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Purification and characterization of coclaurine *N*-methyltransferase from cultured *Coptis japonica* cells

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Abstract

S-Adenosyl-L-methionine (SAM): coclaurine *N*-methyltransferase (CNMT), which catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine to the amino group of the tetrahydrobenzylisoquinoline alkaloid coclaurine, was purified 340-fold from *Coptis japonica* cells in 1% yield to give an almost homogeneous protein. The purified enzyme, which occurred as a homotetramer with a native M_r of 160 kDa (gel-filtration chromatography) and a subunit M_r of 45 kDa (SDS-polyacrylamide gel electrophoresis), had an optimum pH of 7.0 and a pI of 4.2. Whereas (*R*)-coclaurine was the best substrate for enzyme activity, *Coptis* CNMT had broad substrate specificity and no stereospecificity; CNMT methylated norlaudanosoline, 6,7-dimethoxyl-1,2,3,4-tetrahydroisoquinoline and 1-methyl-6,7-dihydroxy-1,2,3,4,-tetrahydroisoquinoline. The enzyme did not require any metal ion. *p*-Chloromercuribenzoate and iodoacetamide did not inhibit CNMT activity, but the addition of Co^{2+}, Cu^{2+} or Mn^{2+} at 5 mM severely inhibited such activity by 75, 47 and 57%, respectively. The substrate-saturation kinetics of CNMT for norreticuline and SAM were of the typical Michaelis–Menten-type with respective K_m values of 0.38 and 0.65 mM.

Keywords: Alkaloid biosynthesis; Berberine; *Coptis japonica*; Ranunculaceae; Isoquinoline alkaloid; *S*-Adenosyl-L-methionine; Coclaurine *N*-methyltransferase

1. Introduction

Berberine, a benzylisoquinoline alkaloid obtained from *Berberis* and *Coptis* species, is an important pharmaceutical alkaloid with antibacterial, stomachic and anti-inflammatory activity (Sato et al., 1994). The biotechnological importance of this alkaloid and the basic interest in elucidating the steps involved in its biosynthesis have led to the complete characterization of this pathway in plant cell cultures (Sato et al., 1993, 1994; Kutchan, 1998). Berberine is derived from L-tyrosine via 13 different enzyme reactions involving an *N*-methyltransferase (NMT) (Frenzel and Zenk, 1990a; Wat et al., 1986), three *O*-methyltransferases (OMTs) (Rueffer et al., 1983; Muemmler et al., 1985; Frenzel and Zenk, 1990b; Sato et al., 1993, 1994), a hydroxylase (Loeffler and Zenk, 1990), a berberine bridge enzyme (Steffens et al., 1984), a methylenedioxy ring-forming enzyme (Rueffer and Zenk, 1985) and a tetrahydroprotoberberine oxidase (Yamada and Okada, 1985; Galneder et al., 1988). Only norcoclaurine 6-*O*-methyltransferase (6OMT) (Rueffer et. al., 1983; Sato et al., 1994), 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (4′OMT) (Frenzel and Zenk, 1990b) and (*S*)-scoulerine 9-*O*-methyltransferase (SMT) (Sato et al., 1993) have been highly purified and characterized.

The enzymological characterization of secondary metabolism and the biotechnological application of these biosynthetic enzymes should prove useful for the biotransformation of fine chemicals. One difficulty in purifying the enzymes in the berberine biosynthetic pathway is that they have similar enzymological properties due to the similarity of their reaction mechanisms and substrates (Sato et al., 1994; Morishige et al., 2000). Recent isolation of the cDNAs of methyltransferases and the investigation of their expression in *Escherichia coli* have provided further information about OMTs (Frick and Kutchan, 1999; Morishige et al., 2000). However, there is little information available on coclaurine *N*-methyltransferase (CNMT), a unique *N*-methyltransferase

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in berberine biosynthesis (Fig. 1) (Frenzel and Zenk, 1990a). CNMT catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to coclaurine with the formation of *N*-methylcoclaurine. So far, of the *N*-methyltransferases involved in secondary plant metabolism, only the putrescine *N*-methyltransferase in nicotine biosynthesis has been well characterized, and its cDNA has been isolated (Hibi et al., 1994). Characterization of the CNMT in berberine biosynthesis should help us understand the molecular aspects of NMT in secondary metabolism, especially alkaloid biosynthesis. We report here the purification and characterization of CNMT from cultured *Coptis japonica* cells and compare it with OMTs in berberine biosynthesis. Its broad substrate specificity and biotechnological applications are discussed.

2. Results and discussion

2.1. Purification of coclaurine N-methyltransferase (CNMT)

An outline of the purification of coclaurine *N*-methyltransferase (CNMT) from cultured *Coptis* cells is shown in Table 1. The enzyme was enriched 343-fold and gave a yield of 1% after ammonium sulfate fractionation, Phenyl Sepharose, Q-Sepharose, Mono Q anion-exchange and Mono P column chromatographies of the crude extract. Preliminary experiments showed that chromatography of the crude enzyme extracts of *Coptis* cells in 20 mM Tris–HCl buffer on a Q-Sepharose column gave two distinct active fractions. Since the activity of the first CNMT fraction was only 25% that of the second fraction, the latter was used for further purification. Although final isoelectric focusing chromatography on a Mono P column considerably decreased the recovery of CNMT activity, this step was essential for removing contaminating OMTs and purifying the enzyme to near homogeneity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and silver staining showed that the fraction purified through the Mono P column gave only two protein bands which had similar mobilities (silver staining) (Fig. 2). Whereas it was not possible to separate these proteins further, careful analysis by SDS-PAGE and enzyme assay

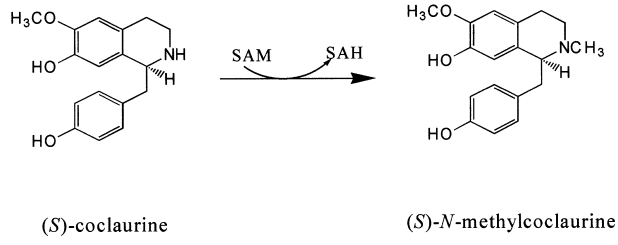

Fig. 1. Reaction catalyzed by coclaurine *N*-methyltransferase (CNMT). SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine.

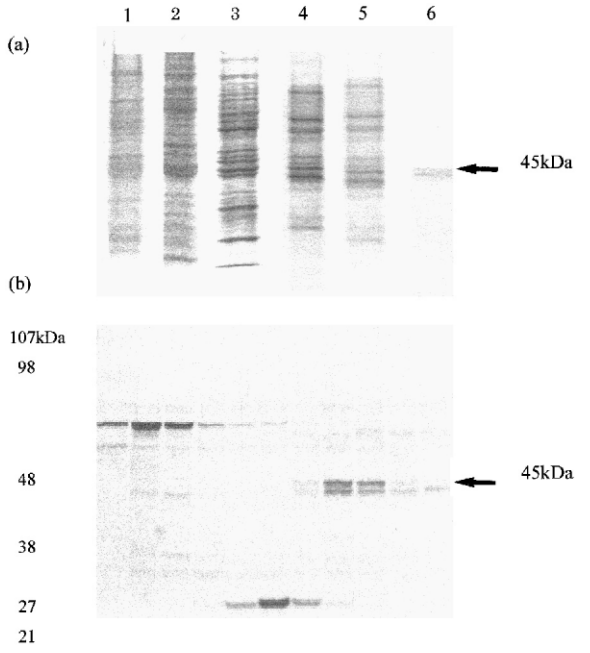

Fig. 2. SDS-PAGE analysis of purified coclaurine *N*-methyltransferase. (a) Analysis of proteins at various stages of purification. Fractions from each purification step were separated by SDS-PAGE and then stained with Coomassie Brilliant Blue: Lane 1, crude extract; Lane 2, 30–50% $(NH_4)_2SO_4$; Lane 3, Phenyl Sepharose; Lane 4, Q-Sepharose; Lane 5, Mono Q; Lane 6, Mono P column purified fraction. (b) Analysis of purified coclaurine *N*-methyltransferase by SDS-PAGE. Fractions were separated from the Mono P column, analyzed by SDS-PAGE in 10% polyacrylamide gel and made visible by silver staining.

Table 1
Purification of coclaurine *N*-methyltransferase from *Coptis japonica* cells[a]

Purification step	Total protein (mg)	Total activity (pkat)	Specific activity (pkat/mg)	Purification fold	Yield (%)
Crude extract	512	428	0.83	1.0	100
30–50% Ammonium sulfate	151	240	1.59	1.91	54
Phenyl Sepharose	65.1	369	5.66	6.81	82
Q-Sepharose	13.2	204	15.4	18.6	45
Mono Q	0.76	34.4	45.3	54.5	8
Mono P	0.02	5.7	285	343	1.2

[a] Two hundred grams of cultured *Coptis* cells were homogenized for purification of the enzyme.

indicated that the larger 45-kDa protein corresponded to CNMT activity (data not shown). Since the CNMT purified in the Mono P column showed no *O*-methyltransferase activity and no positive protein band was detected by Western blotting with the anti-*Coptis* 4′OMT and SMT antibodies, we used this fraction as the purified CNMT fraction (data not shown).

To our knowledge, this is the first study in which coclaurine *N*-methyltransferase has been obtained in high purity and characterized. Since four methyltransferases (4′OMT, 6 OMT, SMT and CNMT) with similar reactions and substrates are involved in berberine biosynthesis (Rueffer et al., 1983; Muemmler et al., 1985; Frenzel and Zenk, 1990a,b; Sato et al., 1993, 1994; Morishige et al., 2000), it is not easy to purify each to homogeneity. In fact, preparation of homogenous 4′OMT and 6OMT required the heterologous expression of enzymes in *E. coli* (Morishige et al., 2000). In our study, also, 4′OMT and SMT could not be separated from CNMT during the early purification steps, even though their primary structures differ considerably (Takeshita et al., 1995; Morishige et al., 2000).

2.2. CNMT characterization

SDS-PAGE analysis showed that the molecular mass of the CNMT monomer is 45 kDa, whereas gel-filtration by Superose 12 column chromatography showed that the molecular mass of native CNMT was approximately 160 kDa. These results suggest that the native enzyme is a homotetramer. Chromatofocusing of CNMT on a Mono P column gave an isoelectric point (pI) of 4.2, and examination of the pH-dependence of enzyme activity showed that the highest activity was at pH 7.0, whereas 50% of the activity was at pH 6.0 and 9.0 (data not shown).

Examination of the effects of metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Li^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} at 5 mM) and potential inhibitors (EDTA, iodoacetamide, *p*-chloromercuribenzoate at 1 or 5 mM) on CNMT activity indicated that the enzyme did not require a divalent cation and the addition of EDTA did not inhibit activity, whereas the addition of Ca^{2+} at 5 mM slightly increased activity by 25%. The addition of some divalent cations (i.e. Co^{2+}, Cu^{2+} or Mn^{2+}), however, severely inhibited enzyme activity (by 75, 47 and 57%, respectively). Whereas some *O*-methyltransferase activities are inhibited by the addition of SH-directed inhibitors, e.g., *p*-chloromercuribenzoate and iodoacetamide at 5 mM, these chemicals did not inhibit CNMT activity.

2.3. Substrate specificity of CNMT

The substrate specificity of CNMT was examined for a wide range of isoquinoline alkaloid substrates (Fig. 3).

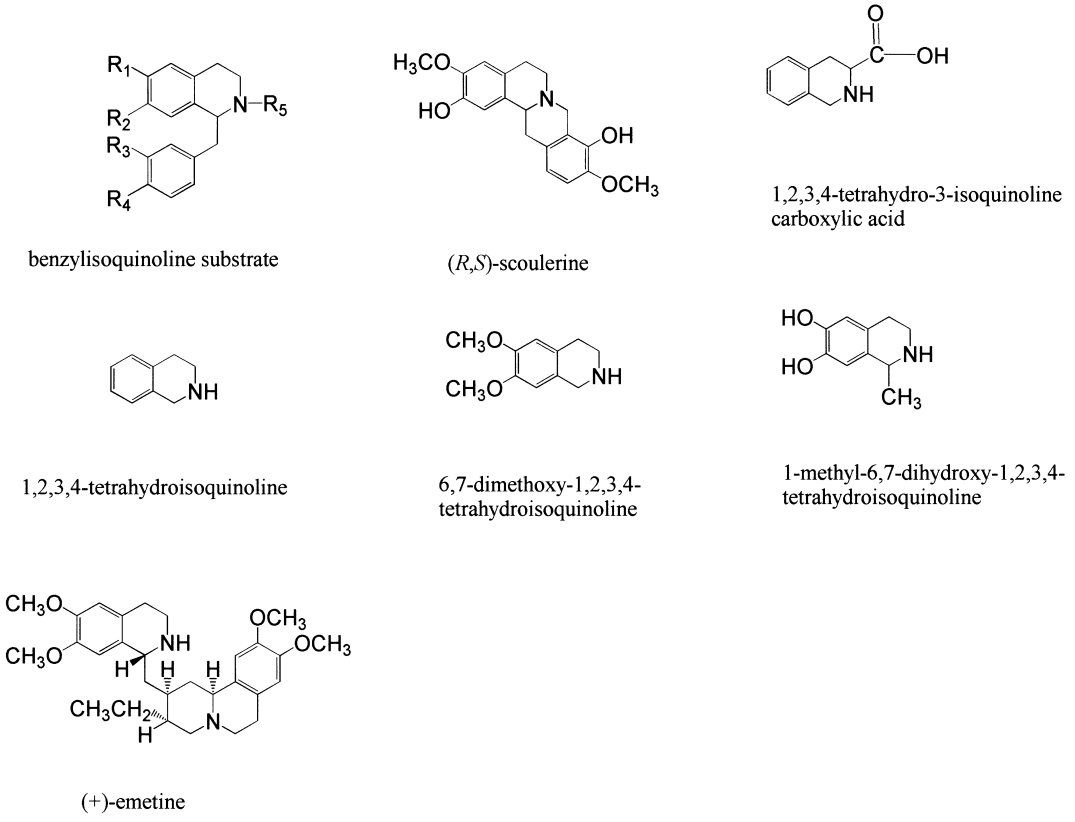

Fig. 3. Structures of isoquinoline alkaloids and chemicals tested as CNMT substrates. The benzylisoquinoline substrates in Table 2 are coclaurine, norreticuline, norlaudanosoline and 6-*O*-methylnorlaudanosoline.

The incorporation of radioactivity from *S*-adenosyl-L-[methyl-^{3}H] methionine into alkaloid products was used to measure enzyme activity, since the radioactivities measured corresponded to those of HPLC measurement for several substrates (data not shown). When (*S*)-coclaurine at 1 mM was chosen as the control substrate (i.e. the relative incorporation was taken as 100%) for *Coptis* CNMT, the respective relative activities with (*R*)-coclaurine, norreticuline, norlaudanosoline and 6-*O*-methylnorlaudanosoline were 122, 55, 48 and 38% (Table 2). Interestingly, 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline was also a good substrate, whereas 1,2,3,4-tetrahydroisoquinoline was not methylated. *Coptis* CNMT transferred the methyl group to a fairly broad range of isoquinoline substrates; i.e. from simple dimethoxy-tetrahydroisoquinoline to more complicated benzylisoquinoline substrates. The similar relative activities of (*R*)-coclaurine and (*S*)-coclaurine suggests that *Coptis* CNMT was not stereospecific. However, the lack of methylation activity with 4-*O*-methyldopamine, tryptamine, histamine and purine suggests that the isoquinoline ring is important for the *Coptis* CNMT reaction (data not shown).

Coptis CNMT has a substrate specificity similar to that of *Berberis* NMT (Frenzel and Zenk, 1990a), in that both enzymes show no stereospecificity and have relatively broad substrate specificity for (*R*)- and (*S*)-coclaurine, norreticuline and 6-*O*-methylnorlaudanosoline. *Coptis* CNMT also *N*-methylates norlaudanosoline, whereas *Berberis* NMT does not. These results indicate that *Coptis* CNMT has a much broader substrate specificity; *O*-methylation of the isoquinoline moiety of the benzylisoquinoline substrate has little effect on *Coptis* CNMT activity. The fact that even 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline was methylated, as well as norreticuline, suggests that the benzyl moiety of norreticuline is not essential for the CNMT reaction. However, the fact that there was no CNMT activity with emetine indicates that replacement of this benzyl moiety with a large group makes this compound non-functional as a substrate. The lack of methylation activity with 1,2,3,4-tetrahydroisoquinoline suggests that hydroxylation of the isoquinoline ring is important for the *Coptis* CNMT reaction. The methylation of 1-methyl-6-7-dihydroxy-1,2,3,4-tetrahydroisoquinoline supports this idea.

2.4. Kinetics

Although (*R*)-coclaurine was the best substrate, we also determined the enzyme characteristics of CNMT with norreticuline, the most readily available substrate, and its similar product formation. CNMT from *Coptis* cells showed Michaelis–Menten-type kinetics for norreticuline and SAM (Fig. 4). Fig. 4 shows that *Coptis* CNMT followed a sequential bi-substrate mechanism (ordered bi bi or rapid equilibrium random bi bi), but further experiments on product inhibition are needed to determine the actual mechanism. The kinetic constants of CNMT (K_m and V_{max}) were calculated from double-reciprocal plots. The V_{max} of CNMT was estimated from Lineweaver–Burk double-reciprocal plots, and the respective apparent K_m values for norreticuline and *S*-adenosyl-L-methionine (SAM) were 0.38 and 0.65 mM (data not shown).

Although the K_m values of *Coptis* CNMT for norreticuline (0.38 mM) and SAM (0.65 mM) are somewhat larger than those of *Berberis* CNMT (ca.0.2 mM for (*R*)-norreticuline and 0.04 mM for SAM) and *Sanguinaria* NMT (0.02 mM for (*R,S*)-tetrahydroberberine and 0.012 mM for SAM) (Frenzel and Zenk, 1990a, O'Keefe and Beecher, 1994), the *Coptis* CNMT values are still smaller than those of 6OMT (2.23 mM for (*R,S*)-norlaudanosoline and 3.95 mM for SAM) (Sato et al., 1994). Enzymological characterization of the purified CNMT protein should be beneficial in the future use of this enzyme with other *O*-methyltransferases to convert various isoquinoline derivatives into *N*-methylated products by enzymological biotransformation; e.g., from norlaudanosoline to reticuline.

Table 2
Substrate specificity of purified *Coptis* coclaurine *N*-methyltransferase[a]

Substrate	Relative activity	R1[b]	R2	R3	R4	R5
(*R*)-Coclaurine	122	OMe	OH	H	OH	H
(*S*)-Coclaurine	100	OMe	OH	H	OH	H
(*R,S*)-Norreticuline	55	OMe	OH	OH	OMe	H
(*R,S*)-Norlaudanosoline	48	OH	OH	OH	OH	H
(*R,S*)-6-*O*-Methylnorlaudanosoline	38	OMe	OH	OH	OH	H
(*R,S*)-Scoulerine	0					
6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline	39					
1,2,3,4-Tetrahydroisoquinoline	0					
1-Methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	10					
1,2,3,4-Tetrahydro-3-isoquinolinecarboxylic acid	0					
(+)-Emetine	0					

[a] The enzyme reaction mixture was incubated for 1 h at 30°C; total volume 50 μl containing 100 mM potassium phosphate (pH 7.0), 2.5 mM sodium ascorbate, 1 mM ^{3}H-SAM and 20 μl of the purified Mono-P fraction.

[b] See Fig. 3 for the numbering system.

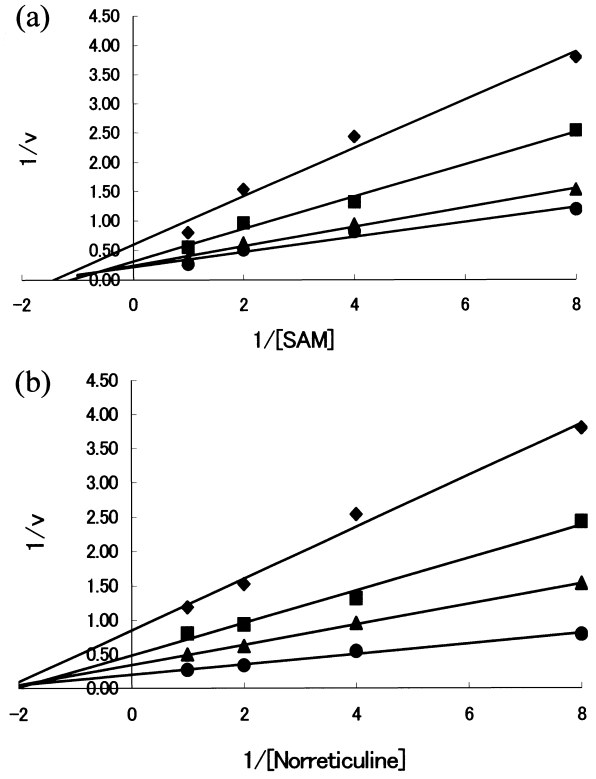

Fig. 4. Double reciprocal plots of CNMT activity. (a) Plots of 1/v versus 1/[SAM] at various constant concentrations of norreticuline. (b) Plots of 1/v versus 1/[norreticuline] at various constant concentrations of SAM. ◆, ■, ▲ and ● respectively represent 0.125, 0.25, 0.5 and 1.0 mM of these substrates.

3. Experimental

3.1. Plant material

Cultured *Coptis japonica* cells with high berberine productivity were used (Sato and Yamada, 1984). Cells were subcultured every 3 weeks on a rotary shaker (ca. 100 rpm) in the dark in Linsmaier-Skoog liquid medium containing 10 μM naphthalene acetic acid and 0.01 μM 6-benzyladenine as described elsewhere (Sato and Yamada, 1984), and 14-day-old cultured cells were used for enzyme purification. Harvested cells were immediately frozen in liquid nitrogen and stored at −80°C until use.

3.2. Chemicals

(*R,S*)-1-[(3,4-Dihydroxyphenyl)methyl]-1,2,3,4-tetrahydro-6,7-isoquinolinediol (norlaudanosoline or tetrahydropapaveroline), 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride, 1,2,3,4-tetrahydroisoquinoline, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline hydrobromide and 1,2,3,4-tetrahydro-3-isoquinoline carboxylic acid hydrochloride were purchased from Aldrich. (*R*)-Coclaurine and (*S*)-coclaurine were gifts from Dr. N. Nagakura, Kobe Women's College of Pharmacy. [Methyl-^{3}H] *S*-adenosyl-L-methionine (SAM) was purchased from NEN^{TM} Life Science, and the other alkaloids were gifts from Mitsui Petrochemical Industries Ltd. The other chemicals used were of the highest purity available.

3.3. Enzyme assay and protein determination

CNMT enzyme activity was measured by HPLC or by the incorporation of [methyl-^{3}H] into the product from [methyl-^{3}H] SAM. Enzyme activities during purification were analyzed by HPLC. The standard assay solution consisted of 0.1 mM norreticuline, 100 mM potassium phosphate buffer (pH 7.0), 2.5 mM sodium ascorbate, 1 mM SAM and 20 μl of the enzyme solution. Norreticuline was selected as the standard substrate because of its availability and similarity of product formation to that of coclaurine, the native substrate. The assay mixture was incubated at 30°C for 60 min, after which the reaction was stopped by adding 50 μl of methanol. After protein precipitation at 10,000×*g* for 10 min at 4°C, the amount of reticuline formed was determined by the following HPLC separation: mobile phase, 22% acetonitrile and 1% acetic acid; column, LiChrosper 100RP-18 (250×4 mm, Cica Merck); flow rate, 1.0 ml/min; detection, at 280 nm.

Transfer of the [methyl-^{3}H] group of [methyl-^{3}H] SAM was investigated for the enzymological characterization of CNMT. The standard assay solution consisted of 1 mM norreticuline, 100 mM potassium phosphate buffer (pH 7.0), 2.5 mM sodium ascorbate, 1 mM [methyl-^{3}H] SAM (1.5 MBq/μmol) and 20 μl of the purified enzyme fraction; total volume 50 μl. This mixture was incubated at 30°C for 20 min, after which the reaction was terminated by adding 150 μl of Na_2CO_3 (0.5 M) and 400 μl of isoamylalcohol. After vigorous shaking, the mixture was centrifuged at 10,000×*g* for 5 min at room temperature, and 150 μl of the organic phase was then used to determine the radioactivity. An assay mixture without the enzyme fraction was used as the control. The radioactivities incorporated corresponded to the activities measured by HPLC assay.

Protein concentration was determined with a Bio-Rad protein assay kit according to the manufacturer's protocols.

3.4. Purification of coclaurine N-methyltransferase

All operations were performed at 0–4°C, and all the buffers contained 20 mM β-mercaptoethanol and 10% glycerol unless stated otherwise. Typically, about 200 g of frozen cells were homogenized in 450 ml 0.2 M Tris–HCl buffer (pH 7.5) in a Waring blender at top speed for 2 min, and then sonicated for 20 min and filtered. The filtrate was centrifuged at 10,000×*g* for 50 min. Part of the supernatant which was desalted through an NAP-10 column (Pharmacia) was designated the crude

extract. The enzyme which precipitated between 30 and 50% $(NH_4)_2SO_4$ saturation was dissolved in 60 ml of the extraction buffer and then centrifuged. The protein solution obtained was desalted in a PD-10 column (Pharmacia) and then applied to a Phenyl Sepharose CL-4B column (Pharmacia, 50 ml of bed volume) that had been equilibrated with a starting buffer (200 mM Tris–HCl (pH 7.5) containing 30% $(NH_4)_2SO_4$). After the column had been washed sufficiently with the same buffer to remove any remaining yellow alkaloids, CNMT was eluted with 120 ml of a linear gradient (30–0%) of $(NH_4)_2SO_4$ solution. The CNMT fractions were then subjected to ion-exchange chromatography in a Q-Sepharose FF column (Pharmacia, 50 ml of bed volume) that had been pre-equilibrated with 200 mM Tris–HCl buffer (pH 7.5). After the column was washed with the same buffer, CNMT was eluted with a linear gradient (0–0.5 M) of NaCl solution (120 ml). Active fractions were collected, desalted and purified in an FPLC system with a Mono Q column (HR 5/5, Pharmacia) that had been pre-equilibrated with 20 mM Tris–HCl buffer (pH 7.5). CNMT was then eluted stepwise with a gradient (0–0.35 M) of NaCl solution (40 ml). The active fractions were combined and chromatofocused in a Mono P column (HR 5/20, Pharmacia) in an FPLC system: starting buffer; 25 mM Bis Tris–IDA (iminodiacetic acid), pH 7.1, eluting buffer; 10% Polybuffer 74 adjusted to pH 4 by IDA. The pHs of the eluted fractions were immediately adjusted with 100 mM Tris–HCl (pH 7.5). The purified enzyme was kept at −20°C in 50% glycerol. Since repeated freezing and thawing markedly reduced CNMT activity, thawed samples were not repeatedly frozen. CNMT was active after 1 year at −20°C when frozen together with 50% glycerol. All FPLC steps were done at room temperature.

3.5. Characterization of coclaurine N-methyltransferase

The CNMT fraction purified on the Mono P column was used for further enzymological characterization. Effects of pH, metal ions and chemicals on CNMT activity were determined with norreticuline as the substrate. Divalent cations were added as their chloride salts. The optimum pH of CNMT activity was determined with the following buffers at concentrations of 100 mM: 2-(*N*-morpholino)ethane sulfuric acid (MES) (pH 5.4, 6.2 and 6.6), potassium phosphate (pH 5.8, 6.2, 6.6, 7.0, 7.4 and 7.8), *N*-2-hydroxyethypiperazine-N-2-ethane sulfonic acid (HEPES) (pH 6.5, 7.0, 7.5, 8.0 and 8.5), Tris–HCl (pH 7.5, 8.0, 8.5 and 9.0), glycine-NaOH (pH 9.0 and 10.0).

3.6. Molecular weight determination

The subunit molecular weight of CNMT was determined by SDS-PAGE (10%) analysis with molecular weight standards (Bio-Rad). The native molecular weight of CNMT was determined by gel filtration chromatography on a calibrated Superose 12 column (HR 10/30, Pharmacia).

3.7. Kinetic properties

Kinetic constants for CNMT activity were determined for various amounts of norreticuline and SAM with 0.2 μg of purified CNMT in a 50 μl reaction mixture. Kinetic data were fitted to the standard equation.

Acknowledgements

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