# Strictosidine synthase from *Catharanthus roseus*: purification and characterization of multiple forms

Anthony DE WAAL, Annemarie H. MEIJER and Robert VERPOORTE*

Division of Pharmacognosy, Leiden/Amsterdam Center for Drug Research, Leiden University, Gorlaeus Laboratories, P.O. Box 9502, 2300 RA Leiden, The Netherlands

Multiple (six) forms of strictosidine synthase from *Catharanthus roseus* cell suspension cultures were purified and characterized. A purification protocol is presented composed of hydrophobic-interaction, gel-permeation and ion-exchange chromatography and chromatofocusing. Four of six isoforms were purified to apparent homogeneity, whereas two others were nearly homogeneous. All strictosidine synthase isoforms were found to be glycoproteins. The isoforms were also found in leaves and roots of the plant, in seedlings and in hairy root cultures. The ratio of the different isoforms differed slightly between these sources. The kinetic parameters of the isoforms showed no significant differences. The maximal velocity (300–400 nkat/mg of protein) is the highest reported so far. It was demonstrated that the apparent Michaelis constant for tryptamine (approx. 9 $\mu$M) is much lower than values reported previously. The presence of weak product inhibition ($K_p$ approx. 35 times $K_m$) was established, whereas substrate inhibition was not detected.

## INTRODUCTION

The enzyme strictosidine synthase (EC 4.3.3.2.) catalyses the stereospecific condensation of the aldehyde function of the secoiridoid glucoside, secologanin, with the amine function of tryptamine, to form 3$\alpha$(*S*)-strictosidine, a key intermediate in the biosynthesis of monoterpenoid indole alkaloids (reviewed in refs. [1–3]). The enzyme is found in the plant families Apocynaceae [4] and Rubiaceae [5,6]. Purification of the strictosidine synthase from *Cinchona robusta* (Rubiaceae) has recently been reported [7]. Purification of strictosidine synthase from *Catharanthus roseus* was first described by Treimer and Zenk [8] and Mizukami et al. [9]. Neither group recognized the presence of isoforms. Later, the groups of Zenk [10,11] and ourselves [12] discovered that six or seven isoforms exist. There is a discrepancy in the reported values for the kinetic parameters of strictosidine synthase from *C. roseus*. The apparent Michaelis constant for tryptamine was reported by Mizukami et al. [9] to be 0.83 mM. In contrast, Zenk's group reported a value of 2.3 mM [8], and later values ranging from 0.9 to 6.6 mM for (partially) purified isoforms were reported [11]. In addition, only Zenk's group mentioned substrate inhibition by tryptamine at concentrations around the Michaelis constant [8,11].

Four isoforms were kinetically characterized by Zenk's group [11] and reported to possess different properties. Here we report on the purification and characterization of six strictosidine synthase isoforms. We demonstrate that all isoforms are kinetically indistinguishable and that the Michaelis constant for tryptamine is much lower than previously reported. A possible cause for the discrepancy is discussed.

The cDNA sequences have been reported for strictosidine synthase from *C. roseus* [13,14], and *Rauvolfia serpentina* [15], both members of the Apocynaceae. The enzyme from *C. roseus* was found to be encoded by a single-copy gene, suggesting that the isoforms are the result of post-translational modifications of a single precursor [14]. We investigated whether the isoforms were differentially glycosylated. We also studied whether the occurrence of isoforms can be related to mechanisms of developmental or tissue-specific regulation, thereby addressing the question of whether the presence of multiple forms of the enzyme has any physiological significance.

## MATERIALS AND METHODS

### Cultures

A cell line of *C. roseus* (L.) G. Don was grown as a cell suspension culture in 300 ml of LS medium [16], containing 2.0 mg/l naphthaleneacetic acid, 0.2 mg/l kinetin and 30 g/l sucrose, under constant light at 25 °C and shaking at 120 rev./min. The cultures were subcultured by 4-fold dilution in fresh medium every week. Cells were harvested at the end of the growth phase by suction through 70 $\mu$m nylon net, immediately frozen in liquid nitrogen, and kept at −80 °C until use.

### Plants and seedlings

*C. roseus* seeds (*Vinca rosea*, var. Morning Mist) were obtained from Kieft (Blokker, The Netherlands), and plants were grown in a greenhouse at 23 °C.

### Enzyme assay

The strictosidine synthase assay was a modification of the h.p.l.c. assay described previously [17]. For routine assay, incubation was at 30 °C in Eppendorf vials, in a volume of 100 $\mu$l, with final concentrations of 0.1 M sodium phosphate, pH 6.8, 1 mM tryptamine and 5 mM secologanin. For analysis of fractions obtained before strictosidine glucosidase was removed by gel-permeation chromatography, 100 mM D(+)-gluconic acid-$\delta$-lactone/100 mM Tris base was present during the incubation to inhibit the activity of the glucosidase. The reaction was stopped by placing the vial in ice/water, followed by the addition of 100 $\mu$l of ice-cold trichloroacetic acid (5%). Before injection into the h.p.l.c. system, 25 $\mu$l of internal standard (8 mM codeine hydrochloride) was added. The column (4 mm × 250 mm) was Lichrosorb RP-8 Select B with 7 $\mu$m particle size (Merck). A pre-column of the same type was used. The eluent consisted of methanol/water (68:32, v/v) containing 25 mM $NaH_2PO_4$ and 7 mM SDS. Strictosidine and codeine were detected by measuring

* To whom correspondence should be addressed.

absorbance at 280 nm. Flow rate was 1 ml/min. Peaks were integrated with an electronic integrator. Calibration of the tryptamine response of the detector, relative to codeine hydrochloride, was carried out using tryptamine and codeine hydrochloride standard solutions. The strictosidine response was determined enzymically with purified strictosidine synthase, by total conversion of a defined amount of tryptamine into strictosidine using an excess of secologanin.

For the recording of progress curves, the assay was slightly modified. The reaction volume was 400 or 500 $\mu$l and the secologanin concentration was raised to 10 mM. Tryptamine concentration was varied between 0.25 and 1.5 mM. Codeine, the internal standard for h.p.l.c., was present at 1.6 mM before the reaction was started by the addition of enzyme to the reaction mixture. The reactions were not stopped, but samples were directly injected on the h.p.l.c. system at intervals of approx. 4–5 min (depending on the elution time of the peaks) using a Gilson 232-401 autosampler, programmed for sampling the same vial repeatedly. Detection of tryptamine and codeine was by fluorescence measurement (excitation at 280 nm, emission at 354 nm). The enzyme concentration was chosen such that the tryptamine was used up after about 20–25 injections. The incubation mixture was kept at 30 °C by perfusing the aluminium rack (code 32; Gilson) of the autosampler with water from a thermostatically controlled water-bath.

A curve of enzyme activity as a function of pH was obtained by adjusting the buffer used in the standard assay. The buffer concentration was 100 mM, and the buffer composition was adapted to give different ranges of pH. For pH 2.75–6 sodium citrate/citric acid was used, for 6–8 sodium phosphate, for 8–9.25 Tris/HCl, and for 9.25–10 glycine/NaOH. Calibration of the buffers was performed at 25 °C. Steps of 0.25 pH unit were measured. Incubation time at 30 °C was 20 min.

### Substrates

Tryptamine hydrochloride was obtained from Serva. Secologanin was purified from the fruits of *Symphoricarpos albus*, modified as described by Tietze et al. [18] or from a preparation obtained from Aldrich. Both preparations were finally purified by column chromatography (silica gel 60; Merck), using step gradient elution with increasing percentage of acetone in ethyl acetate. The secologanin concentration was determined enzymically with purified strictosidine synthase as the amount of strictosidine generated from an excess of tryptamine. Pure strictosidine was enzymically prepared from secologanin and tryptamine on a column of immobilized strictosidine synthase, using a modification of the method of Pfitzner and Zenk [19]. The concentrations of tryptamine and secologanin were 5 mM and 7.5 mM respectively, thereby minimizing the tryptamine concentration in the eluate of the enzyme column. Strictosidine was purified from the eluate by acid/base extraction in ethyl acetate/water. The purity of secologanin and strictosidine was checked by t.l.c. and $^1$H-n.m.r.

### Protein assay

Protein was determined by the Bradford assay, using the Bio-Rad kit with the micro-assay protocol, and the supplied BSA as standard. Samples were measured in duplicate.

### Electrophoresis

Gel electrophoresis was performed with the Pharmacia Phastsystem, using PhastGel media. The gels were developed by staining with Coomassie Brilliant Blue and silver staining using Pharmacia reagents and protocols. Gels were photographed, and black-and-white prints were scanned with a CCD video camera, using the public domain program NIH Image 1.47 (written by Wayne Rasband, available from alw.nih.gov [128.231.128.251] by anonymous file-transfer protocol), on an Apple Macintosh computer. For the native silver-stained gel, the background resulting from local deposition of a film of silver on the gel surface was partially removed electronically, using NIH Image. Because of all the steps (staining, photographing, scanning and background reduction), the scans were regarded as semi-quantitative.

### Glycosylation

The lectin-based glycan-differentiation kit of Boehringer-Mannheim was used. SDS/PAGE [20] with a Mini-Protean II (Bio-Rad) was performed, followed by semidry electroblotting (MultiPhor; LKB) to Immobilon [poly(vinylidene difluoride): Millipore], for screening with *Galanthus nivalis* agglutinin from the kit. The enzyme was also directly applied by dot-blotting to Immobilon, to screen with the complete series of lectins: *Galanthus nivalis* agglutinin, *Sambucus nigra* agglutinin, *Maackia amurenis* agglutinin, peanut agglutinin and *Datura stramonium* agglutinin. The protocol supplied with the kit was followed [21].

### N-Terminal sequence analysis

The major isoforms, B and C (containing C1 and a small amount of C2), of a previous isolation were prepared for amino acid sequencing of the N-terminus, taking precautions to prevent modification of the protein. Protein samples (35 $\mu$g of B, 83 $\mu$g of C) were denatured in sample buffer for 20 min at 50 °C, and run in a 12 % (30:0.8) acrylamide gel [20], polymerized for 24 h, with a Mini-Protean II (Bio-Rad). In the upper-electrode buffer, 0.1 mM thioglycolic acid was included. The protein was transferred to Immobilon with semidry electroblotting with the LKB MultiPhor, using the discontinuous buffer system of the LKB documentation. Bands were visualized on the blot by staining for a few seconds with 0.1 % Amido Black in 50 % methanol/10 % acetic acid, and destaining with 40 % methanol/10 % acetic acid for a few minutes. Approximately two-thirds of the protein was transferred to the first sheet of Immobilon (the rest being trapped in subsequent layers of Immobilon). Bands, containing 216 pmol of B and 512 pmol of C, were excised, and gas-phase sequencing was performed at the Max Planck Institute for Molecular Genetics (Berlin, Germany).

### Enzyme purification

*C. roseus* cells stored at −80 °C (1.6 kg) were homogenized, after cooling with liquid nitrogen, in a Waring blender with stainless-steel bucket, for 1 min at full speed. The powder was stirred into homogenization buffer (30 °C) in a thin-walled glass vessel, at 2 g of powder/g of buffer. Final concentrations were 0.2 M Tris/HCl, pH 8.0, 3 mM EDTA, 7.5 $\mu$g/ml leupeptin, 6 mM dithiothreitol, and 1 mM phenylmethanesulphonyl fluoride. Preswollen polyvinylpolypyrrolidone (Sigma) was added (5 %, w/w) to bind potentially harmful polyphenols. The mixture was rapidly thawed in a 30 °C water-bath. Debris was removed by centrifugation for 40 min (11600 *g*) in a Sorvall GS-3 rotor) at 4 °C. The supernatant was brought to 35 % saturation with powdered $(NH_4)_2SO_4$, stirred for 30 min, and centrifuged for 30 min. The supernatant was made 50 % satd., stirred for 30 min, and centrifuged for 40 min. The pellets were suspended in a minimal amount of 50 mM Tris/HCl, pH 7.5. The resulting

solution (23 ml) was clarified by centrifugation at 10000 *g* in a Hermle swing-out rotor for 15 min. The solution was loaded on a phenyl-Sepharose CL-4B column (2.6 cm × 40 cm). Elution rate was 0.5 ml/min and fraction size 10 ml. Buffer A was 50 mM Tris/HCl, pH 7.5, plus 0.1 M $(NH_4)_2SO_4$. Buffer B was 50 mM Tris/HCl, pH 7.5, plus 5% (w/v) ethylene glycol. The gradient was 150 ml of buffer A, linear gradient of 100% buffer A to 100% buffer B in 300 ml, 300 ml of buffer B.

Strictosidine synthase peak fractions were pooled and concentrated by ultrafiltration (YM-10; Amicon). The concentrate (3.9 ml) was loaded on a Sephacryl S-200 HR column (2.6 cm × 93 cm). Elution buffer was 50 mM Tris/HCl, pH 7.5, at 0.5 ml/min, fraction size 10 ml. Peak fractions were pooled, and concentrated by ultrafiltration to 5 ml.

The concentrate was further fractionated by f.p.l.c., using an analytical Mono Q HR5/5 anion-exchange column. Optimal resolution and stability during chromatography were obtained at pH 6.5. Buffer A was 25 mM BisTris/HCl, pH 6.54, and buffer B was 25 mM BisTris/HCl, pH 6.45, plus 1 M KCl. Elution was at 1 ml/min, and fraction size was 0.5 ml. The gradient was 0–10% buffer B in 5 ml, to 20% buffer B in 40 ml, to 100% buffer B in 10 ml. The first two activity peaks were pooled into pool A. Subsequent peaks were individually collected in respectively pool B, C1, C2 (a shoulder in the major peak C1) and D.

All five preparations were individually subjected to chromatofocusing on a Mono P HR5/20 column, at 0.5 ml/min, fraction size 1 ml. The start buffer was 25 mM BisTris/HCl, pH 7.1, and the elution buffer was Polybuffer 74 [10% (v/v) in start buffer] adjusted to pH 4.0 with HCl. The pregradient was 3 ml of Polybuffer, before the sample was loaded. Elution was with 46 ml of Polybuffer. The pH in the fractions was measured manually with a microelectrode immediately after elution. Fractions were subsequently neutralized with 1 M BisTris/HCl, pH 7, to 50 mM final concentration. All runs were performed on the same day with the same batch of diluted Polybuffer to avoid variations. The drift of the pH-meter was of the order of 0.01 pH unit over the whole day. Peak fractions were pooled, and concentrated in Centricon 10 (Amicon) ultrafiltration units.

Protein elution was monitored in all chromatographic steps by measuring absorbance at 280 nm. All procedures were performed at 4 °C except f.p.l.c., which was performed at room temperature. All chromatography media, columns, Polybuffer 74, low-pressure chromatograph and the f.p.l.c. were obtained from Pharmacia.

## RESULTS

### Enzyme purification

Strictosidine synthase was isolated by extraction, $(NH_4)_2SO_4$ precipitation, hydrophobic-interaction chromatography, gel-permeation chromatography and anion-exchange chromatography. Peak fractions were fractionated separately, by chromatofocusing. The purification scheme is presented in Table 1.

The first chromatographic step, hydrophobic-interaction chromatography, combined high capacity and simultaneous purification and desalting after the $(NH_4)_2SO_4$ precipitation. The low recovery at this step was caused by loss of activity during the ultrafiltration of the fractions, as the pooled fractions before concentration still contained 192 nkat of strictosidine synthase.

The next step was gel-permeation chromatography on Sephacryl S-200 HR. We obtained a molecular mass for strictosidine synthase of 35–37 kDa, relative to the elution volumes of RNAase A, chymotrypsin, ovalbumin and BSA from the Pharmacia marker protein kit. This value is comparable with the values derived from both electrophoresis and cDNA sequencing [14].

At this stage of purification, the amount of total protein was only 3 mg, and therefore we used an analytical Mono Q f.p.l.c. column at high resolution for the nest step. In Figure 1, six isoforms are discernible (A1, A2, B, C1, C2 and D). From this single chromatogram, not all peaks appear to be significant, especially peak C2, which relies on one activity determination. On the other hand, the fractions were measured several times, and the separation was carried out in three independent isolations, and the same elution profile resulted each time. During previous isolations, isoforms A1 and A2 were well separated by chromatofocusing; therefore we pooled these peaks for the next separation. The shoulder (C2) on the fourth peak (C1), on the other hand, was shown previously not to be separated from the main peak on chromatofocusing of the mixture, and was therefore carefully kept apart for an individual chromatofocusing run. The results of all chromatofocusing runs on Mono P are shown in Figure 2. This Figure shows that the use of chromatofocusing without anion-exchange chromatography would have resolved only four isoforms. Isoforms A2 and B were eluted at the same pH, and also C1 and C2. It is tempting to speculate that the four isoforms (I to IV) of ref. [11] are respectively A1, (A2 + B), (C1 + C2) and D. Unfortunately, the present isoforms were eluted at significantly higher pH (half a unit) than those in ref. [11], preventing unambiguous assignment of names. To avoid confusion, we decided therefore not to adopt the roman-numeral nomenclature.

### Analysis of isoforms by electrophoresis

The purity of the pooled Mono P fractions was assessed by SDS/PAGE, using a 12.5% PhastGel developed by silver staining (not shown). Only preparation A2 showed a faint extra band around 43 kDa whereas the rest appeared homogeneous. The molecular mass is estimated as 35 kDa. Typically, we observed that the isoforms that were eluted first from an anion-exchange column have a slightly higher molecular mass (approx. 0.5 kDa). This difference is so small that it can only be seen between adjacent bands. Electrophoresis of mixtures of isoforms revealed that isoforms A1 and A2 have a higher molecular mass than all others (not shown).

Densitometric records of non-denaturing (native) PAGE in 20% homogeneous Phastgel media (Figure 3a) and isoelectric focusing (Figure 3b) are shown. Native PAGE reveals that the isoforms are not as homogeneous as appeared from SDS/PAGE. In particular, the extra band in preparation A2 was now also seen in A1, at $R_F$ approx. 0.6. The strictosidine synthase peaks are situated close to the anode (at $R_F$ approx 0.6–0.8). The broad peaks other than the strictosidine synthase peaks are caused by silver deposition on the gel surface, not by protein in the gel. The enzyme fractions eluted last had the greatest mobility towards the anode. This may correlate with the net charge of the isoforms. Interestingly, isoforms A1 and A2 have a lower pI (Figure 3b) than expected from their behaviour on Mono Q and in native PAGE. Also, the order of elution from Mono P did not strictly correlate with the pI, A2 again being a clear exception with too low a pI. Finally, A2 and B were eluted in the same fraction from Mono P, while behaving differently on native PAGE, Mono Q and isoelectric focusing. The same was observed for C1 and C2. This may be explained by assuming differing conformations of the various isoforms. It is unlikely that the 0.5 kDa reflects the presence or absence of the signal sequence, which is 31 amino acid residues in length [13,14] and therefore would give rise to a difference of around 3 kDa.

**Table 1 Purification of strictosidine synthase from 1.6 kg (wet weight) of *C. roseus* cells**

| Purification step | Volume (ml) | Protein (mg) | Strictosidine synthase (nkat) | Specific activity (nkat/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|---|
| Cell-free extract | 1355 | 2352 | 310 | 0.13 | 1 | 100 |
| 35–50% $(NH_4)_2SO_4$ | 23.0 | 727 | 245 | 0.34 | 2.6 | 79.2 |
| Phenyl-Sepharose CL-4B | 3.9 | 33.3 | 128 | 3.8 | 29.1 | 41.2 |
| Sephacryl S-200 | 5.0 | 3.0 | 153 | 51.0 | 387 | 49.4 |
| Pool A Mono Q | 0.61 | 0.18 | 14.5 | 80.1 | 607 | 4.7 |
| Pool B Mono Q | 0.62 | 0.23 | 14.3 | 61.7 | 468 | 4.6 |
| Pool C1 Mono Q | 0.70 | 0.23 | 55.6 | 245 | 1858 | 17.9 |
| Pool C2 Mono Q | 0.61 | 0.11 | 10.9 | 96.8 | 735 | 3.5 |
| Pool D Mono Q | 0.63 | 0.15 | 17.4 | 113 | 861 | 5.6 |
| Mono Q total | 3.17 | 0.91 | 103 | 114 | 864 | 36.4 |
| A1 Mono P | 0.28 | 0.012 | 5.3 | 439 | 3332 | 1.7 |
| A2 Mono P | 0.26 | 0.023 | 11.2 | 496 | 3763 | 3.6 |
| B Mono P | 0.27 | 0.043 | 26.5 | 617 | 4685 | 8.5 |
| C1 Mono P | 0.29 | 0.100 | 54.7 | 546 | 4142 | 17.6 |
| C2 Mono P | 0.31 | 0.020 | 7.5 | 383 | 2903 | 2.4 |
| D Mono P | 0.39 | 0.018 | 7.9 | 453 | 3434 | 2.6 |
| Mono P total | 1.79 | 0.215 | 113.0 | 527 | 3995 | 36.5 |

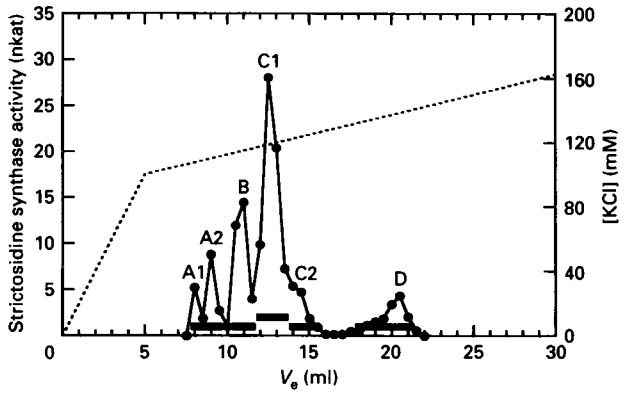


**Figure 1 Anion-exchange chromatography of strictosidine synthase on Mono Q**

A pool of strictosidine synthase isoforms obtained by $(NH_4)_2SO_4$ precipitation, hydrophobic-interaction chromatography and gel-permeation chromatography was fractionated by anion-exchange chromatography. The bars denote the pooled fractions, which were named as indicated in the Figure, except for fractions A1 and A2 which pooled as A. ·····, [KCl]; ●, strictosidine synthase activity.

The mobility differences in native PAGE were only observed in 20% homogeneous PhastGel media. In 12.5% gels, the strictosidine synthase travelled with the front ($R_F$ = 1) (not shown).

### Physiological significance and distribution of isoforms

We were interested in the physiological significance of the presence of isoforms. It has already been reported that four isoforms are present in both cell cultures and leaves of *C. roseus* [1]. We wanted to extend these studies to different organs of the plant and different developmental stages. To this end we subjected a number of tissues to part of the isolation procedure described above. Dialysis was used for desalting instead of hydrophobic-interaction chromatography. The experiment was divided into two sets, each with a cell suspension culture as a reference. In Figure 4 the results of the comparison of cells, roots and leaves are shown, and in Figure 5 cells, hairy roots and seedlings are compared. It is clear that all isoforms of strictosidine are present in all sources tested. Isoform C2 appears to be more abundant in roots and leaves than in seedlings and cultures. For a more detailed investigation of isoform C2 it would therefore be more appropriate to use whole plants than cell suspension cultures as the source. It can also be seen in Figures 4 and 5 that isoform C1 is most abundant in cultured cells, especially in the cell suspensions. In the other sources, isoform B is the most abundant. There is also some variation between the two experiments with cell suspensions in the relative abundance of isoforms, caused by biological variation or inaccuracy in the determination of enzyme activity in the column fractions.

In conclusion, we have shown that the presence of isoforms is not the result of the culture conditions, such as cells becoming polyploid or modification of enzymes in cultured cells that are dead or dying. The isoforms are present in leaves and roots of plants, as well as in seedlings. The difference in relative abundance between roots and leaves of differentiated plants is small.

### Glycosylation

As the strictosidine synthase gene is present as a single copy in the haploid genome [14], there is one primary product. In the case of allelic variation there could be more primary products, but analysis of cDNA clones and genomic clones does not support this. As a consequence, it is likely that the isoforms are the result of post-translational modification(s). As strictosidine synthase is a vacuolar enzyme [22,23], glycosylation will play a role in intracellular routing. Therefore we investigated the extent and type of glycosylation of all isoforms.

All strictosidine synthase isoforms reacted with *G. nivalis* agglutinin, indicating terminally bound mannose(s) (not shown). Not all isoforms reacted to the same extent. In the dot-blots, A1, C2 and D in particular reacted, although more enzyme was present in the form of B and C1. With electroblots from SDS/PAGE, all bands showed up when developed with *G. nivalis* agglutinin. In this case only A2 reacted significantly less than the other isoforms. This may reflect (in)accessibility of the sugar residues, as the dot-blots contained the native enzyme

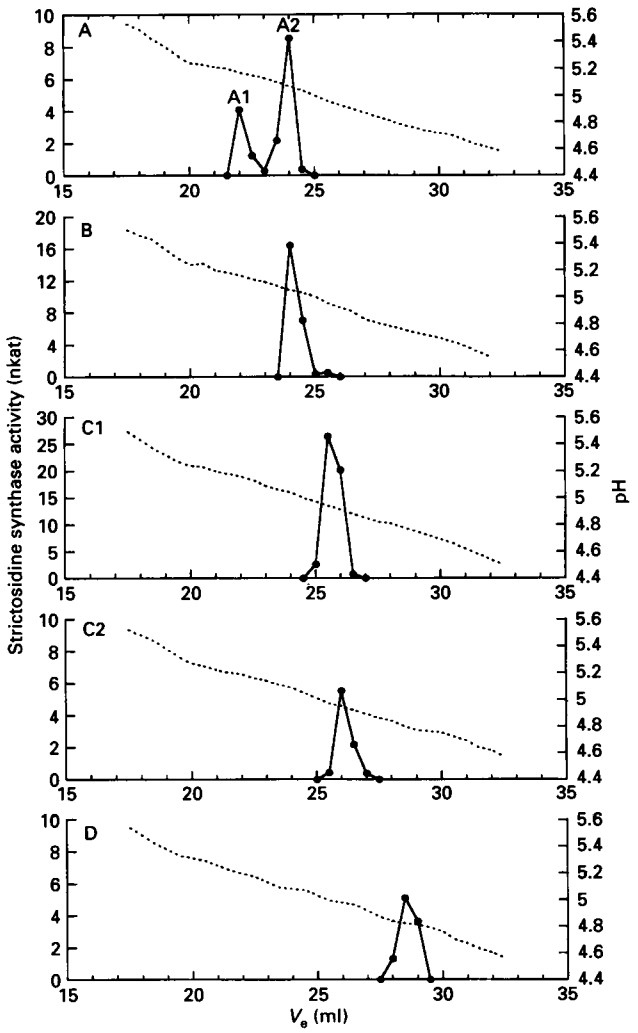


**Figure 2 Chromatofocusing of strictosidine synthase isoforms on Mono P**

Pools of strictosidine synthase peak fractions from Mono Q (see Figure 1) were individually fractionated by chromatofocusing. The designation of the pool loaded is given in the top left corner of each chromatogram. The peaks eluted were named after the fraction loaded, except for the fractions originating from pool A, which were named A1 and A2 in order of elution.

protein. All other lectins tested showed no response. This may mean the absence or inaccessibility of the sugar or lack of sensitivity. Reaction with *G. nivalis* agglutinin indicates the presence of terminally bound mannose(s), as in high-mannose or hybrid-type oligosaccharides. This could not be confirmed, however, because preincubation with endoglycosidase H had no effect on the mobility on SDS/PAGE (not shown). Again, inaccessibility of the sugar to the glycosidase or lack of sensitivity in the detection of a minor mobility change may explain the negative result. Alternatively, the presence of an O-linked mannose, such as in yeast or unusual oligosaccharides, is unlikely but not excluded. When the strictosidine synthase protein follows the general route of transport in plants (reviewed in ref. [24]), the glycosylation pattern probably does not determine the signal for the vacuolar localization. The glycosylation of isoforms more probably reflects the stages of glycosylation. Within the resolving power of these experiments, the isoforms are indistinguishable.

## N-terminal sequence analysis

The major isoforms, B and C1, were compared. Combining the two sequencing runs, the cDNA sequence could be confirmed up to 21 amino acids [14]. Amino acid residue 1 of isoform B was not sequenced because of a technical problem. Residues 2–9 were identical in the two isoforms. Thereafter the sequence data were ambiguous, apparently because of cleavage of the protein between Lys-5 and Lys-6 during the sequencing. The consequential presence of a second N-terminus gave rise to two amino acid residues per sequencing cycle. We can therefore only conclude that there are no major differences, such as proteolytic processing of the N-terminus, between isoforms B and C1. Point mutations cannot be excluded from this experiment, but are unlikely, taking into account the fact that the gene is present as a single copy [14]. The other isoforms were not obtained in sufficient amounts to be analysed by amino acid sequencing.

## Enzyme activity as a function of pH

The relationship between pH and strictosidine synthase activity has been published [8]. A sharp optimum was found at pH 6.8. At the low pH side of the curve, 50% and 5% activity was at pH 5 and 4 respectively. At the high pH side, 50% and 5% activity was at pH 7.5 and 8.2 respectively.

We measured the pH optimum of isoform B, and found a different, more symmetrical, type of curve. The activity was highest between pH 6 and 7.5, and almost pH-independent in between. At the low pH side of the curve, 50% and 5% activity was at pH 4.5 and 3.2 respectively. At the high pH side, 50% and 5% activity was at pH 9.2 and 10.0 respectively.

The discrepancy with the published curve is probably caused by the contribution of pH-dependent denaturation, which we minimized by incubation at 30 °C and reducing incubation time to 20 min, instead of 35 °C and 45 min [8]. In addition we used homogeneous enzyme protein, reducing the risk of proteolysis during incubation.

## Stability

The stability of strictosidine synthase was found to decrease when the enzyme was very highly diluted. As it was necessary to dilute the enzyme extensively in order to record progress curves, we needed to improve the thermal stability. We checked whether the addition of various compounds to partially purified enzyme diluted 1000-fold in 50 mM Tris/HCl (pH 7.0) improved the stability during incubation at 30 °C for 60 min. The addition of 1 mM secologanin or tryptamine, 250 mM glycine and 1 M glycerol had no or little effect. Sucrose at 1 M protected against inactivation. BSA at 1 mg/ml improved the long-term stability by a factor of at least 2 (not shown). Moreover, BSA caused an almost 2-fold stimulation of activity compared with the control without additions, presumably by reversing previous inactivation. Therefore for enzyme kinetic experiments we diluted the enzyme in 50 mM Tris/HCl, pH 7, containing 1 mg/ml BSA.

## Kinetics

The tryptamine concentration of 1 mM routinely used in our enzyme assay was chosen on the basis of the Michaelis constant and substrate inhibition constant reported by others [8,11]. It was considered to be a compromise between non-linearity because of operating in the range below the Michaelis constant and loss of sensitivity as the result of substrate inhibition. In practice, the assay was found to be remarkably linear. No substrate inhibition was ever observed, up to 5 mM tryptamine. On the other hand, when the tryptamine was nearly exhausted, we could still see

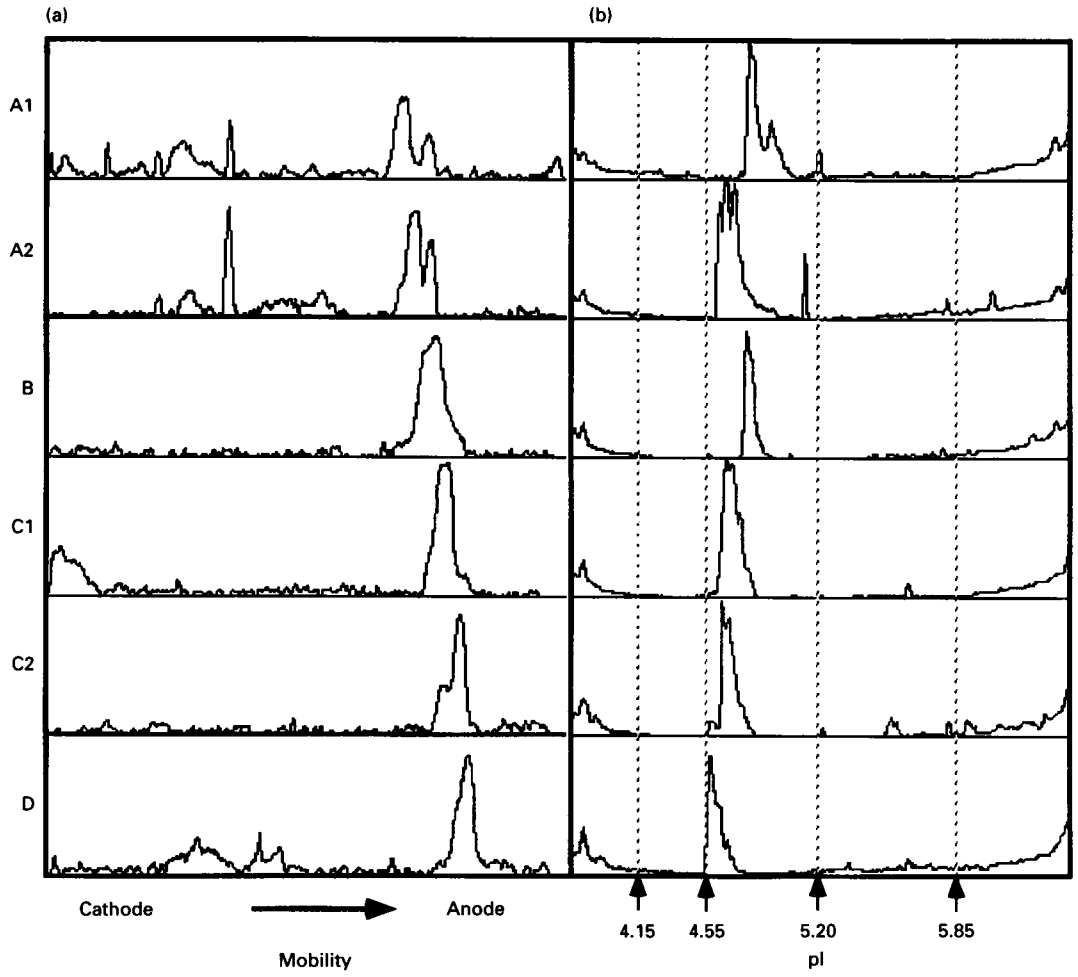


**Figure 3 Densitograms of (a) native PAGE and (b) isoelectric focusing of strictosidine synthase isoforms**

Pooled peak fractions from chromatofocusing were applied to a 20% homogeneous PhastGel with non-denaturing bufferstrips (**a**), and to PhastGel 4–6.5 isoelectrofocusing gel (**b**). The amount (pkat) of enzyme applied to the gels (PAGE; isoelectric focusing) was: A1 (19.2; 5.7), A2 (23.8; 12.9), B (29.1; 6.0), C1 (23.9; 11.5), C2 (15.2; 7.2), D (14.5; 6.1). The left and right ends of the traces in (**a**) indicate the beginning of the separating gel and the front respectively. The pI indicated in (**b**) (arrows with dotted lines) is the position of marker proteins from the low-pI marker kit from Pharmacia. The density is in arbitrary units, and only approximately linearly related to the true protein concentration in the gel.

significant activity. We could observe this phenomenon because both substrate and product concentration are measured in the h.p.l.c. assay. Clearly, our observations were not compatible with literature data, and this prompted us to investigate the kinetic properties of the enzyme as we had isolated it.

Although the stopped enzyme assay is useful for rapid screening purposes, it has a drawback when accurate data for kinetic analysis must be obtained. To detect either the decrease in substrate concentration or the increase in product concentration, a significant amount of substrate must be converted. As a consequence, it is not possible to determine accurately the Michaelis constant with the stopped type of assay, especially if product can react back or inhibit the reaction. To overcome this limitation we modified the assay to enable construction of a reaction progress curve. We increased the volume of the reaction mixture, and measured portions at regular time intervals by direct injection into the h.p.l.c. system. To remove the random experimental error associated with the pipetting of the internal standard, it was added before the start of the reaction. This was possible because codeine hydrochloride was proved not to influence the strictosidine synthase reaction (not shown). Now only the (small) random error of the h.p.l.c. system remained.

Pipetting error in the addition of the enzyme and substrates does not show up as random error in a progress curve, but introduces bias in estimated parameters. This makes it necessary to perform a number of independent measurements, although determination of the Michaelis constant and maximal velocity is possible from one incubation. We used the method of Yun and Suelter [25] to extract velocity–substrate concentration pairs of data, which can be analysed as initial-velocity data, e.g. with a Lineweaver–Burk plot. The results we obtained using this approach indeed pointed to a $K_m$ for tryptamine in the low micromolar range: below 10 $\mu$M for a curve that had a starting concentration of 250 $\mu$M.

It is advisable to make progress curves at several initial substrate concentrations to detect the presence of deviations from simple Michaelis–Menten kinetics. Indeed we found that doubling the initial tryptamine concentration led to almost a doubling of the apparent $K_m$ for tryptamine, whereas $V_{max.}$ remained approximately the same. This is an indication of

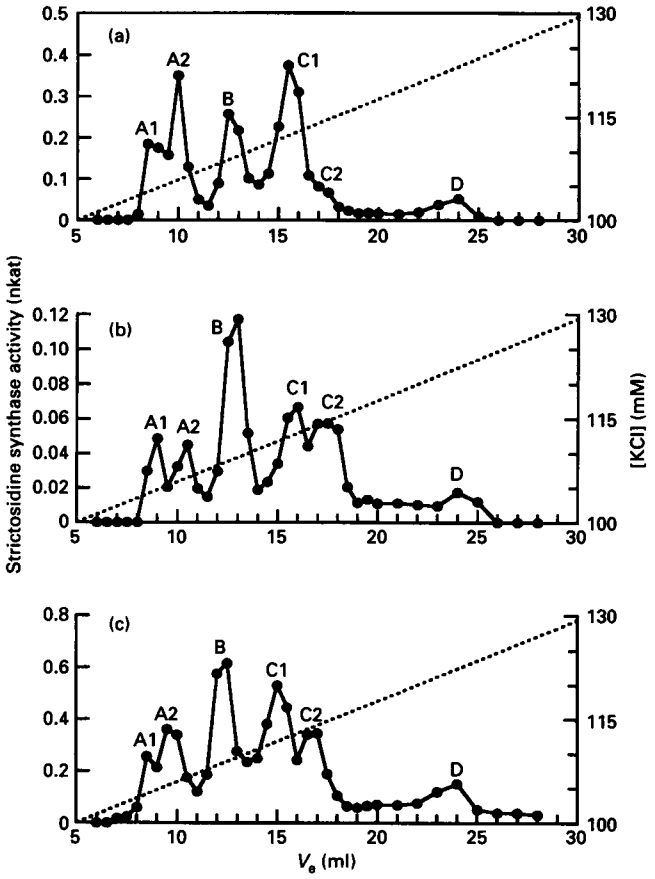


**Figure 4 Distribution of strictosidine synthase isoforms in (a) cell culture, (b) roots and (c) leaves of *C. roseus***

A pool of strictosidine synthase isoforms obtained by $(NH_4)_2SO_4$ precipitation and gel-permeation chromatography was fractionated by anion-exchange chromatography. ·····, [KCl]; ●, strictosidine synthase activity. Starting material, in wet weight of source [nkat after $(NH_4)_2SO_4$ precipitation] was : cell culture 150 g (14), roots 63 g (8.8) and leaves 150 g (48.9).

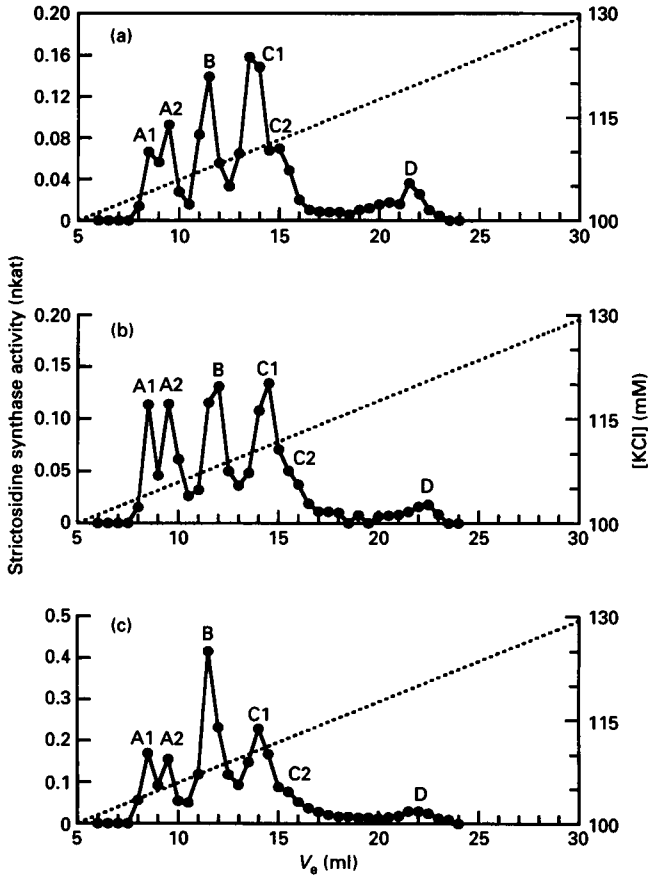


**Figure 5 Distribution of strictosidine synthase isoforms in (a) cell culture, (b) hairy roots and (c) seedlings of *C. roseus***

A pool of strictosidine synthase isoforms obtained by $(NH_4)_2SO_4$ precipitation and gel-permeation chromatography was fractionated by anion-exchange chromatography. ·····, [KCl]; ●, strictosidine synthase activity. Starting material, in wet weight of source (nkat of extract) was: cell culture 30 g (6.3), hairy roots 10.7 g (4.4) and seedlings (5 days) 4 g (5.3).

competitive product inhibition, not substrate inhibition. The 'true' parameters can be determined from a secondary plot of the slope from the Lineweaver–Burk plots against initial substrate concentration [25]. To exclude the possibility that other factors might lead to apparent product inhibition (inactivation of enzyme, inhibition by codeine or others), we tested the effect of adding purified strictosidine before the start of the reaction. As expected, the addition of 0.5 mM strictosidine to a reaction mixture containing 0.5 mM tryptamine shifted the Lineweaver–Burk plot to the position of that obtained with an initial tryptamine concentration of 1 mM (not shown), meaning that the data all lie on the straight line obtained in the secondary plot.

Being fast and simple, the above procedure was quite useful as a diagnostic tool for product inhibition. Much better, however, is the use of a non-linear least-squares minimization method to fit the raw data to the integrated rate equation for the proper reaction mechanism, avoiding all transformations of data that introduce bias. Each progress curve yields a full set of parameters (although as it is obtained from a single pipetting of enzyme, it is likely to contain systematic error in the estimate of $V_{max.}$). Averaging of parameters from a number of progress curves, preferably with different substrate concentrations, yields parameters with standard deviations. We used CCv5.1, a recent version of the program COSY [26], made available by the author without charge. It uses the Marquardt algorithm for curve fitting and contains equations for 30 frequently used enzyme mechanisms and binding studies. The models we used were taken from ref. [27]. CCv5.1 calculates goodness-of-fit criteria for model discrimination. The Marquardt-fit procedure requires rather accurate estimates for the parameters, which were obtained by the procedure described in ref. [25], outlined above.

The kinetic parameters we obtained for our strictosidine synthase isoforms are shown in Table 2. The average specific activity is 234 (±64) nkat/mg of enzyme. This variation in specific activity does not necessarily reflect different turnover numbers for the isoforms. It may also arise from scatter in the protein determination data of the final preparations, as amounts as low as 0.4 $\mu$g had to be determined. Also, the enzymes were extensively diluted to record the progress curves, giving rise to some loss of activity. In conclusion, we believe that within experimental error the values for $V_{max.}$ are the same for all isoforms. The specific activity is higher than the highest value reported in the literature (104 nkat/mg). This may mean that our enzyme is purer, better preserved or both. Certainly, it is unlikely that our purified enzyme preparations consist of ill-defined breakdown products of the native enzyme. The value for

**Table 2 Estimates of kinetic parameters of strictosidine synthase from reaction progress curves**

The reaction progress curves of purified strictosidine isoforms were measured by on-line h.p.l.c. analysis. The data were fitted to the rate equation for a Uni-Uni reaction mechanism with competitive product inhibition, weighted by the square root of their $y$ value. The first three datapoints were discarded to allow for temperature equilibration and enzyme activation after addition of the enzyme. The initial product concentration was estimated by subtracting the substrate concentration from the concentration present before the start of the reaction. Approximately 18 datapoints were used per progress curve. Each run yields a full set of parameters, but without a reliable error estimate. Data are given as the mean ± S.D. of the parameter values obtained from runs with four (except B: three) initial tryptamine concentrations: 0.25, 0.5, 1.0 and 1.5 mM. Secologanin concentration was kept in excess: 10 mM. The average of the kinetic parameters of all isoforms was calculated as the average of all the individual values, without weighting by their S.D., or the abundance of the isoforms.

| Isoform | $V_{max.}$ (nkat/mg) | $K_m^{tryptamine}$ ($\mu$M) | $K_p$ ($\mu$M) |
|---|---|---|---|
| A1 | 215 ± 3 | 8.7 ± 0.6 | 260 ± 34 |
| A2 | 238 ± 14 | 8.2 ± 0.9 | 442 ± 145 |
| B | 312 ± 3 | 9.2 ± 0.3 | 261 ± 14 |
| C1 | 303 ± 11 | 9.3 ± 1.1 | 325 ± 33 |
| C2 | 153 ± 15 | 8.4 ± 2.6 | 248 ± 56 |
| D | 180 ± 2 | 9.4 ± 1.1 | 316 ± 33 |
| Average of all | 234 ± 64 | 8.9 ± 0.5 | 309 ± 73 |

**Table 3 Error analysis of fitting procedure**

Data were generated by Monte-Carlo simulation with 10% random error, normally distributed around zero. Model functions with and without product inhibition were used. The symbols used are $S_i$ for initial substrate concentration, $V_{max.}$ for maximal velocity, $K_m$ for Michaelis constant, $K_p$ for product inhibition constant and $P_i$ for initial product concentration. All units are arbitrary. Per analysis, 16 progress curves were generated. Means ± S.D. are given.

| Parameter | Generated | Fitted with product inhibition | Fitted without product inhibition |
|---|---|---|---|
| (A) Comparison of model functions | | | |
| $S_i$ | 200 | – | – |
| $V_{max.}$ | 200 | 199.8 ± 4.6 | 206.6 ± 7.7 |
| $K_m$ | 10 | 9.7 ± 0.9 | 18.6 ± 5.1 |
| $K_p$ | 300 | 344.5 ± 114.9 | – |
| $P_i$ | 50 | – | – |
| (B) Test for fitting with high initial substrate concentration | | | |
| $S_i$ | 1000 | – | – |
| $V_{max.}$ | 200 | 200.2 ± 0.9 | – |
| $K_m$ | 10 | 10.0 ± 0.4 | – |
| $K_p$ | 300 | 299.2 ± 14.6 | – |
| $P_i$ | 500 | – | – |
| (C) Test for generation of product inhibition as artifact of model function | | | |
| $S_i$ | 250 | – | – |
| $V_{max.}$ | 200 | 202.6 ± 6.8 | 202.8 ± 6.8 |
| $K_m$ | 20 | 24.2 ± 13.2 | 24.3 ± 13.2 |
| $K_p$ | – | 35896.0 ± 8947.0 | – |
| $P_i$ | – | – | – |

the product inhibition constant is 309 ± 73 $\mu$M (Table 2). This constant shows some parameter redundancy, i.e. variation in this parameter contributes less to the goodness-of-fit than $K_m$ and $V_{max.}$. The standard deviation is therefore high. Within experimental error, this means that the isoforms cannot be distinguished. The product inhibition is weak relative to the Michaelis constant for tryptamine ($K_p$ approximately 35 times higher than $K_m$). The possible relevance for the plant's physiology will be discussed below.

Table 2 shows that the $K_m$ for tryptamine is 8.9 (±0.5) $\mu$M. Within experimental error, this means that all isoforms are similar.

## DISCUSSION

We purified and characterized six isoforms of strictosidine synthase. The isoforms showed differences in pI, molecular mass and elution position in anion-exchange chromatography and chromatofocusing. No differences were detected in the type of glycosylation, and no correlation was found with distribution among tissues or developmental stage. The isoforms were indistinguishable by their kinetic parameters.

### Kinetics

Our estimates of the $K_m$ for tryptamine disagree with the values in ref. [11] by at least two orders of magnitude. This seemed to us too large a discrepancy to be attributed to biological variation alone. Therefore we compared the methodology used. We inspected the goodness-of-fit criteria of fitting to our data. Runs tests (to detect trends in the residuals) and Wilcoxon's signed-ranks tests (for normal distribution of the residuals) passed at the 5% significance level, indicating that the model fitted the data well. This means that the data reflect an apparent Uni-Uni mechanism with or without competitive product inhibition. No other properties, such as an exponential decay of enzyme activity during the assay or non-competitive inhibition, are likely as these would produce a trend in the residuals.

We tested the fitting of simulated data to the model functions describing progress curves with and without product inhibition for the ability to yield accurately fitted parameter estimates, as shown in Table 3. From part A it follows that the 'wrong' model gave reasonable estimates. Also goodness-of-fit has no model-discriminating value in this case, because with the different parameters the same function can be described, yielding the same residuals (on which goodness-of-fit tests are performed). Additional experimental information (variation of $S_i$ and $P_i$) is therefore needed to choose the right model function. In part B we confirmed that the fit procedure also gives reliable results at high initial substrate concentrations. In part C we tested for the possibility that the use of the model function for product inhibition on data that do not contain this inhibition artificially creates an inhibition constant. The product inhibition constant thus obtained is much higher than our experimentally obtained value.

The model functions we used are derived for apparently irreversible reactions. To test whether this is a reasonable assumption for our system, we measured the backward reaction, using purified strictosidine as substrate. The formation of a small peak was observed during the h.p.l.c. analysis, which may be tryptamine, when 100 mM of semicarbazide was added to the reaction mixture to remove secologanin from the equilibrium. Even then, the backward rate with 1 mM strictosidine was only 0.02% of the forward rate with 1 mM tryptamine and 5 mM secologanin. We therefore conclude that the non-linear least-squares fitting of progress curves is a good approach using the specific set of parameters we have analysed.

We also attempted to analyse the data found in the literature. The data in ref. [9] could not be scrutinized because no details were given. The paper by Pfitzner and Zenk [11], on the other hand, is most carefully documented, allowing us to perform calculations and simulations. From the description of their method, and data in their Figure 4, it follows that up to 70% of

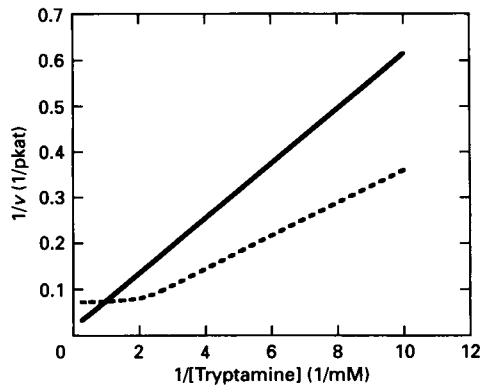


**Figure 6 Simulation of strictosidine synthase assay**

The stopped assay (in 0.1 ml, incubation time 60 min) described by Pfitzner and Zenk [11] was simulated using the rate equation for an apparent Uni-Uni mechanism without product inhibition (——, $V_{max.}$ = 48 nkat, $K_m$ = 1.9 mM) and the rate equation with product inhibition (– – – –, $V_{max.}$ = 14 nkat, $K_m$ = 0.01 mM; $K_p$ = 0.3 mM).

the substrate, tryptamine, was converted into tritiated water, especially at low substrate concentrations. Therefore the criteria for initial velocity (substrate concentration not changed, and no product accumulated) were not met for all samples. To estimate the possible consequences of using the assay conditions described in ref. [11], we simulated progress curves using the program CCv5.1 [26]. The amount of product formed after 1 h was used to calculate the velocity, as in [11]. Lineweaver–Burk plots (Figure 6) of the simulations reveal that, as expected, the parameters from ref. [11] (48 nkat, 1.9 mM) can largely reproduce the linear part of their Figure 4, which was used to calculate the parameters. Our simulations cannot, however, explain the parts of the Lineweaver–Burk plots in the Figures from both ref. [11] and [8] that show substrate inhibition at 1 mM tryptamine. We also ran a simulation with our parameters ($K_m$ = 10 $\mu$M, $K_p$ = 300 $\mu$m) and $V$ = 14 nkat (since the standard assay in ref. [11] would give values near $V_{max.}$ when our $K_m$ is correct), which also gave rise to a Lineweaver–Burk plot compatible with the experimental data. The points at high substrate concentration extrapolate to the 'true' $K_m$ and $V_{max.}$ but at low substrate concentrations, substrate is exhausted during the incubation. As a result, the apparent activity becomes an almost linear function of the initial substrate concentration. Therefore the points at low substrate concentration are on a line extrapolating almost through the origin (giving a far too high estimate for $V_{max.}$ and $K_m$ in a Lineweaver–Burk plot). This may be the case in the treatment of the data from both [11] and [8].

## Physiological significance

Having established that the isoforms of strictosidine synthase in *C. roseus* are kinetically indistinguishable and are present in all tissues and sources investigated, and considering that the closely related enzyme from *R. serpentina* has no isoforms [11], the question arises of what is the function of isoforms of this enzyme? The possibility that specific isoforms exist in specific tissues or developmental stages is ruled out by our finding that all isoforms are present in all tissues studied. This is in line with the findings that the level of strictosidine synthase activity in general has no correlation with the alkaloid-biosynthesis rate in the development of seedlings or the induction of alkaloid production in cell suspension cultures (see ref. [3]).

Another possibility is that the intracellular distribution is different for the different isoforms and that the isoforms carry information aimed at a specific intracellular target. Experiments by McKnight et al. [22] showed that all immunoreactive protein is localized in the vacuole in roots of *C. roseus* plants. As all isoforms are present in the roots, this seems to argue against a role of isoforms in targeting of the enzyme.

If they have no obvious function, are the multiple forms really all present *in vivo* or are they an artifact of the procedures? We believe that they are not an artifact of the cell-suspension culture; we have shown that if there is a difference with plant material, the cell culture rather has one isoform less abundantly expressed (isoform C2) than one that is generated. It is also highly unlikely that the isolation procedure generates the isoforms, as we observed quite reproducible patterns in our isolations, and we took many precautions to prevent covalent modification of the enzyme by secondary metabolites and proteases. Also, other workers have found no effect of inhibitors of proteolysis, and have observed the same number of isoforms using a rapid procedure including immunoaffinity chromatography [11]. Another factor in favour of the existence of the isoforms *in vivo* is that the same workers isolated homogeneous enzyme from *R. serpentina* [28], but multiple forms from *C. roseus* [11]. Interestingly, the strictosidine synthase from *Cinchona robusta* was also found to have four isoforms [23].

It appears that efforts to express strictosidine synthase in heterologous systems in order to construct a system for production of precursors of indole alkaloids are not likely to suffer from the heterogeneity of the enzyme. As all available evidence indicates that the enzyme is encoded by a single gene [14], it is also not likely that an undesired gene will be erroneously cloned.

Finally the question arises of what is the significance of the low Michaelis constant for tryptamine and product inhibition for the indole alkaloid pathway? We believe that a low $K_m$ may be advantageous in this case, enabling concentrations upstream in the pathway to remain low, as tryptophan may be toxic at high concentrations [29]. The product inhibition seems to be insignificant at first sight, the $K_p$ being 35 times the $K_m$. This inhibition is comparable with the 10% inhibition by ajmalicine and catharanthine at four times the concentration of tryptamine [9]. The localization of a significant part of the pathway to secondary metabolites in vesicular structures, such as the vacuoles in the case of strictosidine synthase, gives a new viewpoint on this matter. It may be that the product concentration in the vacuole becomes very high without affecting primary metabolism, thereby reaching concentrations that do inhibit strictosidine synthase activity significantly.

We thank Wim Snoeijer, Bert Goosen and Karl Weisz for culturing plants and producing cell suspension cultures. Luc Stevens is acknowledged for making purified secologanin available. Thanks are due to Marc Eberhard for providing the fitting program and for helpful discussions. We thank Volker Kruft (Berlin) for performing the amino acid sequencing.

## REFERENCES

1 Stöckigt, J. (1980) in Indole and Biogenetically Related Alkaloids (Phillipson, J. D. and Zenk, M. H., eds.), pp. 113–141, Academic Press, London
2 Kutchan, T. M. (1993) Phytochemistry **32**, 493–506
3 Meijer, A. H., Verpoorte, R. and Hoge, J. H. C. (1993) J. Plant Res. Special Issue **3**, 145–164
4 Treimer, J. F. and Zenk, M. H. (1979) FEBS Lett. **97**, 159–162
5 Skinner, S. E., Walton, N. J., Robins, R. J. and Rhodes, M. J. C. (1987) Phytochemistry **26**, 721–725
6 Aerts, R. J., van der Leer, T., van der Heijden, R. and Verpoorte, R. (1990) J. Plant Physiol. **136**, 86–91
7 Stevens, L. H., Giroud, C., Pennings, E. J. M. and Verpoorte, R. (1993) Phytochemistry **33**, 99–106

8 Treimer, J. F. and Zenk, M. H. (1979) Eur. J. Biochem. **101**, 225–233
9 Mizukami, H., Nordlöv, H., Lee, S. L. and Scott, A. I. (1979) Biochemistry **18**, 3760–3763
10 Pfitzner, U. and Zenk, M. H. (1982) Planta Med. **46**, 10–14
11 Pfitzner, U. and Zenk, M. H. (1989) Planta Med. **55**, 525–530
12 de Waal, A., Pennings, E. J. M., Goddijn, O. J. M., Hoge, J. H. C. and Verpoorte, R. (1989) Planta Med. **55**, 665
13 McKnight, T. D., Roessner, C. A., Devagupta, R., Scott, A. I. and Nessler, C. L. (1990) Nucleic Acids Res. **18**, 4939
14 Pasquali, G., Goddijn, O. J. M., de Waal, A., Verpoorte, R., Schilperoort, R. A., Hoge, J. H. C. and Memelink, J. (1992) Plant Mol. Biol. **18**, 1121–1131
15 Kutchan, T. M., Hampp, N., Lottspeich, F., Beyreuther, K. and Zenk, M. H. (1988) FEBS Lett. **237**, 40–44
16 Linsmaier, E. M. and Skoog, F. (1965) Physiol. Plant. **18**, 100–127
17 Pennings, E. J. M., van den Bosch, R. A., van der Heijden, R., Stevens, L. H., Duine, J. A. and Verpoorte, R. (1989) Anal. Biochem. **176**, 412–415
18 Tietze, L. F., Henke, S. and Remberg, G. (1986) Leibigs Ann. Chem. **1986**, 1413–1327
19 Pfitzner, U. and Zenk, M. H. (1987) Methods Enzymol. **136**, 342–350
20 Laemmli, U. K. (1970) Nature (London) **227**, 681–685
21 Haselbeck, A., Schickaneder, E., von der Eltz, H. and Hösel, W. (1990) Anal. Biochem. **191**, 25–30
22 McKnight, T. D., Bergey, D. R., Burnett, R. J. and Nessler, C. L. (1991) Planta **185**, 148–152
23 Stevens, L. H., Blom, T. J. M. and Verpoorte, R. (1993) Plant Cell Rep. **12**, 573–576
24 Vitale, A. and Chrispeels, M. J. (1992) BioEssays **14**, 151–160
25 Yun, S. L. and Suelter, C. H. (1977) Biochem. Biophys. Acta **480**, 1–13
26 Eberhard, M. (1990) Comput. Applic. Biosci. **6**, 213–221
27 Duggleby, R. G. and Morrison, J. F. (1977) Biochim. Biophys. Acta **481**, 297–312
28 Hampp, N. and Zenk, M. H. (1988) Phytochemistry **27**, 3811–3815
29 van der Leer, T., Wijnsma, R., van der Heijden, R., Verpoorte, R. and Svendsen, A. B. (1991) Plant Physiol. Biochem. **29**, 91–98

Received 3 June 1994/20 October 1994; accepted 3 November 1994