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# Expression of enzymatically active cloned strictosidine synthase from the higher plant *Rauvolfia serpentina* in *Escherichia coli*

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The cDNA for strictosidine synthase, the enzyme catalyzing the stereospecific condensation of tryptamine with secologanin producing strictosidine, the key intermediate in indole alkaloid biosynthesis, has been expressed in an enzymatically active form in *Escherichia coli*. The cDNA trimmed of its 3'- and 5'-flanking regions was inserted into the vector pKK223-3 by addition of a synthetic adapter containing the ribosome binding site derived from the β-galactosidase gene. Strictosidine synthase activity (138 nkat·l$^{-1}$) could be measured in both whole bacteria and in bacterial protein extracts. Strictosidine synthase represents the first enzyme of plant secondary metabolism to be actively expressed in a microorganism.

Strictosidine synthase; Indole alkaloid biosynthesis; Cloned enzyme expression; *(Rauvolfia serpentina, Escherichia coli)*

## 1. INTRODUCTION

Strictosidine, the key intermediate leading to over 1800 indole alkaloids, was initially identified as such more than 20 years ago [1]. Subsequent studies led to a discrepancy concerning the correct stereochemistry of the precursor [2,3] which was resolved as the 3α(*S*)-epimer only after the discovery of the enzyme which catalyzes the condensation of tryptamine and secologanin to form strictosidine (fig.1) [4,5]. The enzyme, strictosidine synthase, from *Rauvolfia serpentina* has been purified to homogeneity and characterized [6]. In immobilized form, the enzyme [6,7] is extremely stable such that it is possible to produce strictosidine in gram quantities with as little as 24 nkat in a 10 × 50 mm column. The precursors, tryptamine and secologanin, are attainable commercially and through a simple isolation procedure, respectively [8]. The potential to produce known indole alkaloids through biomimetic syntheses or to develop potentially new drugs by modification of the alkaloidal glucoside [9] then depends only on the availability of the biocatalyst, strictosidine synthase. The cDNA for this enzyme from *R. serpentina* has recently been attained, found to contain an open reading frame of 1032 base pairs encoding 344 amino acids and the protein has been expressed in *Escherichia coli* [10]. Expression of the active enzyme in a microorganism would lead to the unlimited amounts of catalyst required for production of strictosidine and for a thorough analysis of the reaction mechanism and regulation of this central enzyme in indole alkaloid metabolism.

Reported here is the expression of enzymatically active strictosidine synthase in *E. coli*.

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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

The cDNA clone for strictosidine synthase and the anti-strictosidine synthase antibodies were obtained as previously described [10]. Restriction enzymes, T4 DNA ligase and Exonuclease III were from Boehringer Mannheim, and the eukaryotic expression vector pKK223-3 was from Pharmacia. Sequenase was purchased from United States Biochemical Corp.

### 2.2. *Construction of expression vector*

The 3'-flanking region of the cDNA for strictosidine synthase cloned into the *Eco*RI restriction site in pUC18 was removed by digestion with *Pst*I (in polylinker) and *Ava*I (unique site 84 base pairs downstream from the stop codon). The remainder of the region was digested to within 32 base pairs of the UAA codon with Exonuclease III, the ends filled in with Klenow polymerase and the vector circularized and transformed into *E. coli* DH5. The plasmid DNA from

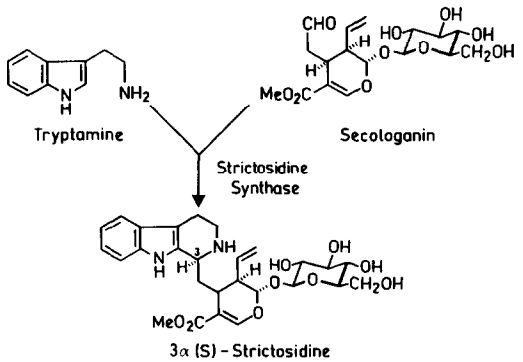


Fig.1. Condensation of tryptamine and secologanin catalyzed by strictosidine synthase to form the 3α(*S*)-glucoalkaloid, strictosidine.

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this preparation was then digested with *Hin*dIII (in polylinker) and *Bal*I (unique site immediately 3′ to the translational start codon) and the restriction fragment purified by agarose gel electrophoresis. The *Hin*dIII-*Bal*I fragment was ligated into *Eco*RI-*Hin*dIII restricted pKK223-3 together with a synthetic adapter containing a sequence derived from the ribosome binding site of the β-galactosidase gene and the ATGG region which had been removed from the open reading frame of the strictosidine synthase cDNA through *Bal*I digestion. This final construct (pKSS1) was introduced initially into *E. coli* DH5 and subsequently into *E. coli* SG935 for expression.

2.3. *Enzyme assay*

*E. coli* SG935 containing pKSS1 was grown in a modified Luria-Bertani medium composed of 2% Bacto Tryptone, 1% Bacto Yeast Extract, 1% NaCl, 5% glycerol with 100 μg/ml ampicillin to an $A_{590}$ of 12.0. Cells were collected by centrifugation (7000 × *g*,5 min, 4°C), washed with 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, and resuspended in 4 ml of 20 mM potassium phosphate, pH 6.5, and either used directly for enzyme assays or were broken by sonication, centrifuged (30.000 × *g*, 20 min, 4°C) and the supernatant used for enzyme assays. The enzyme assay for strictosidine synthase was performed as previously described [11].

2.4. *Immunological procedures*

*E. coli* SG935 containing pKSS1 was grown in Luria-Bertani medium containing 100 μg/ml ampicillin to an $A_{590}$ of 2.0 at which time chloramphenicol was added to the growth medium to a final concentration of 100 μg/ml. A 30 μl aliquot of cells was removed at 0,10 and 40 min. after chloramphenicol addition and immediately frozen at −80°C. Total bacterial protein was fractionated on an SDS-polyacrylamide gel (11%) and blotted onto nitrocellulose. Filters were treated with strictosidine synthase-specific antibodies and $^{125}$I-protein A as in [10] with the following modification: the antibody was pretreated with a protein extract (1 mg/ml final protein concentration) of *E. coli* SG935 containing the empty vector pKK223-3 for 30 min at room temperature prior to addition to the nitrocellulose filters. This served to reduce background due to cross reaction of the crude antisera with bacterial proteins.

2.5. *Structure elucidation of reaction product*

Strictosidine was produced by incubation (1 h, 37°C) of a solution containing 15 mM secologanin, 15 mM tryptamine, 100 mM potassium phosphate pH 6.5 and either whole bacteria (*E. coli* SG935 containing pKSS1) or a crude protein extract (representing 14 nkat enzyme) of this same bacterial strain obtained as described in 2.3. Bacteria were removed by centrifugation (7000 × *g*, 5 min, 4°C) and the reaction mixture lyophilized. Strictosidine was isolated from the methanol-soluble fraction by thin layer chromatography (TLC) on SIL G/$UV_{254}$ (Machery and Nagel) plates in solvent system I (acetone/methanol/diethylamine, 7:2:1) and acetylated [12]. The penta-acetate of the glucoalkaloid was purified by consecutive chromatography in the following 3 solvent systems: II (benzene/ethylacetate, 2:1); III (ethylacetate/*n*-hexane 4:6); IV (benzene/acetone/petroleum ether, 5:2:3). The bacterially produced strictosidine was further characterized by derivatization to the lactam followed by acetylation according to [12].

2.6. *General methods*

The oligonucleotides were synthesized with an Applied Biosystems 380B DNA synthesizer. NMR measurements were made on a 360 MHz Brucker Aspect 3000 NMR spectrometer. Supercoil DNA sequencing was performed according to the dideoxy chain termination method of Sanger [13]. Protein determinations were as described by Bradford [14].

## 3. RESULTS

### 3.1. *Construction of expression vector*

The cDNA clone for strictosidine synthase from *Rauvolfia serpentina* contained a 1032 base pair open reading frame together with 60 base pairs at the 5′ end and 120 bases of 3′-flanking region [10]. The cDNA, as originally subcloned into pUC18, out of frame with respect to the β-galactosidase gene exhibited enzyme activity. In order to increase the total amount of enzyme produced in the bacterium, the cDNA was modified as follows. The 5′-flanking region of the cDNA clone (fig.2) was removed by digestion with *Bal*I which recognized a unique restriction site immediately 3′ to the first ATG and then with *Eco*RI. The *Eco*RI/*Bal*I fragment was replaced by a synthetic oligodeoxynucleotide adapter which contained the ribosome binding site sequence of the β-galactosidase gene together with the ATGG sequence of the reading frame which had been removed through *Bal*I digestion. The 3′-flanking region of the cDNA clone was digested with Exonuclease III to within 32 base pairs of the stop codon to remove the poly A tail and stabilize the messenger RNA. Ultimately, the trimmed cDNA and the adapter were ligated into *Eco*RI/*Hin*dIII digested pKK223-3 to form the expression vector pKSS1.

### 3.2. *Expression of active enzyme*

A series of vector constructs, bacterial strains and culture conditions were tested, the results of several of which are represented in table 1. In general, the expression vector pKSS1 yielded a 2-fold increase in the amount of enzyme produced as compared to pUC18 with the cDNA in the correct reading frame expressed as a β-galactosidase fusion protein (pUC18-4). Northern blot analysis of RNA from bacteria containing either construct indicated that the messenger RNA transcribed from pKSS1 was more stable than that from pUC18-4 presumably due to the presence of the transcription terminator, $rrnBT_1T_2$ (data not shown). The *lon*$^-$, *htpr*$^-$ *E. coli* strain SG935 accumulated 5 times more enzyme than did *E. coli* DH5, suggesting that the cloned gene product is more stable in the protease deficient strain. This stability was monitored in *E. coli* SG935 with $^{125}$I-protein A-treated immunoblots of bacterial protein extracts under conditions of protein synthesis followed by treatment with chloramphenicol. The eukaryotic protein was stable for at least 40 min (fig.3A) after the addition of chloramphenicol. It became apparent through this analysis that the bacterium was processing the cloned gene product. This same phenomemon was observed with the enzyme expressed as a β-galactosidase fusion protein in pUC18 (fig.3B). Enzyme assays performed at various time points after chloramphenicol addition indicated that the processing did not significantly affect the catalytic activity.

After the establishment of the expression system, whole bacteria containing pKSS1 were tested for the capacity to condense secologanin and tryptamine to strictosidine. It was found that when *E. coli* SG935 was either resuspended in potassium phosphate buffer, pH 6.5 or maintained in the growth medium, the addi-

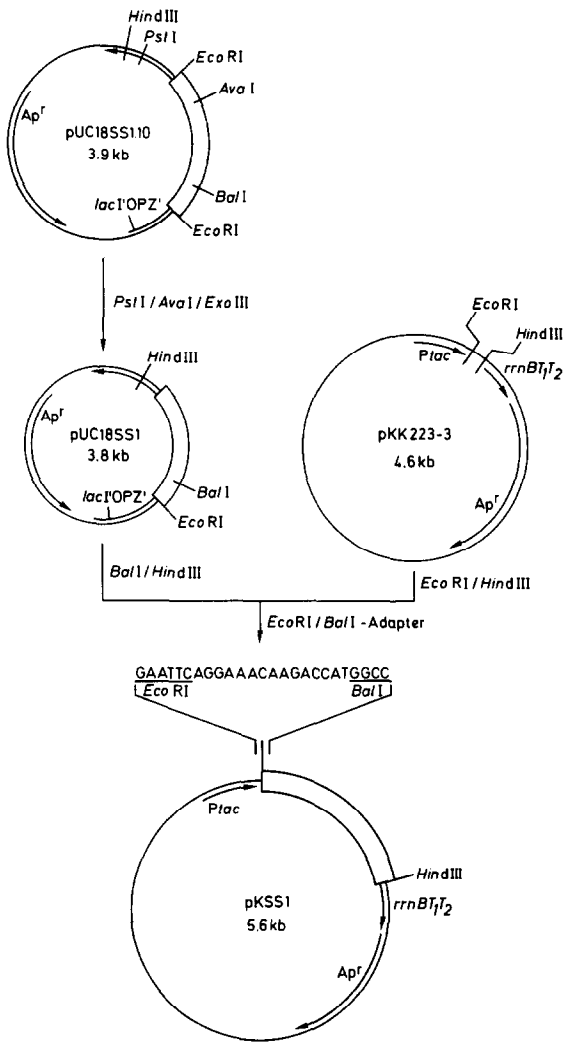


Fig.2. Construction of the strictosidine synthase expression vector pKSS1.

Table 1

Catalytic activity of cloned strictosidine synthase in *E. coli*

| Vector construct | Activity (nkat·l$^{-1}$) | Relative activity | % Soluble protein |
|---|---|---|---|
| pUC18SS1.10/DH5[a] | 0.45 | 1 | 0.0002 |
| pKSS1/DH5[b] | 1.93 | 4.3 | 0.0008 |
| pUC18-4/SG935[c] | 4.91 | 10.9 | 0.002 |
| pKSS1/SG935 (2)[d] | 9.72 | 21.6 | 0.004 |
| pKSS1/SG935 (12)[e] | 138[f] | 330 | 0.063 |

[a] Construct represents the original cDNA with both the 5'- and 3'-flanking regions subcloned out of frame with respect to the β-galactosidase gene in pUC18 expressed in *E. coli* DH5 grown to $A_{590}=2.0$

[b] *E. coli* DH5 containing pKSS1 grown to $A_{590}=2.0$

[c] *E. coli* SG935 containing pUC18-4 grown to $A_{590}=2.0$

[d] *E. coli* SG935 containing pKSS1 grown to $A_{590}=2.0$

[e] and grown to $A_{590}=12.0$. The percentage of soluble bacterial protein was calculated based on a specific activity equivalent to that of the plant-produced enzyme

[f] Equivalent to 7.9 fkat strictosidine synthase activity per $10^6$ bacteria and a production of 19.7 nkat·$^{-1}$ per hour of culture time. Production of the enzyme in *R. serpentina* cell suspension culture is 1.0 nkat·l$^{-1}$ per hour of culture time [6].

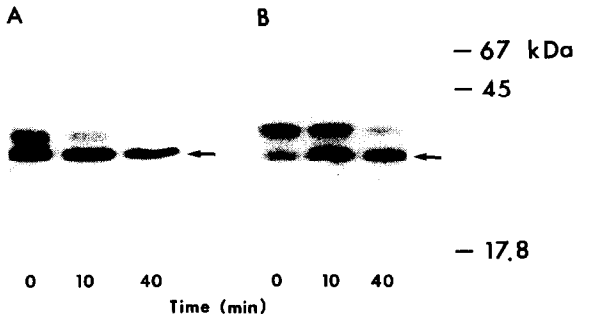


Fig.3. Autoradiogram of $^{125}$I-protein A-treated immunoblot of total protein from *E. coli* SG935 containing (A) pKSS1 and (B) pUC18-4 (β-galactosidase fusion protein). The time after the addition of chloramphenicol to the growth medium at which aliquots were removed for electrophoresis is as indicated. (Arrow indicates position of bacterially-processed strictosidine synthase protein.)

tion of equimolar (15 mM) concentrations of secologanin and tryptamine to the suspension resulted in the quantitative conversion of the precursors to strictosidine. Analysis of the medium, soluble protein and membrane bound protein indicated that the enzyme was in a soluble form exclusively within the bacterium but the product, strictosidine, accumulated in the medium.

### 3.3. *Characterization of reaction product*

To produce sufficient quantities of the reaction product for verification of the structure and stereochemistry of the glucoalkaloid, equimolar concentrations of secologanin and tryptamine were added to either intact *E. coli* SG935 containing pKSS1 or to a protein extract of this strain. Within one hour, the tryptamine has been exhausted. When the reaction was performed with isotopically labeled tryptamine, only one product was formed in quantitative yield ($R_f$ 0.68 in TLC solvent I, superimposable with authentic strictosidine). The bacterially produced strictosidine was readily purified by TLC and either directly acetylated or first cyclized to the lactam, then acetylated. The $^1$H-NMR spectrum of the penta-acetyl derivative was in every respect identical to that of authentic standard as was the $^1$H-NMR spectrum of the purified tetra-acetate of strictosamide [12] ($CDCl_3$, $(CH_3)_4Si$, 360 MHz) δ 2.07, 1.99, 1.88 (9H, s, $OCOCH_3$) which unambiguously contained the characteristic anomalous acetate signal at δ 1.22 (3H, s, $OCOCH_3$) indicative exclusively of the 3α(*S*)-epimer.

## 4. DISCUSSION

The cDNA clone for strictosidine synthase from *Rauvolfia serpentina* has been expressed in an enzymatically active form in *E. coli*. This represents the first example of a gene of plant secondary metabolism which has been expressed in an active form in a bacterium. As such, it has opened a new field in the study of alkaloid metabolism and its biotechnological

applications. Virtually unlimited quantities of strictosidine can now be readily and rapidly produced by intact, non-permeabilized bacteria. The system is superior to the plant *R. serpentina* in that on a time basis, 20 times more enzyme per liter is produced in the microbial culture than in the plant suspension culture [6]. In addition, strictosidine is not modified in any way by the bacterium, resulting in an accumulation of the genuine glucoalkaloid. Biomimetic synthesis of pharmacologically active indole alkoids starting from strictosidine and a detailed biochemical analysis of the reaction catalyzed by strictosidine synthase have now become more attainable.

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