An Assay for Secologanin in Plant Tissues Based on Enzymatic Conversion into Strictosidine

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The secoiridoid glucoside secologanin is the terpenoid building block in the biosynthesis of terpenoid indole alkaloids. A method for its determination in plant tissues and in cell suspension cultures has been developed. This assay is based on the condensation of secologanin with tryptamine, yielding strictosidine, in a reaction catalysed by the enzyme strictosidine synthase (STR; E.C. 4.3.3.2). Subsequently, the formation of strictosidine is quantified by high performance liquid chromatography (HPLC). STR was isolated from transgenic *Nicotiana tabacum* cells expressing a cDNA-derived gene coding for STR from *Catharanthus roseus*. The high specificity of STR for secologanin, in combination with a sensitive and selective HPLC system, allows a simple extraction of secologanin from plant tissue. The detection limit of this method is 15 ng secologanin. Using this assay, secologanin contents were determined in tissues of various plant species; *Lonicera xylosteum* hairy roots were found to contain 1% of secologanin on a dry weight basis. © 1998 John Wiley & Sons, Ltd.

INTRODUCTION

In the biosynthesis of plant terpenoid indole alkaloids, the secoiridoid glucoside secologanin is condensed with tryptamine in a stereo-specific reaction catalysed by the enzyme strictosidine synthase (STR; E.C. 4.3.3.2). The product, strictosidine, constitutes the universal precursor of at least 3000 indole and quinoline alkaloids. STR has been well characterized from some Apocynaceous and Rubiaceous plants and its molecular properties have been the subject of a number of reviews (Kutchan *et al.*, 1991; Kutchan, 1993; Hashimoto and Yamada, 1994; Scott, 1994; Verpoorte *et al.*, 1997).

In the course of our studies on the bottlenecks hampering terpenoid indole alkaloid accumulation (Verpoorte *et al.*, 1991), we found that the biosynthesis of iridoids represents a limiting factor in the accumulation of alkaloids in some cell cultures of *Catharanthus roseus.* Feeding of loganin or secologanin to the cultures strongly increased alkaloid (strictosidine and ajmalicine) production (Moreno *et al.*, 1993). Other laboratories have made similar observations (Zenk *et al.*, 1977; Mérillon *et al.*, 1986; Stafford and Smith, 1986; Naudascher *et al.*, 1989).

For such experiments, secologanin (which, unlike loganin, is not commercially available) can be isolated from leaves of *Lonicera* species (Kinast and Tietze, 1976; Hermans-Lokkerbol and Verpoorte, 1987), from berries of *Symphoricarpos albus* (Tietze *et al.*, 1986) or from leaves of *Weigela* hybrids (Jensen *et al.*, 1975).

The secologanin content of plant tissues have been determined by gas chromatography after acetylation or silylation (Inouye *et al.*, 1976; Naudascher *et al.*, 1989), by high performance liquid chromatography (HPLC) (Dabiné Lengyel *et al.*, 1986; Dagnino *et al.*, 1996) and by radioimmunoassay (Deus-Neumann and Zenk 1984). In addition to a method for the determination of loganin, secologanin, tryptophan and tryptamine concentrations in plant cell cultures (Dagnino *et al.*, 1996), we present here an assay specifically for secologanin. This method allows the use of a standard UV detector (280 nm) instead of a photodiode array detector, short chromatography run times and a less aggressive eluent. Furthermore, the use of an aqueous extraction reduces the risk of artefact formation particularly since alcoholic extraction may result in the formation of acetalic iridoids (Tomassini *et al.* 1995).

Our studies on *C. roseus* have resulted in the development of an HPLC assay for STR (Pennings *et al.*, 1989) and cDNA cloning of the gene coding for STR (Pasquali *et al.*, 1992). These achievements provided the basis for an enzymatic HPLC assay. After extraction and addition of tryptamine, secologanin was enzymatically converted into strictosidine, in a reaction catalysed by partially purified STR from a transgenic *Nicotiana*
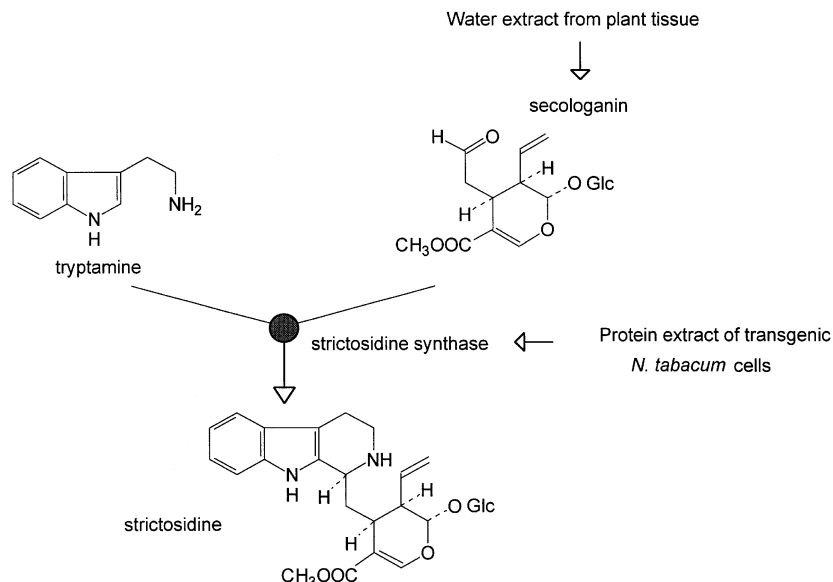
Figure 1. The reaction catalysed by strictosidine synthase is shown together with the principle of the secologanin assay and the origin of secologanin and strictosidine synthase.

tabacum cell suspension culture expressing *C. roseus str* cDNA (Fig. 1). Strictosidine formation was quantified by HPLC. This assay was applied to the determination of secologanin content in samples from various plant species belonging to the Caprifoliaceae, Apocynaceae, Rubiaceae, Nyssaceae and Loganiaceae families known to contain secologanin (Jensen, 1991).

EXPERIMENTAL

Plant materials. Apocynaceae: *Catharanthus roseus* — obtained as seed from Botanical Garden (Boedakalesk, Hungary) with herbarium reference 890171; used as cell cultures A12A2 (Moreno *et al.*, 1993) and 11CR58/9 (Dagnino *et al.*, 1996) and also as hairy root cultures (Hoekstra, 1993); Caprifoliaceae: *Lonicera xylosteum* — obtained as seed from Horticenter (Boskoop, The Netherlands) with herbarium reference 940081, used as hairy root cultures (Hallard *et al.*, 1997); *Symphoricarpos albus 'Laevigatus'* — purchased as small shrubs from Horticenter with herbarium reference 810224; *Weigela 'Styriaca'* — obtained from public plantings in the Gorlaeus laboratory area and probably purchased from Horticenter in 1990 (herbarium specimen available). *Weigela* cultures were initiated from disinfected leaves and cultured on Gamborg B_5 medium supplemented with 2,4-D (1 mg/L), kinetin (0.2 mg/L) and sucrose (30 g/L). Loganiaceae: *Strychnos potatorum* — purchased as seed from Silverhill Seeds (Kenilworth, Republic of South Africa) with herbarium reference 940025; Nyssaceae: *Camptotheca acuminata* — received as seed from Amsterdamsche Chinine Fabriek (ACF, Maarssen, The Netherlands) with herbarium reference 970003; Rubiaceae: *Cinchona officinalis 'Ledgeriana'* used as hairy root culture (Hallard *et al.*, 1997).

Cell culture. The suspension culture of transgenic tobacco cells (cell line WTTG), expressing a *Cathar-*

anthus roseus cDNA clone coding for STR, was grown at 25°C in the light on LS medium (Linsmaier and Skoog, 1965) supplemented with 1-naphthalene acetic acid (2.0 mg/L), kinetin (0.2 mg/L) and sucrose (30 g/L) (Hallard *et al.*, 1997). Subculturing was performed every 7 days with a twofold dilution with fresh medium. The cells for the STR preparation were harvested 10 days after subculture from a 2 L flask containing 500 mL of medium. After harvesting, the cells were immediately frozen in liquid nitrogen and stored at −80°C.

Preparation of crude STR. Frozen cell material [450 g fresh weight (fwt); line WTTG] was powdered in a Waring blender. After the addition of polyvinylpolypyrrolidone (50 mg/g fwt) and 1 mL/g fwt of 2 mM ethylenediamine tetraacetic acid (EDTA) and 4 mM 1,4-dithiothreitol (DTT; freshly added) in 0.1 M sodium phosphate (pH 7; buffer A), the mixture was thawed and subsequently clarified by centrifugation (10,000 *g*; 30 min). From this crude extract an ammonium sulphate precipitate (30–55% saturation) was prepared and resuspended in 10 mL of buffer A. For desalting, the sample was applied to PD10 columns (Pharmacia, Uppsala, Sweden) and eluted with buffer A in the absence of DTT. This eluate (the crude STR fraction) was used for secologanin determination or stored at −80°C. Protein concentrations were assayed in duplicate by the method of Peterson (1977) using bovine serum albumin as standard; STR activity was assayed according to the method of Pennings *et al.* (1989).

Secologanin assay. Secologanin was initially purified from the leaves of *Weigela* hybrids (S.R. Jensen, unpublished results) and its identity and purity (>95%) were determined by proton nuclear magnetic resonance spectroscopy.

For the assay of secologanin in plant samples, the dry powder of the plant tissue (100 mg) was incubated with boiling water (2 mL) for 2 min in a 100°C water bath, and subsequently secologanin was extracted using an Ystral

homogeniser for 2–3 min. After centrifugation (5000 *g*; 20 min), the pellets were extracted again as described above. The supernatants were combined, adjusted to 5 mL in volume and assayed for secologanin as described below. For cell suspension cultures, the supernatant was concentrated by freeze drying and the residue was re-suspended in 200 μL of water and centrifuged before being submitted to the secologanin assay.

For the determination of secologanin concentrations, sample extracts (25 μL) were incubated with the crude STR fraction (>15 pkat; ca. 25 μL) together with 1 mM tryptamine in 0.1 mM sodium phosphate buffer (pH 6.8) containing 1 mM EDTA and 3 mM DTT in a total volume of 100 μL. All incubations were carried out in duplicate for 30 min at 30°C. The incubation was stopped by the addition of 5% trichloroacetic acid (100 μL): for each sample a control incubation was performed in which 5% trichloroacetic acid (100 μL) was added at zero time. For samples and controls, after the addition of internal standard (8 mM codeine hydrochloride; 20 μL) and centrifugation, the mixture (10 μL) was analysed using the HPLC system described by Pennings *et al.* (1989) with the difference that the eluent was modified to be 25 mM sodium phosphate in water: 7 mM sodium dodecyl sulphate in methanol (37:63).

RESULTS AND DISCUSSION

The assay of secologanin in plant tissues is based on the enzymatic condensation of secologanin and tryptamine to produce strictosidine, followed by quantification of this product by HPLC (Pennings *et al.*, 1989). Basically, the assay comprises three components:

(1) Preparation of strictosidine synthase

STR, the enzyme catalysing strictosidine formation, is suitable for this assay as it is specific and stable. STR is highly specific for both substrates (Treiner and Zenk, 1979; Pfitzner and Zenk, 1989), although at is also able to accept 9,10-dihydrosecologanin and 3'-*O*-methylsecologanin, both of which are unknown as natural products. Furthermore, it can be obtained in sufficient amounts by overexpressing gene constructs encoding STR in organisms of choice (Kutchan *et al.*, 1991).

The crude STR fraction obtained from STR transgenic tobacco cells typically has an STR activity of about 0.5–1 nkat/ml. The enzyme can be stored at −80°C for at least 1 year without appreciable loss of catalytic activity. It can also be lyophilized with little loss of activity. An advantage of isolating STR from *N. tabacum* cells over isolating it from *C. roseus* cells, is that the tobacco preparation is free from strictosidine glucosidase activity, which may interfere with the assay (see below).

(2) Extraction of secologanin

The specificity of STR and the selectivity of the HPLC system permit a simple purification procedure. Secologanin was extracted from plant tissues and cell cultures by two sequential extractions with hot water (> 90°C) which gave a reproducible recovery of > 90% (data not
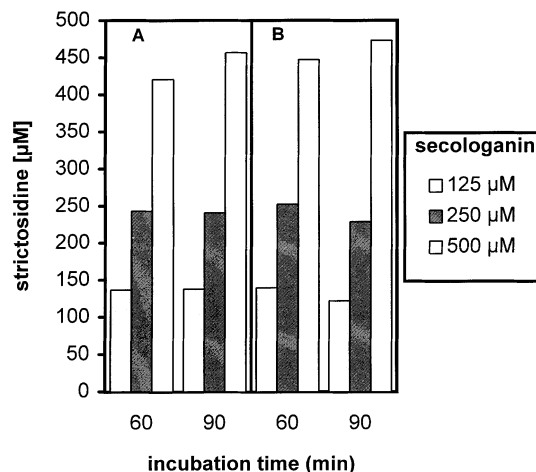

Figure 2. The formation of strictosidine catalysed by 15 pkatal strictosidine synthase in relation to the incubation time (60 or 90 min), the secologanin concentration (125, 250 or 500 μM) and the tryptamine concentration (**A** = 1 mM or **B** = 2 mM). (The values represent the averages of three measurements).

shown). This extract can be directly used for incubation with STR and tryptamine or it can be concentrated (by freeze-drying) to increase the sensitivity of the assay by name 25-fold. Secologanin was stable for at least 2 weeks in the water extract when stored at −80°C.

By extracting plant tissues with water proteins will also be recovered, and endogenous glucosidases may interfere with the assay since they reduce the accumulation of strictosidine and thus result in low estimations of the secologanin content. For example, strictosidine glucosidase from *C. roseus* is relatively stable at higher temperatures and is present in *C. roseus* in relatively high activity (Stevens *et al.*, 1992). For this reason samples were incubated in boiling water for 2 min and subsequently extracted with hot water to deactivate all catabolic enzymes.

(3) Incubation of secologanin with tryptamine and strictosidine synthase; quantification of strictosidine formation

For the quantitative enzymatic conversion of (extracted) secologanin to strictosidine, sufficient tryptamine and STR activity has to be supplemented to the incubation mixtures. The high activity of the STR preparation allows addition of at least 10 pkat of STR in relatively small volumes to the incubation mixtures. Theoretically, 10 pkat of STR is able to convert 54 nmol of secologanin and tryptamine during an incubation period of 90 min. Strictosidine formation was quantified by HPLC, after incubating 100 and 200 nmol tryptamine with 12.5, 25 and 50 nmol secologanin, for 60 and 90 min in the presence of 15 pkat STR (Fig. 2). Doubling of the tryptamine concentration did not result in significantly higher yields of strictosidine. In further assays tryptamine was therefore used at a concentration of 1 mM (100 nmol per incubation). Within the 60 min incubation period, 12.5 and 25 nmol of secologanin were quantitatively converted to strictosidine (Fig. 2). For the highest concentration of secologanin (50 nmol), incubation for 90 min resulted in a higher incorporation into strictosi-
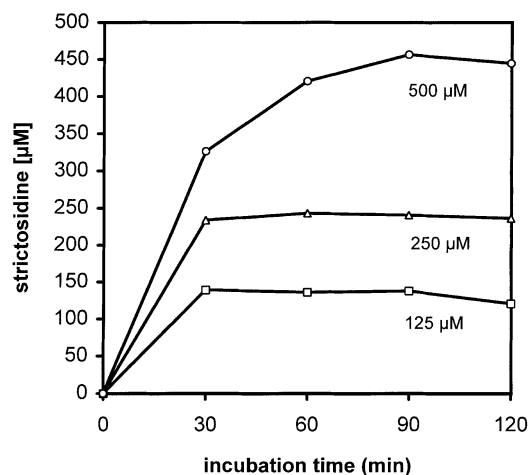
Figure 3. The time course of strictosidine formation catalysed by 24 pkatal strictosidine synthase incubated in the presence of 1 mM tryptamine (100 nmol/incubation) and three concentrations of secologanin (125, 250 or 500 μM). (The values represent the averages of three measurements).

Figure 4. Calibration curve of the secologanin assay, indicating the relationship between the formation of strictosidine and the amount of secologanin present in the incubation mixture. (Different concentrations of secologanin were incubated with 1 mM tryptamine and 15 pkatal strictosidine for 30 min at 30°C: the data show the average of six analyses).

dine, although 100% conversion was not reached (see below).

In a subsequent experiment, progress curves of strictosidine formation were determined for three concentrations of secologanin, 125, 250 and 500 μM (12.5, 25 and 50 nmol/incubation, respectively), incubated in the presence of 1 mM (100 nmol/incubation) tryptamine and 24 pkat STR (Fig. 3). After 30 min of incubation, secologanin present in 125 μM and 250 μM concentrations was quantitatively converted. For 500 μM secologanin, this required more than 90 minutes, but strictosidine appeared not to be completely stable under these conditions.

Based on the previous results, a calibration curve (Fig. 4) was made by incubating different secologanin concentrations with 1 mM (100 nmol) tryptamine and 15 pkat STR for 30 min. This yielded a linear relation for
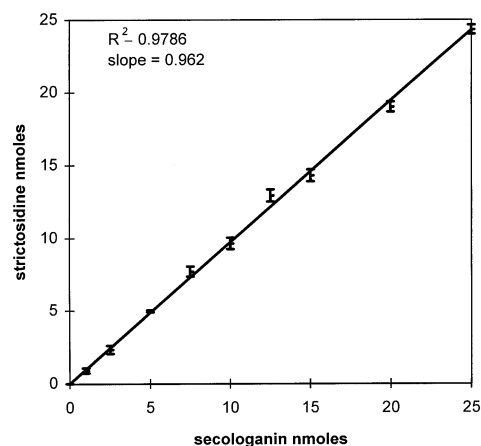
up to at least 25 nmol secologanin per sample. The slope of the mathematical function is close to 1 (0.962), indicating that all of the secologanin is converted to strictosidine (based on the recognition that secologanin was only 95% pure). Hence the reversible hydration of the aldehyde function of secologanin in aqueous solution has no influence on the conversion of secologanin to strictosidine. The detection limit of the assay as determined from a peak height of three times the noise level at 280 nm, was equivalent at 15 ng.

Determination of the secologanin content in various plant tissues

Samples collected from various plant species known to contain secologanin were analysed for their secologanin

Table 1. Secologanin content of tissues from various plant species

Family/species	Tissue	Secologanin content (mg/g dry weight)[a]
Apocynaceae		
Catharanthus roseus	leaf	3
	hairy root	0.04
	cell suspension (11CR58/9)	0.9–1.9
	cell suspension (A12A2)	n.d.[b]
Caprifoliaceae		
Lonicera xylosteum	leaf	13.5
	hairy root (dark)	2
	hairy root (light)	10
Weigela 'Styriaca'	leaf	7.6
	callus	0.03
	cell suspension	n.d.
Symphoricarpos albus	berries	2.1
Loganiaceae		
Strychnos potatorum	leaf of seedling	n.d.
Nyssaceae		
Camptotheca acuminata	leaf	0.66
Rubiaceae		
Cinchona officinalis L.	hairy root	0.02–0.05

[a] Average value ($n = 2$).
[b] n.d. - non-detected.

contents (Table 1). Owing to the selectivity of the HPLC system, which is based on ion pair reversed-phase chromatography and UV detection (Pennings *et al.*, 1989), in all cases clean chromatograms showing tryptamine, codeine and strictosidine were obtained (Fig. 5A). When analysing the 25-fold concentrated extracts, some additional peaks were detected but they did not interfere with the quantification (Fig. 5B). For strictosidine-accumulating plants, an appropriate blank must be performed (Fig. 5B).

As expected, secologanin was found in high concentrations in the leaves of *Lonicera* and *Weigela*. Furthermore, secologanin was detected in the hairy root culture of *L. xylosteum:* after 30 days of culture the roots accumulated 2 and 10 mg/g dry weight in the dark and light grown cultures, respectively (Table 1).

Secologanin could not be detected in *C. roseus* suspension cultured cells (A12A2) as expected (Moreno *et al.*, 1993). On the other hand, cell line (11CR58/9) accumulated 0.9–1.9 mg secologanin/g dry weight depending on culture age and inoculum density (A. Contin, unpublished results). Dagnino *et al.* (1996) reported a content of secologanin of 1.5 mg/g dry weight for this cell line which was explained by the inability of the line to accumulate tryptamine.

Secologanin is found in relatively large amounts in many plant species but little is known about its biological function. A protective function against organisms feeding on *W. 'Styriaca'* is suggested by the high concentrations found in young (and potentially vulnerable) tissues. For loganin, an antifeedant activity has been reported (Rimpler, 1991). Moreover, our recent results show the presence of an enzyme degrading secologanin in *Lonicera* and *Weigela* species. Present research focuses on degradation and may lead to a further understanding of the biological activity of secologanin.

In summary, the assay described allows a sensitive and highly selective determination of secologanin contents and will be useful in the studies on secologanin biosynthesis, and on the accumulation in plant tissues and cell suspension cultures of terpenoid indole alkaloid producing plants. Furthermore it could be used to screen plants for new sources of secologanin.

Acknowledgements

Figure 5. Chromatograms obtained from a determination of secologanin in **A:** *Weigela 'Styriaca'* leaf, and **B:** *Catharanthus roseus* cell suspension: in each case **A** shows a control incubation which was stopped at $t = 0$ min, whilst **B** shows the sample (30 min incubation). Peak identity: **1**-codeine (internal standard; $R_t = 4.4$ min), **2**-tryptamine ($R_t = 6.8$ min), **3**-strictosidine ($R_t = 10.8$ min). Detection at 280 nm.

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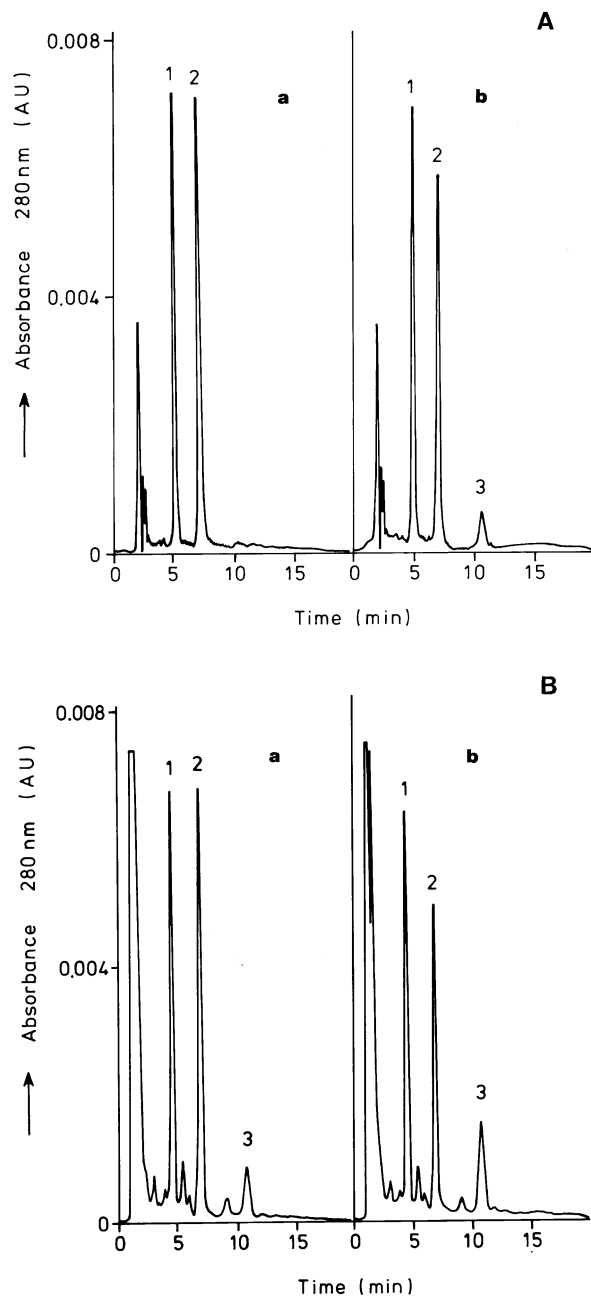
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