.1	6.5	0.08	0.23
Hydroxyapatite Ultrogel	15.7	34.5	2.20	303.6	19.3	0.24	0.20
o-Aminooctylagarose	6.1	17.8	2.92	308.6	50.6	0.62	0.20
TSK Phenyl-5PW	0.36	1.7	4.72	187.0	519.4	6.35	0.12

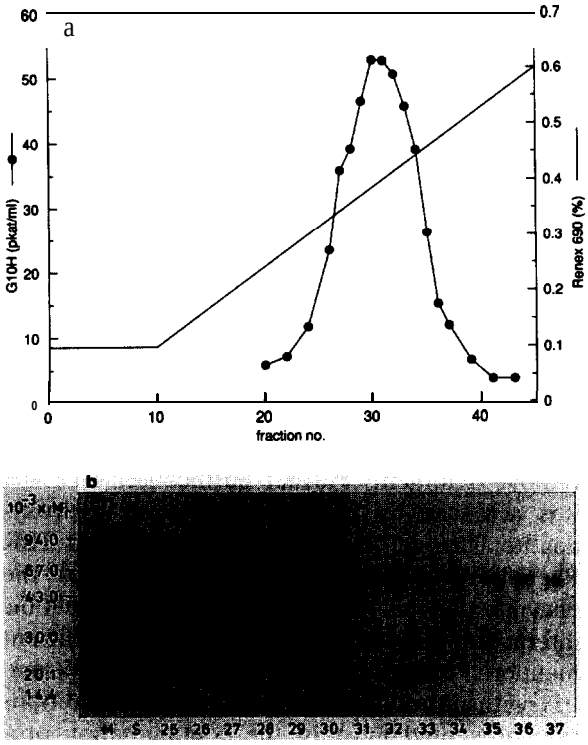

Fig. 3. TSK Phenyl-5PW chromatography of G10H. (a) Elution profile from TSK Phenyl-5PW. The column was equilibrated in 20 m*M* potassium phosphate buffer (pH 7.7) containing 15% (v/v) glycerol, 0.1% (v/v) Renex 690, 1 m*M* EDTA and 0.1 m*M* DTT. Elution was with 10 ml of equilibration buffer, followed by a linear gradient from 0.1 to 0.6% (v/v) Renex 690 in 35 ml of equilibration buffer. The fraction size was 1 ml. (b) SDS-PAGE of fractions from TSK Phenyl-5PW. SDS-PAGE was performed with a PhastSystem in PhastGel homogeneous medium 12.5. Detection was with silver staining. M = low-molecular-mass markers; S = sample applied on TSK Phenyl-5PW; 25–37 = fractions from TSK Phenyl-5PW [elution profile in (a)]; fractions 31-34 were pooled.

The four homogeneous fractions were pooled. The purification scheme is given in Table I. N-Terminal sequence analysis indicated that the M_r 56 000 protein band consisted of a single polypeptide. A partial amino acid sequence of sixteen residues starting with methionine was obtained. The sequence consisted of hydrophobic residues, which is in agreement with a function as membrane insertion signal peptide.

In the crude G10H membrane preparation at least one other cytochrome P-450 species is present, *trans*-cinnamate 4-hydroxylase. In the G10H pool from DEAE-Sephacel *trans*-cinnamate 4-hydroxylase activity could be measured after reconstitution with NADPH:cytochrome P-450 reductase and lipid by the same method as used for G10H. Analogous with G10H activity, a low *trans*-cinnamate Chydroxylase activity was realised in the reconstituted system. On hydroxyapatite Ultrogel and o-aminooctylagarose, *trans*-cinnamate 4-hydroxylase co-purified with G10H, with high recovery. The G10H pool from TSK Phenyl-5PW, however, was free of *trans*-cinnamate Chydroxylase activity. We verified that the high concentrations of Renex 690 present in TSK Phenyl-5PW fractions do not interfere with reconstitution of *trans*-cinnamate Chydroxylase activity. Possibly this enzyme is bound more tightly to TSK Phenyl-5PW than G10H, as the activity was also not detected in the other fractions, eluting between 0.1 and 0.6% of the Renex 690 gradient, or in the unbound fraction.

The specific activity of the purified G10H for geraniol was 519 pkat/mg, measured in the reconstituted system. The purified enzyme also accepted nerol, the *cis* isomer of geraniol, as a substrate. Conversion of the structurally related C_{15} and C_{30} terpenoids farnesol and squalene under the standard assay procedures was not observed. Licht et *al.* [34] reported that no hydroxylation of the pyrophosphates of geraniol or nerol was observed with G10H from both C. *roseus* or animal membrane preparations. We tested geraniol monophosphate and geraniol pyrophosphate as substrates for crude G10H and observed no hydroxylation of these compounds.

The purified G10H had a specific P-450 content of 4.7 nmol/mg protein, which was much higher than that for the homogeneous 3,9-dihydropterocarpan 6a-hydroxylase from *Glycine mux* (*0.64* nmol/mg protein) [14], but lower than that for purified cytochrome P-450 from *Tulipa gesneriana* (*6.7* nmol/mg protein) [13] and *Persea americana* (*17.5* nmol/mg protein) [10]. Specific contents reported for highly purified cytochrome P-450 isoforms from animal sources vary between cu. 4 and 18 nmol/mg protein [35]. The specific content can show slight variations between individual purifications, owing to differences in the loss of haeme. A CO-difference

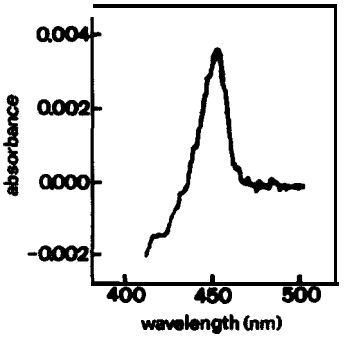

Fig. 4. CO-difference spectrum of purified G10H. A sample of the pooled G10H-containing fractions from TSK Phenyl-5PW was diluted to 8.2 μg/ml in 20 m*M* potassium phosphate buffer (pH 7.7) containing 15% (v/v) glycerol and a difference spectrum (dithionite-reduced *versus* dithionite-reduced, CO-saturated) was measured.

spectrum of the purified enzyme preparation is shown in Fig. 4.

The described purification procedure was reproducible. In terms of enzyme activity the recovery was low, but probably this was due to incomplete removal of inhibitory detergents or a less efficient interaction between NADPH:cytochrome P-450 reductase and cytochrome P-450 in the reconstituted system. Despite the low yield of enzyme activity, the amounts of purified G10H protein obtained from C. *roseus* cultures grown on alkaloid production medium were relatively high for a membrane-bound plant enzyme. The purification of cytochrome P-450-dependent G10H is the first step towards cloning of the corresponding gene, which will permit further studies concerning the regulation of this enzyme.

CONCLUSIONS

The membrane-bound cytochrome P-450 enzyme geraniol 10-hydroxylase (G10H) was purified from *Catharanthus roseus* cell cultures by an efficient procedure consisting of four chromatographic steps. The purified enzyme had an M_r of 56 000, a specific cytochrome P-450 content of 4.7 nmol/mg and catalysed the hydroxylation of both geraniol and nerol. The N-terminal amino acid residues of the purified protein were hydrophobic and may serve as a membrane anchor. As cytochrome P-450 enzymes in plants are present at low levels, an important step in G10H purification was the selection of cell cultures from which membrane preparations with a high G10H specific activity could be obtained. C. *roseus* cells grown on alkaloid production medium were identified as a suitable enzyme source. After cholate solubilization an almost complete separation of cytochrome P-450 and NADPH:cytochrome P-450 reductase was achieved by ion-exchange chromatography on DEAE-Sephacel. Reconstitution of G10H activity by the addition of the reductase and a C. *roseus* lipid extract was found to occur most efficient when Extracti-gel D was used to remove detergent. Further purification of G10H was achieved by chromatography on hydroxyapatite Ultrogel, o-aminooctylagarose and TSK Phenyl-5PW. The hydrophobic nature of the enzyme allowed the application of hydrophobic interaction chromatography in a highly efficient manner. Presaturation of TSK Phenyl-5PW matrix with non-ionic detergent created conditions that prevented most of the contaminating proteins in the sample from binding, while G10H was still retained on the column. Subsequent elution of G10H by further increasing the detergent concentration resulted in an electrophoretically homogeneous protein preparation. Application of this method may be useful for the purification of other membrane-bound enzymes.

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