Phytochemistry, Vol. 21, No. 7, pp. 1537–1540, 1982.
Printed in Great Britain.

0031-9422/82/071537-04$03.00/0

CHARACTERIZATION OF THREE DISTINCT FLAVONOL *O*-METHYLTRANSFERASES FROM *CHRYSOSPLENIUM AMERICANUM**

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(*Received* 20 *November* 1981)

Key Word Index—*Chrysosplenium americanum*; Saxifragaceae; *O*-methyltransferase; polymethylated flavonols; enzymatic synthesis; 3-, 6- and 3′-*O*-methylation.

Abstract—The partially purified *O*-methyltransferase (OMT) system of *Chrysosplenium americanum* was found to catalyse the stepwise *O*-methylation of quercetin to its mono-, di- and trimethyl derivatives. It also utilized the partially methylated flavonol intermediates to form the next higher order of *O*-methylated products; thus indicating the involvement of several OMTs. The latter were resolved by chromatofocusing into three distinct peaks of enzyme activity which focused at pI values 4.8, 5.4 and 5.7. The former enzyme *O*-methylated quercetin at the 3-position, whereas the latter two *O*-methylated 3, 7-di-*O*-methyl quercetagetin at the 3′- and 6-positions, respectively. None of the focused enzymes accepted caffeic acid, or other flavonoids such as kaempferol or luteolin, as substrates; thus indicating specificity towards flavonols with 3′, 4′-substitution. The three OMTs had similar MWs and the K_m values for their substrates were of the same order of magnitude. The biochemical role of these novel enzymes is discussed in relation to the biosynthesis of polymethylated flavonols in this tissue.

INTRODUCTION

Several *O*-methyltransferases (OMTs) (EC 2.1.1.6) have been detected in plant tissues involving the biosynthesis of phenylpropanoid and flavonoid compounds ([1, 2] and references cited therein). In most cases, *O*-methylation was reported to take place predominantly at the hydroxyl group *meta* to the side chain of an *o*-dihydroxyphenol, but not to the exclusion of *O*-methylating *p*-hydroxy compounds [3, 4]. Few reports have appeared recently on the *O*-methylation of different hydroxyl groups on the phenolic ring system. In only two cases, however, have discrete enzymes been shown to catalyse these methylations [5–7].

Polymethylated flavonoids are known for their limited occurrence in the Plant Kingdom [8]. Those of *Chrysosplenium americanum* consist of tri-, tetra- or penta-methyl derivatives‡ of quercetin and 6-hydroxyquercetin (quercetagetin) with the methyl groups being located at positions 3, 6, 7, 2′, 4′ or 5′ [9]. Recent work in this laboratory has demonstrated the sequential *O*-methylation of several flavonoid compounds, including quercetin, by cell-free extracts of Calamondin orange [10, 11] and *C. americanum* [9], thus indicating the possible involvement of a number of OMTs.

This report describes, for the first time, the isolation and some properties of three distinct OMTs which exhibited strict position specificity towards 3-, 6- and 3′ positions of the flavonol ring system and seem to be involved in the biosynthesis of polymethylated flavonols of this tissue.

RESULTS AND DISCUSSION

Purification of the Chrysosplenium *OMT system*

The enzyme system of *C. americanum* was purified by fractional precipitation with ammonium sulphate and chromatography of the resulting pellet on Sephadex G-100 or DEAE-cellulose columns. OMT activity was recovered from the gel filtration step as a discrete peak with an apparent MW of 65000 ± 5000 daltons (Fig. 1). When the desalted ammonium sulphate pellet was chromatographed on DEAE-cellulose using a 0–150 mM potassium chloride gradient two peaks of activity were obtained (not shown). One fraction catalysed the *O*-methylation of quercetin and 3, 7-di-*O*-methylquercetagetin, whereas the other accepted only the latter substrate for further *O*-methylation. Since both activity peaks exhibited no definite substrate specificity, a one-step elution of the enzyme activity from DEAE-cellulose was normally used. The partially purified enzyme preparations (Sephadex G-100 or DEAE-cellulose eluates) were found to catalyse the efficient *O*-methylation of quercetin, but not quercetagetin, to its 3-methyl,

*Part 2 in the series "Polymethylated Flavonols of *Chrysosplenium americanum*". For Part 1 see ref. [9].

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‡The highest order of *O*-methylated products are 5, 2′-dihydroxy-3, 7, 4′, 5′-tetramethoxyflavone - 2′ - *O* - glucoside and 5, 5′-dihydroxy-3, 6, 7, 2′, 4′ - pentamethoxyflavone - 5′ - *O* - glucoside. Whenever quercetin or quercetagetin is substituted at the 2′-position, numbering of its B-ring substitution pattern becomes 4′, 5′ instead of 3′, 4′, where the *meta* positions 3′ and 5′ are analogous.

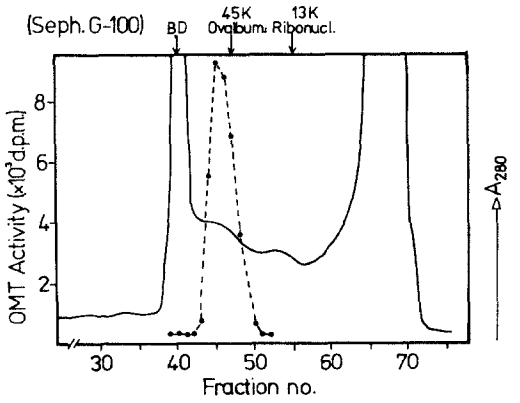

Fig. 1. Elution profile of OMT activity after chromatography on Sephadex G-100, using quercetin as substrate, and elution volumes of ribonuclease and ovalbumin as marker proteins.

3, 7-dimethyl and 3, 7, 3'-trimethyl derivatives, though with different rates (Table 1). They also utilized the partially *O*-methylated derivatives of quercetin or quercetagetin to form the next higher order of *O*-methylated products (Table 1); thus indicating the existence of several OMTs in these preparations.

Further purification of the enzyme protein was achieved by chromatofocusing on Polybuffer exchanger and resulted in three peaks with OMT activity (Fig. 2). These activity peaks exhibited distinct pI values and substrate specificities, though they had similar MWs and had K_m values of the same order of magnitude (Table 2).

Substrate specificity

One enzyme activity (peak I) *O*-methylated quercetin at the 3-position and gave only one product identified as 3-methylquercetin (Fig. 2). The fact that this enzyme did not accept any of the flavonols tested (galangin, kaempferol, quercetagetin or gossypetin) or any of the di- or trimethyl substrates for further *O*-methylation, strongly suggests its designation as quercetin - 3 - *O* - methyltransferase. Activity peaks II and III, on the other hand, catalysed further *O*-methylation of 3, 7 - di - *O* - methylquercetagetin at the 3'- and 6-positions, respectively (Fig. 2, Table 2). Neither of these two activity peaks utilized quercetin as substrate. Furthermore, activity peak III *O*-methylated 3, 7, 3' - methylquercetagetin to its 3, 6, 7, 3'-tetramethyl ether, whereas peak II did not; thus indicating the specificity of both enzymes towards positions 6 and 3', respectively. It should be noted, however, that the substrates used with activity peaks II and III may not necessarily be those utilized *in vivo*; since the final products that accumulate in this tissue are usually substituted at the 2'-position [9].

None of the three enzymes reported here reacted with phenylpropanoids (caffeic or 5-hydroxyferulic acids) or other flavonoids (luteolin or dihydroquercetin) indicating a high degree of specificity towards the 3', 4-disubstituted flavonols and a requirement for *quasi*-planar configuration.

Other properties

The three focused enzymes exhibited fairly low K_m values indicating high affinity towards their substrates and specificity for the positions they methylate. This correlates well with the absence of mono- and dimethyl intermediates *in vivo* [9]. Their pH optima and their requirement for Mg^{2+} are consistent with those reported for other flavonoid OMTs [12–14]. The fact that the three purified enzymes have similar MWs may raise the question as to whether they are components of a larger, loosely associated protein

Table 1. *O*-Methylation of flavonols by partially purified enzyme preparations from *Chrysosplenium americanum**†

Substrate	*O*-Methylated products‡
Quercetin	3-Methylquercetin 3, 7-Dimethylquercetin 3, 7, 3'-Trimethylquercetin
3-Methylquercetin	3, 7-Dimethylquercetin
3, 7-Dimethylquercetagetin	3, 7, 3'-Trimethylquercetagetin 3, 6, 7-Trimethylquercetagetin
3, 7, 4'-Trimethylquercetin 3, 7, 3'-Trimethylquercetagetin 3, 6, 7-Trimethylquercetagetin	Tetramethyl ethers§

*Sephadex G-100 or DEAE-cellulose eluates.

†The standard enzyme assay was used as described in the Experimental.

‡Identified by co-chromatography with reference compounds and autoradiography.

§Distinguished by their R_f values as compared with those of the substrates.

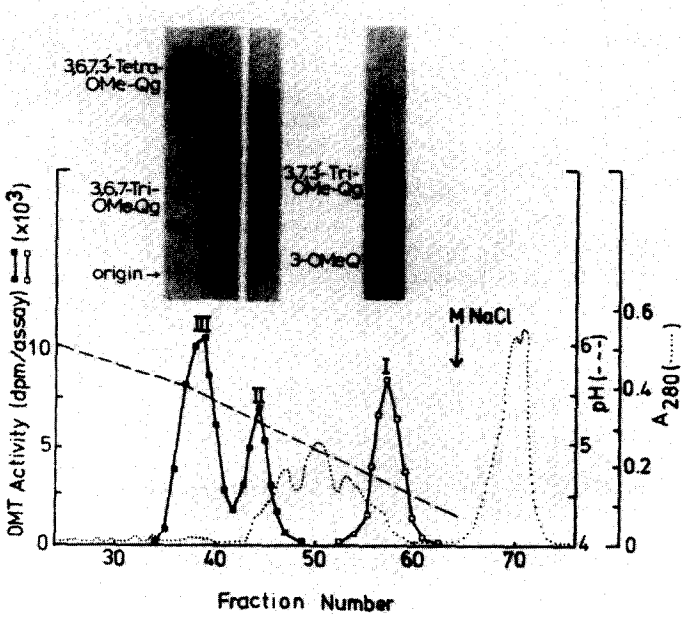

Fig. 2. Elution profile of OMT activity (peaks I–III) after chromatofocusing on Polybuffer exchanger which generated a gradient of PH 6–4 (below) and a print of the autoradiographed reaction products of the three activity peaks (above). The substrates used in the OMT assay and their reaction products are given in Table 2.

Table 2. Characteristics of *C. americanum* O-methyltransferases

Characteristic	Activity peaks		
	I	II	III*
pI value	4.8	5.4	5.7
Substrate	Quercetin	3, 7-Dimethyl-quercetagetin	3, 7-Dimethyl-quercetagetin
Product	3-Methyl-quercetin	3, 7, 3'-Trimethyl-quercetagetin	3, 6, 7-Trimethyl-quercetagetin
K_m (μM)			
Substrate	1.4	0.5	2.5
S-adenosyl-L-methionine	70	90	50
Mg^{2+} requirement (5 mM)			
at 5 μM SAM	No	Yes	No
at 30 μM SAM	Yes	Yes	Yes
pH optimum	7.5–8.5	7.5–8.5	7.5–8.5
MW (thousand daltons)	65 ± 5	65 ± 5	65 ± 5
Designation	3-OMT	3'-OMT	6-OMT

*Also utilized 3, 7, 3'-trimethyl-quercetagetin to form the 3, 6, 7, 3'-tetramethyl ether.

aggregate that remains to be isolated in an intact form.

The biochemical role of the three OMTs reported here appears to mediate the sequential O-methylation of quercetin at the 3-position and of 3, 7-dimethylquercetagetin at the 3'- and 6-positions, respectively. These enzymes together with other OMTs, yet to be identified constitute the complement involved in polymethylated flavonol synthesis in *C. americanum*. These enzymes are distinct from other previously reported flavonoid OMTs[12–14] which catalyse ring B O-methylation only. Furthermore, the 3'- and 6-OMTs utilize partially methylated flavonols for further O-methylation. These results clearly indicate that O-methylation of flavonoids does occur at the C_{15} level[15] both on rings A and B as well as the heterocyclic ring[11]. It is interesting that although the partially purified enzyme preparations catalysed the sequential O-methylation of quercetin to its 3, 7, 3'-trimethyl ether (Table 1), attempts to detect the 7-OMT activity in the chromatofocused fractions were unsuccessful. This may have been due to instability of the purified enzyme or to the lack of some factor that may be required for its activity.

In order to formulate the biogenetic sequence for *Chrysosplenium* flavonoids there remain a number of

problems to be investigated, namely: (a) to isolate and characterize other OMTs that appear to be specific for positions 7, 2′ and 4′; (b) to determine which of the partially *O*-methylated intermediates of quercetin is further hydroxylated at positions 6 and/or 2′ and investigate the nature of the hydroxylases involved; (c) to study the glucosylation steps at positions 2′ and 5′ of the *O*-methylated flavonols in view of determining whether this reaction is a final step in polymethylated flavonoid synthesis; and (d) localization and regulation of the various enzymes involved in this biosynthetic pathway.

Nomenclature

The results reported here have clearly established the existence of at least three distinct OMTs that are involved in flavonoid biosynthesis. We propose, therefore, the following designations: *S* - adenosyl - L - methionine:quercetin 3 - *O* - methyltransferase; *S* - adenosyl - L - methionine:3, 7 - dimethylflavonol 3′ - *O* - methyltransferase and *S* - adenosyl - L - methionine:3, 7 - dimethylflavonol 6 - *O* - methyltransferase, for activity peaks I, II and III, respectively.

EXPERIMENTAL

Plant material. Chrysosplenium americanum Schwein. ex. Hooker (Saxifragaceae) was collected from Sutton Junction, Québec and was maintained under greenhouse conditions.

Preparation of crude extracts. Young shoot tips (*ca* 20 g) were frozen in liquid N_2 and mixed with Polyclar AT (10% w/w) before being homogenized with 100 mM Pi buffer, pH 7.6 (1:3, w/v) containing 5 mM EDTA, 10 mM diethylammonium diethyldithiocarbamate and 14 mM β-mercaptoethanol (buffer A). The slurry was filtered through nylon mesh and the filtrate was centrifuged at 20000 *g* for 15 min. The supernatant was stirred with Dowex 1×2 (10%, w/v) which had previously been equilibrated with the same buffer, then filtered. The filtrate was fractionated with solid $(NH_4)_2SO_4$ and the protein fraction which ppted between 35–70% was collected by centrifugation and desalted on a Sephadex G-25 column using 40 mM Pi buffer, pH 7.6 containing 10 M EDTA and 10% glycerol (buffer B).

Purification of OMT activity. The desalted $(NH_4)_2SO_4$ pellet was chromatographed on either Sephadex G-100 (Pharmacia) or DEAE-cellulose (Bio-Rad) columns which had previously been equilibrated with buffer B. In the latter case, the column was washed with the same buffer followed by a linear gradient of 0–150 mM KCl in buffer B. Since the two eluted peaks of OMT activity exhibited no definite substrate specificity, a one-step elution of the enzyme protein was carried out using 125 mM KCl in buffer B. Protein fractions with OMT activity were combined and ppted with 70% $(NH_4)_2SO_4$. The dissolved pellet was desalted on a Sephadex G-25 column with 25 mM imidazole buffer, pH 7.4, containing 10% glycerol (buffer C). The desalted protein was further purified by chromatofocusing on Polybuffer exchanger (1×20 cm column, Pharmacia) which had previously been equilibrated with buffer C. The protein was eluted with Polybuffer-94 (after being diluted to manufacturer's specification) which generated a linear, 200 ml gradient of pH 6–4 and 3 ml fractions were collected for OMT assay against different substrates.

OMT assay and identification of reaction products. The standard enzyme assay was the same as that described previously [6, 11] using *S* - adenosyl - L - [^{14}C - methyl] - methionine as the methyl donor (sp. act. 50 mCi/mmol, New England Nuclear). The *O*-methylated products were extracted in a mixture of C_6H_6–EtOAc (1 : 1) and co-chromatographed with reference compounds on Polyamide-6 TLC plates, using toluene–HCO_2Et–BuOAc–HCO_2H (25 : 50 : 23 : 2), then autoradiographed.

MW determination. An estimation of the MWs of the partially purified (Sephadex G-100) and the chromatofocused enzymes was obtained by determination of their elution vols from a calibrated Sephadex G-100 (superfine) column [16, 17].

Acknowledgements—We wish to thank Drs. M. Jay, B. Voirin and B. A. Bohm for their generous gifts of *O*-methylated flavonols and Dr. F. W. Collins for helpful discussions. This work was supported by an operating grant (A4549) from the Natural Science and Engineering Research Council of Canada.

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