Volume 237, number 1,2, 40–44 FEB 06278 September 1988

The cDNA clone for strictosidine synthase from *Rauvolfia serpentina*

DNA sequence determination and expression in *Escherichia coli*

T.M. Kutchan, N. Hampp, F. Lottspeich*, K. Beyreuther+ and M.H. Zenk

*Lehrstuhl für Pharmazeutische Biologie, Universität München, Karlstrasse 29, D-8000 München 2, *Gentechnologische Arbeitsgruppe am Max-Planck-Institut für Biochemie, D-8033 Martinsried* and *+Institut für Genetik, Universität Köln, Weyertal 121, D-5000 Köln 40, FRG*

Received 20 July 1988

The cDNA clone for strictosidine synthase, the enzyme which catalyzes the stereospecific condensation of tryptamine with secologanin to form the key intermediate in indole alkaloid biosynthesis, strictosidine, has been identified with a synthetic oligodeoxynucleotide hybridization probe in a λgt11 cDNA library of cultured cells of *Rauvolfia serpentina*. The DNA has been sequenced, revealing an open reading frame of 1032 base pairs encoding 344 amino acids. The sequence of 60 nucleotides in the 5'-flanking region has been determined by primer extension analysis. The encoded protein has been expressed in *E. coli* DH5 as detected by immunoblotting of protein extracts with antibodies raised against the native enzyme.

Strictosidine synthase; cDNA cloning; cDNA sequence; Protein expression; (*Rauvolfia serpentina*)

1. INTRODUCTION

The alkaloidal glucoside, strictosidine, was recognized in 1968 as a biosynthetic precursor of monoterpenoid indole alkaloids [1]. There was, however, much confusion about the stereochemistry of this first intermediate in the pathway [2,3] which was only clarified after the enzyme responsible for the stereospecific condensation of tryptamine with secologanin was discovered [4,5] and characterized [6,7]. This precursor of over 1800 indole alkaloids, some of high commercial value [8], has been unequivocally shown to possess a 3α(*S*) configuration [9]. The enzyme catalyzing the formation of strictosidine was named strictosidine synthase [5,6]. A convenient assay has been developed to monitor this reaction [6] and the enzyme from *Rauvolfia serpentina* has been purified to homogeneity [10]. Subsequent characterization identified it as a single polypeptide containing 5.3% carbohydrate. In immobilized form, the enzyme exhibits such excellent stability [10,11] that is has become possible to produce gram quantities of strictosidine for biomimetic syntheses or chemical modification [12]. The only limiting factor in the application of this process is the availability of the synthase, the biocatalyst; tryptamine is commercially available and secologanin is readily attainable in large quantities through a simple isolation procedure which has recently been described [13]. Biotechnological production of strictosidine synthase in microorganisms would potentially lead to unrestricted quantities of the enzyme.

We report here the first steps toward the production of this important catalyst: the isolation and sequence analysis of the cDNA clone for strictosidine

Correspondence address: T.M. Kutchan, Lehrstuhl für Pharmazeutische Biologie, Universität München, Karlstraße 29, D-8000 München 2, FRG

The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession no. Y00756

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50 © 1988 Federation of European Biochemical Societies

synthase from *R. serpentina* and expression of the protein in *E. coli* DH5. The cDNA clone for strictosidine synthase is the first which has become available from a pathway leading to an alkaloid and the nucleotide sequence is one of the very few examples for an enzyme involved in secondary plant metabolism.

2. MATERIALS AND METHODS

2.1. *Tryptic peptide purification and amino acid sequence determination*

Tryptic peptides were prepared from 1–10 nmol homogeneous strictosidine synthase [10] with TPCK-trypsin as described [14]. The peptide mixture was resolved by reversed-phase HPLC [15] [columns: Baker Bakerbond RP8 and RP4 (4.1 × 250 mm); solvent system: (A) 0.1% TFA, (B) 0.1% TFA, 60% acetonitrile; gradient 1%/min; flow rate 1 ml/min] with dual detection at 293 and 210 nm. In this manner, eight peptides were purified sufficiently for amino acid sequence determination. Microsequencing was accomplished with an Applied Biosystems model 470 A gas-phase sequencer.

2.2. *Isolation of poly(A$^+$) RNA*

Poly(A$^+$) RNA was extracted by standard techniques [16] from cultured cells of *R. serpentina* (L.) Benth. [10] on the third day after transfer to alkaloid production medium [17]. Typically, 30 g fresh wt cells yielded 100 μg poly(A$^+$) RNA which had been twice fractionated on oligo(dT)-cellulose.

2.3. *Primer extension and DNA sequence analyses*

Primer extension indirect RNA sequencing reactions were carried out exactly as in [18]. Sequencing of the deletion derivatives [19] generated by exonuclease III digestion of the cDNA for strictosidine synthase subcloned into pUC18 was carried out according to [20].

2.4. *Screening of the λgt11 library*

Preparation of cDNA from *R. serpentina* poly(A$^+$) RNA and insertion into the λgt11 cloning vector were performed according to standard techniques [21]. Plaque hybridization with a ^{32}P end-labeled oligodeoxynucleotide derived from a strictosidine synthase tryptic peptide sequence was carried out overnight at 42°C with subsequent washes at 50°C in a tetramethylammonium chloride solution as described [22].

2.5. *Immunological procedures*

Homogeneous strictosidine synthase from *R. serpentina* [10] was utilized in the production of antibodies. The enzyme (25–100 μg) in PBS was mixed with complete Freund's adjuvant, emulsified (Branson S 125 sonicator) and injected subcutaneously into rabbits at weekly intervals. After 4 weeks, booster injections were administered with 50–200 μg enzyme. Serum was collected through the ear vein and used unpurified for the blotting procedure.

E. coli DH5 containing either the clone for strictosidine synthase or empty vector (pUC18) was grown in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin to an A_{590} of 1.0. Cells were collected by centrifugation (7000 × *g*, 5 min, 4°C), resuspended in 4 ml of 10 mM potassium phosphate (pH 7.5) and lysed by sonication. Debris was removed by centrifugation (30000 × *g*, 20 min, 4°C) and the subsequently obtained 20–70% ammonium sulfate fraction was desalted on a Sephadex G-25 column (8 × 120 mm, 10 mM potassium phosphate, pH 7.5). Extracts of *R. serpentina* cell cultures (30 g fresh wt) were prepared in 45 ml of 10 mM potassium phosphate (pH 7.5) with a mortar and pestle. Cell debris was removed by centrifugation (10000 × *g*, 10 min, 4°C) and the supernatant treated identically to the bacterial extracts.

Protein (50 μg) was fractionated on an SDS-polyacrylamide gel (11%), blotted onto nitrocellulose and treated with antibody and ^{125}I-protein A as in [23].

3. RESULTS

3.1. *Design of an oligodeoxynucleotide as hybridization probe*

Of the eight strictosidine synthase tryptic peptides which were purified and sequenced, two were found to be suitable for the design of synthetic oligodeoxynucleotides. From a segment of one of these two (Gln-Gln-Ile-Met-Asp-Thr), an oligodeoxynucleotide of length 17 nucleotides was synthesized in three subsets, each having a degeneracy of 8. Hybridization to Northern blots of *R. serpentina* poly(A$^+$) RNA failed to yield a discrete band. To improve the quality of the oligodeoxynucleotide, indirect RNA sequencing primed by each of the three subsets of mixed oligodeoxynucleotides was performed. A specific non-degenerate, sequence was obtained from one of the subsets and the following hybridization probe 52 nucleotides in length possessing a degeneracy of only four was designed:

5′-TCCATAATT/CTGT/CTGGACACCTCTG-
TCATCATATAAGGTGCTAACATCGGTGA-3′

To test the efficacy and specificity of this oligodeoxynucleotide as a hybridization probe and to determine the length of the transcript for strictosidine synthase, glyoxal-denatured *R. serpentina* poly(A$^+$) RNA was fractionated by agarose gel electrophoresis and blotted onto a nylon filter by capillary transfer. Hybridization to the ^{32}P end-labeled oligodeoxynucleotide produced a single band by autoradiography corresponding to an approximate length of 1200–1300 nucleotides (not shown).

```
                                   30                                  60
CTACTAGTTGAGCTCCACTCATCGTTAAATCCCAGTCCTTCGTCCGTCTGTCCCCCTACT

                                   90                                 120
ATGGCCAAACTTTCTGATTCGCAAACTATGGCACTGTTCACCGTCTTCCTTCTTTTCCTC
MetAlaLysLeuSerAspSerGlnThrMetAlaLeuPheThrValPheLeuLeuPheLeu
                                  150                                 180
TCCTCTTCGCTCGCTCTCTCCTCTCCAATCTTGAAAGAGATTTTGATTGAGGCTCCTTCC
SerSerSerLeuAlaLeuSerSerProIleLeuLysGluIleLeuIleGluAlaProSer
                                  210                                 240
TATGCCCCCAATTCCTTCACCTTCGACTCAACCAACAAAGGGTTCTACACCTCCGTCCAA
TyrAlaProAsnSerPheThrPheAspSerThrAsnLysGlyPheTyrThrSerValGln
                                  270                                 300
GATGGCCGAGTTATCAAGTACGAAGGACCCAACTCCGGTTTCGTCGACTTCGCCTATGCA
AspGlyArgValIleLysTyrGluGlyProAsnSerGlyPheValAspPheAlaTyrAla
                                  330                                 360
TCTCCCTACTGGAACAAAGCGTTCTGTGAGAACAGCACAGATGCAGAGAAAAGACCCTTG
SerProTyrTrpAsnLysAlaPheCysGluAsnSerThrAspAlaGluLysArgProLeu
                                  390                                 420
TGTGGGAGGACATATGATATTTCATATAACTTGCAAAACAACCAGCTTTACATTGTTGAT
CysGlyArgThrTyrAspIleSerTyrAsnLeuGlnAsnAsnGlnLeuTyrIleValAsp
                                  450                                 480
TGCTATTATCATCTTTCTGTGGTTGGTTCTGAAGGTGGGCATGCTACCCAACTCGCCACC
CysTyrTyrHisLeuSerValValGlySerGluGlyGlyHisAlaThrGlnLeuAlaThr
                                  510                                 540
AGCGTTGATGGAGTGCCATTCAAGTGGCTCTATGCAGTAACAGTTGATCAGAGAACTCGG
SerValAspGlyValProPheLysTrpLeuTyrAlaValThrValAspGlnArgThrGly
                                  570                                 600
ATTGTTTACTTCACCGATGTTAGCACCTTATATGATGACAGAGGTGTCCAACAAATTATG
IleValTyrPheThrAspValSerThrLeuTyrAspAspArgGlyValGlnGlnIleMet
                                  630                                 660
GATACAAGCGATAAAACAGGAAGACTAATAAAGTATGATCCCTCCACCAAAGAAACAACA
AspThrSerAspLysThrGlyArgLeuIleLysTyrAspProSerThrLysGluThrThr
                                  690                                 720
CTACTGTTGAAAGAGCTACACGTTCCAGGTGGCGCAGAAGTCAGTGCAGATAGCTCCTTT
LeuLeuLeuLysGluLeuHisValProGlyGlyAlaGluValSerAlaAspSerSerPhe
                                  750                                 780
GTTCTTGTGGCTGAGTTTTGAGCCATCAAATTGTCAAATATTGGCTAGAAGGGCCTAAG
ValLeuValAlaGluPheLeuSerHisGlnIleValLysTyrTrpLeuGluGlyProLys
                                  810                                 840
AAGGGCACTGCGGAGGTTTAGTGAAAATCCCAAACCCAGGAAATATAAAGAGGAACGCT
LysGlyThrAlaGluValLeuValLysIleProAsnProGlyAsnIleLysArgAsnAla
                                  870                                 900
GATGGACATTTTTGGGTTTCCTCAAGTGAAGAATTAGATGGAAATATGCACGGAAGAGTT
AspGlyHisPheTrpValSerSerSerGluGluLeuAspGlyAsnMetHisGlyArgVal
                                  930                                 960
GATCCTAAAGGAATAAAATTTGATGAGTTTGGGAACATTCTTGAAGTTATCCCACTCCCA
AspProLysGlyIleLysPheAspGluPheGlyAsnIleLeuGluValIleProLeuPro
                                  990                                1020
CCACCATTTGCAGGTGAACACTTCGAACAAATTCAAGAGCATGATGGTTTGCTGTACATT
ProProPheAlaGlyGluHisPheGluGlnIleGlnGluHisAspGlyLeuLeuTyrIle
                                 1050                                1080
GGAACCCTGTTCCATGGCTCTGTGGGCATATTAGTATATGATAAGAAGGGAAATTCTTTT
GlyThrLeuPheHisGlySerValGlyIleLeuValTyrAspLysLysGlyAsnSerPhe
                                 1110                                1140
GTTTCAAGTCATTAAATTTTCCACGAACCAGATTGGTTTTGTTTTTGATTTGTATAACAC
ValSerSerHisEnd
                                 1170                                1200
TCTTTAAAGGTTTTGTATTCGAATCACGTCATCTCAGCCTCGAGGAATAAGAAAAAAGCA

GCAGAATAACTTCTC
```

Fig.1. Nucleotide sequence of the cDNA clone of strictosidine synthase from *R. serpentina*. The coding strand of the DNA is presented in the 5′ to 3′ orientation along with the translation of the only contiguous open reading frame. Underlined sequences are those obtained from the amino acid sequencing of eight tryptic peptides from strictosidine synthase.

3.2. *Isolation and subcloning of the cDNA for strictosidine synthase*

From the initial screening of only 2000 plaques of the λgt11 cDNA library for *R. serpentina* with the ^{32}P end-labeled, low-degeneracy 52-mer oligodeoxynucleotide designed from the primer extension experiments, a single, positive clone was identified by autoradiography after a 3 h exposure period. This plaque was picked and rescreened twice again before the λDNA was isolated and the insert was partially sequenced for positive identification. Sequencing reactions with avian reverse transcriptase primed by the original oligodeoxynucleotide (17-mer, 8-fold degeneracy) utilized in the indirect RNA sequencing experiments yielded a sequence identical to the 52-nucleotide-long oligodeoxynucleotide used as the hybridization probe in screening the library. The insert, approx. 1200 base pairs in length, was excised with *Eco*RI, purified by agarose gel electrophoresis and subcloned into the *Eco*RI site in the polylinker of pUC18 for complete sequence analysis.

3.3. *DNA sequence of the cDNA clone*

Subclones in pUC18 possessing the strictosidine synthase cDNA in opposite orientations (designated pUC18SS1.10 and pUC18SS1.25) were identified by partial restriction map analysis and subsequently sequenced. The initial sequence was 1177 base pairs in length and contained an open reading frame (fig.1) of 1032 base pairs, corresponding to 344 amino acids and a polypeptide molecular mass of 38 119 Da. Within the translated region, the sequences of all eight tryptic peptides obtained from strictosidine synthase could be found. A putative carbohydrate attachment site (Asn-Ser-Thr) was located at nucleotide positions 331–339. To determine the sequence of the 5′-flanking region, an oligodeoxynucleotide was designed (5′-CATAGTTTGCGAATCAG-3′) which was complementary to the coding strand in the region beginning 13 nucleotides 3′ to the tentative translational start. Indirect RNA sequencing was performed utilizing this synthetic oligodeoxynucleotide as primer. After the 60th nucleotide, the sequencing ladder as determined by primer extension stopped. The position of the AUG start codon could be assigned based on the presence of a sequence highly homologous to the eukaryotic translation start consensus sequence ACCAUGG [24]. In the region 5′ to the translational start signal, neither regulatory sequences nor a ribosome-binding site could be identified, suggesting that such consensus sequences may be positioned further upstream.

3.4. *Expression of strictosidine synthase in* E. coli *DH5*

As proof of the identity of the clone, in addition

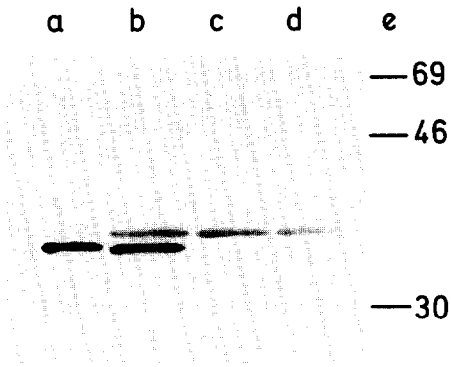

Fig.2. Autoradiogram of ^{125}I-protein A-treated immunoblot of protein extracts from (a) *R. serpentina* cell cultures, (b) *E. coli* DH5 containing pUC18SS1.10, (c) *E. coli* DH5 containing pUC18SS1.25, (d) *E. coli* DH5 containing pUC18, (e) molecular mass markers (in kDa).

to the localization of the eight tryptic peptide sequences, and as a preliminary test for subsequent active expression studies, protein extracts of *E. coli* DH5 containing either pUC18SS1.10 or pUC18-ss1.25 were tested with antibodies raised against the purified enzyme from *R. serpentina* for the presence of the protein. Although strictosidine synthase is a glycoprotein, the antibodies reacted with the periodate-treated enzyme, suggesting that there should also be a reaction with the nonglycosylated protein that would be produced in the bacterium. When the bacterial protein extracts were fractionated by SDS-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose, incubated with antibody and reacted with ^{125}I-protein A, a unique band appeared (fig.2, lane b) upon autoradiography. This protein was slightly smaller in molecular mass than the strictosidine synthase in crude extracts of *R. serpentina* suspension cultures (fig.2, lane a) corresponding to the absence of carbohydrate in the bacterially produced protein. This protein was totally absent from crude extracts of the bacteria containing either the cDNA in the alternative orientation or empty vector (fig.2, lanes c,d, respectively) indicating that it originates specifically from the cloned sequence. A cross-reaction between the antibodies in the crude serum and a protein of bacterial origin is evident in all three bacterial extracts (fig.2, lanes b–d).

4. DISCUSSION

We have reported here the cloning of the cDNA for strictosidine synthase from *R. serpentina* and the expression of the protein in *E. coli*. This represents the first clone obtained for an enzyme involved in alkaloid metabolism. As such, it potentially opens a new field in the study of alkaloid biosynthesis and its application to biotechnology. Although many pharmaceutically important [8] and structurally interesting molecules occur in the alkaloid class, often only limited quantities accumulate in the plant and frequently secondary metabolites are completely absent from cultured cells [25]. The functional expression of strictosidine synthase in a microorganism would lead to a virtually unlimited amount of this key enzyme to be utilized in the biomimetic synthesis of pharmacologically active alkaloids and in a detailed analysis of the catalytic site and reaction mechanism. Ultimately, the gene for this enzyme would provide the system with which to begin the elucidation of the regulatory mechanisms of alkaloid metabolism in plant cell cultures.

Acknowledgements: This work was supported by the Bundesminister für Forschung und Technologie, Bonn. We thank Professor A. Böck and Dr Horst Domdey for their continual helpful discussions and Dr R. Mertz for the synthesis of the deoxyoligonucleotides. We gratefully acknowledge the excellent technical assistance of Ms Angela Schmid.

REFERENCES

[1] Smith, G.N. (1968) Chem. Commun. 912–914.
[2] Battersby, A.R., Burnett, A.R. and Parsons, P.G. (1969) J. Chem. Soc. C, 1193–1200.
[3] Battersby, A.R. (1972) Acc. Chem. Res. 5, 148–154.
[4] Stöckigt, J. and Zenk, M.H. (1977) FEBS Lett. 79, 233–237.
[5] Stöckigt, J. and Zenk, M.H. (1977) Chem. Commun. 646–648.
[6] Treimer, J.F. and Zenk, M.H. (1979) Eur. J. Biochem. 101, 225–233.
[7] Mizukami, H., Nordlöv, H., Lee, S.L. and Scott, A.I. (1979) Biochemistry 18, 3760–3763.
[8] Farnsworth, N.R. (1984) in: Natural Products and Drug Development (Krogsgaard-Larsen, P. et al. eds) pp. 17–28, Munksgaard, Copenhagen.
[9] Nagakura, N., Rueffer, M. and Zenk, M.H. (1979) J. Chem. Soc. Perkin Trans. I, 2308–2312.
[10] Hampp, N. and Zenk, M.H. (1988) Phytochemistry, in press.
[11] Pfitzner, U. and Zenk, M.H. (1982) Planta Med. 46, 10–14.
[12] Brown, R.T. (1979) in: Stereoselective Synthesis of Natural Products (Bartmann, W. and Winterfeldt, E. eds) pp. 62–70, Excerpta Medica, Amsterdam.

[13] Ruyter, C.M., Schübel, H. and Stöckigt, J. (1988) Z. Naturforsch. 43c, in press.
[14] Beyreuther, K., Adler, K., Fanning, E., Murray, C., Klemm, A. and Geisler, N. (1975) Eur. J. Biochem. 59, 491–509.
[15] Stüber, K. and Beyreuther, K. (1981) in: High Performance Liquid Chromatography in Protein and Peptide Chemistry (Lottspeich, F. et al. eds) pp. 205–221, De Gruyter, Berlin.
[16] Lichtenstein, C. and Draper, J. (1985) in: DNA Cloning, Vol. II (Glover, D.M. ed.) pp. 67–119, IRL, Oxford.
[17] Schübel, H., Ruyter, C.M. and Stöckigt, J. (1988) Phytochemistry, in press.
[18] Kührer, K., Kutchan, T.M. and Domdey, H. (1988) DNA, submitted.
[19] Henikoff, S. (1984) Gene 28, 351–359.
[20] Chen, E.Y. and Seeburg, P.H. (1985) DNA 4, 165–170.
[21] Huynh, T.V., Young, R.A. and Davis, R.W. (1985) in: DNA Cloning, vol. I (Glover, D.M. ed.) pp. 49–78, IRL, Oxford.
[22] Wood, W.I., Gitschier, J., Lasky, L.A. and Lawn, R.M. (1985) Proc. Natl. Acad. Sci. USA 82, 1585–1588.
[23] Burnette, W.N. (1981) Anal. Biochem. 112, 195–203.
[24] Kozak, M. (1984) Nucleic Acids Res. 12, 857–872.
[25] Böhm, H. (1982) in: Plant Tissue Culture 1982 (Fujiwara, A. ed.) pp. 325–328, Maruzen, Tokyo.