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SEPARATION OF THE *N*-7 METHYLTRANSFERASE, THE KEY ENZYME IN CAFFEINE BIOSYNTHESIS

SIMONE S. MÖSLI WALDHAUSER, FIONA M. GILLIES,* ALAN CROZIER* and THOMAS W. BAUMANN†

Institute of Plant Biology, University of Zürich, CH-8008 Zürich, Switzerland; * Bower Building, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, U.K.

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Key Word Index—*Coffea arabica*; Rubiaceae, caffeine; 7-methylxanthine; 7-methylxanthosine; 7-methyl-XMP; theobromine; purine alkaloids; SAM; *N*-methyltransferase; photoaffinity labelling.

Abstract—Caffeine biosynthesis comprises sequential methylations at *N*-7, *N*-3 and *N*-1 of the xanthine ring catalysed by *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase activities that, to date, have not been resolved. Enzyme extracts were prepared from young, emerging coffee leaflets and following anion exchange chromatography, chromatofocusing facilitated the clear separation of the *N*-7-methyltransferase from the *N*-3- and *N*-1-methyltransferase activities. All three *N*-methyltransferases co-eluted when analysed by gel filtration chromatography and their native molecular mass was *ca* 67 kDa. Photoaffinity labelling with [methyl-^{3}H]SAM followed by SDS-PAGE of a chromatofocusing-purified preparation containing only *N*-7-methyltransferase activity demonstrated the presence of a single labelled band of 40 kDa. Similar analysis of a gel filtration purified preparation containing all three *N*-methyltransferase activities revealed the presence of three labelled bands at 49, 43 and 40 kDa. It remains to be determined whether the 49 and 43 kDa bands are associated with the *N*-3 and *N*-1-methyltransferases or whether they are unrelated SAM-dependent methyltransferases or other SAM-binding proteins. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Caffeine biosynthesis in coffee (*Coffea arabica* L.) begins with an *N*-7-methyltransferase (7NMT) converting XMP to 7-methyl-XMP [1]. Dephosphoribosylation of 7-methyl-XMP yields 7-methylxanthine, which is further methylated by an *N*-3-methyltransferase (3NMT) to produce theobromine. The final and third methylation step in the pathway is mediated by an *N*-1-methyl-transferase (1NMT), that converts theobromine to caffeine [2, 3]. The results of mixed substrate experiments have indicated that separate enzymes catalyse the *N*-3 and *N*-1 methylations [4]. This is in line with reports that theobromine, but not caffeine, is synthesised in leaves and flower buds of *Camellia irrawadiensis* [5, 6] and that the leaves of *Theobroma cacao* clone ‘Pound 12’ contain only theobromine rather than the more typical mixture of theobromine and caffeine [T. W. Baumann and P. J. Fritz, unpublished data]. These and other observations suggesting the existence of discrete *N*-methyltransferases (NMTs) can, however, also be interpreted as the actions of a single enzyme that carries out all three methylation steps. This view may be supported by the paralleled 3- and 1NMT activities observed during the culture cycle of coffee cell suspensions [4] and during coffee leaf development [7]. Clearly one form of decisive evidence on this point would be the purification and separation of individual methyltransferase activities. To date this has not been achieved, primarily because of the extreme instability of the methyltransferases which has both prevented effective purification and caused such a marked decrease in specific activity that the success of claimed isolations is questionable [8]. In this paper we report on the use of a novel purification protocol with coffee leaf extracts that yielded substantial NMT purification coupled with marked increases in specific activity. The data obtained demonstrate for the first time the presence of an independent 7NMT.

RESULTS AND DISCUSSION

Purification of NMT

NMT activities from coffee leaves were found to be stable at all stages of purification in the presence of

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† Author to whom correspondence should be addressed.

Table 1. Purification of *N*-methyltransferases from coffee leaves

Purification step	Total protein [mg]	Total activity [pkat]	Specific activity [pkat/mg]	Purification [x-fold]
			7NMT	
Crude homogenate	1050	2070	2	1
40–70% $(NH_4)_2SO_4$ precipitation	462	2300	5	2
Q-Sepharose	89*	702*	8	4
Mono P Pool I	6	177	30	15
Mono P Pool II	10	229	23	11
Superdex 200 (Pool II)	1	19	19	9
			3NMT	
Crude homogenate	1050	24600	24	1
40–70% $(NH_4)_2SO_4$ precipitation	462	12400	27	1
Q-Sepharose	89*	9310	105	4
Mono P Pool I	6	0	0	0
Mono P Pool II	10	3760	380	16
Superdex 200 (Pool II)	1	927	927	39
			1NMT	
Crude homogenate	1050	9000	9	1
40–70% $(NH_4)_2SO_4$ precipitation	462	3820	8	1
Q-Sepharose	89*	2120	24	3
Mono P Pool I	6	0	0	0
Mono P Pool II	10	620	62	7
Superdex 200 (Pool II)	1	219	219	24

* Not the entire Q-Sepharose pool was loaded onto the Mono-P column. Therefore, the values for the following purification steps are calculated yields. See also Experimental.

The 7NMT activity was measured using XMP and [^{14}C]SAM as substrates.

10 mM dithiothreitol (DTT) and 20% ethylene glycol (or glycerol) as reported previously [7, 9]. In the present study, extracts were stored in liquid N_2 for at least 8 months without any activity loss. Nevertheless, each step in a sequential purification procedure resulted in the disappearance of considerable amounts of NMT activity. In order to develop more efficient purification procedures for these labile enzymes, a wide array of methods was tested systematically as outlined in the Experimental. The resulting purification protocol involved the sequential use of $(NH_4)_2SO_4$ precipitation (40–70%), anion-exchange chromatography, chromatofocusing and gel filtration chromatography. The data obtained when these procedures were used to purify NMT from coffee leaf extracts are summarised in Table 1. Overall, the 7-, 3- and 1NMT activities were enriched 9-, 39- and 24-fold, respectively, with estimated yields of 0.9, 3.8 and 2.4%.

All the three NMT activities co-eluted as a double peak from a Q-Sepharose column, with the major peak at *ca* 0.21 M NaCl (Fig. 1). When subjected to chromatofocusing, 7NMT activity eluted first and was well resolved from the other two NMTs. Arguably, there was a slight separation of 3NMT and 1NMT activities [Fig. 2(A)]. The narrower the pH gradient, the closer the enzymes eluted and the sharper their activity peak [Fig. 2(B), (C)]. The isoelectric point (p*I*) of the individual activities varied between 4.7 and 5.2 (7NMT), 4.5 and 5.1 (3NMT), and 4.0 and 4.9 (1NMT). This is in line with previously reported p*I* values for 3NMT activity from coffee leaves [7] and 1NMT activity from coffee endosperm [8].

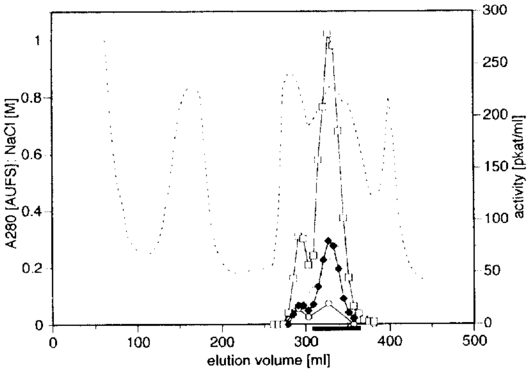

Fig. 1. Protein (---) and 7NMT (–○–), 3NMT (–□–) and 1NMT (–◆–) activities eluting from a Q-Sepharose anion-exchange column developed with a linear gradient of 0–0.5 M NaCl (▨) in buffer A at 2 ml min^{-1}. Fractions of 6 ml were collected. Proteins were monitored at 280 nm. The horizontal bar indicates fractions that were pooled for further purification. The 7NMT activity was measured using XMP as the methyl group acceptor and [^{14}C]SAM as the methyl group donor.

Chromatofocusing pool I fractions were assayed by

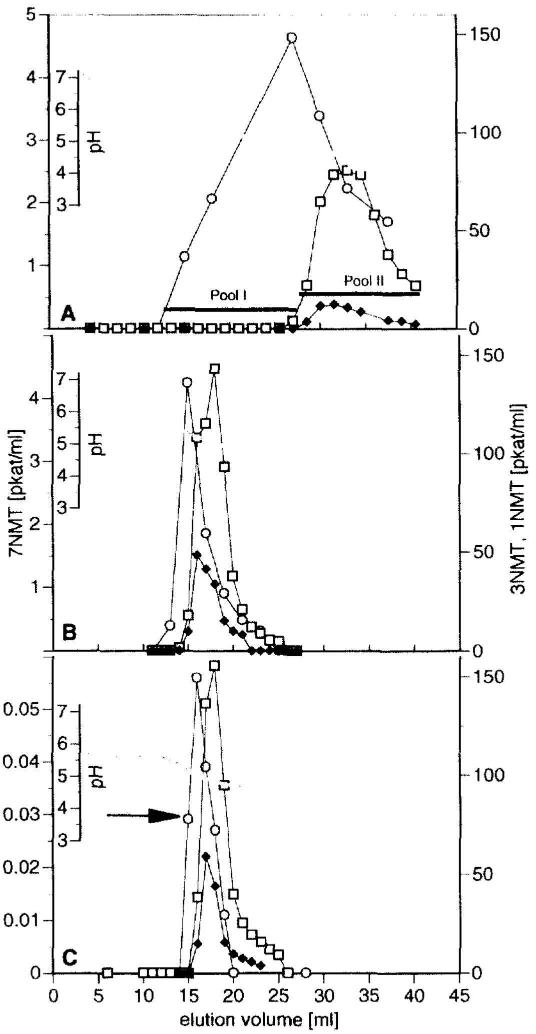

Fig. 2. Chromatofocusing profiles of 7NMT (–○–), 3NMT (–□–) and 1NMT (–◆–) activities eluted from a Mono P HR 5/20 column with the following pH gradients (A) pH 7.0–4.0, (B) pH 6.0–3.5 and (C) 5.5–4.0. The pH gradient (▭) was established with Polybuffer 74 in buffer B at 0.5 ml min^{-1} and successive 1 ml fractions were collected and aliquots assayed for NMT activity. The 7NMT activity was measured using XMP and either [^{14}C]SAM [Fig. 2(A) and (B)] or [^{3}H]SAM [Fig. 2(C)] as substrates. The lower 7NMT activity in Fig. 2(C) is a consequence of the lower substrate concentration. The arrow indicates the single fraction that was used subsequently for photoaffinity labelling (see Fig. 6, lane 6) while the horizontal bars indicate the fractions that were pooled (pool I and pool II). Pool II contained all three NMT activities and was analysed further by gel filtration chromatography.

measuring NMT activity with XMP as the substrate [Fig. 2(A)], as outlined in the Experimental. At the end of the assay period, the reaction mixture was hydrolysed to convert any 7-methyl-XMP and 7-methylxanthosine present to 7-methylxanthine, which was then analysed quantitatively by HPLC in order to obtain an accurate estimate of *N*-7 methylation activity. In a further experiment with chromatofocusing pool I using XMP and xanthosine as substrates, the post-assay hydrolysis step was omitted to enable the products of the *N*-7 methylation reaction to be analysed in more detail. Xanthosine was readily methylated to 7-methylxanthosine. XMP was likewise converted to 7-methylxanthosine although trace quantities of 7-methyl-XMP were also detected. Thus, in addition to the 7NMT, the Pool I fraction also contained nucleotidase activity which would facilitate the conversion of 7-methyl-XMP to 7-methylxanthosine and/or XMP to xanthosine. Other substrates, 7-methylxanthine, theobromine (3,7-dimethylxanthine), theophylline (1,3-dimethylxanthine), and paraxanthine (1,7-dimethylxanthine) did not serve as methyl group acceptors for chromatofocusing pool I. This confirms that the 7NMT was successfully separated from the other NMT, and that it is a distinct enzyme.

Pool II from chromatofocusing chromatography contained all three NMTs [Table 1; Fig. 2(A)] and when subjected to gel filtration chromatography (Fig. 3), the individual activities were not resolved, eluting as a single peak at a retention time corresponding to a M_r of *ca* 67 kDa. This value is consistent with those reported for the 3NMT (60 kDa, unpublished data) and 1NMT (54–60 kDa) from coffee endosperm [8], and with the M_r of 3NMT from tea leaves (61 kDa) [10]. The purification summarised in Table 1 was monitored by SDS-PAGE, which revealed the enrichment of a band near 30 kDa (Fig. 4). However, as will be outlined below, neither this nor any other prominent bands can be considered as candidates for any of the three NMT activities.

Native gel electrophoresis, from which enzyme activities could be assayed from gel slices, was used as an additional purification step [Fig. 5(A)]. All three enzymes were detected in the same region of the gel. As the protein content of the gel slices was not measured, the yield and purification factor was not determined. Slices of the native gel, as numbered in

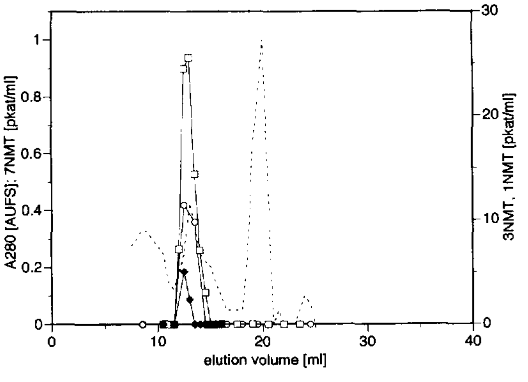

Fig. 3. Gel filtration chromatography elution profiles of protein (- - -) and 7NMT (–○–), 3NMT (–□–) and 1NMT (–◆–) activities. A 500 μl aliquot of the chromatofocusing Pool II was chromatographed on a Superdex 200 column equilibrated in 0.15 M NaCl in buffer A and eluted at a flow rate of 0.25 ml min^{-1}. Fractions of 0.5 ml were collected. Proteins were monitored at 280 nm. The 7NMT activity was measured using XMP and [^{14}C]SAM as substrates.

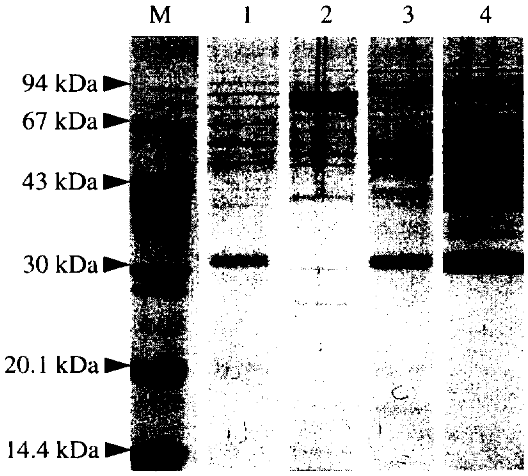

Fig. 4. Analysis of purified proteins by SDS-PAGE. Proteins from the last four purification steps (see Table 1) were separated by SDS-PAGE with a 12.5% acrylamide resolving gel and visualised by silver staining. M: *Mr* marker. Lane 1: Q-Sepharose, 1 μg. Lane 2: chromatofocusing pH 7–4, Pool I, 1 μg. Lane 3: chromatofocusing pH 7–4, Pool II, 1 μg. Lane 4: gel filtration, *ca* 2 μg.

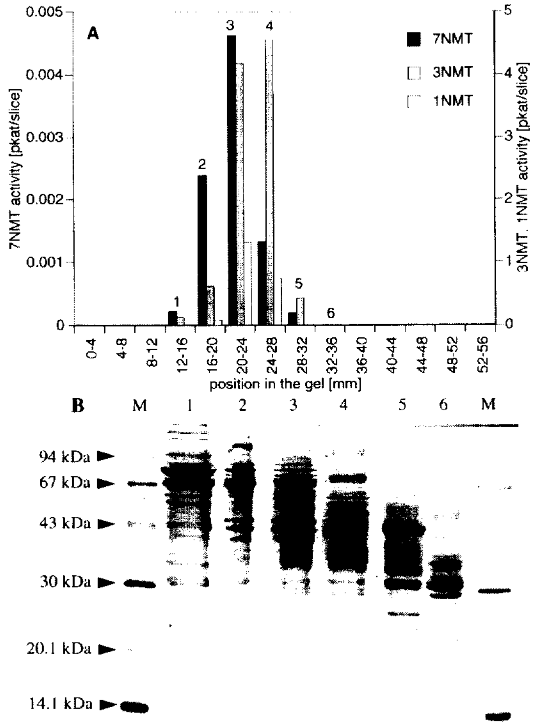

Fig. 5. (A) Native gel electrophoresis run on a gradient gel (7.5–15%, 5% plateau) using a continuous buffer system as described in the Experimental. For each NMT and for the analysis by SDS-PAGE, 15 μl concentrated Q-Sepharose Pool was applied on the gel comprising 0.023 pkat 7NMT, 16 pkat 3NMT and 2.3 pkat 1NMT. Slices of 4 mm were assayed for individual NMT activities. The 7NMT activity was measured using xanthosine and [^{3}H]SAM as substrates. The numbers correspond to the lane numbers in Fig. 5B. (B) SDS-PAGE analysis of active fractions from the native gel. M: M_r markers. Lane 1: 12–16 mm slice; lane 2: 16–20 mm slice; lane 3: 20-24 mm slice; lane 4: 24–28 mm slice; lane 5: 28–32 mm slice; lane 6: inactive 32–36 mm slice.

Fig. 5(A), were subjected directly to SDS-PAGE [Fig. 5(B)]. As with the gels in Fig. 4, a prominent band with a M_r of *ca* 30 kDa can be seen in lanes 5 and 6 [Fig. 5(B)]. The corresponding gel slices were more or less devoid of any NMT activity [Fig. 5(A)], indicating that the 30 kDa band and also that at *ca* 43 kDa are not the NMT proteins. Even after chromatography on Q-Sepharose and electrophoresis on a native gel, each of the zones showing NMT activities [Fig. 5(A)] consisted of a vast number of proteins and peptides [Fig. 5(B), lanes 2–4], making an assignment of the NMT activities impossible. It appears that NMT are present in the coffee leaflet at low concentrations and that an additional, selective method such as photoaffinity labelling is required for their detection.

Photoaffinity labelling with [methyl-^{3}H]SAM

Initially, control experiments were carried out in order to confirm the photochemical cross-linking of methyl-^{3}H-labelled SAM to the NMT in coffee (data not shown). Essentially, the results obtained were in keeping with the original reports [11, 12]. When the photoaffinity-labelled enzyme extract was separated by IEF, three major radioactive bands were detected, one corresponding exactly to the 3NMT activity peak (data not shown). In addition, the Q-Sepharose pool labelled with [^{3}H]SAM and run on a native gel yielded six radioactive bands, two of which corresponded to the NMT activity peaks (data not shown).

Enzyme extracts at all purification steps were photoaffinity-labelled and run on a SDS-PAGE (Fig. 6). The number of radioactive bands decreased to three (lane 7, gel filtration) during purification as other SAM-dependent methyltransferases are removed. These bands, a, b and c have a respective M_r of *ca* 49, 43 and 40 kDa. A very similar banding pattern, consisting of bands a–c and two additional bands, was found in coffee endosperm purified by anion-exchange and hydrophobic interaction chromatography (HIC) containing all three NMT activities (unpublished data).

Interestingly, in the chromatofocusing Pool I of coffee leaves, containing 7NMT activity, only band c was present (Fig. 6, lane 3). 7NMT activity was similarly only associated with the presence of band c in lane 6 (Fig. 6), which is a single fraction from a separate chromatofocusing experiment from pH 5.5–4.0 [Fig. 2(C), arrow]. Thus, band c with a M_r of 40 kDa could be a subunit of 7NMT since gel filtration chromatography yielded a native M_r of *ca* 67 kDa. We can therefore speculate that the native NMT in coffee leaves is a multimer, with at least one subunit containing the binding site for SAM. Based upon the native M_r of *ca* 60 kDa found in coffee endosperm, as well as in leaves of coffee and tea, the existence of a dimeric NMT is most likely. Nevertheless, it should be borne in mind that the native M_r determined by gel

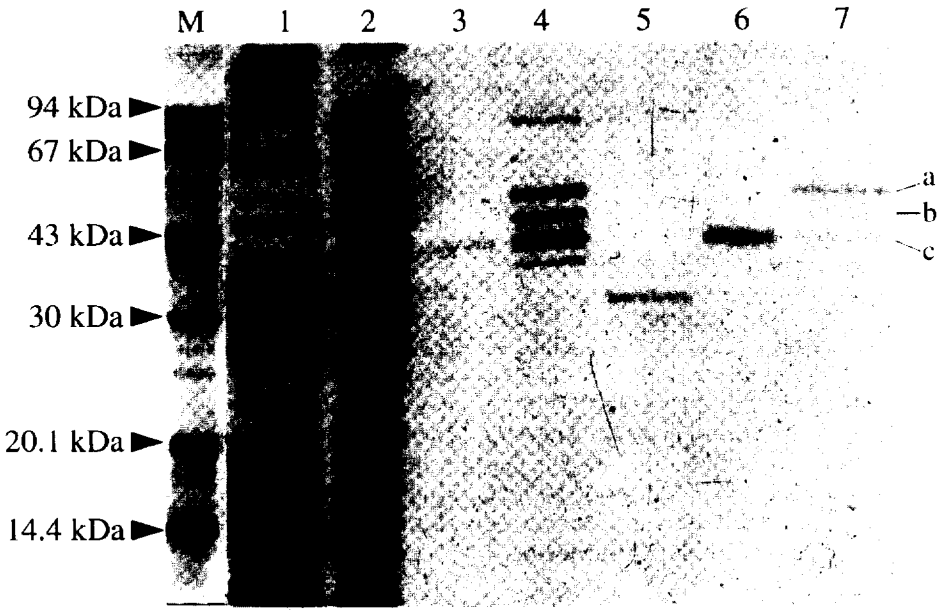

Fig. 6. Fluorographs of a SDS-PAGE gel of purified coffee leaf NMT preparations photoaffinity-labelled with [^{3}H]SAM for 15 min. M: Coomassie Brilliant Blue stained LMW marker. Lane 1: $(NH_4)_2SO_4$ precipitation 40–70%, comprising all three NMT activities. Lane 2: Q-Sepharose, pool comprising all three NMT activities. Lane 3: chromatofocusing pH 7–4, Pool I comprising 7NMT activity only. Lane 4: chromatofocusing pH 5.5–4.0, pool comprising all three NMT activities. Lane 5: chromatofocusing pH 5.5–4.0, inactive fraction. Lane 6: chromatofocusing pH 5.5–4.0, single fraction with 7NMT activity only. Lane 7: gel filtration, pool comprising all three NMT activities. The bands a, b, and c represent three peptides with a M_r of *ca* 49, 43 and 40 kDa, respectively. For further details see the text.

filtration chromatography applies to globular proteins. If the NMT does not have a spherical globular shape, a monomeric enzyme with a M_r of *ca* 40 kDa on SDS-PAGE would still be conceivable. Accordingly, Mazzafera *et al.* [8] came to the conclusion that the 1NMT in coffee endosperm is a monomeric enzyme since they observed only one band on SDS-PAGE. While bands a and b in Fig. 6 may be the 3- and 1NMT involved in caffeine biosynthesis, they could well be unrelated SAM-dependent methyltransferases or other SAM-binding proteins. Another possibility is that band c could also be associated with all three NMTs. Further study is required to clarify this point.

EXPERIMENTAL

Chemicals. *S*-Adenosylhomocysteine (SAH), *S*-adenosyl-L-methionine (SAM) and XMP from Sigma. Caffeine, theobromine, paraxanthine, theophylline, 7-methylxanthine and xanthosine from Fluka. Adenosine deaminase from Boehringer. Q-Sepharose Fast Flow, PD-10 gel filtration columns (Sephadex G25-M), Sephadex G25 Fine, Mono P HR 5/20, Superdex 200 HR 10/30, Polybuffer 74, gel filtration calibration kit, LMW calibration kit for electrophoresis and ampholine from Pharmacia. IEF standard and Bio-Rad Protein Assay from Bio-Rad. [Methyl-^{3}H]SAM (specific activity 87 Ci mmol^{-1}) and [methyl-^{14}C]SAM (specific activity 55 mCi mmol^{-1}) from Amersham. 7-Methyl-XMP was synthesised in our laboratory as described in ref. [1].

Buffers. Extraction buffer (EB): 0.2 M Na-Pi (pH 7.5) containing 50 mM $NaCl_2$, 10 mM dithiothreitol (DTT), 2 mM NaEDTA, 0.6% Na-ascorbate, 20% (v/v) ethylene glycol. Storage buffer (SB): same as EB but with 0.1 M Na-Pi and 40% (v/v) ethylene glycol. Assay buffer (AB): 50 mM Tris–HCl pH 8.0, 0.06% Na-ascorbate. PAGE-SDS sample buffer (SSB): 80 mM Tris, 3% (w/v) SDS, 16% (v/v) glycerol, 25% (v/v) 2-mercaptoethanol, 0.1% (w/v) Bromophenol Blue. Buffer A: 20 mM Tris–HCl pH 8.0, 5 mM DTT, 5 mM NaEDTA, 20% (v/v) glycerol. Buffer B1: 25 mM Bis–Tris–iminodiacetic acid pH 7.1, 5 mM DTT, 20% (v/v) ethylene glycol. Buffer B2: 25 mM Bis–Tris–HCl acid pH 6.3, 5 mM DTT, 20% (v/v) ethylene glycol. Buffer B3: 25 mM piperazine-iminodiacetic acid pH 6.3, 5 mM DTT, 20% (v/v) ethylene glycol. Buffer C1: 1 : 10 diluted Polybuffer 74, pH 4.0 adjusted with satd iminodiacetic acid, 5 mM DTT, 20% (v/v) ethylene glycol. Buffer C2: 1 : 10 diluted Polybuffer 74, pH 3.5 adjusted with HCl, 5 mM DTT, 20% (v/v) ethylene glycol. Buffer C3: 1 : 10 diluted Polybuffer 74, pH 4.0 adjusted with satd iminodiacetic acid, 5 mM DTT, 20% (v/v) ethylene glycol. Buffer D: 0.5 M Tris–HCl pH 7.5, 5 mM DTT, 20% (v/v) glycerol. Buffer E: 50 mM Tris–HCl pH 8.5, 10% (v/v) glycerol.

Plant material. Seeds obtained from parent trees of *Coffea arabica* L. var. *catuai* kept in an environmental chamber under short day (8 hr; 24°), flower-inducing conditions [13, 14], were germinated and seedlings raised in a greenhouse (natural photoperiod, with supplementary lighting during wintertime). Young expanding leaves were obtained from plants *ca* 2 years old.

The NMT assay is based on the transfer of the methyl group from SAM to the substrate. The assays for the *N*-3 and *N*-1-methylations were performed according to ref. [7]. The *N*-7-methylation assay was a modification of the procedures of ref. [1], using: 50 μl enzyme extract, 20 μl 0.5 M tricine–NaOH buffer pH 7.5, 10 μl 10 mM $MgCl_2$, 10 μl XMP or xanthosine (both 50 mM) and 5 μl adenosine deaminase (2 units) with either 8 μl H_2O and 2 μl [^{3}H]SAM (1 μCi μl^{-1}; 87 Ci mmol^{-1}) or 6 μl H_2O and 4 μl [^{14}C]SAM (0.025 μCi μl^{-1}; 55 mCi mmol^{-1}). When the methylating capacity towards XMP was studied 10 μl 10 mM Na_2MoO_4 (nucleotidase inhibitor) was added to the incubation mixt. All assays were carried out at 30° for 20 min. Assays for 3NMT and 1NMT were stopped by the addition of 10 μl 1.2 M $HClO_4$, while 7NMT assays were terminated by two different procedures: (a) addition of 10 μl concd HCl and hydrolysis of the reaction products at 80° for 60 min, followed by neutralisation with satd Na_2CO_3; (b) addition of 10 μl of 5 mM 7-methyl-XMP and placing the reaction tube for 45 sec into a silicone oil bath at 150° in order to study the formation of [^{3}H]7-methyl-XMP [1].

Native gel slices were assayed for 3- and 1NMT (7NMT) activity by adding one gel slice (4 × 8 × 1.5 mm, corresponding to a vol. of *ca* 48 μl) to 55 μl 7-methylxanthine or theobromine (both 5 mM) (10 μl 50 mM xanthosine), 5 μl adenosine deaminase (2 units), 10 μl 16.65 mM SAM in 0.01 N H_2SO_4 (2 μl [^{3}H]SAM), 25 μl 50 mM AB (78 μl). The reaction was terminated by the addition of $HClO_4$.

HPLC analysis and radiodetection. All samples were centrifuged prior to analysis on a Hewlett–Packard liquid chromatograph equipped with a diode array and, where necessary, a radioactivity monitor (Flo-one, Canberra Packard; Zürich, Switzerland). The column temp. was set at 40° and radioactivity was determined on-line by the addition of liquid scintillant (Pico-Aqua, Canberra Packard) pumped at a flow rate of 1 ml min^{-1} to the HPLC effluent emerging from the diode array detector. Reverse phase sepns were carried out on Nucleosil 100-5 ODS (ChromCart, Macherey-Nagel, Oensingen, Switzerland) columns (5 μm) 125 × 4 mm (i.d.) (methylxanthines as substrates) and 250 × 4 mm (i.d.) (XMP or xanthosine as substrates). Both columns were fitted with a 8 × 4 mm precolumn. Peaks were identified by comparing UV spectra and R_t values with those of authentic standards. In addition, the products 7-methylxanthosine and 7-methyl-XMP were identified following hydrolysis, leading to the formation of 7-methylxanthine, with 10% (v/v) conc. HCl for 60 min at 80°.

Theobromine was quantified using the following gradient of MeOH in H_2O: 0–6 min (8–13% MeOH), 6–8 min (13–25% MeOH), 8–9 min (25% MeOH), 9–9.5 min (25–8% MeOH), 9.5–12 min (8% MeOH). The R_ts (min) for SAM, 7-methylxanthine, theobromine, caffeine and methylthioadenosine (MTA) were 1.3, 3.4, 6.1, 10.8, and 11.2 min, respectively. The flow rate was 1 ml min^{-1}, the injection vol. 60 μl and the diode array was set at 272 nm.

Caffeine formation was monitored using the following gradient of MeOH–MeCN in H_2O, which separates caffeine from MTA: 0–4 min with 0–7.5% MeOH and 0–2.5% MeCN, 4–20 min with 7.5% MeOH and 2.5% MeCN. The R_ts for SAM, 7-methylxanthine, theobromine, caffeine and MTA were 1.5, 4.9, 6.9, 15.1 and 18.2 min, respectively. The flow rate was 1.1 ml min^{-1}, the injection vol. 80 μl and the diode array was set at 272 nm.

Methylated products of XMP or xanthosine were sepd using the following gradient of MeOH–MeCN (1:1; v/v) in 100 mM NH_4OAc pH 5.6: 0–5 min (0% MeOH–MeCN), 5–14 min (0–1.5% MeOH–MeCN), 14–25 min (1.5–10% MeOH–MeCN), 25–30 min (10% MeOH–MeCN). The R_ts for XMP, 7-methyl-XMP, SAM, xanthine, 7-methylxanthosine, xanthosine, 7-methylxanthine, theobromine, caffeine and MTA were 3.3, 3.8, 6.7, 7.7, 11, 12, 17.2, 22.8, 28.2 and 28.9 min, respectively. The flow rate was 1 ml min^{-1}, the injection vol. 80 μl and the diode array was set at 254 nm.

Extraction and partial purification of the NMT. All operations were carried out at 4°. The buffers were filtered and degassed before use. Chromatography steps, with the exception of the desalting, were carried out using a FPLC (Pharmacia). Protein concn was monitored with an UV detector operating at 280 nm. Frs and pools were stored in liquid N_2.

Protein was extracted according to ref. [7] with slight modifications: young, emerging coffee leaflets, about 6–8 mm in length [7, 15] were homogenised in a prechilled mortar in the presence of EB without the addition of PVPP. After brief centrifugation, the pellet was re-extracted, the supernatants were combined and stirred for 20 min in the presence of 30% (w/fr. wt) equilibrated PVPP. The extract was filtered through one layer of fine-mesh nylon, and a 40–70% satd $(NH_4)_2SO_4$ cut prepd which was dissolved in a minimum vol. of SB and stored in liquid N_2. Generally, 1 g coffee leaves yielded *ca* 1 ml enzyme extract. For the purification work, 8 harvests corresponding to 50 g fr. wt were thawed, combined and desalted on PD-10 gel filtration columns equilibrated with buffer A. The eluate was pooled and loaded onto a 13 × 1.6 cm (i.d.) Q-Sepharose FF column equilibrated with buffer A. The column was washed with buffer A until no further absorption was detected at 280 nm. Protein was eluted with a 65 min linear gradient of 0–0.5 M NaCl in buffer A at a flow rate of 2 ml min^{-1}. Frs of 6 ml were collected and NMT activity was determined in 0.15 ml aliquots. Conductivity was measured in each fr. using a conductometer (Metrohm, Herisau, Switzerland). Frs comprising the main peak of activity were pooled and a 5 ml aliquot was desalted on two

PD-10 columns equilibrated with buffer B1 (2 or 3). The eluates were combined and loaded onto a Mono P HR 5/20 column equilibrated with buffer B1 (pH gradient 7–4), B2 (pH gradient 6.0–3.5) or B3 (pH gradient 5.5–4.0). The column was washed with 3 column vols of buffer B1 (2 or 3) and protein elution was achieved with 100% buffer C1 (2 or 3) which established a linear pH gradient from 7–4 (C1), 6.0–3.5 (C2) or 5.5–4.0 (C3) at a flow rate of 0.5 ml min^{-1}. Frs of 1 ml were collected, the pH adjusted to 7.5 with 300 μl buffer D and 0.15 ml aliquots used for determination of NMT activity. Using a gradient from 7–4, the 7NMT was well sepd from the two other activities and was pooled separately (Pool I). Pool II comprised peak frs of 3NMT and 1NMT contaminated with traces of 7NMT activity. Pool II was concd 2.5-fold using a Centriplus-30 Concentrator (Amicon) and a 500 μl aliquot was loaded on a Superdex 200 HR 10/30 column equilibrated with 0.15 M NaCl in buffer A and eluted at 0.25 ml min^{-1}. Frs of 0.5 ml were collected and assayed for individual NMT activities.

Several purification techniques and media were screened for their effectiveness. These included (i) additional precipitating agents such as EtOH, MeOH, Me_2CO, polyethylene glycol 6000, pH change and even heat were examined; (ii) ion-exchange chromatography on matrices such as CM-Sephadex C-25 (cation-exchange; Pharmacia), SP-Toyopearl (cation-exchange; Tosohaas, Philadelphia, USA), DEAE-Sephacel (anion-exchange; Pharmacia); (iii) a variety of affinity matrices ranging from the commercially available dyematrex screening kit (Blue A, Red A, Orange A, Green A and Blue B; Amicon), to xanthine-agarose (Sigma) and adenosine-agarose (dephosphorylation of ADP-agarose from Sigma) and self-prepared affinity media with ligands including 7-methylxanthine (epoxy-activated-Sepharose 6B; Pharmacia), SAH (coupling through the α-amino group using HiTrap N-hydroxysuccinimide-activated Sepharose; Pharmacia, coupling through the cis-diol group of the ribose using phenylboronate agarose; Amicon, and coupling through the α-carboxy group using EAH Sepharose 4B; Pharmacia). However, in no case was a significant purification achieved; (iv) hydrophobic interaction chromatography (HIC) using a HiTrap HIC test kit (Pharmacia) and an Econo-Pac t-Butyl HIC cartridge (Bio-Rad); (v) hydroxyapatite chromatography (Econo-Pac CHT-II cartridge, Bio-Rad). Of these varying purification methods, dye-ligand affinity chromatography with Blue B, HIC and hydroxyapatite chromatography provided the most satisfactory purifications and yields and, therefore, may be incorporated into the sequential purification protocol.

M_r *determination.* The native M_rs of the NMTs were determined by comparison with a calibration of standard proteins on a gel filtration column. Ribonuclease A (13 700), chymotrypsinogen A (25 000), ovalbumin (43 000), and albumin (67 000) were used as standards. The void vol. of the column was determined with Blue Dextran 2000. The K_{av} (x-axis) was calculated for each protein and plotted against the log of the M_r (y-axis). The best fitting line was drawn by computer and the M_rs of the NMTs were determined for a calculated K_{av} of 0.43.

Polyacrylamide gel electrophoresis. Proteins were sepd under denaturing conditions using SDS-PAGE according to the method of ref. [16] or under the following non-denaturing conditions: 7.5–15% plateau, continuous buffer system with Tris–Tricine pH 8.75, 20 mM Tris. The sample buffer contained 20% (v/v) glycerol. The gels were pre-run for 1 hr at 150 V. Electrophoresis was carried out at 200 V.

IEF gels were cast using a horizontal glass plate sandwich (12.4 × 6.5 cm, silanized glass) with two 1 mm spacers. A gel support film (GelBond PAG film, Bio-Rad) was attached to one of the two glass plates. The gel media (1.3 ml 30% monomer [30% T, 2.7% C]. 1 ml glycerol, 0.45 ml ampholine pH 3.5–10, 13 μl TEMED, 40 μl 10% (w/v) APS, H_2O to a total vol. of 8 ml) was pipetted between the two plates and polymerised for 1 hr. The gel was run on a horizontal electrophoresis cell at *ca* 4° for 4 hr (4 W gel^{-1}) with H_3PO_4 as the anode soln and NaOH as cathode soln (Electrode strips [Serva] were soaked with these solns and placed onto the gel). The isoelectric point (pI) of the protein was determined by comparison with coloured IEF standards, according to the manufacturer's instructions. The gel was silver stained as described by ref. [17]. For the 3NMT assay, the gel was cut into 2 mm slices with a gel slicer. Each slice was transferred to an Eppendorf tube, 20 μl 0.5 M Tris–HCl, pH 8 was added and the assay performed as described above. For localisation of radioactivity, the gel was sliced into 2-mm sections, transferred to vials containing scintillant (Ready Safe, Beckmann, Fullerton, U.S.A.) and the radioactivity was measured by liquid scintillation spectrometry.

Photoaffinity labelling was conducted in the dark in a round-bottom 96-well microtiter plate floating on an ice–H_2O slurry, as described in refs [11, 12]. Prior to irradiation, the enzyme was spin-desalted through a 5 ml Sephadex G25-fine column equilibrated with buffer E [7, 18]. The reaction mixt. contained 20 μl desalted enzyme extract and 2 μCi [^{3}H]SAM. After 10 min incubation in the dark at room temp., the samples were irradiated for 1–10 min with a short-wave UV light (8 W at 254 nm, with a 1.5 nm filter; Desaga, Heidelberg, Germany). The distance between the UV lamp and samples was adjusted to *ca* 5 mm. Immediately after photolabelling, samples were transferred to Microcon-50 Microconcentrators (Amicon) to separate unbound [^{3}H]SAM from the photolabelled enzyme. The enzyme was washed × 3 with 300 μl buffer E and concd to *ca* 20 μl. To each sample 5 μl SSB was added, before heating for 3 min at 100° followed by SDS-PAGE gel electrophoresis (12.5% resolving gel, 5% stacking gel). Control experiments were carried out with: (a) SAH, a known inhibitor of

NMT (final concn 2 mM); (b) unlabelled SAM (final concn 3 mM and 20 mM); (c) heat-inactivated enzyme; (d) varying enzyme concn; (e) varying [^{3}H]SAM concs (1–5 μCi); (f) no UV; (g) varying UV irradiation time (1–30 min).

Fluorography of SDS-PAGE gels was performed using slight modifications of the published procedures [19, 20]. After gel electrophoresis, the gel was fixed in MeOH–HOAc–H_2O (45:10:45, v/v) for 30 min and soaked in DMSO for 30 min followed by a second 30-min immersion in fresh DMSO. The gel was then immersed in 3% PPO (w/v) in DMSO for 1 hr, washed in several changes of H_2O for at least 1 hr before being dried under vacuum for 2 hr. A Fuji RX film was placed in direct contact with the gel in a light-tight box and exposed for 14–28 days at −80°. After fluorography, the dried gel was stained by immersion in 5% glycerol in H_2O (v/v) and soaking in DMSO for *ca* 1 hr to remove pptd PPO. The clear gel was then washed in several changes of H_2O and stained with Coomassie Brilliant Blue or silver stain [17].

Protein content was determined by the method of ref. [21] with a prepd dye binding reagent (Bio-Rad) and modified for the use in 96-well microtiter plates. Bovine serum albumin was used as the standard.

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