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INDOLETHYLAMINE *N*-METHYLTRANSFERASES OF *PHALARIS TUBEROSA*, PURIFICATION AND PROPERTIES

JOSEPH P. G. MACK* and M. SLAYTOR†
Department of Biochemistry, The University of Sydney, N.S.W., 2006 Australia

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Abstract—Two *S*-adenosylmethionine-dependent indolethylamine *N*-methyltransferase activities were purified from soluble extracts of *Phalaris tuberosa* by fractionation with $(NH_4)_2SO_4$, and chromatography on DEAE-Sephadex. One enzyme methylated the primary indolethylamines and the other methylated the secondary indolethylamines in the plant. These two enzymes were similar in catalytic and bulk physical properties and could not be separated during purification or by A 1.5 Agarose or hydroxylapatite chromatography. The products of enzymic reactions were identified by TLC.

INTRODUCTION

The pasture grass *Phalaris tuberosa* synthesizes two main alkaloids, DMT‡ and 5-MeODMT, at concentrations of *ca* 1 μmol/g [1]. Bufotenine, 5-OHDMT, is also present in smaller amounts. The unmethylated tryptamine intermediates, T and 5-OHT, are detectable but at much lower concentrations [2]. The alkaloids are derived from tryptophan by decarboxylation, hydroxylation and *N*- and *O*-methylation [2]. As shown by trapping and feeding experiments, several pathways are involved, although T appears to be the normal precursor of the alkaloids. Similarly, 5-hydroxy- and 5-methoxytryptophan fed to seedlings caused decreased synthesis of the labelled alkaloids from labelled tryptophan [2]. It thus appears that the enzymes in the pathway will also function when there is a substituent in the indole 5-position.

The following is a report on the *N*-methyltransferases in the leaves involved in the synthesis of the tryptamine alkaloids in *P. tuberosa.*

RESULTS AND DISCUSSION

Preliminary experiments

Preliminary experiments showed that there was enzyme activity present in homogenates of *P. tuberosa* which would convert tryptamine and *S*-adenosylmethionine-[methyl-^{14}C] (SAM) to DMT-[^{14}C]. The activity was distributed evenly along the leaves and was not found in the roots. The crude homogenate would convert T to MT and to DMT, MT to DMT, 5-MeOT to 5-MeOMT and to 5-MeODMT. 5-OHT was converted to 5-MeOT but no 5-MeOMT or 5-MeODMT was produced, presumably because of the small amount of 5-MeOT formed. The behaviour of the crude enzyme preparation with respect to activity on storage suggested that there were two activities present, the less stable primary indolethylamine *N*-methyltransferase (PIM) and the more stable secondary indolethylamine *N*-methyltransferase (SIM).

Purification of the enzymes

The enzymes were isolated and purified according to the procedure outlined in Experimental. The results of this procedure are summarized in Table 1.

2-Mercaptoethanol (0.14 M) was added for the initial homogenization to prevent oxidation of phenolic compounds in the preparation. The preparation at step 1 could not be purified by heat treatment.

The step 3 preparation was exhaustively dialysed to remove inhibitors (as indicated by increased linearity of enzyme activity at higher protein concentration) and to prevent yellowing of the preparation on storage. Although some loss of activity occurs on dialysis, the resulting preparation lost activity more slowly than if not dialysed. Dialysis for 2 days was not sufficient to prevent yellowing or to remove inhibitors. PIM and SIM were not separated on DEAE-Sephadex at pH 7, 7.6, 8, 8.5 or 9. Rechromatography of the step 3 preparation on DEAE-Sephadex at pH 8 resulted in a further 7-fold purification but the preparation rapidly lost activity with time. Chromatography of the step 3 preparation on hydroxylapatite gave no separation and poor recovery (15%) of the activities.

None of the purification steps separated PIM and SIM. The activities methylating 5-methoxy derivatives were not followed through the purification, but were found in the same ratio as that at step 1, namely

*Present address: Department of Molecular Biophysics and Biochemistry, Yale University, 333 Cedar Street, New Haven, CT 06510, U.S.A.

†To whom all correspondence should be addressed.

‡The following abbreviations are used for tryptamine and its derivatives. Tryptamine, T; 5-hydroxytryptamine, 5-OHT; 5-methoxytryptamine, 5-MeOT; *N*-methyltryptamine, MT; *N,N*-dimethyltryptamine, DMT; 5-hydroxy-*N,N*-dimethyltryptamine, 5-OHDMT; 5-methoxy-*N,N*-dimethyltryptamine, 5-MeODMT.

Table 1. Isolation of methyltransferase activities from *Phalaris tuberosa*. Summary of results of purification

	Activity (nmol/hr) PIM	SIM	Recovery (%) PIM	SIM	Activity (nmol/hr) SIM	Recovery (%)	Protein (mg)	Volume (ml)	Purification
Step 1 High speed supernatant	1.1	2.2	100		2.2	100	160	60	1
Step 2 50–60% $(NH_4)_2SO_4$ Cut	0.30	0.87			0.87		40	1	4
Step 3 DEAE-Sephadex A-25 eluate	0.21*	0.80	43	29	0.80	29	2.1	90	76

*Estimation high due to presence of SIM (see Assay).

T/5-MeOT = 1/1 and MT/5-MeOMT = 1/1, and assumed to be due to the same enzymes which catalysed the unsubstituted tryptamines. That is, PIM can convert T to MT and 5-MeOT to 5-MeOMT, while SIM can convert MT to DMT and 5-MeOMT to 5-MeODMT. The ratio of *N*-methyltransferase activity towards the primary and secondary amines varied between 0.3 and 0.5 in different fresh preparations.

PIM and SIM also could not be separated by affinity chromatography on immobilized *S*-adenosylhomocysteine–Agarose (SAH–Agarose)[3] indicating that PIM and SIM have similar kinetic constants for SAH in the pH range 6–10.5.

The step 3 preparation was used for studying the properties of the enzymes. No side products were detected and the reactions were linear with time for 4 hr with protein concentration up to 1 mg/ml, and with conversion of reactants up to 15%. This conversion is based on SAM, the limiting reactant.

Unless otherwise stated, results for the activities of PIM with T and 5-MeOT as substrates are discussed together, similarly for SIM with MT and 5-MeOMT as substrates. Both activities of each enzyme were routinely assayed.

Effects of pH and buffers

PIM retained full catalytic activity in the pH range 6–9 and SIM from 5 to 10 under the assay conditions of 25° for 1 hr. The pH for maximal catalytic activity is 9.25 for PIM and 9.75 for SIM (in glycine buffer). The activity profile is skewed, with activity falling off more rapidly at higher than at lower pH values. At pH 10.5 and 6.5 there is 10% of the optimal activity. That the pH optima are so close to the pH of inactivation would indicate that the pH optima could be at higher pH than that detected by this experiment.

Of the 9 buffers used to determine the activity profile, sodium phosphate, Tris–HCl, Tris–glycine, glycine–NaOH, sodium barbiturate, ammonium chloride–NaOH, sodium glutamate, sodium borate and sodium bicarbonate, the last 4 are inhibitors of enzyme activity. The buffer composition and pH for optimal activity could not be used for storage due to rapid loss of enzyme activity. Tris–HCl, phosphate and ammonium chloride (tested at pH 8.5, 7 and 9, respectively) were the only buffering ions in which at least 80% of enzyme activity was retained after one week of storage. Tris–HCl is the only one of these buffers in which PIM and SIM are appreciably active. Tris–HCl was not inhibitory to either activities at concentrations up to 1 M. The advantages of using one buffer for preparation, storage and assay of the enzymes made Tris–HCl, pH 8.5 the buffer of choice.

Table 2. Effect of cations

Ion	Activity PIM (%)	Activity SIM (%)
NH_4^+, Li^+		
Na^+, K^+, Rb^+	100	100
Cs^+	90	100
Ca^{2+}, Mg^{2+}	75	85
Mn^{2+}	50	70
Co^{2+}	13	25
Ni^{2+}	2	4
Fe^{2+}, Sn^{2+}	1	1
Fe^{3+}, Al^{3+}, Cu^{2+}		
Cd^{2+}, Zn^{2+}	<0.5	<0.2

The step 3 preparation was dialysed against assay buffer without EDTA or 2-mercaptoethanol. Cations were added to a final concentration of 10 mM.

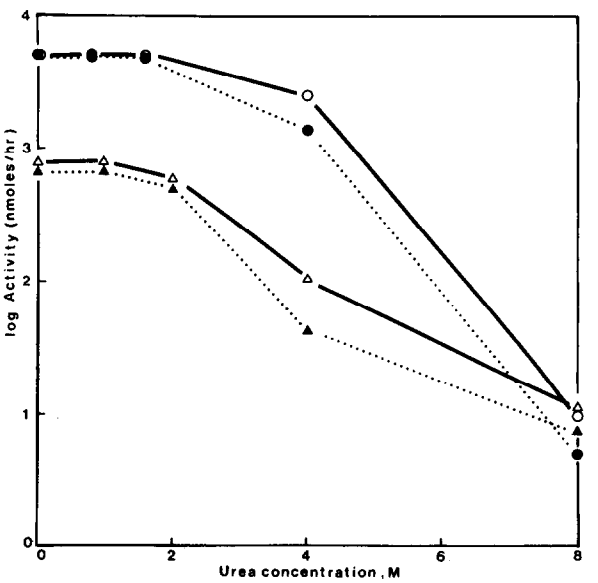

Fig 1. The effect of urea on *N*-methyltransferase activity. △,▲ PIM; ○,● SIM. A step 3 preparation was dialysed against two sets of buffers: 50 mM Tris–HCl, pH 7.6, containing urea at the concentrations indicated (▲,●); or 50 mM Tris–HCl, pH 7.6, containing urea at the concentrations indicated together with T (1 μM) and MT (1 μM) (△,○). Both preparations were then dialysed against 50 mM Tris–HCl, pH 8.5 (100 vol × 16 hr) before assaying.

Table 3. Effect of substrate on the temperature of inactivation of the indolethylamine *N*-methyltransferases

Enzyme	Temperature of inactivation (°) Substrate added			
	None	Indolethylamine	SAM	Indolethylamine and SAM
PIM	30	45	30	45
SIM	40	40	25	45

The step 3 enzyme preparation was incubated for 1 hr at 5° intervals from 0 to 50° in the presence of none, one or two of the substrates. The incubates were cooled in ice and the remaining substrate(s) added. The mixture was then assayed at 25° in the usual way. Data for both substrates were taken from the point at which the Arrhenius plot became non-linear at high temperature. Boiled enzyme blanks were assayed at each temperature. Activity was constant up to a certain temperature and then decreased linearly with temperature. The point of intersection of these two lines gave the temperature of inactivation.

Table 4. The heat of activation, ΔH^*, of the *N*-methyltransferases

Substrate	ΔH^* (kcal/mol)	
	PIM	SIM
Tryptamine	9.0 ± 0.3†	
5-Methoxytryptamine	7.0 ± 0.6‡	
N-Methyltryptamine		9.0 ± 0.9†
5-Methoxy-*N*-methyltryptamine		6.3 ± 0.6‡

†Not significantly different ($P = 0.1$).
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The reaction velocity was determined for temperature in the range 0–50° and fitted to the Arrhenius equation using an unweighted linear regression. The data for temperatures above 40° were not used due to inactivation of the enzymes at these temperatures.

MWs

The MW of both *N*-methyltransferases is 63 000 ± 11 000, as determined by gel filtration.

Effect of cations

All cations tested were without effect or were inhibitory (Table 2). The results for the substrates T and MT were the same, and similarly for the substrates 5-MeOT and 5-MeOMT, and were not recorded separately.

Effect of urea

The effect of urea on PIM and SIM activity is shown in Fig. 1. The activity of PIM was constant up to 2 M and then decreased with increasing concentration of urea. The inactivation concentration for SIM was 3 M. Both PIM activities and both SIM activities behaved similarly. Tryptamine protects both PIM activities against inactivation by 4 M urea. Similarly, MT protects both SIM activities against inactivation by 4 M urea. This indicates that the two PIM activities are due to the same enzyme and that both SIM activities are due to a second enzyme. At 8 M urea T and MT do not protect the enzyme against urea inactivation.

Temperature stability of enzyme activities

The temperature stability of the enzymes in the presence and absence of substrates is shown in Table 3. Since the enzymes are saturated by substrates [4] in these experiments, the data represent the stability of the appropriate enzyme–substrate complexes. With respect to loss of activity at high temperatures, PIM is less stable than PIM alone whereas the SIM–MT complex is as stable as SIM. The effect of the substrate SAM is opposite to the indolethylamine substrates. The PIM–SAM complex has the same stability as PIM alone, whereas the SIM–SAM complex is less stable than SIM alone. Both ternary complexes are more stable than any of the other complexes. In these respects the two enzymes exhibit quite different properties.

It is interesting to note that the heats of activation of the *N*-methyltransferase reactions (Table 4) are dependent on the substituent in the 5-position of the indole nucleus but not the degree of *N*-methylation for the ethylamine side chain, as could be expected if different enzymes were responsible for *N*-methylating tryptamines with differing numbers of *N*-methyl groups. A methoxy group in the 5-position of the substrate, which will increase the hydrophobicity of the substrate, decreases the heat of activation for both PIM and SIM. PIM methylates phenylethylamine but not the less hydrophobic histamine. Thus both enzymes prefer hydrophobic substrates.

Effects of thiol group blocking agent

*p*CMB (1 mM) inhibited all methyltransferase activity. Dialysis of the inactive product against excess 2-mercaptoethanol (0.1 M, 2 days) in assay buffer in order to remove the *p*CMB did not restore activity.

Substrate specificity

The specificities of the amine substrates to accept methyl groups using the step 3 preparation are listed in Table 5. 5-Methyltryptamine and 5-MeOT are methylated at the same rate as T. Other substituents in the indole nucleus cause methylation to proceed at a lower rate, e.g. 6-methoxytryptamine is methylated at 30% of the rate at which tryptamine is methylated. MT and 5-MeOMT are methylated at *ca* 2.5 times the tryptamine rate, reflecting the higher levels of SIM activity compared to PIM in the preparation. Phenylethylamines are methylated at a lower rate than the tryptamines. The ratio of activity towards *N*-methylphenylethylamine and phenylethylamine is 3:1, about the same as that found for the corresponding tryptamine substrates. Thus it seems that both PIM and SIM have the ability to accept a benzene ring instead of the normal indole nucleus. Substitution of the phenyl ring by bulky methoxy groups does not change the ability to methylate these compounds. The methoxy groups would occupy the space normally occupied by the benzene ring of the indole nucleus, and thus they offer no steric hindrance for binding to PIM. None of the tetrahydro-β-carbolines, the ring-closed analogues of the tryptamines were methylated, i.e. the ethylamine side chain must be in a position other than that found in the tetrahydro-β-carbolines. Consistent with this is the fact that the

Table 5. Specificity of the indolethylamine *N*-methyltransferases from *P. tuberosa*

Substrate	Activity*	Substrate	Activity
Tryptamine	100	3-Hydroxy-4, 5-dimethoxyphenylethylamine	3.1
N-Methyltryptamine	260	Tetrahydroharman	0.12
5-Methoxytryptamine	88	1-Methyl-6-hydroxytetrahydro-β-carboline	0.38
5-Methoxy-*N*-methyltryptamine	220	1-Methyl-6-methoxytetrahydro-β-carboline	0.31
6-Methoxytryptamine	31	6, 7-Dimethoxy-1, 2, 3, 4-tetrahydroisoquinoline	0.59
5-Methyltryptamine	76	1-Methyl-6-hydroxy-7-methoxy-1, 2, 3, 4-tetrahydroisoquinoline	0.93
6-Hydroxytryptamine	24	Salsoline	2.3
Phenylethylamine	10	Anhalonidine	0.90
N-Methylphenylethylamine	30	Histamine	<0.12
4-Hydroxyphenylethylamine	1.2	Ethylamine	0.31
3, 4-Dihydroxyphenylethylamine	0.2	Diethylamine	0.19
3-Hydroxy-4-methoxyphenylethylamine	8.1	Piperidine	<0.12
3, 4-Dimethoxyphenylethylamine	13	Tetrahydropyrrole	0.12

*Activities are expressed as percentages relative to tryptamine. Each substrate (1 mM) was assayed in duplicate. The limit of detection of activity in this experiment is 0.12%.

tetrahydroisoquinolines were also not methylated. The rate of methylation of histamine, which is much less hydrophobic than phenylethylamine, although having a similar charge at pH 8.5 and being only slightly smaller, was 100 times less.

The 5-hydroxyindole derivatives seem to be as good substrates as the unsubstituted or 5-methoxy derivatives, allowing for the lower solubility of the product in the extracting toluene. Since 5-OHDMT is produced in the plant [1], it seems most likely that 5-OHT and 5-OHMT are normal substrates of PIM and SIM. A similar conclusion is reached from trapping experiments with whole plants [2].

The specificity of the SAM site was tested using homocysteine and adenosine as inhibitors in the normal assay. There was no inhibition of PIM or SIM activity by adenosine (1 mM) or homocysteine (1 mM).

EXPERIMENTAL

Materials. Seeds of *P. tuberosa* L. cv Australian Commercial were purchased from Rumsey's seeds, Sydney. SAM-[methyl-^{14}C] was purchased from the Radiochemical Centre, Amersham, U.K., tryptamine-[α-^{14}C] was synthesized [5] from indolylacetonitrile-[α-^{14}C] [6]. The synthesized tryptamine-[α-^{14}C] was pure by TLC and autoradiography. The activity was 300 μCi/mol. All derivatives of tryptamine were synthesized [7]. 3-Hydroxy-4-methoxyphenylethylamine and 3-hydroxy-4,5-dimethoxyphenylethylamine were synthesized [8]. Other compounds in the substrate specificity experiment were purchased.

General methods. Purification procedures were carried out at 0–4°. All buffers contained 2-mercaptoethanol (10 mM) and EDTA (1 mM). All buffers were 50 mM with respect to the buffering ion unless otherwise specified. pH refers to the value at 25°. Protein was assayed [9] using BSA as standard.

Germination and growth conditions. These were critical owing to heat sensitivity of the seedlings and the necessity for a diurnal temperature rhythm. The seedlings were grown in a glass house under a diurnal regime of 24°/19°–12/12 hr with natural daylight filtered to 28% by plastic mesh [10].

Purification of enzyme. Preparation of crude extract (step 1). The leaves of 8- to 10-day-old seedlings were harvested with scissors and homogenized in a mortar and pestle with acid-washed sand and assay buffer (1.5 v/w). 2-Mercaptoethanol (1% v/v; 140 mM) was added to the homogenate which was filtered through muslin and centrifuged at 140 000 *g* for 1 hr to give the crude extract.

$(NH_4)_2SO_4$ fractionation (step 2). The protein which precipitated between 50 and 60% satn was dissolved in 0.01 × original vol. in 50 mM Tris–HCl, pH 7.6 and dialysed against assay buffer to give the $(NH_4)_2SO_4$ purified prepn.

Chromatography on DEAE-Sephadex (step 3). The dissolved sample from step 2 without dialysis was chromatographed on DEAE-Sephadex A-25 (12 × 2.1 cm). Protein was eluted with a continuous gradient of 0–250 mM NaCl in 50 mM Tris–HCl, pH 7.6. Enzyme activity was eluted between 125 and 175 mM NACl and the fractions containing greater than 25% of the activity of the most active fractions were pooled. The pH was increased by addition of 1 M Tris–HCl, pH 8.5 (0.1 v/v) to a pH 8.25 where the enzyme prepn was more stable. The enzyme was concd by ultrafiltration (Amicon, PM 30 filter) and the concentrate exhaustively dialysed against assay buffer. Buffer (100 vol.) were firstly changed twice a day for 2 days and then after 1 week. This DEAE-purified enzyme prepn was used for the characterization of the enzymes.

Chromatography on hydroxylapatite. The step 3 prepn was chromatographed on a column of hydroxylapatite (1.1 × 15 cm) equilibrated with 50mM NaPi, pH 7, with EDTA omitted. Protein was eluted with a linear gradient of the same buffer from 50 to 300 mM (100 ml).

MW determination on A 1.5 M Agarose. The MWs of the enzymes were determined on an A 1.5 M Agarose column (80 × 2.1 cm). Protein was eluted with 50 mM Tris–HCl, pH 7.6 and 150 mM NaCl. Bovine thyroglobulin, bovine gamma-globulin, BSA, ovalbumin and cytochrome *c* were used as reference proteins.

Enzyme storage. The dialysed step 3 prepn was frozen in liquid N_2. It maintained activity on immediate thawing, but had little activity after thawing following storage for 3 weeks at −25°. The step 3 prepn was thus stored at 4° and required 2-mercaptoethanol for maintenance of activity. Under these conditions there was sufficient enzyme activity for assays over 10 days.

Enzyme assays. The enzyme was routinely assayed with 0.1 ml of reaction mixture containing enzyme in Tris–HCl, pH 8.5, 1 mM indolethylamine, 0.20 mM SAM-[methyl-^{14}C] (10 pCi) at 25°/1 hr in a sealed 15 ml centrifuge tube. The reaction was stopped by addition of 1 M H_3BO_3–Na_2CO_3, pH 10 (200 μl), excess dry Na_2SO_4 was added and the mixture freeze-dried. The solid residue was ground in a pestle and mortar and the powder placed in a glass column (10 × 1 cm) and washed with $CHCl_3$ (3 × 1 ml). The eluant, containing the enzymic products, was evapd under N_2 at room temp. The residue was dissolved in CH_2Cl_2 and separated by TLC. The alkaloids were detected by comparison with standards, by UV absorption (200 nm) and initially by autoradiography. With crude enzyme prepns, i.e. steps 1 and 2 prepns, many non-alkaloidal radioactive com-

pounds were formed. The required products were separated by TLC. Usually, T was the substrate for PIM and MT the substrate for SIM. PIM activity was calculated from the amount of MT produced plus half the DMT produced. With the step 3 prepn, only the required enzyme products were formed and the assay was performed in a scintillation vial, terminated as above, H_2O (2 ml) (to lower the assay blank) and scintillant added. The radioactive products were extracted into scintillant by agitation on a vortex mixer and the activity determined directly by scintillation counting (blank level 100 ± 30 pmol) or after freezing the mixture and decanting (blank level 4 ± 2 pmol). This is a modification of a previous method [11]. With this direct extraction, MT and DMT are not separated and the assay for PIM in the presence of SIM leads to a systematically high estimate of PIM due to further conversion of the MT produced to DMT. This overestimation was *ca* 30% and was not considered to be a major drawback for quantitative work with the first methylation, since the ratio of these two activities did not vary much between different enzyme prepns.

TLC. System I: Alumina F_{254} neutral (type E), (Merck 5550/0025) solvent, C_6H_6–Et_2O (1:4), satd with 18 M NH_4OH. This separated the tryptamines according to the number of Me groups, but not the 5-methoxy- from the unsubstituted derivatives. R_f:T, 0.1; MT, 0.5; DMT, 0.9. This was used when tryptamine-[α-^{14}C] was the labelled substrate. System II: Si gel F_{254} (Merck 5554/0025); solvent, $CHCl_3$–MeOH–NH_4OH (40:7:1). This was used when it was not necessary to separate T from MT. R_f:T and MT, 0.4; DMT, 0.9.

Scintillation counting. ^{14}C was measured by counting in toluene scintillant containing PPO (4 g/l.) and POPOP (100 mg/l.) and C_6H_6 (100 mg/l.). Samples on Si gel or Al_2O_3 were suspended in thixotropic gel powder (0.2 g/10 ml).

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