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S-adenosyl-L-methionine: (S)-scoulerine 9-O-methyltransferase, a highly stereo- and regio-specific enzyme in tetrahydroprotoberberine biosynthesis

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ABSTRACT

Suspension cultures of *Berberis* species are useful sources for the detection and isolation of a new enzyme which transfers the methyl group from S-adenosyl-L-methionine specifically to the 9-position of the (S)-enantiomer of scoulerine, producing (S)-tetrahydrocolumbamine. The enzyme was enriched 27-fold; it is not particle bound, has a pH optimum of 8.9, a molecular weight of 63 000 and shows a high degree of substrate specificity.

ABBREVIATIONS

SAH = S-Adenosyl-L-homocysteine; SAM = S-Adenosyl-L-methionine, STOX = (S)-Tetrahydroprotoberberine oxidase.

INTRODUCTION

Previous studies have demonstrated that (S)-reticuline and (S)-scoulerine, when administered to shoots of plants containing isoquinoline alkaloids, are readily converted to protoberberines (see Cordell, 1981). Subsequent experiments with cell-free extracts of *Macleaya microcarpa* callus have shown the presence of an enzyme which catalyses the formation of scoulerine from reticuline, which was named berberine bridge enzyme (Rink and Böhm, 1975). This enzyme was purified to homogeneity, characterized (Steffens et al., 1984), and shown to catalyse the conversion of (S)-reticuline to (S)-scoulerine in the presence of oxygen. In the course of the biosynthesis of berberine-type alkaloids, (S)-scoulerine is expected to undergo various methylation steps as well as the oxidation of ring C.

We report here on a new enzyme and some of its characteristics which catalyses the stereo- and regiospecific O-methylation at the 9-position of (S)-scoulerine using SAM (Fig. 1). This enzyme transforms (S)-scoulerine to (S)-tetrahydrocolumbamine, which, in turn, serves as the precursor of most protoberberine alkaloids, including columbamine, palmatine, berberine, jatrorrhizine, etc. This methyltransferase therefore occupies a central role in protoberberine biosynthesis.

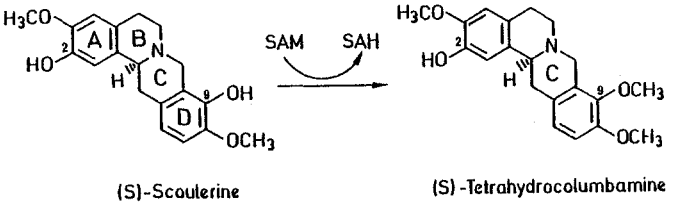

Fig. 1 Reaction sequence catalysed by (S)-scoulerine 9-O-methyltransferase

MATERIALS and METHODS

Suspension cultures provided by our cell culture laboratory were used, especially those of *Berberis wilsoniae* var. *subcaulialata* Schneid., and grown as previously reported (Hinz and Zenk, 1981). Cells were harvested after reaching the stationary phase, frozen in liquid nitrogen and stored at -20°C. Potassium phosphate buffer (0.1 M, pH 7.5, 200 ml) containing β-mercaptoethanol (20 mM) was added to 100 g fresh weight of frozen cells and the mixture was stirred, until a homogeneous mass was obtained. This was pressed through cheesecloth, and the resulting liquid was centrifuged for 10 min at 13 500 x g. Solid $(NH_4)_2SO_4$ was added to the supernatant until 70% saturation was reached. The precipitate was centrifuged down as above and taken up in 10 ml of the original phosphate buffer and centrifuged again (30 000xg, 10 min). The supernatant of this step was applied to a AcA 44 gel filtration column (2.3 x 87 cm) and the protein fractionated with the same buffer (elution speed 0.3 ml min^{-1}; fraction vol. 4.5 ml). The fractions containing the enzyme (typically fractions 30-60), were pooled and applied to a DEAE-Sephacel column (2.3 x 6 cm) at a speed of 0.6 cm min^{-1}. The column was washed with phosphate buffer and the eluent was collected in 9-ml fractions. The methyltransferase was found in the initial fractions. This fluid was concentrated by ultrafiltration (Amicon membrane YM 10) to a final volume of 6 ml. The enzyme was divided into three equal portions and then subjected to high pressure liquid chromato-

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graphy (LKB system, G 300 SW column, 2.15 x 60 cm, potassium phosphate 25 mM buffer (pH 7.0) containing 25 mM NaCl, 3.0 ml min^{-1}). 3-ml fractions were collected and examined for protein absorption at 280 nm, then fractions containing protein were assayed for enzyme activity, fractions containing the enzyme were pooled and concentrated as above.

Standard Enzyme Assay

The reaction mixture (200 µl) included 500 µmole glycine-NaOH buffer (pH 9.0); 20 µmole sodium ascorbate (to prevent oxidation of the phenolic substrates), 50 µmole (S)-scoulerine; 20 µmole [C^3H_3]-SAM (24 x 10^3 cpm) and enzyme. The assay mixture was incubated for 20 min at 30°C. The reaction was terminated by addition of 200 µl Tris-HCl, 1 M, pH 8.5, and 400 µl isoamyl alcohol. The sample was shaken mechanically for 15 min. The suspension was centrifuged, and then a 200-µl aliquot of the organic layer removed for liquid scintillation counting. Controls were run with heat-denatured protein. The kinetic constants for the enzyme were determined using the standard assay but in keeping the concentration of one of the reactants under investigation fixed at 5 mM. The enzyme (ca. 10 µg) used usually had a specific activity of 260 pkat/mg. The molecular weight of the enzyme was determined by standard HPLC and gel filtration methods; values obtained by both techniques agreed within 3%. Protein determinations were performed according to Bradford (1976) or, on purified samples, by the 260/280-nm method.

Alkaloids

(S)- and (R)-scoulerine were synthetized according to standard procedures (Battersby et al., 1966). All other alkaloids used were synthetized by known procedures or were isolated from plant material. For the characterization of reaction products 10-to 20-fold larger incubation mixtures in the standard proportions containing ^{14}C-SAM were used. The labelled alkaloid was extracted into ethyl acetate and subjected to TLC using the solvent system ethyl acetate: 2-butanone: formic acid : H_2O = 5 : 3 : 1 : 1. Labelled tetrahydrocolumbamine thus formed was oxidized on a preparative scale using (S)-tetrahydroprotoberberine oxidase (Amann et al., 1984), giving columbamine, which was diluted with unlabelled carrier material and subsequently crystallized to const. specific activity.

RESULTS

The new enzyme was assayed for its catalytic activity by monitoring the transfer of the tritium labelled methyl group of SAM to (S)-scoulerine. Extraction of the labelled alkaloid into isoamyl alcohol left unreacted radioactive SAM in the aqueous phase. Radioactivity found in the organic phase was taken as evidence for methyl group transfer. Using this assay system the presence of O-methyltransferase(s) in crude enzyme extracts of plant cell cultures of different taxonomic origin was investigated. The highest activity was observed in *Berberis wilsoniae* var. *subcaulialata*, from which the enzyme was purified. The results are shown in the Table 1.

Purification step	Total activity pkat	Total protein mg	Specific activity pkat/mg	Recovery (%)	Purification -fold
Crude extract	2160	225	9.6	100	1
$(NH_4)_2SO_4$ (0-70%)	2550	161	15.8	118	1.6
Gel Filtr. (AcA 44)	2670	85	31.4	124	3.3
DEAE-cellulose	2270	50	45.7	105	4.8
HPLC	1230	4.7	260	57	27

Table 1 Purification procedure for (S)-scoulerine 9-O-methyltransferase.

A typical HPLC profile showing the distribution of protein as well as that of the O-methyltransferase is depicted in Fig. 2.

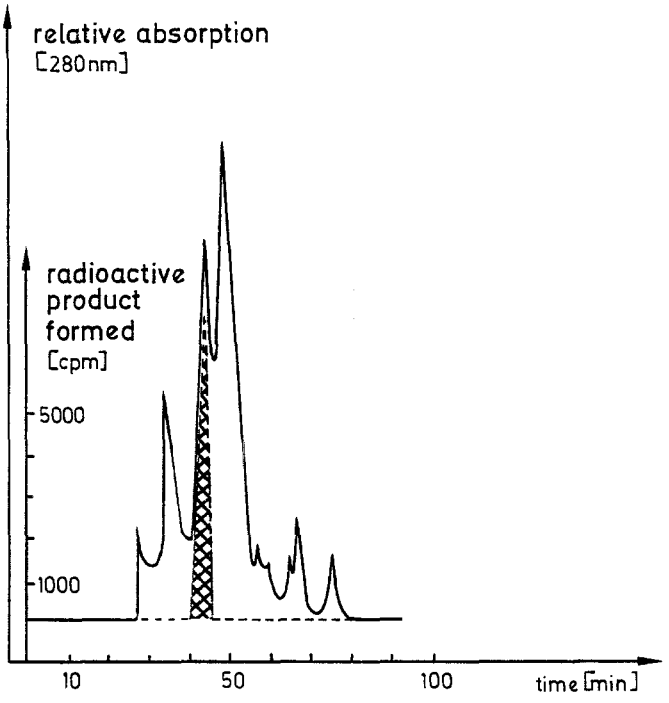


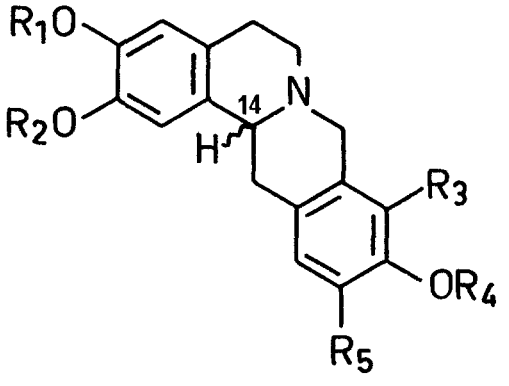
Fig. 2 Typical HPLC profile of the partially purified enzyme, (——) = distribution of protein, (---) = distribution of enzyme activity

The protein fraction purified 27-fold (57% yield), proved to be satisfactory for all of our purposes, since none of the other known enzymes of the isoquinoline pathway with potential for interference in the experiment described here (Zenk, in press) was present. The partly purified enzyme also did not contain any contaminating methyltransferase activity for transforming tetrahydrocolumbamine to tetrahydropalmatine (Beecher and Kelleher, 1984). For this

reason, no further purification of our enzyme was attempted. A plot of the tetrahydrocolumbamine production vs. time was linear for the first 20 min, when 3 pkat of the enzyme was used in the standard assay. Dependence of the reaction on protein concentration (enriched 27-fold) was linear over a range from 0 to 9 µg and then gradually fell off. The pH optimum of the reaction was at 8.9. The temperature optimum was found to be at 37°C. The molecular weight was determined to be 63±2 kD. After storage for 7 months at -20°C in glycerol (20%), the enzyme still retained more than 50% of its original activity. The possible O-methylation products of (S)-scoulerine include tetrahydrocolumbamine, tetrahydropalmatrubine or, if two methyl groups are transferred, tetrahydropalmatine. The TLC and HPLC (Nucleosil- C18, Macherey and Nagel) behaviours of the labelled product were identical with those of tetrahydrocolumbamine. The radioactive product was transformed by the action of STOX into a product identical with columbamine; dilution with carrier columbamine and crystallisation yielded a labelled product containing the expected radioactivity within experimental error (± 10%). The fact that STOX acts on the product shows that the tetrahydrocolumbamine obtained by this enzyme reaction has the (S)-configuration at C-14 (Amann et al., 1984). These procedures establish the product of enzymatic methylation of scoulerine to be (S)-tetrahydrocolumbamine. Of a series of 12 metal salts tested at 5 mM concentration only Cu^{++} inhibited the enzyme (residual activity 3%). The kinetic constants of the enzyme were measured as described in the materials and methods section, *vide intra*. The K_m values determined for (S)-scoulerine and SAM were 1.6 mM and 0.042 mM respectively; the K_i for SAH was 0.01 mM. Of crucial importance for the position of this enzyme in the biosynthesis of protoberberine alkaloids was its substrate specificity. No reaction could be observed by using (R)-scoulerine or dehydroscoulerine, its ring C aromatized counterpart. The enzyme was also (Fig. 3) totally inactive towards a number of other berbines including (S)-coreximine, (R,S)-desmethylenetetrahydroberberine, (R,S)-discretamine, (S)-corydalmine, (R,S)-stepholidine, (R,S)-tetrahydrojatrorrhizine, (S)-coramine, (R,S)-tetrahydroberberrubine (nandinine). Also, no reaction was observed with (R)- and (S)-reticuline, boldine, magnoflorine, and the simple phenols caffeic acid, dopamine, and quercetin. Two other substrates, which are not natural products, were methylated by the enzyme: (R,S)-2,3,9,10-tetrahydroxy-tetrahydroprotoberberine (at 50% of the rate of (S)-scoulerine) and (S)-3,9-hydroxy-2,10-methoxy tetrahydroprotoberberine (at 4% of the rate of (S)-scoulerine).

DISCUSSION

S-Adenosyl-L-methionine : (S)-scoulerine 9-O--methyltransferase which has been purified and characterized in this work is a highly stereo- and regiospecific methyltransferase. A variety of closely related tetrahydroprotoberberines was tested and none of the naturally occurring substrates served as methyl group acceptor except for (S)-scoulerine. The (R)-enantiomer of scoulerine and dehydroscoulerine were not used in the enzyme reaction. This shows an unusual degree of specificity of the methyltransferase for the (S)-form of the substrate. It has been unequivocally established that only one of the hydroxyl groups of (S)-scoulerine, namely the one at position 9, is methylated, the product being (S)-tetrahydrocolumbamine. As we have shown, tetrahydrocolumbamine and several other tetrahydroprotoberberines with differing O-methyl or methylenedioxy substitution patterns in both rings A and D can serve as intermediates for the formation of ring C aromatic protoberberines (particularly columbamine), by action of the STOX enzyme (Amann et al., 1984).



Coramine : $R_1=R_3=H$; $R_2=R_4=CH_3$; $R_5=OH$

Coreximine : $R_1=R_4=CH_3$; $R_2=R_3=H$; $R_5=OH$

Corydalmine : $R_1=R_2=CH_3$; $R_3=OCH_3$; $R_4=R_5=H$

Desmethylene-tetrahydroberberine : $R_1=R_2=H$; $R_3=OCH_3$; $R_4=CH_3$; $R_5=H$

Discretamine : $R_1=R_4=H$; $R_2=CH_3$; $R_3=OCH_3$; $R_5=H$

Nandinine : $R_1+R_2=-CH_2-$; $R_3=OH$; $R_4=CH_3$; $R_5=H$

3,9-Hydroxy-2,10-methoxy-tetrahydroprotoberberine : $R_1=R_5=H$; $R_2=R_4=CH_3$; $R_3=OH$

Scoulerine : $R_1=R_4=CH_3$; $R_2=R_5=H$; $R_3=OH$

Tetrahydrojatrorrhizine : $R_1=R_5=H$; $R_2=R_4=CH_3$; $R_3=OCH_3$

2,3,9,10-Tetrahydroxy-tetrahydroprotoberberine : $R_1=R_2=R_4=R_5=H$; $R_3=OH$

Tetrahydrostepharanine (Stepholidine) : $R_1=CH_3$; $R_2=R_4=R_5=H$; $R_3=OCH_3$

14 H = α=(S); 14 H = β=(R)

Fig. 3 Protoberberines tested for substrate specificity of the enzyme

Columbamine in turn, can be used as a substrate by the methylenedioxy bridge-forming enzyme (berberine synthase) to yield berberine (Rueffer and Zenk, in press). Berberine can additionally undergo ring-opening at the methylenedioxy bridge to give jatrorrhizine (Beecher and Kelleher, 1983; Rueffer et al., 1983). Beecher and Kelleher (1984) have shown that tetrahydrocolumbamine can also be methylated by a different methyltransferase to form tetrahydropalmatine; the stereochemical course and substrate specificity of this reaction have not been determined. The 9-O-methyltransferase reported here has a pH optimum of pH 8.9, like the berberine bridge and the STOX enzymes. The berberine bridge and the STOX enzymes are found exclusively in particles with the density of = 1.14 g ml^{-1}, while the 9-O-methyltransferase could not be compartmented. Density gradient centrifugation and ultracentrifugation studies demonstrated this enzyme to be in the cytosol. The main end product out of (S)-scoulerine in protoberberine biosynthesis in *Berberis* cell cultures is jatrorrhizine. The question remains then, as to how a portion of the (S)-scoulerine escapes the particle in order to be methylated at position 9 prior to the formation of columbamine.

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REFERENCES

Amann M, Nagakura N, Zenk MH (1984) Tetrahedron Lett. 25: 953.
Battersby AR, Southgate R, Staunton J,Hirst M (1966) J.Chem.Soc. (C): 1052-1057.
Beecher CWW, Kelleher WJ (1983) Tetrahedron Lett. 24: 469.
Beecher CWW, Kelleher WJ (1984) Tetrahedron Lett. 25: 4595.
Bradford MM (1976) Anal.Biochem. 72: 248.
Cordell GA (1981) Introduction to Alkaloids. John Wiley Interscience New York.
Hinz H, Zenk MH (1981) Naturwiss. 67: 620.
Rink E, Böhm H (1975) FEBS Lett. 49: 396.
Rueffer M, Ekundayo O, Nagakura N, Zenk MH (1983) Tetrahedron Lett. 24: 2643.
Rueffer M, Zenk MH. Tetrahedron Lett. in press.
Steffens P, Nagakura N, Zenk MH (1984) Tetrahedron Lett. 25: 951.
Wiltshire HR (1975) J.C.S. Perkin I: 1147.
Zenk MH, in: The Chemistry and Biology of Isoquinoline Alkaloids (Phillipson JD et al. eds.) Springer Verlag Heidelberg, in press.