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Isolation and Characterization of *S*-Adenosyl-L-Methionine:Tetrahydroberberine-*cis*-*N*-Methyltransferase from Suspension Cultures of *Sanguinaria canadensis* L.[1]

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As part of a continuing study of the induction of alkaloid biosynthesis, we report the isolation to homogeneity and characterization of *S*-adenosyl-L-methionine:tetrahydroberberine-*cis*-*N*-methyltransferase from suspension cultures of *Sanguinaria canadensis* that were induced to produce alkaloids by hormone depletion. This enzyme catalyzes the stereospecific transfer of a methyl group from *S*-adenosyl-L-methionine to the tertiary nitrogen of the protoberberine alkaloid tetrahydroberberine (canadine). The enzyme was purified 315-fold by ammonium sulfate precipitation, gel permeation chromatography, affinity dye chromatography, and both diethylaminoethyl and Mono-Q ion-exchange chromatography. The enzyme was further purified to an optimum specific activity of 225 nkat/mg of protein (3500-fold) and electrophoretic homogeneity by native polyacrylamide gel electrophoresis (PAGE). In contrast to previous reports with partially purified enzyme, the isolated protein was found to have a pH optimum of 7.0, a temperature optimum of 25 to 30°C, and an isoelectric point of 5.1. Furthermore, the molecular weight of the homogeneous protein was found to be 39,000 by sodium dodecyl sulfate-PAGE. The homogeneous enzyme preferred tetrahydroberberine over all other substrates tested, showing an apparent K_m of 2.1 μM, but also showed partial activity with tetrahydrojatrorrhizine and tetrahydropalmatrubine.

Sanguinaria canadensis L. (Papaveraceae) is a common woodland plant throughout the eastern and middle western United States and Canada. The benzophenanthridine alkaloids produced by *S. canadensis* (bloodroot) are of interest because of their various pharmaceutical properties, which include antibacterial (sanguinarine) and antineoplastic (sanguinarine, chelerythrine) activity (Lewis and Elvin-Lewis, 1977). For example, the alkaloid sanguinarine has been commercialized for its activity against oral plaque-forming bacteria (Dzink and Socransky, 1985), and recent studies have shown that chelerythrine is a potent protein kinase C inhibitor (Herbert et al., 1990). These more recent uses for the quaternary alkaloids produced by *S. canadensis* and several other species of the Papaveraceae have stimulated continued interest in the plant cell tissue culture of these plant species and in the biosynthesis of their biologically active alkaloids.

The biosynthesis of the benzylisoquinoline alkaloids has been widely investigated and reviewed (Cordell, 1981; Beecher and Kelleher, 1988). The biosynthetic pathway leading from Phe to the benzophenanthridine alkaloid sanguinarine involves approximately 17 different enzymic steps of which at least five are methyltransferases. Many putative methyltransferases from this pathway have been partially purified and characterized, including SAM:(*R,S*)-norlaudanosoline O^6-methyltransferase (Rueffer et al., 1983a), SAM:6-*O*-methyl-norlaudanosoline $O^{5'}$-methyltransferase (Rueffer et al., 1983b), SAM:norreticuline *N*-methyltransferase (Wat et al., 1985), SAM:(*R,S*)-tetrahydrobenzylisoquinoline *N*-methyltransferase (Frenzel and Zenk, 1990a), SAM:3′-hydroxy-*N*-methyl-(*S*)-coclaurine $O^{4'}$-methyltransferase (Frenzel and Zenk, 1990b), SAM:(*S*)-scoulerine O^9-methyltransferase (Muemmler et al., 1985; Sato et al., 1993), and SAM:THB *cis*-*N*-methyltransferase (Rueffer and Zenk, 1986). A later revision of the biosynthetic scheme (Stadler et al., 1987) determined that the trioxygenated alkaloid norcoclaurine, and not norlaudanosoline, was the precursor to the key intermediate reticuline. This resulted in a change in the proposed biosynthetic pathway that has, in turn, necessitated a re-evaluation of the characteristics of the first three of these early methyltransferases.

The benzophenanthridine alkaloids are formed later in the biosynthetic pathway following the *N*-methylation of a variety of tetrahydroprotoberberine substrates (Cordell, 1981). Previous work (Takao et al., 1983) showed that only the *cis*-*N*-methyl derivatives of the tetrahydroprotoberberines can be further metabolized to form the protopine and benzophenanthridine skeletons. The stereospecific *N*-methylation of THB occurs via transfer of a methyl group from the substrate *S*-adenosyl-L-Met (Fig. 1) and has been shown to be catalyzed by a highly specific enzyme that has been partially purified from both *S. canadensis* (O'Keefe and Beecher, 1992) and *Corydalis vaginans* (Rueffer and Zenk, 1986). More recent reports of partially purified protein from *Eschscholtzia californica* and *C. vaginans* have revised the characteristics of the enzyme by describing the partial purification (90-fold) of a single enzyme responsible for the *N*-methylation of not only (*S*)-THB but other tetrahydroproto-

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Abbreviations: HPEC, high performance electrophoretic chromatography; NMTHB, *N*-methyltetrahydroberberine; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine; THB, tetrahydroberberine.

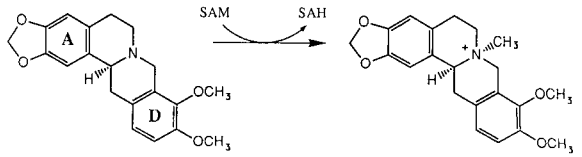


Figure 1. The enzyme THB-*N*-methyltransferase catalyzes the stereospecific transfer of a methyl group from SAM to the tertiary nitrogen of THB, resulting in the quaternary protoberberine alkaloid *cis*-NMTHB.

berberines (i.e. stylopine) as well (Rueffer et al., 1990). We now report the isolation, to homogeneity, of a similar enzyme that resides at a committed branch point in the biosynthesis of the benzophenanthridine alkaloids, SAM:THB-*cis*-*N*-methyltransferase, and has been purified from suspension cultures of *S. canadensis*.

MATERIALS AND METHODS

Chemicals

SAH was purchased from Sigma. [*methyl*-^{3}H]SAM was purchased from either Amersham, Sigma, or ICN. THB, tetrahydrojatrorrhizine, tetrahydropalmatrubine, tetrahydrophenol base, tetrahydrocolumbamine, and tetrahydropalmatine were produced by sodium cyanoborohydide (Sigma) reduction of their quaternary equivalents immediately prior to use. Reticuline, norreticuline, coclaurine, and the remaining tetrahydroprotoberberine substrates were kindly supplied by Dr. Hélène Guinaudeau of the Centre D'Étude Des Plantes Medicinales. All other chemicals were purchased from Sigma, Aldrich, or Bio-Rad.

Plant Material

Cell-suspension cultures of *Sanguinaria canadensis* L. were grown in roller bottles containing 100 mL of Gamborg B5 media (Gamborg et al., 1968) augmented with 0.5 mM 2,4-D at 23°C, and 5 g of inoculum were transferred to fresh medium every 14 d (Mahady and Beecher, 1990). Cells were induced to produce alkaloids by transfer into medium lacking all phytohormones and harvested 12 to 16 h later. Following suction filtration, cells were immediately frozen in liquid nitrogen and stored at −83°C until used for the enzyme isolation.

Methyltransferase Assay

To assay for methyltransferase activity a standard assay solution consisting of 0.2 mM (*R,S*)-THB, 0.28 μM [^{3}H-*methyl*] SAM (9.5 mCi mmol^{-1}), 3 mM DTT, 1% glycerol, and 50 mM sodium phosphate buffer (pH 7.0) in a total volume of 100 μL was freshly prepared. Reactions were initiated by the addition of 20 μL of the protein sample to the assay mix and were incubated for 30 min at 30°C. The reaction was quenched by the addition of 100 μL of a saturated NaCl solution. Products of the reaction were extracted by the addition of 1 mL of chloroform, followed by mechanical agitation and centrifugation (3000*g*, 1 min). After the sample was centrifuged, 100 μL of the organic phase was placed in a scintillation vial and diluted with 3.5 mL of scintillator (Scintilene, Fisher), and the radioactivity was measured on a Beckman LS5801 scintillation counter. Assays with boiled enzyme served as controls.

For the native gel assays, the capillary gel was extruded from its tube and cut into 3-mm pieces. Each piece was then incubated with the standard assay mixture in a total volume of 100 μL at 23°C and allowed to react for 16 h (essentially exhausting the radiolabeled substrate).

In the substrate specificity assays, the individual substrates replaced THB in the standard assay mixture and were incubated with 30 μL of native gel-purified protein for 3 h at 30°C. For determination of metal ion requirements the various divalent cations were added to the reaction mixture in the standard buffer as their chloride salts. In addition, a second nonionic buffer system (20 mM Hepes, 3 mM DTT, 1% glycerol, pH 7.0) was also used with all of the substrate concentrations and reaction conditions remaining the same. In the kinetics assays, reactions were measured at 5-, 10-, and 30-min intervals at the indicated concentrations of SAM and THB using protein purified through at least the Mono-Q step (Table I). All results are the average of at least three experiments.

Chromatography of Reaction Products

To determine the stereospecificity of the methyl group transfer, 100 μL of the Mono-Q-purified protein was incubated in the standard assay system to produce enough reac-

Table I. *Purification of THB-N-methyltransferase*

Purification Step	Total Protein	Total Activity	Specific Activity	Purification
	mg	*nkat*	*pkat/mg*	*-fold*
1. Crude supernatant	12,200	790	64	1
2. 75% $(NH_4)_2SO_4$ precipitation	4520	741	164	3
3. Affinity dye	2520	1000	396	6
4. 30–75% $(NH_4)_2SO_4$ precipitation	774	685	885	14
5. Gel filtration	368	398	1080	17
6. DEAE anion exchange	69	192	2800	43
7. Mono-Q anion exchange	4.6	94	20,300	315
8. Native gel electrophoresis	0.12	27	225,000	3500

tion product for characterization by TLC. Assay conditions were identical with the standard system except that the reaction was allowed to run to exhaustion and the total volume of the reaction was increased to 1 mL (with all substrate concentrations unchanged).

After the assay was complete the products were extracted from the buffer system as before except that the chloroform extraction step was repeated three times. The extracts were dried at room temperature under nitrogen, spotted on glass-backed silica gel plates (E. Merck), and co-chromatographed with a mixture of authentic *cis*- and *trans*-NMTHB with chloroform:methanol:ammonium hydroxide (140:40:1) as a developing solvent. Following chromatography one side of the plate was sprayed with Dragendorff's reagent to locate the separated *cis* and *trans* alkaloids. The corresponding nonsprayed alkaloids were recovered, placed into a scintillation vial, diluted with 3.5 mL of scintillation fluid, and counted for radioactivity.

Enzyme Preparation and Purification

All enzyme extraction and purification procedures were performed at 0 to 4°C unless otherwise stated. Frozen *S. canadensis* cells (1.9 kg) were ground under liquid nitrogen with a mortar and pestle and then placed in a 50 mM sodium phosphate buffer (pH 7.5) containing 3 mM DTT, 5 mM $MgCl_2$, and 1% glycerol.The cellular slurry was homogenized on a Beckman Polytron for 30 s, and then the crude homogenate was centrifuged at 12,000*g* for 15 min (model A-2; Lourdes Instrument Corp.). Following centrifugation, the supernatant was brought to 75% saturation with $(NH_4)_2SO_4$, the protein was allowed to precipitate for 60 min, and the solution was centrifuged at 20,000g for 15 min. The supernatant was discarded, and the pellets were taken up in a minimum volume of 10 mM sodium phosphate buffer (3 mM DTT, 5 mM $MgCl_2$, 1% glycerol, pH 7.5). This mixture was desalted on a YM-30 membrane (Amicon). The resulting solution (70 mL) was immediately placed on a Cibacron blue 3GA-agarose column (20 × 2.5 cm, Sigma) and washed through with an additional 400 mL of the starting buffer. The eluate (540 mL) was brought to 30% saturation with $(NH_4)_2SO_4$ and, following precipitation, was centrifuged at 20,000*g* for 15 min. The supernatant was collected and brought to 75% saturation with $(NH_4)_2SO_4$, the protein was allowed to precipitate for 60 min, and the mixture was again centrifuged at 20,000*g* for 15 min. The pellets were combined and taken up in a minimal volume of the 50 mM sodium phosphate buffer and placed on a Sephadex G-150 column (100 × 2.5 cm, Pharmacia). The column was eluted at a flow rate of 12 mL h^{-1}. The active fractions were combined and concentrated, and the buffer was exchanged by ultrafiltration (YM-30 membrane, Amicon). This resulting active protein was then subjected to ion-exchange chromatography on a DEAE-Sephacel column (20 × 2.5 cm, Pharmacia) that had been pre-equilibrated with a 10 mM sodium phosphate starting buffer (3 mM DTT, 1% glycerol, pH 7.5).

After application of the protein the column was washed with 40 mL of the starting buffer and then eluted with a linear gradient from 0 to 0.5 M KCl over a volume of 160 mL. The enzyme activity eluted at approximately 0.25 M KCl. The active fractions were concentrated and desalted as before (YM-30 membrane, Amicon) and then applied at 23°C to a Mono-Q fast protein liquid chromatography column (HR 5/5, Pharmacia) pre-equilibrated with the 10 mM sodium phosphate buffer. The column was washed with 20 mL of the starting buffer, followed by a 20-mL wash with starting buffer containing 0.1 M KCl. The active protein was then eluted with a linear gradient from 0.1 to 0.5 M KCl over a volume of 40 mL. Finally, the column was washed with 1.0 M KCl to remove any residual protein.

Electrophoresis

Active fractions eluted from the Mono-Q column were further purified on a native PAGE system consisting of a 12.5% acrylamide (Sigma) tube gel (60 × 0.75 mm), adjusted to pH 7.5, and performed at a constant current of 0.35 mA gel^{-1} at 4°C using standard buffer systems according to the method of Laemmli (1970) augmented with 5 mM DTT.

SDS-PAGE was carried out using 12.5% polyacrylamide (Sigma) slab gels adjusted to pH 8.8 with stacking gels adjusted to pH 6.8 and buffer systems according to the method of Laemmli (1970). HPEC was performed on native gels with both 7 and 10% polyacrylamide (2.5 × 50 mm) on a model 230 HPEC system (Applied Biosystems) according to the manufacturer's protocols.

Protein Determinations

Protein concentrations were established by colorimetric assay using a Coomassie brilliant blue-based system (Bio-Rad) with BSA as a standard according to procedures of Bradford (1976). In the case of HPEC-purified protein, concentrations were determined by amino acid analysis on a automated amino acid analyzer (Applied Biosystems, model 420A) according to the manufacturer's protocols.

M_r Determination

The mol wt of the protein was determined by comparison to standards (SDS-6H, Sigma) run on the standard SDS-PAGE system. The mol wt markers used were carbonic anhydrase (M_r 29,000), ovalbumin (M_r 45,000), BSA (M_r 66,000), phosphorylase B (M_r 97,400), and β-galactosidase (M_r 116,000). The native molecular radius of the protein was determined by comparison to a calibration of standard proteins on a fast protein liquid chromatography gel filtration Superose 12 column (HR 10/30, Pharmacia) at a flow rate of 500 μL min^{-1}. The molecular radius markers used were the same as those used in the SDS-PAGE system except that in this instance the standard proteins were analyzed in their native state. Active enzyme fractions purified through the G-150 (17-fold), Mono-Q (315-fold), and HPEC (3500-fold) steps were analyzed by this procedure.

IEF

IEF was done using a non-urea polyacrylamide tube gel on the Bio-Rad MiniProtean II system at 4°C. The isoelectric point of the protein was determined by comparison to a standard calibration done using standards (IEF Mix II, Sigma)

and ampholites (Ampholine 3.5–9.5, Sigma) according to the manufacturer's protocols.

RESULTS

Purification

The protocol adopted to purify THB-*N*-methyltransferase and results of the various procedures used are summarized in Table I. The enzyme was enriched 315-fold using ammonium sulfate fractionation, Cibacron blue 3GA-agarose, Sephadex G-150, and DEAE-Sephacel and Mono-Q anion exchange. Of particular note in the early purification procedures is the Cibacron blue 3GA-agarose affinity dye gel. This column bound approximately 50% of the total protein but did not bind the *N*-methyltransferase, thereby resulting in the removal of the majority of the unwanted protein with a simple bulk wash. Also of interest is that the results of the DEAE-Sephacel column indicated the possible presence of more than one enzyme active in our assay system (data not shown). Because of this apparent heterogeneity, the active fractions from this column were rechromatographed on a Mono-Q column, which resulted in the separation of two active protein peaks; the most active of these peaks was carried on to the final purification procedures. Later analysis of the second peak revealed that the active protein present had a mol wt similar to that of the protein from the the first peak but that it possessed only 25% of the relative activity.

The final step in the purification was native gel electrophoresis, and two different systems were used. First the Mono-Q-purified protein was separated on minitube gels in a Bio-Rad MiniProtean II gel electrophoresis system according to the procedures reported earlier. The tube gels were then cut into 3-mm sections and tested for activity. The results of a typical experiment are shown in Figure 2A. SDS-PAGE analysis of the active sections revealed a single band of protein, corresponding to a mol wt of approximately 39,000 (Fig. 2B). The homogeneity of the native protein was later confirmed by capillary gel electrophoresis (Fig. 2C). The native gel system was then adapted to preparative HPEC, which allowed the active protein to be eluted from the gel matrix and analyzed for specific activity (Fig. 3). The HPEC-purified protein was found to be fully active and was purified 3500-fold, exhibiting a specific activity of 225 nkat mg^{-1}.

The HPEC-purified protein was found to be stable for 30 d at 4°C in the presence of DTT (3 mM) and 1% glycerol. Furthermore, this protein was still active after 1 year when stored at −83°C in the same buffer system, although, when thawed, it remained active for only 3 d at 4°C.

Temperature and pH Optima

The temperature dependence of the enzyme was examined over the range from 0 to 50°C, and a broad maximum was found between 25 and 30°C with a distinct decrease in activity outside the range of 20 to 35°C (Fig. 4). The pH dependence of the enzyme activity was also examined over the range pH 4.5 to 9.0 in the standard 50 mM phosphate buffer system. In this case, a sharp peak of activity was found at pH 7.0, with 50% of the activity lost at pH 6.5 and 8.0. (Fig. 5).

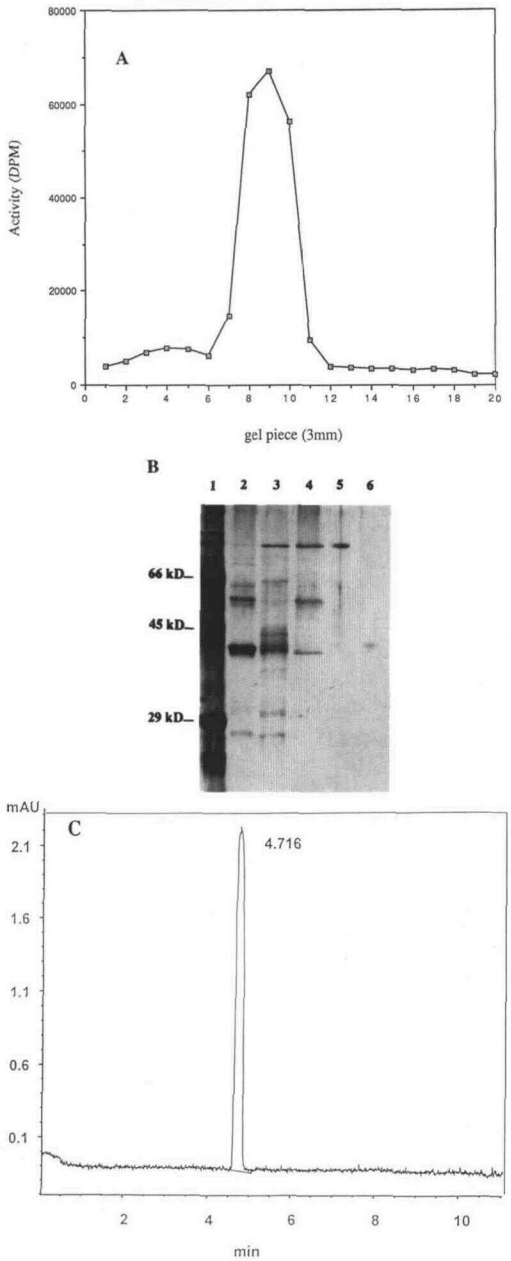


Figure 2. Native tube gel electrophoresis of NMTHB-*N*-methyltransferase. A, Sections of the tube gel (3 mm) were assayed for activity and yielded a single peak that was found to be fully active with THB. B, SDS-PAGE analysis of active fractions from both analytical and preparative native gel electrophoresis: Lane 1, mol wt standards; lane 2, HPEC-purified active fraction; lane 3, partially active fraction from HPEC; lane 4, inactive fraction from HPEC purification; lane 5, gel piece 8 from analytical native gel in A; lane 6, gel piece 9 from analytical native gel in A. C, Capillary gel electrophoresis of the most active native tube gel section shows only a single homogeneous peak of protein (absorbing at 280 nm) responsible for the activity. mAU, Milli-absorbance unit.

Both the temperature and pH maxima for the *N*-methyltransferase are similar to likely physiological conditions, although both are lower than previously reported for the partially purified enzyme (Rueffer et al., 1990). In both cases, these discrepancies may be accounted for by the large background levels that were seen in the control experiments at basic pHs and at temperatures greater than 30°C.

Mol Wt

The mol wt of the homogeneous protein was determined by comparison with the mobility of commercially available standards on SDS-PAGE. The protein was run in both the presence and absence of reducing agents (DTT or β-mercaptoethanol) to ensure that disulfide bridge formation was not altering the results. In both cases the homogeneous protein showed a single band at approximately 39,000. This mol wt is much lower than that reported for this enzyme in other plant species (78,000 and 72,000 for *E. californica* and *C. vaginans*, respectively) (Rueffer et al., 1990).

Because of the differences between the previously reported mol wts and our results, we sought to determine the native molecular radius of the *N*-methyltransferase by gel permeation chromatography on a high-performance Superose 12 HR column. Earlier attempts to resolve the active protein by gel permeation on analytical Sephadex G-150 and G-200 columns revealed that the activity was eluting at a retention volume corresponding to a mol wt of approximately 70,000. The molecular radius was determined by comparison with the same mol wt standards used for the SDS-PAGE analysis, except that all of the proteins were chromatographed in their native state. The results indicated that, whereas all of the standard proteins eluted at rates relative to their mobility in the SDS-PAGE system, the *N*-methyltransferase did not. It was determined that in this system, as in the earlier experiments, the *N*-methyltransferase eluted at a retention volume corresponding to a molecular radius of 70,000 (±3%). Furthermore, the retention volume of the active protein did not change with increasing levels of purification, because enzyme fractions purified 17-, 315-, or 3500-fold all displayed similar retention volumes. The results indicate either that the native protein is a homodimer or that the protein is not globular.

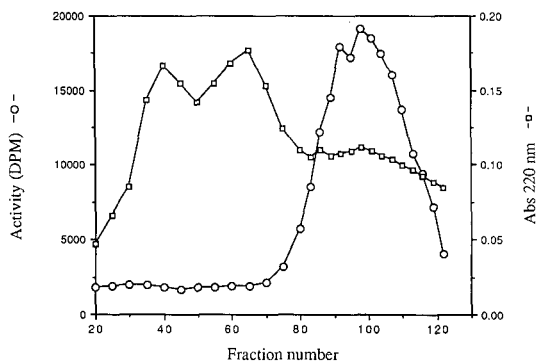


Figure 3. HPEC of THB-*N*-methyltransferase. Enzyme, purified through the Mono-Q anion-exchange step, was applied to a 10% polyacrylamide native gel column (2.5 × 50 mm) and eluted at a constant current of 2.0 mA. Abs, *A*.

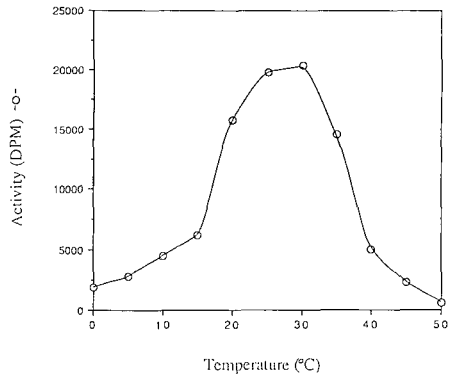


Figure 4. Temperature profile of the catalytic activity of THB-*N*-methyltransferase. The 315-fold purified enzyme was assayed in 50 mM sodium phosphate buffer (3 mM DTT, 5 mM $MgCl_2$, 1% glycerol, pH 7.0).

Isoelectric Point

The isoelectric point of the enzyme was determined by IEF on a polyacrylamide-based system, followed by SDS-PAGE. The native gel-purified protein was compared with commercially available standards and was found to have an isoelectric point of 5.1. The protein did not focus particularly well and usually gave a slightly smeared spot with the great majority of the protein residing at the isoelectric point.

Metal Ion Interactions

The divalent metal ion interactions of the *N*-methyltransferase were examined, and the results were found to be greatly dependent on the buffer system used for the analysis. Initial studies were conducted in a 50 mM sodium phosphate buffer (pH 7.0), and the results indicated a slight increase in enzyme activity with increasing Mg^{2+} concentrations, whereas the heavy metal ions Co^{2+} and Zn^{2+} were found to be inhibitory. The increased activity of the enzyme in response to Mg^{2+} was similar to previously published reports of other plant-derived methyltransferases (Jay et al., 1983; Knogge and Weissenböck, 1984). However, when the same

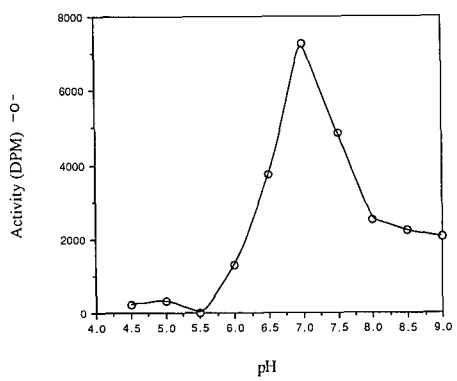


Figure 5. The pH dependence of the catalytic activity of THB-*N*-methyltransferase. Enzyme purified through the Mono-Q step was assayed as described in "Materials and Methods."

interactions were examined in a non-ionic buffer system (20 mM Hepes, pH 7.0), the results were completely different. As detailed in Table II, when placed in the Hepes buffer system the enzyme was not only inhibited by the heavy metal cations Co^{2+} and Zn^{2+} but also by Ca^{2+}, Mn^{2+}, and Mg^{2+}. Finally, it was shown that the addition of 10 mM EDTA increased *N*-methyltransferase activity in crude enzyme preparations using either the Hepes or the phosphate buffer systems.

Substrate Specificity

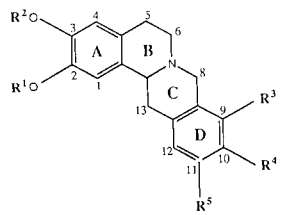
The substrate specificity of the enzyme was examined to determine the range of isoquinoline substrates that could be methylated. Of the 14 tetrahydroprotoberberine substrates tested (Fig. 6), none showed the level of activity displayed by THB (100%). Furthermore, only tetrahydropalmatrubine (45%) and tetrahydrojatrorrhizine (7%) showed partial activity with the enzyme, whereas tetrahydropalmatine, tetrahydrocolumbamine, tetrahydrophenolbase, kikemanine, scoulerine, stylopine, cheilanthifoline, thalictracavine, anibacanine, xylopinine, and pseudo-anibacanine were shown to be inactive. In addition, five benzylisoquinoline alkaloids (reticuline, coclaurine, norreticuline, norlaudanosoline, and *N*-methylcoclaurine) were also tested, and none of these compounds was found to be an acceptable substrate for the *N*-methyltransferase.

The purified enzyme shows a very high degree of specificity with very strict limitations on the acceptable substitution patterns on rings A and D of the protoberberine skeleton. At first it was surprising that no activity for stylopine was found with the homogeneous protein, because such activity had been reported earlier (Rueffer et al., 1990). Upon re-analysis we did see the stylopine-*N*-methyltransferase activity in earlier, less highly purified protein extracts, but all of this activity was removed during the final stages of purification.

Table II. *Influence of divalent cations on THB-N-methyltransferase activity*

Enzyme was assayed in either 20 mM Hepes buffer (pH 7.0) or 50 mM sodium phosphate buffer (pH 7.0) with 3 mM DTT, 1% glycerol, and the indicated additions.

Addition	Buffer	Concentration	Relative Activity
		mM	%
None	Hepes		100
None	PO_4		109
Na_2EDTA	Hepes	10	145
$CaCl_2$	Hepes	1	37
$CaCl_2$	Hepes	5	0
$CaCl_2$	PO_4	5	104
$MnCl_2$	Hepes	1	9
$MnCl_2$	Hepes	5	2
$MnCl_2$	PO_4	5	125
$MgCl_2$	Hepes	1	18
$MgCl_2$	Hepes	5	2
$MgCl_2$	PO_4	5	141
$CoCl_2$	Hepes	1	0
$CoCl_2$	Hepes	5	0
$CoCl_2$	PO_4	5	24
$ZnCl_2$	Hepes	1	0
$ZnCl_2$	Hepes	5	0
$ZnCl_2$	PO_4	5	21



	R¹	R²	R³	R⁴	R⁵
Tetrahydroberberine	-CH_2-		-OCH_3	-OCH_3	-H
Tetrahydrocolumbamine	-H	-CH_3	-OCH_3	-OCH_3	-H
Tetrahydrojatrorrhizine	-CH_3	-H	-OCH_3	-OCH_3	-H
Tetrahydropalmatine	-CH_3	-CH_3	-OCH_3	-OCH_3	-H
Tetrahydropalmatrubine	-CH_3	-CH_3	-OH	-OCH_3	-H
Tetrahydrophenol base	-H	-H	-OCH_3	-OCH_3	-H
Scoulerine	-H	-CH_3	-OH	-OCH_3	-H
Stylopine	-CH_2-		-OCH_2O-		-H
Cheilanthifoline	-H	-CH_3	-OCH_2O-		-H
Thalictracavine	-CH_2-		-OCH_3	-OCH_3	-H
Pseudoanibacinine	-H	-CH_3	-H	-H	-OH
Xylopinine	-CH_3	-CH_3	-OCH_3	-OCH_3	-OCH_3
Kikeminine	-CH_3	-CH_3	-OCH_3	-OH	-H
Anibacinine	-H	-CH_3	-OH	-H	-H

Figure 6. Structural relationships among the tetrahydroprotoberberine substrates tested for activity with THB-*N*-methyltransferase.

Stereospecificity of the Methylation Reaction

To determine the position of the *N*-methyl group on the product NMTHB, a large-scale assay was run and the products of the reaction were separated by TLC. The *cis* and *trans* isomers of standard NMTHB were separated using a chloroform:methanol:ammonium hydroxide (140:40:1) solvent system and silica gel plates (R_F = 0.24 and 0.41, respectively). When the radiolabeled enzyme reaction product was co-chromatographed with standard compounds, followed by scintillation counting of the excised spots, we found that all of the alkaloid-associated radioactivity resided in the spot corresponding to the *cis* conformation of the product NMTHB (Fig. 7).

Kinetics

Kinetic analysis was done to examine the affinity of the enzyme for the two main substrates THB and SAM. The K_m for (*R,S*)-THB was found to be 2.1 μM (Fig. 8A), and the K_m for SAM was found to be 1.2 μM (Fig. 8B). Both figures compare favorably to those previously published for methyltransferases along this secondary biosynthetic pathway (Frenzel and Zenk, 1990a; Rueffer et al., 1990). The known methyltransferase inhibitor SAH was also examined for competitive inhibition of the enzyme and, as expected, displayed classic competitive inhibition giving a K_i of 7.5 μM (data not shown).

Other inhibitors of the *N*-methyltransferase activity were

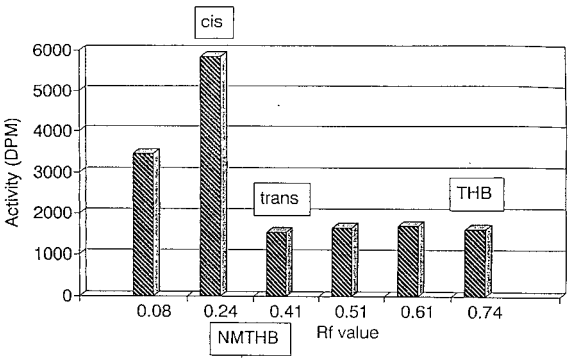


Figure 7. Thin-layer co-chromatography of the reaction product resulting from incubation of THB with [*methyl*-^{3}H]SAM and standard *cis*- and *trans*-NMTHB. Solvent system used chloroform: methanol:ammonium hydroxide (140:40:1).

the alkaloid berberine and the sulfhydryl-modifying agent *N*-ethylmaleimide. The inhibition by *N*-ethylmaleimide correlates well with the reducing agent requirement for this enzyme and suggests that reduced Cys residues may be involved with the active site of this enzyme.

DISCUSSION

The enzyme SAM:THB-*cis*-*N*-methyltransferase has been purified to homogeneity from suspension cultures of *S. canadensis*. This enzyme catalyzes the stereospecific transfer of a methyl group from the substrate SAM to the tertiary nitrogen of the protoberberine alkaloid THB. The enzyme was purified 3500-fold to an extremely high specific activity of 225 nkat mg^{-1}, despite the fact that this protein was present in only minute concentrations (0.001% of total protein). The specific activity of the purified THB-*N*-methyltransferase from *S. canadensis* is in the same range as that reported for dihydrobenzophenanthridine oxidase from this plant (Arakawa et al., 1992) but approximately 200 times greater than that reported for the tetrahydroprotoberberine-*N*-methyltransferase from *E. californica* (Rueffer et al., 1990). The final native gel electrophoresis step in the purification was the key to obtaining active homogeneous enzyme. Throughout the purification procedures two bands of protein were continually co-eluting in the active fractions, one at approximately 39,000 and one at approximately 80,000. Although many other methods of purification were attempted, including SAH affinity, hydroxylapatite, gel permeation, and hydrophobic interaction chromatography, only the native gel technique provided electrophoretically pure protein.

The isolated protein was shown to have a mol wt of approximately 39,000 by SDS-PAGE. This value is smaller than previously reported for this enzyme (Rueffer and Zenk, 1986) but similar to that reported for other plant-derived methyltransferases (Preisig et al., 1989; Bugos et al., 1991; Sato et al., 1993). The native molecular radius, as determined by gel filtration, was found to be greater than that of BSA (M_r 66,000), indicating that the protein either is not globular or is a homodimer in its native state. This discrepancy between the mol wt and molecular radius has been reported previously for plant-derived methyltransferases (Preisig et al., 1989; Bugos et al., 1991; Sato et al., 1993). The fact that both the reduced and oxidized forms of the homogeneous native enzyme are indistinguishable by capillary gel electrophoresis, together with evidence that both the native and urea-denatured forms of the enzyme show identical two-dimensional electrophoretic positions, indicates that, although the possibility of a dimer cannot be completely ruled out at this time, the probability is that the protein is not globular. Mass spectral analysis of both the native and denatured forms of the protein, presently being undertaken in our laboratory, should differentiate between these two possibilities.

The purified *N*-methyltransferase was found to be stable at 4°C for several weeks when kept in buffer augmented with 3 mM DTT and 1% glycerol and was still active after 1 year when stored at −83°C. The temperature and pH optima of 25 to 30°C and 7.0, respectively, are very close to physiological conditions, although significantly lower than those reported for a less highly purified enzyme from *E. californica* and *C. vaginans* (Rueffer et al., 1990).

Previous reports of divalent metal ion interactions with methyltransferases appear almost equally split between those that display enhanced activity in the presence of Mg^{2+} and those that show no metal ion requirements (for a review, see Poulton, 1981). In the case of THB-*N*-methyltransferase the interaction with metal ions was shown to be greatly influ-

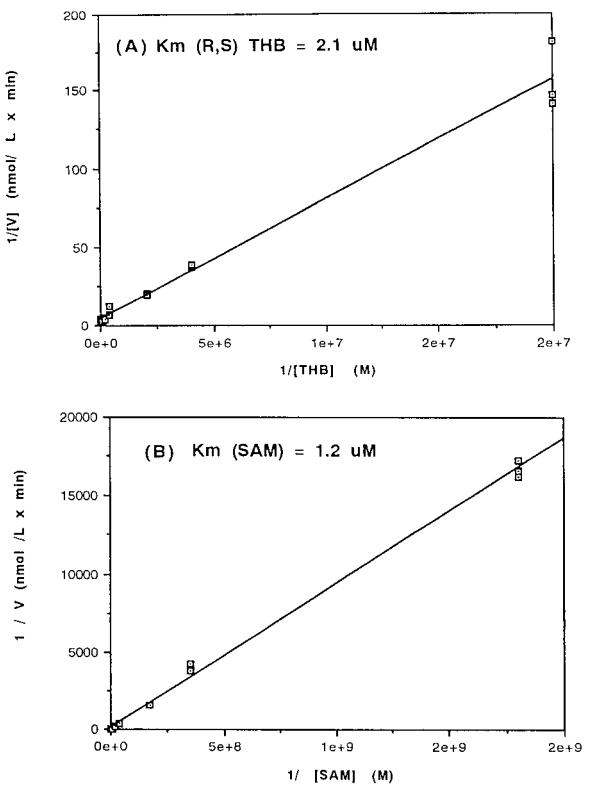


Figure 8. Double-reciprocal plots showing the relationship between the velocity of the *N*-methyltransferase catalyzed reaction and the concentration of THB (A) and SAM (B).

enced by the buffer system used. When an ionic buffer such as sodium phosphate was used, the buffer interacted with the divalent metal ions, leading to the spurious result that Mg^{2+} cations enhanced enzyme activity. Upon examination of the same interactions in a noninteracting Hepes buffer, it was clearly demonstrated that, not only did the metal ions not increase enzyme activity, they strongly inhibited it.

THB-*N*-methyltransferase was found to be stringently specific with regard to acceptable tetrahydroprotoberberine substrates. Of the 15 tetrahydroprotoberberine and 5 benzylisoquinoline compounds tested, only THB was fully active, whereas tetrahydrojatrorrhizine and the unnatural substrate tetrahydropalmatrubine showed partial activity with the homogeneous enzyme. Neither of the early protoberberine intermediates scoulerine or tetrahydrocolumbamine were active with the enzyme. Because most of the tetrahydroprotoberberine substrates tested differed only in the substitution pattern on the A and D ring systems, it is apparent that these regions are key to the specificity of the enzyme.

The lack of methyltransferase activity with the substrate stylopine was not expected, because earlier descriptions of this enzyme had indicated equivalent activity for stylopine and THB (Rueffer et al., 1990). This ability to differentiate between stylopine and THB, however, has been reported with other enzymes along this biosynthetic pathway (Galneder et al., 1988; De-Eknamkul et al., 1992). Because crude enzyme preparations from *S. canadensis* do show the ability to *N*-methylate stylopine, whereas the homogeneous protein does not, the probability is that another enzyme is responsible for the activity demonstrated for this substrate.

The kinetic analysis of THB-*N*-methyltransferase was initially hampered by the fact that both of the major contaminants in the substrates SAM and THB were inhibitory to the methylation reaction (SAH and berberine, respectively). When these impurities were removed, the purified enzyme displayed a high degree of affinity for both substrates, indicated by the low K_m values of 2.1 μM for (*R,S*)-THB and 1.2 μM for SAM. The competitive inhibition of the enzyme by the reaction product SAH was expected, and the K_i of 7.5 μM is slightly lower than that reported for the enzyme from *E. californica* and *C. vaginans* (Rueffer et al., 1990). This product inhibition by SAH is in contrast to the other product of the reaction, NMTHB, which was not found to be inhibitory even at levels of 100 μM (data not shown).

The requirement for reducing agents such as DTT and β-mercaptoethanol indicated that one or more reduced Cys residues was involved in enzyme activity. This hypothesis was confirmed by the ability of the sulfhydryl-alkylating compound *N*-ethylmaleimide to completely inhibit the enzyme. Similar requirements for reduced sulfhydryl groups by plant-derived methyltransferases have been reported frequently in the literature (for a review, see Poulton, 1981).

The results presented here show that THB-*N*-methyltransferase is a very specific branch point enzyme in the biosynthesis of the benzophenanthridine alkaloids by *S. canadensis*. The enzyme was purified to the highest level of specific activity by native gel electrophoresis, and this active protein was shown to be homogeneous by both SDS-PAGE and capillary gel electrophoresis. Because of the differences between the characteristics of the homogeneous enzyme from *S. canadensis* reported here and those described previously (e.g. mol wt, pH, and temperature optima, substrate specificity; Rueffer and Zenk, 1986; Rueffer et al., 1990), it is apparent that the analysis of partially purified enzymes can lead to results representative of a mixture of proteins that do not necessarily represent the enzyme of interest. Continued work toward a more detailed analysis of the physical and kinetic characteristics of THB-*N*-methyltransferase, as well as a more thorough understanding of the regulation of this biosynthetic pathway, is presently being undertaken in our laboratory.

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