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S-ADENOSYL-L-METHIONINE:NORCOCLAURINE 6-*O*-METHYLTRANSFERASE FROM *THALICTRUM MINUS* CELL CULTURES

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Key Word Index—*Thalictrum minus*; Ranunculaceae; cell culture; SAM:norcoclaurine 6-*O*-methyltransferase; berberine biosynthesis.

Abstract—*S*-Adenosyl-L-methionine (SAM): norcoclaurine 6-*O*-methyltransferase (NCMT), which controls berberine biosynthesis in *Thalictrum minus* cell cultures, was purified to homogeneity. The purified NCMT had a native M_r of 72 000 consisting of two subunits of 37 000 each, a pH optimum of 9.0 and a p*I* of 4.3. The enzyme did not require Mg^{2+} for activation, but was strongly inhibited by divalent cations of heavy metals and by SH reagents. Kinetic studies indicated a sequential mechanism with K_m values of 56 μM for norcoclaurine and 260 μM for SAM. *S*-Adenosyl-L-homocysteine (SAH) was a potent inhibitor of NCMT and its K_i value for SAH vs SAM was 89 μM.

INTRODUCTION

Thalictrum minus var. *hypoleucum* cell suspension cultures produce berberine and excrete it into Linsmaier–Skoog (LS) medium [1] containing both 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) [2, 3]. Although berberine biosynthesis is completely suppressed in a LS medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), it can be recovered by adding BAP to the 2,4-D medium [4].

Tracer experiments using L-[U-^{14}C] tyrosine as well as measurements of enzyme activities showed that the suppression of berberine biosynthesis by 2,4-D was a result of inhibition of two enzymes, norcoclaurine 6-*O*-methyltransferase (NCMT) and (*S*)-tetrahydroberberine oxidase [4–6]. NCMT, which is a crucial enzyme controlling benzylisoquinoline biosynthesis [5, 7], was partially purified and characterized in *Argemone platyceras* by using (*R*,*S*)-norlaudanosoline as a substrate [8]. This enzyme converted norlaudanosoline to 6-*O*-methylnorlaudanosoline unstereospecifically. NCMT was also isolated from *Berberis koetineana* cell cultures, but could not be completely separated from 3′-hydroxy-*N*-methyl-(*S*)-coclaurine 4′-*O*-methyltransferase [9]. This paper reports the purification of NCMT from *T. minus* cell suspension cultures and describes its characterization and reaction mechanism.

RESULTS AND DISCUSSION

Purification of NCMT

To prepare *Thalictrum* cells showing a high NCMT activity, suspension cultures were transferred to LS medium supplemented with 1 μM 2,4-D and 10 μM BAP. These cultured cells were harvested 6 days after inoculation when they showed the highest NCMT activity (*ca* 15 pkat g^{-1} fr. wt).

Purification procedures for NCMT are summarized in Table 1. The most efficient method of purification was found to be the affinity chromatography on SAH-Sepharose, which had often been applied to the purification of methyltransferases [10, 11] (Fig. 1). An active fraction

Table 1. Purification of NCMT from *Thalictrum minus* cell suspension cultures

Purification step	Protein (mg)	Total activity (pkat)	Recovery (%)	Specific activity (pkat mg^{-1})	Purification(-fold)
Crude extract	1030	4670	100	4.5	1
Ammonium sulphate precipitation (35/70%)	583	4010	86	6.9	1.5
DEAE-Toyopearl	52.1	2690	58	51.6	11
SAH–Sepharose	0.72	484	10	672	149
Shim-pack DEAE	0.06	89.5	1.9	1490	332

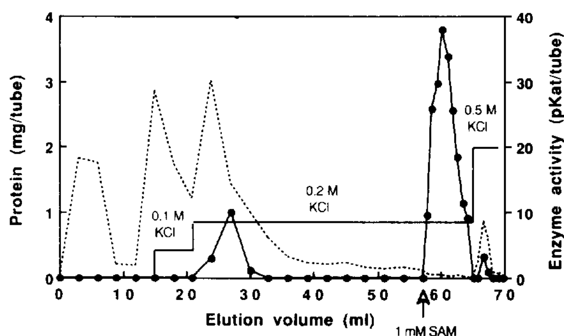

Fig. 1. Purification of NCMT (norcoclaurine 6-*O*-methyltransferase) from *T. minus* cell cultures treated with 10 μM BAP by affinity chromatography on SAH–Sepharose. (···) Protein content, (—●—) NCMT activity. Fraction volumes: 3.0 ml (before the affinity elution with 1 mM SAM) and 0.8 ml (the affinity elution).

obtained by eluting the column with 1 mM *S*-adenosyl-L-methionine (SAM) consisted of a major protein and some minor proteins, as revealed by SDS–PAGE with silver staining. After affinity chromatography, NCMT was purified to homogeneity on Shim-pack DEAE. The final purification was 332-fold and the yield was 1.9%.

Properties of NCMT

The M_r of the denatured NCMT was 37 000 on SDS–PAGE, whereas that of the active NCMT was determined to be 72 000 by gel filtration chromatography on Asahipak GFA-50. This suggested that the active protein should be a homodimer. Its isoelectric point was estimated to be 4.3 by chromatofocusing on Mono P. The pH optimum was 9.0 and the half maximal activities were found at pH 6.0 and 9.5.

Inhibitors of NCMT

Effects of inhibitors on the activity of NCMT are shown in Fig. 2. The enzyme activity was strongly inhibited by cations of heavy metals, in particular Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} at 1 mM. Magnesium ion, which was reported to activate both catechol *O*-methyltransferase [12] and luteolin *O*-methyltransferase [13], did not affect the NCMT activity. SH reagents, *p*-chloromercuribenzoate (PCMB) and *N*-methylmaleimide (NMM), completely inhibited the enzyme activity at 1 mM, whereas iodoacetamide (IOA) was inhibitory at 5 mM. These results suggested that an SH group plays a vital role in the NCMT activity. Not only berberine and magnoflorine which are two major products of *T. minus* cell cultures, but also the related isoquinoline alkaloids (jatrorrhizine, coptisine and sanguinarine) more or less suppressed the NCMT activity at 0.5 mM, suggesting a possible feedback control of NCMT by final biosynthetic products. However, it seems likely that *T. minus* cells may escape such a feedback inhibition by rapidly excreting berberine synthesized into the medium through a unique active transport system [3].

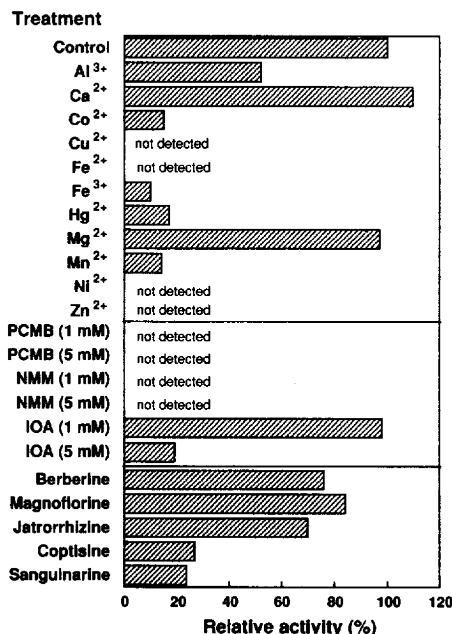

Fig. 2. Effect of metal ions, SH reagents and alkaloids on the activity of NCMT. Inorganic cations were used as chlorides. Concentrations of metal ions and alkaloids administered were 1 and 0.5 mM, respectively. The relative activity of 100% corresponds to 0.646 pkat. PCMB: *p*-chloromercuribenzoate, NMM: *N*-methylmaleimide, IOA: iodoacetamide. Mean of three replicates.

S-Adenosyl-L-homocysteine (SAH), a potent inhibitor of NCMT, competitively inhibited the methylation of norcoclaurine into coclaurine by SAM (Fig. 3), the K_i value of NCMT for SAH being 89 μM.

Kinetics and substrate specificity of NCMT

In general, *O*-methyltransferases of plant origin, including caffeic acid 3-*O*-methyltransferase in *Medicago sativa* [14], 3-methylquercetin 7-*O*-methyltransferase in *Chrysosplenium americanum* [15] and scoulerine 9-*O*-methyltransferase in *Coptis japonica* [16], exhibited a reaction mode of sequential mechanism. Kinetic studies of NCMT in *T. minus* have also indicated a mechanism of sequential binding (Fig. 4). The K_m values for norcoclaurine and SAM were determined to be 56 μM and 260 μM, respectively, implying that NCMT in *T. minus* cells might be regulated by phenolic substrate availability.

NCMT from *T. minus* converted norcoclaurine non-stereospecifically. When 5.7 nmol of (*R*,*S*)-norcoclaurine was added to a reaction mixture containing purified NCMT, the whole substrate was methylated to yield 5.8 nmol of coclaurine. An examination of the substrate specificity of NCMT by the [^{14}C-Me]SAM assay showed that NCMT was able to transfer the labelled methyl group of SAM not only to (*R*,*S*)-norcoclaurine but also to (*R*,*S*)-norlaudanosoline and (*R*,*S*)-laudanosoline equally (Table 2). By contrast, neither phenethyl amines nor any

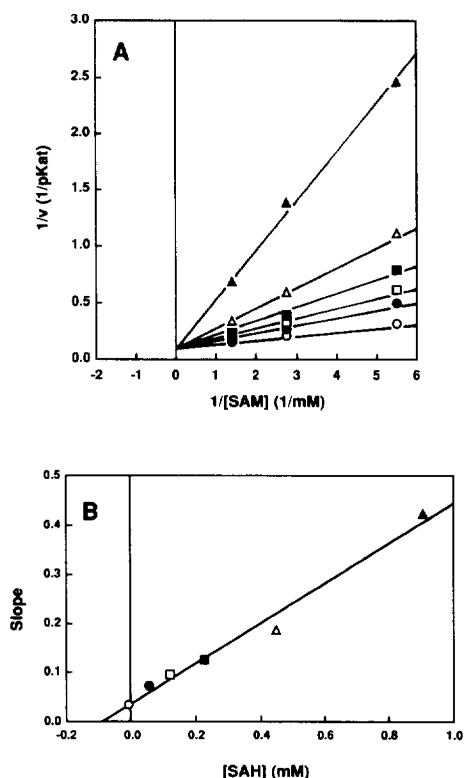

Fig. 3. (A) Double-reciprocal plot against SAM (0.182, 0.364 and 0.727 mM) in the presence of different concentrations of SAH (*S*-adenosyl-L-homocysteine): 0 mM (○), 0.057 mM (●), 0.114 mM (□), 0.227 mM (■), 0.455 mM (△), and 0.909 mM (▲). The norcoclaurine concentration was 0.41 mM. (B) [SAH]-slope plot to determine the K_i value for SAH. Mean of three replicates.

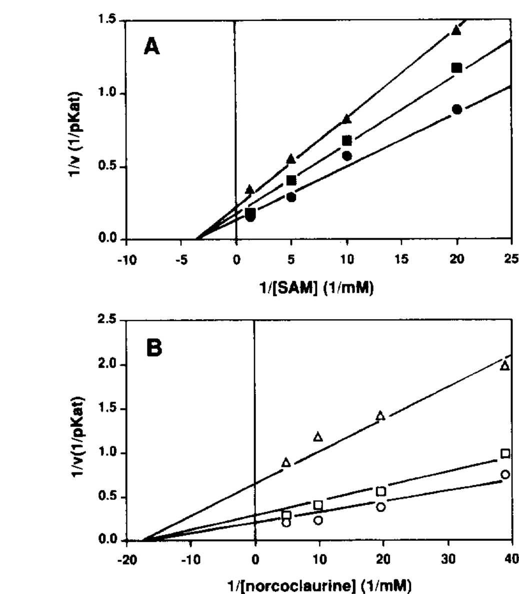

Fig. 4. Double-reciprocal plots for initial velocities of NCMT reaction. (A) The concentration of norcoclaurine was fixed at 0.05 mM (▲), 0.11 mM (■) and 0.21 mM (●). (B) The concentration of SAM was fixed at 0.05 mM (△), 0.2 mM (□), and 0.4 mM (○). Mean of three replicates.

other intermediates of berberine biosynthesis were utilized as substrates by NCMT. Furthermore, the enzyme could methylate neither caffeic acid nor quercetin.

Comparison of NCMTs

Characteristics of NCMT from *T. minus* were compared with those of norlaudanosoline 6-*O*-methyltransferase, which was isolated by Rüffer *et al.* [8] from *Argemone platyceras* cell suspension cultures (Table 3). These enzymes were different in M_r, optimum pH and K_m values for substrates, but were similar in substrate specificities. Both enzymes could methylate (*R*)- and (*S*)-substrate equally, recognizing norlaudanosoline and laudanosoline as substrates.

NCMT is thought to be essential to the biosynthesis of benzylisoquinoline alkaloids [9], which have been found in *ca.* 40 families [17], suggesting its wide distribution in the plant kingdom. However, several differences in the characteristics of NCMT between *Argemone* and *Thalictrum* seem to reflect partial changes in the gene structure which would have occurred during the process of diversification of plants.

EXPERIMENTAL

Chemicals. S-Adenosyl-L-methionine (SAM), *S*-adenosyl-L-homocysteine (SAH), dopamine HCl, tyramine

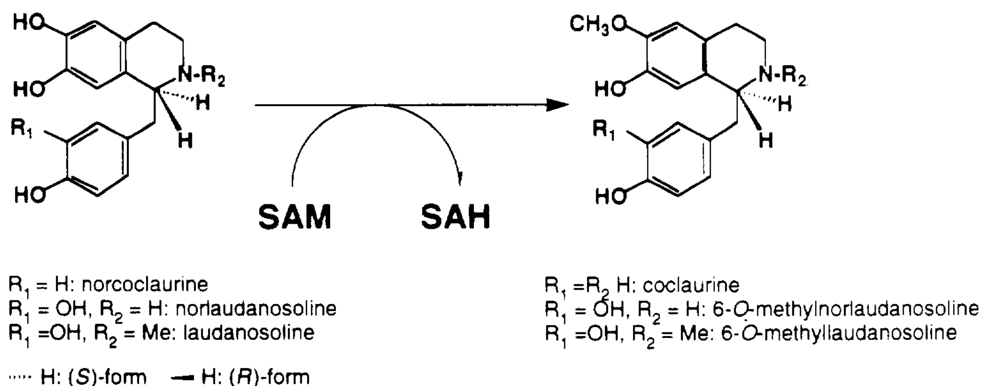

Table 2. Substrate specificity of NCMT isolated from *T. minus* cell cultures

Substrate	Enzyme activity (pkat)	Relative activity (%)
(*R*, *S*)-Norcoclaurine	0.81	100
(*R*,*S*)-Norlaudanosoline	0.96	119
(*R*,*S*)-Laudanosoline	1.17	145
Dopamine	0	0
Tyramine	0	0
(*R*,*S*)-Reticuline	0	0
(*R*,*S*)-Scoulerine	0	0
Caffeic acid	0.01	1
Quercetin	0	0

The enzyme activity for (*R*,*S*)-norcoclaurine was 0.81pkat per assay. The concentration of substrates was 0.5 mM each. Assays were performed by the [^{14}C-Me] SAM assay (see Experimental for details).

Table 3. Comparison of NCMT between *Thalictrum minus* and *Argemone platyceras*

	T. minus	*A. platyceras**
M_r		
native	72 000	47 000
subunit	37 000	not determined
p*I*	4.3	not determined
Optimum pH	9.0	7.5
Substrate stereospecificity	non-specific	non-specific
K_m (μM)	56 (norcoclaurine)	200 (norlaudanosoline)
	260 (SAM)	50 (SAM)
K_i for SAH (μM)	89	10
Inhibitors	Cu^{2+}, Zn^{2+}, Co^{2+}, Hg^{2+}, Fe^{2+}, Mn^{2+}, PCMB, NMM	Cu^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Hg^{2+}, Fe^{2+}, PCMB

*Data cited from ref. [8].

HCl and sanguinarine were purchased from Sigma. [^{14}C-Me]SAM was obtained from ICN (U.S.A). Caffeic acid, quercetin and berberine were purchased from Wako (Japan) and (*R*,*S*)-norlaudanosoline and (*R*,*S*)-laudanosoline from Aldrich. (*R*,*S*)-Reticuline and (*R*,*S*)-scoulerine were generous gifts of Mitsui Petrochemical Industries, Ltd (Japan). (*R*,*S*)-Norcoclaurine was synthesized as described in ref. [5]. Magnoflorine, jatrorrhizine and coptisine were isolated from dry rhizomes of *Coptis japonica* Makino.

Plant cell culture. Thalictrum minus L. var. *hypoleucum* Miq. cell suspension cultures (inoculum size: 3 g fr. wt) [2] were subcultured every two weeks in Linsmaier–Skoog (LS) medium (100 ml) [1] containing 1 μM 2,4-D. For induction of berberine biosynthesis, BAP (10 μM) was added to the culture medium at the beginning of culture. The cultures were agitated on a reciprocal shaker at a speed of 100 strokes min^{-1} at 25° in the dark.

Enzyme assay. For enzyme purification and kinetic studies, the NCMT activity was assayed as previously described [5] with slight modifications. The assay mixt. contained 1 mM (*R*,*S*)-norcoclaurine, 20 mM Na ascorbate, 1 mM SAM and 35 μl of enzyme soln in a total vol. of 50 μl. After incubation for 15 min at 30°, the reaction was stopped by the addition of 5 μl satd Na_2CO_3. Coclaurine formed was then extracted with 40 μl isoamylalcohol and the organic phase was analysed by HPLC; column: TSK-GEL ODS 120A (4.6 × 150 mm), solvent system: 50 mM tartaric acid–10 mM SDS–MeCN–MeOH (4:4:4:1), flow rate: 1.5 ml min^{-1}, detection: 280 nm. The substrate specificity of NCMT was investigated by measuring the radioactivity transferred from [^{14}C-Me]SAM to each substrate, according to ref. [8]. The reaction mixt. consisted of 17.5 μl of purified enzyme (0.81 pkat), 0.5 mM substrate, 20 mM Na ascorbate and 0.5 mM [^{14}C-Me]SAM (0.025 μCi) in a final vol. of 25 μl. Samples were incubated for 20 min at 30°, stopped by adding 2.5 μl of satd Na_2CO_3 (for alkaloids and amines) or 50% TCA (for caffeic acid and quercetin), and extracted with 40 μl of isoamylalcohol (for alkaloids and amines) or EtOAc (for caffeic acid and quercetin). The radioactivity of the organic phase (20 μl) was measured by a liquid scintillation counter.

Purification of NCMT. BAP-treated cells (300 g fr. wt, 6-day-old culture) frozen in liquid N_2 were allowed to thaw in 600 ml of 100 mM Tris–HCl buffer (pH 7.5) containing 60 mM 2-mercaptoethanol and 3% (w/v) polyvinyl polypyrrolidone and homogenized with a pestle. The homogenate was centrifuged for 20 min at

12 000 *g*. The supernatant was adjusted to 35% satn with $(NH_4)_2SO_4$. After centrifugation the supernatant was adjusted to 70% satn and the protein pellet was collected by centrifugation. The ppt. was resuspended in 70 ml of 50 mM Tris–HCl buffer (pH 8.5) containing 20 mM 2-mercaptoethanol (buffer A) and desalted by passing through a PD-10 column (Pharmacia) equilibrated with buffer A. The extract was loaded onto DEAE-Toyopearl 650 M (15 × 280 mm) equilibrated with buffer A and washed with the same buffer. Bound protein was eluted at a rate of 40 ml hr^{-1} by a linear gradient of KCl (0–500 mM) in buffer A (200 ml). Frs showing a single peak of NCMT activity were pooled and the buffer was changed to 50 mM Tris–HCl buffer (pH 8.5) containing 5 mM dithiothreitol (DTT) (buffer B). The sample was then applied to an affinity column of SAH–Sepharose prepared by coupling SAH to EAH-Sepharose 4B (Pharmacia). After washing the column with buffer B containing 200 mM KCl (buffer C), an active fr. was obtained by affinity elution with buffer C containing 1 mM SAM.The eluate was immediately injected to HPLC of the Shimadzu LC-10A system connected to a Shim-pack DEAE column equilibrated with buffer B. The bound protein was eluted at a speed of 1 ml min^{-1} by a linear gradient of NaCl (0–500 mM). Active frs were combined and stabilized by adding 10% glycerol and 5 mM EDTA, and frozen at −80°. The native and subunit M_r were estimated by Asahipak GFA-50 and 12.5% SDS–PAGE, respectively. The isoelectric point of NCMT was measured by Mono P (Pharmacia). The protein content was determined by the method of ref. [18] using BSA as a standard.

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