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FAST PROTEIN LIQUID CHROMATOGRAPHIC PURIFICATION AND SOME PROPERTIES OF A PARTIALLY *O*-METHYLATED FLAVONOL GLUCOSIDE 2′-/5′-*O*-METHYLTRANSFERASE*

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Key Word Index—*Chrysosplenium americanum*; Saxifragaceae; *O*-methyltransferase; purification; FPLC; polymethylated flavonols; 2′-/5′-*O*-methylation.

Abstract—A flavonol *O*-methyltransferase was partially purified from *Chrysosplenium americanum* by fractional precipitation with ammonium sulphate followed by gel filtration and chromatofocusing using an FPLC system. The enzyme which was purified 420-fold catalysed the transfer of the methyl group of SAM to the 2′- or 5′-positions of partially methylated flavonol glucosides, the two terminal methylation steps in the biosynthesis of *Chrysosplenium* flavonoids. The enzyme had a pH optimum of 7 in Pi buffer, a pI of < 5, an M_r of 57 000, no Mg^{2+} requirement and was inhibited by both *N*-ethylmaleimide and phenylmercuriacetate. The K_m value for the flavonol substrate was 2 μM and that for SAM was 100 μM. The role of this enzyme is discussed in relation to the biosynthesis of polymethylated flavonols in this tissue.

INTRODUCTION

Chrysosplenium americanum accumulates a variety of tri- to penta-*O*-methylated flavonol glucosides [1] which are derived from 2′-hydroxyquercetin and its 6-hydroxy derivative, 2′-hydroxyquercetagitin (Fig. 1). Very recently, we isolated and characterized four, distinct, position-specific *O*-methyltransferases (OMT) (EC 2.1.1.—) which catalysed the coordinated sequence of methylation of quercetin → 3-methylquercetin → 3,7-dimethylquercetin → 3,7,4′-trimethylquercetin; and of 3,7-dimethyl or 3,7,3′-trimethylquercetagetin to their 6-*O*-methyl derivatives [2, 3].

We describe in this report the partial purification and some properties of another enzyme which catalyses the terminal steps of methyl transfer in the biosynthesis of *Chrysosplenium* flavonoids, namely 2′-*O*-methyltransferase. In contrast with the four previously reported OMTs [2, 3] which accepted aglycones as substrates, this enzyme accepts only its natural glucosylated substrate, 5,2′-dihydroxy-3,6,7,4′-tetramethoxy flavone-5′-*O*-glucoside. Furthermore, the 2′-OMT preparation catalyses the *O*-methylation of 5,5′-dihydroxy-3,7,4′-trimethoxyflavone-2′-*O*-glucoside with the formation of its 5′-*O*-methyl derivative. It was considered important, therefore, to investigate whether both methylation steps are catalysed by one or two, distinct enzymes. These 2′- and 5′-methylated products as well as their respective substrates (Fig. 1) constitute the four major flavonol glucosides of this tissue [1].

RESULTS AND DISCUSSION

Enzyme purification

The enzyme was purified by fractional precipitation with ammonium sulphate, gel filtration on a Superose® 12 HR 10/30 column (Fig. 2A) and chromatofocusing on a Mono P® HR 5/20 column (Fig. 2B). The purified enzyme preparation was equally active against both substrates **1** (5,2′-dihydroxy-3,6,7,4′-tetramethoxy-flavone-5′-*O*-glucoside) and **2** (5,5′-dihydroxy-3,7,4′-trimethoxyflavone 2′-*O*-glucoside); thus demonstrating the methylation at the 2′- and 5′-positions of the partially methylated flavonol glucosides **1** and **2**. The combined purification steps described above resulted in an increase in specific activity of 420-fold of the enzyme preparation as compared with the crude extract (Table 1). In contrast with the enzyme purification by conventional gel filtration and chromatofocusing techniques, the use of FPLC almost quadrupled the extent of purification of this enzyme (Table 1). However, the recovery of total activity was comparable with the 3-, 6- and 4′-OMTs, which were purified by conventional methods from the same tissue [2, 3].

It is interesting to note that enzyme purification using a linear pH gradient between 7 and 4 resulted in complete loss of both the 2′- and 5′-*O*-methylating activities. Therefore, we resorted to the use of a shallower gradient (pH 7–5), whereby both activities were recovered by elution with high salt (Fig. 2B). This prompted us to look

* Part III in the series "Enzymatic Synthesis of Polymethylated Flavonols of *Chrysosplenium americanum*". For Parts I and II see refs [3] and [4].

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Abbreviations: DTE, dithioerythritol; FPLC, fast protein liquid chromatography; 2-ME, 2-mercaptoethanol; NEM, *N*-ethylmaleimide; OMT, *O*-methyltransferase; SAM, *S*-adenosyl-L-methionine.

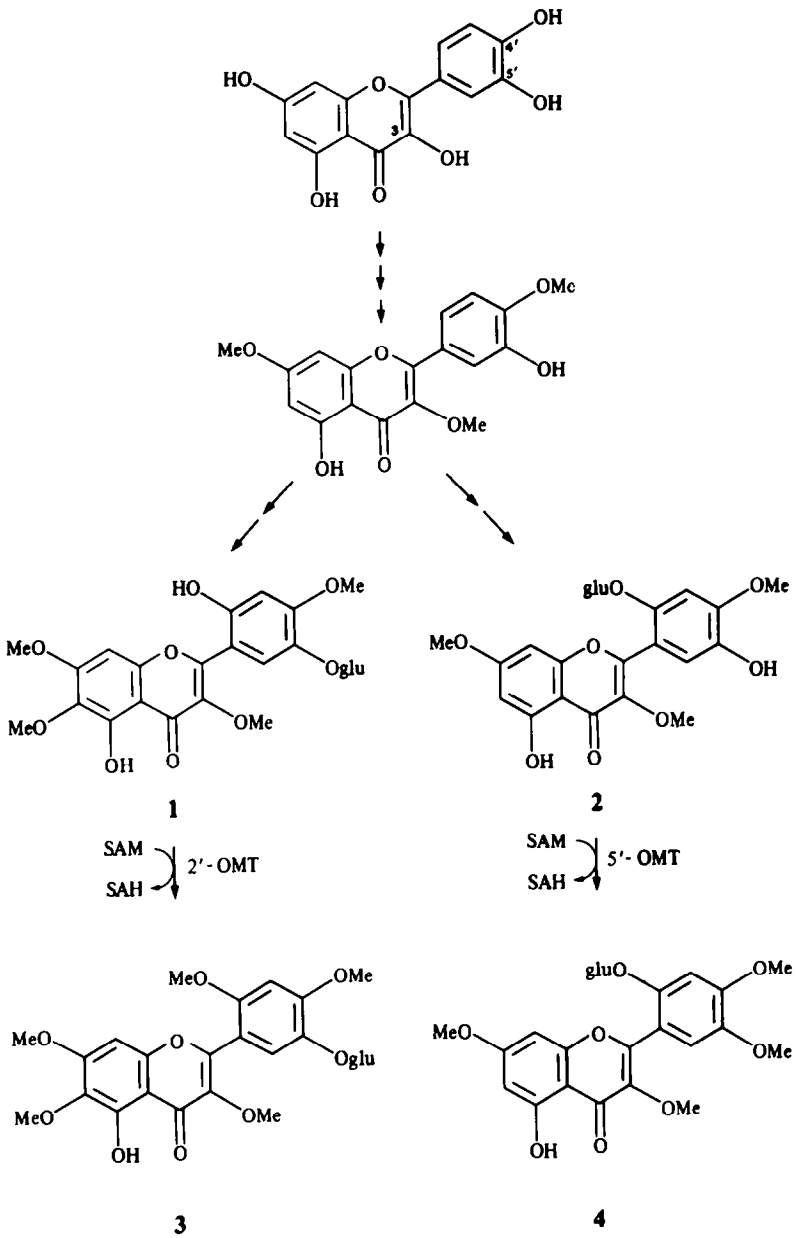

Fig. 1. Structural formulae of four of the major, partially *O*-methylated flavonol glucosides (1–4) which accumulate in *C. americanum*. Compounds 1 and 2 were used as the natural substrates for the 2'- and 5'-OMTs, respectively.

for the 7-OMT activity which was lost during previous chromatofocusing (pH 7–4) of other OMTs of this system [3]. The fact that the 7-OMT activity was recovered with those of the 2'- and 5'-OMTs following chromatofocusing (pH 7–5) indicated that the pI of these enzymes is < 5.

In contrast with the previously reported OMTs [5–10], including those of *Chrysosplenium* [1–4], this enzyme preparation accepted partially *O*-methylated flavonol glucosides as substrates, namely 1 or 2, and gave rise to their respective 2'- and 5'-methyl derivatives (3 and 4). On the other hand, neither of the corresponding aglycones of compounds 1 or 2 nor any of the hydroxylated or partially methylated flavonols tested acted as methyl acceptors. Two other OMTs have been reported to mediate the methylation of 3'- and/or 5'-positions of anthocyanins [11] and the 7-position of the C-glycoflavone, vitexin [12]. These enzymes, however, accepted non-methylated flavonoid glycosides and catalysed single methylation steps.

Enzyme properties

The 2'-/5'-*O*-methylation reaction was linear with time up to 90 min and with protein concentration up to 30 μg, at 10 μM of either substrate. The pH optimum of both methylations was studied in several buffers between pH 6 and 9. Optimum activity was found to be at pH 7 in Pi buffer for both the 2' and 5' methylation reactions. A change of pH of one unit resulted in a loss of 50% of

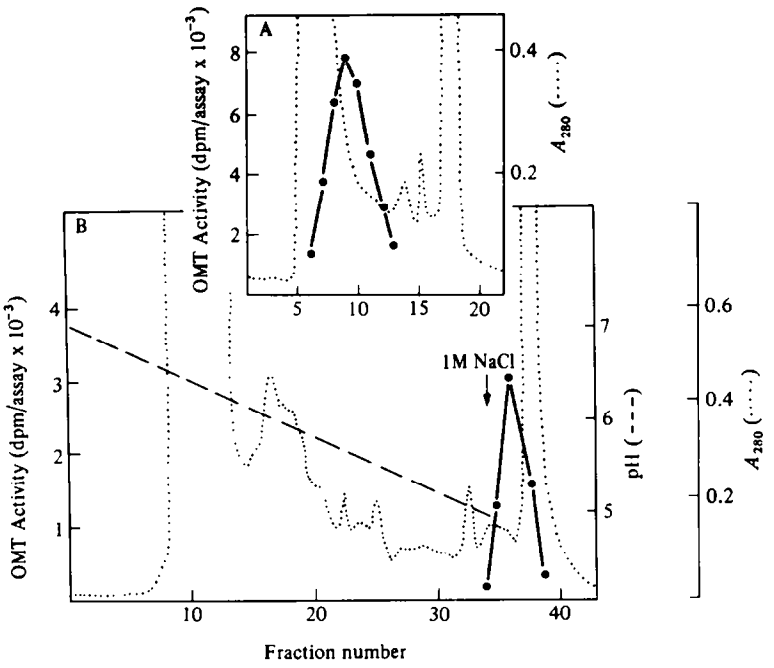

Fig. 2. Elution profile of OMT activity after gel filtration (A) and chromatofocusing (B). Substrates **1** and **2** gave similar activity peaks.

Table 1. Purification of *Chrysosplenium* 2'-/5'-*O*-methyltransferase*

Purification step	Total protein (mg)	Sp. act. (pkat/mg)	Purification (-fold)	Recovery (%)	Purification† (-fold)
Dowex 1 × 2	105	0.53	—	100	—
Ammonium sulphate‡ (40–70%)	88	0.61	1.2	96	—
Gel filtration§	2.5	11.3	21.3	51	18.7
Chromatofocusing‖	0.05	222	419	20	123

*Purification procedure was conducted with 15 g fresh tissue.
†By conventional column chromatography.
‡Desalted on Sephadex G-25.
§On Superose 12 HR 10/30 column.
‖On Mono P HR 5/20 column.

enzyme activity. This pH optimum is in closer agreement with those of the OMTs which accept glycosylated flavonoids [11, 12] than flavonoid aglycones [2, 3, 5, 6, 9, 13].

The M_r of the 2'-/5'-OMT was estimated to be 57 000, similar to other *Chrysosplenium* OMTs. The K_m values for the flavonoid substrates **1** and **2** were similar (2 μM) and those for the methyl donor, SAM, were 100 μM. These values compared well with those of previously reported flavonoid-ring B OMTs [4, 11, 13].

The effect of divalent cations and SH-group inhibitors on the 2'-/5'-*O*-methylation was studied. The results (Table 2) show that in contrast with the 6-OMT of *Chrysosplenium* [3], there was no requirement for Mg^{2+} with either substrate. Furthermore, there were no significant differences observed for the effect of other divalent cations on the methylation of either substrate, except for Ca^{2+}, Co^{2+} and Cu^{2+} (Table 2). SH-group reagents, such as NEM and phenylmercuriacetate, were found to inhibit the *O*-methylation of both substrates to the same extent. However, in contrast with other reagents used (Table 2), inhibition with NEM was partially prevented by the addition of 14 mM 2-ME. Neither ME nor DTE when added to the reaction mixture, had any effect on the methylation of either substrate (Table 2).

These results demonstrate the presence of an enzyme system which catalysed the two terminal methylation steps in the biosynthesis of polymethylated flavonol glucosides in *C. americanum*. It is important to note that the two substrates used (**1** and **2**) as well as their respective

Table 2. Effect of divalent cations and SH-group reagents on *O*-methyltransferase activity*

Additions	Concentration (mM)	Relative activity 2'-OMT	5'-OMT
None	—	100	100
Mg^{2+}	1	100	92
Mg^{2+}	10	76	50
Mn^{2+}	1	73	62
Mn^{2+}	10	14	41
Ca^{2+}	1	110	88
Ca^{2+}	10	84	48
Co^{2+}	1	27	87
Cu^{2+}	1	77	42
Zn^{2+}	1	14	7
EDTA	1	99	87
EDTA	10	87	90
2-ME	14	95	89
DTE	10	102	92
Iodoacetamide	1	59	39
Iodoacetate	1	95	86
NEM	1	7	4
NEM + 14 mM 2-ME	1	82	59
Phenylmercuriacetate	1	31	3
p-Chloromercuribenzoate	1	100	84

*The chromatofocused enzyme and the standard assay were used, as described in the Experimental section.

O-methylated derivatives (**3** and **4**) comprise the four major flavonoid constituents of this tissue (Fig. 1). In view of the strict position specificity of the four, previously studied OMTs of this system [3], the question arises whether the 2'- and 5'-*O*-methylations are catalysed by one or two, distinct enzymes. The fact that the two methylating activities were eluted in the same fractions during purification and exhibited similar enzyme properties suggests that both methylations are mediated by the same enzyme. Further attempts to separate these two activities by affinity chromatography on *S*-adenosyl-L-homocysteine agarose were unsuccessful. The involvement of a single enzyme is further supported by the fact that the 2'- and 5'-positions are *para* oriented to each other, which makes it possible for attack by the same enzyme. However, this does not exclude the possibility of the presence of two distinct enzymes, a 2'- and a 5'-OMT, which may have similar chromatographic and physical properties. We have recently reported a similar situation [14, 15] in which we postulated that *O*-glucosylation at the 5'- and 2'-positions of compounds **3** and **4**, respectively, was catalysed by one *O*-glucosyltransferase. That postulate was based on the similarities in chromatographic behaviour and enzyme properties [14] as well as similarities of the kinetic constants and kinetic mechanism [15].

The use of immunological methods may provide evidence for the involvement of one or two distinct enzymes in the two methylation steps of this system. The 3-*O*-glucosylation of flavonols and anthocyanidins has recently been shown to be catalysed by the same glucosyltransferase based on the behaviour, towards both substrates, of antibodies raised against the partially purified enzyme preparation [16].

EXPERIMENTAL

Plant material. Chrysosplenium americanum Schwein ex Hooker was collected from Sutton Junction, Province of Quebec, and was maintained under controlled conditions of light, temperature and humidity.

Buffers. The following buffers were used: A, 25 mM imidazole, pH 7.2 containing 14 mM 2-ME and 10% glycerol; B, Polybuffer(74)–H_2O (1:10), pH 5.0, containing 14 mM 2-ME and 10% glycerol; and C, buffer A containing 1 M NaCl. All buffers were passed through a 0.22 μm Millipore filter and degased before use.

Protein extraction. Protein was extracted as previously described [2]. The protein was fractionated with solid $(NH_4)_2SO_4$ and the protein fraction that precipitated between 40 and 70% salt saturation was collected by centrifugation.

Enzyme purification. The *O*-methyltransferase activity was purified by FPLC (Pharmacia). The protein pellet was suspended in the minimal amount of buffer A and chromatographed on a Superose® 12 HR 10/30 column, which had previously been equilibrated with the same buffer. The column was developed with buffer A at a flow rate of 0.5 ml/min (pressure of 2.5 MPa) and one-ml fractions were collected and assayed for the 2'- and 5'-*O*-methylating activity using substrates **1** (5,2'-dihydroxy-3,6,7,4'-tetramethoxyflavone-5'-*O*-glucoside) and **2** (5,5'-dihydroxy-3,7,4'-trimethoxyflavone-2'-*O*-glucoside), respectively. The active fractions were pooled and subjected to chromatofocusing on a Mono P® HR 5/20 column which had previously been equilibrated with buffer A. Elution of the bound proteins was first performed with 50 ml of buffer B which generated a linear gradient between pH 7 and 5; and subsequently with 10 ml of buffer C. The flow rate was 0.4 ml/min (pressure of 3.0 MPa) and one-ml fractions were collected and assayed for OMT activity. In order to remove the polybuffer, BSA (1 mg/ml) was added to the active fractions; the protein was precipitated with $(NH_4)_2SO_4$ and subsequently desalted on Sephadex G-25 in buffer A.

OMT assay. The standard enzyme assays were performed as described previously [2, 3] using *S*-adenosyl-L-[Me-^{14}C]methionine as methyl donor.

M_r *determination.* An estimate of the M_r of the partially purified enzyme preparation was obtained by determining its elution vol. on gel filtration.

Protein determination. Protein was determined according to the method of Bradford [17] using the Bio-Rad protein reagent and bovine serum albumin as standard.

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