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PURIFICATION AND IMMUNOLOGICAL CHARACTERIZATION OF A RECOMBINANT TRIMETHYLFLAVONOL 3′-*O*-METHYLTRANSFERASE

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Key Word Index—*Chrysosplenium americanum*; Saxifragaceae, flavonol *O*-methyltransferase; heterologous expression; purification; antibody production; properties.

Abstract—A flavonol *O*-methyltransferase cDNA clone (p*F3′OMT*) from *Chrysosplenium americanum* was expressed in *Escherichia coli* Top 10 and the recombinant protein was purified to near homogeneity by affinity chromatography on chelation resin and gel filtration on Superose 12 columns. The purified protein was enzymatically active as a 42 kDa monomer and exhibited strict specificity for position 3′ of 3,7,4′-trimethylquercetin. It did not accept the mono- or dimethyl analogs, the parent aglycone quercetin or the phenylpropanoids, caffeic and 5-hydroxyferulic acids as substrates; thus indicating its involvement in the later steps of polymethylated flavonol synthesis in this plant. The K_m values of the enzyme for 3,7,4′-trimethylquercetin as substrate and *S*-adenosyl-L-methionine as co-substrate were 7.2 and 20 μM, respectively. The enzyme activity was strongly inhibited by both Ni^{2+} and ρ-chloromercuribenzoate and was restored by the addition of EDTA or β-mercaptoethanol, respectively. Antibodies raised against the F3′OMT recombinant protein recognized a protein band migrating at the expected molecular mass of the enzyme on SDS-polyacrylamide gels of protein extracts prepared from various sources. This implies a high degree of structural similarity among these enzymes that is also corroborated by their hydropathy profiles.

INTRODUCTION

Flavonoid compounds are natural constituents with an ubiquitous distribution in the plant kingdom. Apart from their frequently implied functions as UV protectants and insect-attracting flower pigments, flavonoid compounds have been suggested to play other roles, such as the regulation of auxin transport [1], signalling molecules in plant–symbiont interactions [2], determinants of pollen tube growth and pollen functionality [3] and as precursors of phytoalexins following microbial attack [4, 5]. Enzymatic methylation of flavonoids involves the transfer of the methyl group of *S*-adenosyl-L-methionine (AdoMet) to the hydroxyl group of a flavonoid acceptor, with the formation of its methyl ether derivative and *S*-adenosyl-*L*-homocysteine as products. *O*-Methylation of flavonoid compounds not only reduces the chemical reactivity of their phenolic hydroxyl groups but also modifies their solubility and, hence, increases their antimicrobial activity [6]. In addition, *O*-methylation of flavonoids, such as quercetin, is believed to reduce their mutagenicity and, thus, prevents tumor formation in mammalian tissues [7].

Chrysosplenium americanum, a saxifragaceous semi-aquatic weed, accumulates a variety of tetra- to penta-*O*-methylated flavonol glucosides [8]. Their synthesis has been shown to be catalyzed by a family of five, substrate-specific and position-oriented *O*-methyltransferases (OMTs; E.C. 2.1.1.6x) and two distinct *O*-glucosyltransferases (E.C. 2.4.1.x) [9]. The localization of these metabolites within the apoplastic cellular compartment of *Chrysosplenium* leaves [10, 11] suggested their role as protective compounds against invasion by microorganisms [12].

In a previous paper [13] we reported the isolation of a cDNA clone (p*FOMT*3′) encoding an enzyme which catalyzed the 3′/5′-*O*-methylation of partially methylated flavonols in *C. americanum* and characterized it at the molecular level. This paper describes the purification to near homogeneity and some phy-

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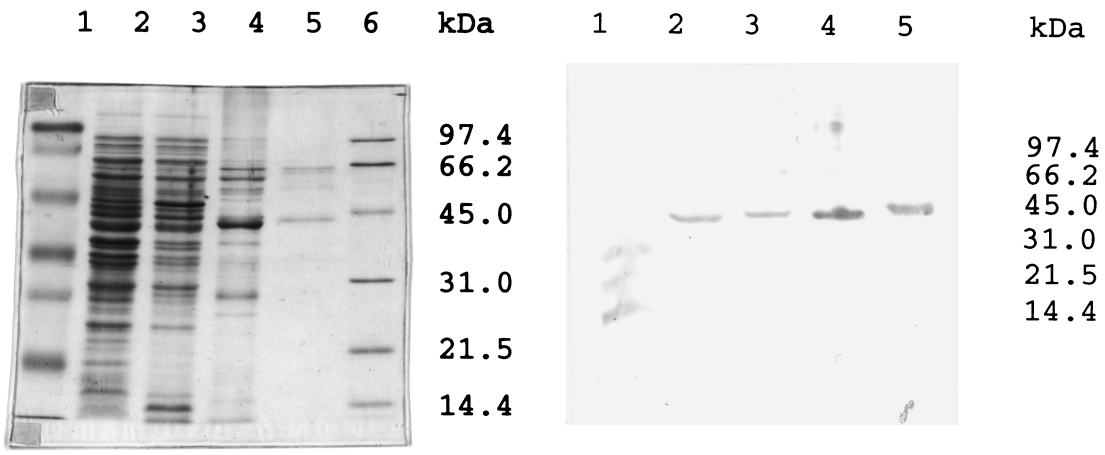

Fig. 1. (A) SDS-PAGE of the F3′OMT recombinant protein fractions from various purification steps after Coomassie blue staining: lane 1, prestained protein standards; lane 2, non-induced bacterial lysate (10 μg); lane 3, IPTG-induced bacterial lysate (10 μg); lane 4, Ni–NTA fraction (5 μg); lane 5, Superose-12 (0.5 μg); lane 6, Coomassie-stained protein M_r markers (kDa): lysozyme (14.4), trypsin inhibitor (21.5), carbonic anhydrase (31.0), ovalbumin (45.0), bovine serum albumin (66.2) and phophorylase B (97.4). (B) Western blot analysis after SDS-PAGE and electroblotting onto nitrocellulose membrane. The immune reaction was revealed with anti-F3′OMT antiserum as described in Section 3. Lanes 1–5, as described in (A).

sicochemical and immunochemical characteristics of the recombinant protein expressed in *Escherichia coli*. A polyclonal antibody to the purified protein was produced and used to study the distribution of the enzyme within the plant, as well as to investigate its cross reactivity with OMTs from other sources. The results obtained suggest a high degree of structural similarity among the enzyme proteins as corroborated by their hydropathy profiles.

RESULTS AND DISCUSSION

Expression and purification of F3′OMT

Expression of the recombinant form of F3′OMT was carried out using the pTRC-His vector which gave rise to a fusion product that possessed a six-histidine tag, as part of a short leader sequence of the N-terminus of the protein [14]. A 2 h-induction with isopropyl β-thiogalactopyranoside (IPTG) of the pTRC-His-*F3′OMT* in *E. coli* Top 10 resulted in a significant expression of an approximately 42 kDa protein, as can be seen on Coomassie-stained SDS-polyacrylamide gels [Fig. 1(A), lanes 3 and 4]. The molecular mass of this protein is consistent with addition of the 3 kDa His-Tag leader sequence [14] fused to the 37.6 kDa F3′OMT [13]. The calculated M_r of the active fraction of the fusion protein, as determined by FPLC gel filtration on a calibrated Superose-12 column, was estimated to be 42 ± 0.5 kDa (Fig. 2), which suggests that the enzyme protein is composed of a single polypeptide chain. This finding contradicts the previous suggestion [13] that the native form of F3′OMT may exist as a 76 kDa dimer, which was formerly estimated by gel filtration of the bacterial lysate in the absence of β-mercapto-ethanol. The latter conditions may have favoured copurification of the bacterial proteins through the formation of disulfide bonds with the protein of interest during cell lysis [15]. Furthermore, the M_r of the recombinant protein (*ca.* 38 kDa) is 19 kDa lower than that previously reported for the intact *C. americanum* shoot OMTs, which was determined by gel filtration on Sepharose S-200 column [16]. It is possible that the F3′OMT gene product is produced in *E. coli* in an immature form, that would normally be processed post-translationally in the intact tissue. A similar situation has been reported for a methyltransferase of the benzylisoquinoline alkaloid pathway, scoulerine OMT [17]. Nevertheless, the M_r of the F3′OMT fusion protein is quite similar to those reported for the alfalfa chalcone 2′-OMT (43 kDa) [18] and isoflavone 7-OMT (41 kDa) [19] recombinant proteins. In contrast, the native molecular mass of the recombinant aspen, lignin-specific caffeic acid OMT [20] and that of *Clarkia breweri* (iso)eugenol OMT [21] have been reported to be 80 and 88 kDa dimers, as determined by native gradient-gel electrophoresis and gel filtration chromatography, respectively.

The use of pTRC-His vector made it possible to purify the fusion protein to near homogeneity by affinity chromatography on a Ni–NTA chelation resin, followed by gel filtration on Superose 12 [Fig. 1(A), lane 4]. Both purification steps resulted in a 148-fold enrichment of the enzyme specific activity, with a 38.5% yield (Table 1). The significant loss of enzyme activity after affinity chromatography can be attributed to the inhibiting effect of Ni^{2+} (Table 3) which may leach from the column and remains in contact with the enzyme protein. However, the addition of both 10 mM EDTA and 14 mM β-mercaptoethanol to the protein extract in the steps following affinity chromatography prevented further loss of enzyme activity.

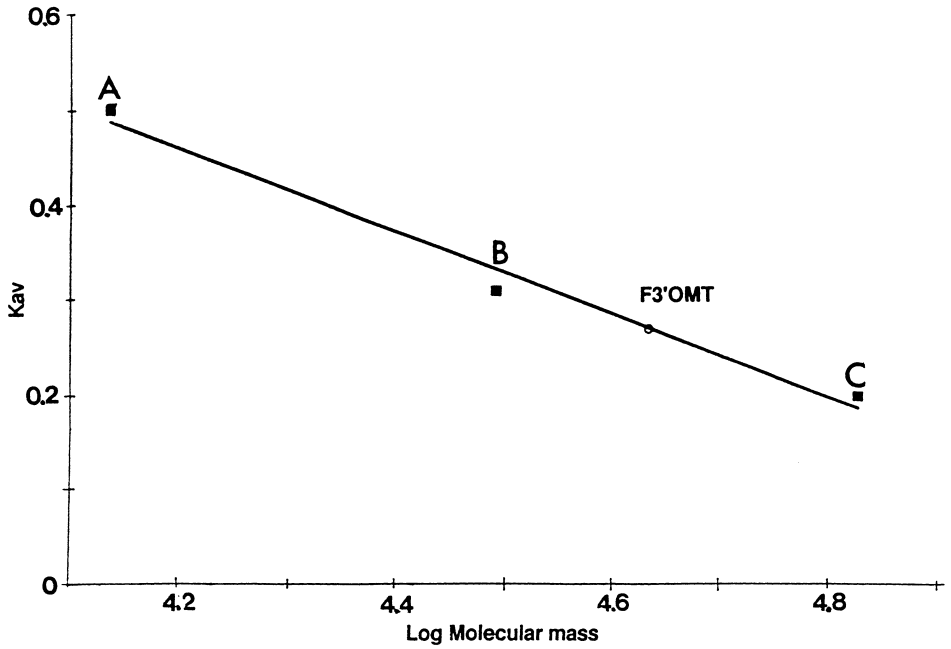

Fig. 2. M_r estimation of the native recombinant F3′OMT by chromatography on Superose-12 column calibrated with (kDa): (A) ribonuclease (13.7); (B) carbonic anhydrase (31.0) and (C) bovine serum albumin (67.0).

Table 1. Purification of the recombinant F3′OMT protein*

Fraction	Protein (mg)	Specific activity (nkat/mg)	Total activity (nkat)	Yield (%)	Purification (-fold)	Recovery (%)
Crude extract	15.4	0.45	6.93	100	–	100
Ni–NTA column	0.35	29.56	10.35	149	65.7	2.30
Superose 12	0.04	66.75	2.67	38.5	148.3	0.26

*The purification steps were carried out on the recombinant protein obtained from a 500-ml IPTG-induced bacterial culture. Enzyme assays were conducted as described in Section 3.

Specificity of the anti-F3′OMT antibody

The specificity of the antisera raised against the Superose 12-purified protein (Table 1) was verified by ELISA and further confirmed by Western blot analysis of the SDS-electrophoresed proteins. Immunodetection of the blotted protein fractions, eluted from the various purification steps [Fig. 1(B), lanes 2–5], revealed that the anti-F3′OMT antiserum (1:1000 dilution) reacted with a protein band migrating at the position of the purified enzyme [Fig. 1(B), lanes 2–5]. No protein bands could be detected when the blots were incubated with the preimmune serum (result not shown). However, it is interesting to note that the non-induced bacterial cells exhibit F3′OMT activity which amounts to about 50% of that of the IPTG-induced cells (Table 2), which seems to be a characteristic feature of the *E. coli* Top 10 cells grown in LB medium. This result is further corroborated by the immunoblot shown in Fig. 1(B), lane 2.

The anti-F3′OMT antibody was used for the immunodetection of other purified recombinant OMTs, as well as the OMTs of the vegetative parts of *C. americanum* and *Serratula tinctoria*. The latter species is known to accumulate 3-*O*-methylquercetin [22] and we have verified the existence of a quercetin 3-OMT activity in its leaves (unpublished data). The anti-F3′OMT antibody recognizes protein bands corresponding to the expected M_r of the partially purified *Serratula* leaf [Fig. 3(A), lanes 1 and 2], *Chrysosplenium* leaf [Fig. 3(A), lane 3] and root [Fig. 3(A), lane 4] OMTs, as well as those of two purified recombinant proteins, OMT1 and OMT2 (GenBank Accession No. U16793) obtained from *C. americanum* [Fig. 3(B), lanes 2 and 3]. The two latter OMTs, whose amino acid sequence differs in only 3 amino acid residues and share *ca.* 85% amino acid similarity with the F3′OMT, catalyze the 3′-*O*-methylation of flavonoids as well as the 3/5-*O*-methylation of caffeic and ferulic acids, respectively, albeit to a different extent [23]. These results suggest epitope similarities among the OMTs from different sources, which is not unexpected

Table 2. Recombinant F3′OMT activity and protein induction of non-induced and IPTG-induced cells*

Source of extract	Specific activity (nkat/mg)		Protein induction by IPTG (% of non-induced)
	−IPTG	+IPTG	
pTRC-His	0	0	35
pTRC-His/F3′OMT	0.12	0.27	65

*The recombinant protein was expressed in *E. coli* Top 10 cells. IPTG induction (1 mM) was carried out for 2 h as described in Section 3.

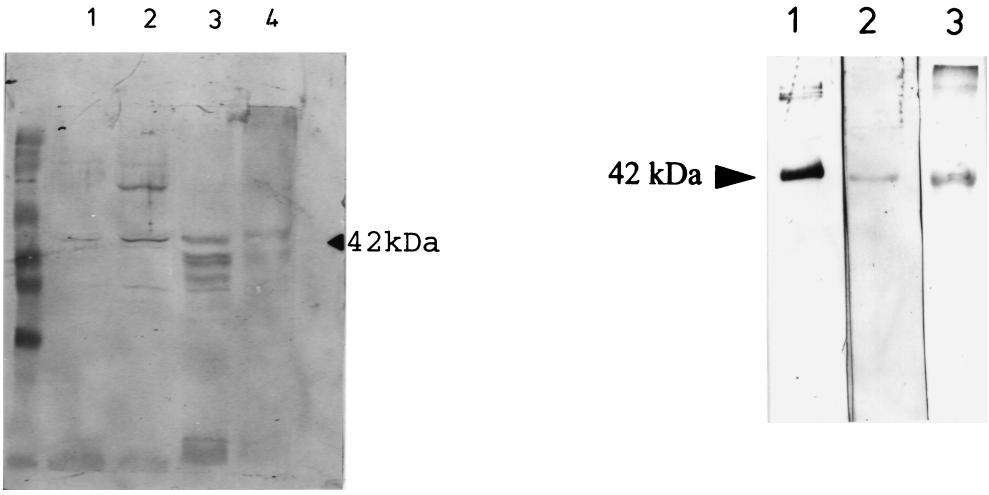

Fig. 3. (A) Immunoblot detection of OMTs in soluble protein extracts (20 μg each): Lanes 1 and 2: two cultivars of *Serratula tinctoria* leaves; lanes 3 and 4: *Chrysosplenium americaum* leaves roots, respectively. Pre-stained protein markers are shown on the left. (B) As in (A), but using the recombinant protein extracts (0.3 μg each) shown in lane 1, F3′OMT; lane 2, OMT1 and lane 3, OMT2. The immunoreactive bands (indicated by an arrow heads) migrate at the expected M_r of 42 kDa.

in view of their similar catalytic functions and molecular mass. However, it is interesting to note that the anti-F3′OMT antibody, which was raised against the non-denatured protein, did not inhibit the recombinant F3′OMT activity to any significant extent when it was added at varying concentrations between 100 μg and 3 mg protein to assay mixtures containing 1–200 μg of the purified enzyme protein (data not shown). This may be due to the fact that the epitopes characteristic of the catalytic sites are not available to the antibody, thus preventing immunoprecipitation of the enzyme protein during antigen–antibody incubation [24].

Physicochemical properties of recombinant F3′OMT

Substrate specificity. The purified recombinant F3′OMT exhibits specificity for 3,7,4′-trimethylquercetin as substrate for further methylation at the 3′-position and gave rise to the expected tetramethyl derivative in the presence of [^{14}C]AdoMet [Fig. 4(A) and (B)]. The enzyme protein accepted neither of the low methylated analogs (3-methyl- or 3,7-dimethylquercetin), the parent aglycone quercetin nor the phenylpropanoids caffeic- or 5-hydroxyferulic acids as substrates. This indicates the strict substrate- and position specificities of this OMT and its involvement in the later steps of polymethylated flavonol synthesis in *C. americanum* [9].

Other properties. The pH optimum of the methylation reaction was 7.8 in 50 mM phosphate buffer. The enzyme reaction had no absolute requirement for divalent cations, but was strongly inhibited in the presence of 1 mM $NiCl_2$ or $NiSO_4$. This inhibition was fully restored by the addition of 1 mM EDTA (Table 3). While the thiol reagent β-mercaptoethanol had no significant effect on OMT activity, ρ-chloromercuribenzoate strongly inhibits it. The fact that such inhibition was restored by the addition of 14 mM β-mercaptoethanol (Table 3) indicates the requirement for SH groups for catalytic activity. These results are similar to those reported for the recombinant, lignin-specific aspen OMT [20]. In fact, the latter

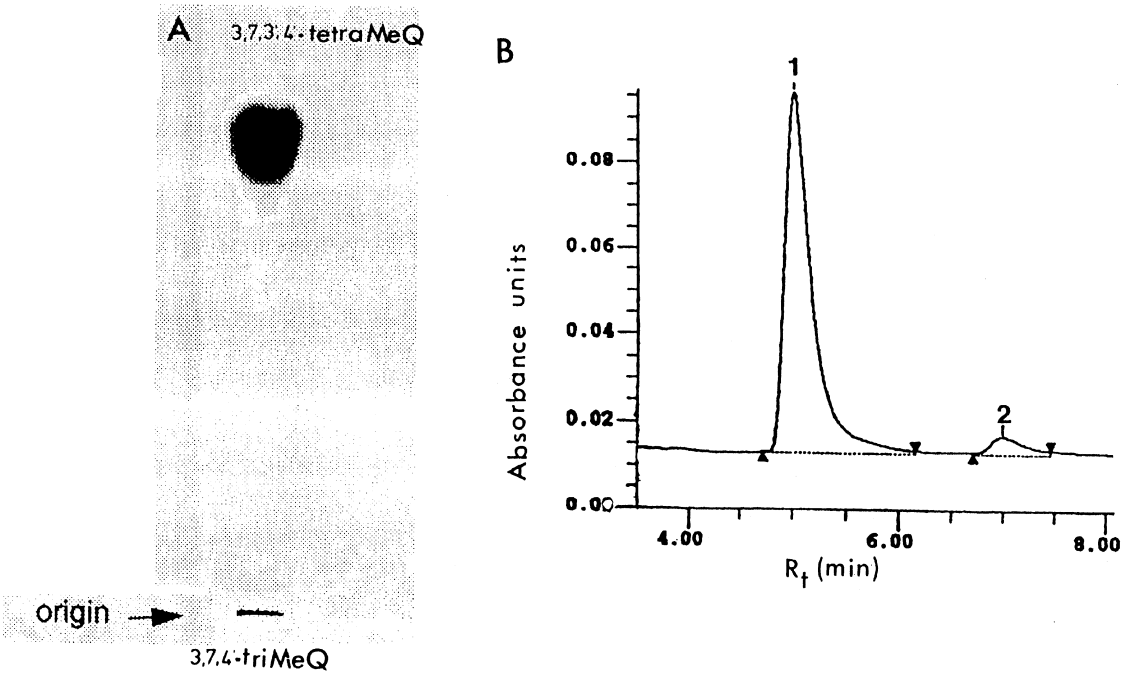

Fig. 4. (A) Computer enhanced image of the TLC separation of the ^{14}C-labelled reaction products obtained with the recombinant F3′OMT assayed with 3,7,4′-trimethylquercetin as substrate, in presence of AdoMet. The radioactive spot comigrates with the 3′-methyl derivative as a reference compound. (B) HPLC elution profile of 3,7,4′-trimethylquercetin as the substrate (peak 1)) and its radioactive 3′-methyl derivative as the F3′OMT reaction product (peak 2), coeluting with an authentic sample of 3,7,3′,4′-tetramethylquercetin from an RP C_{18} semi-prep silica column, using 85% MeOH, 14% H_2O, 1% HOAc as solvent in isocratic mode.

Table 3. Effects of Ni^{2+} and ρ-CMB on F3′OMT activity*

Addition	Concentration (mM)	Relative activity† (% of control)
None (control)	–	100
$NiCl_2/NiSO_4$	1.0	30
Ni^{2+} + EDTA	1.0	100
p-CMB‡	0.1	100
p-CMB	1.0	13
1 mM pCMB + 14 mM β-mercaptoethanol	14	100

*Enzyme assays were conducted as described in Section 3.
†Enzyme activity of the control amounted to 120 pkat/mg protein.
‡Assayed in the absence of β-mercaptoethanol.

authors have recently demonstrated, by site directed mutagenesis, that thiols were not required for the catalytic mechanism of this class of OMTs and that the enzyme sensitivity to inhibition may be due to the reaction of cysteine thiol(s) near the sterically hindered surface of the active site [20]. The apparent K_m value of the enzyme for 3,7,4′-trimethylquercetin, at a saturating concentration of AdoMet, was estimated to be 7.2 μM and that for AdoMet at a saturating concentration of the flavonoid substrate was 20 μM which are consistent with those previously reported for the partially purified enzyme from the intact tissue [16].

Hydropathy profiles. The structural similarity of the recombinant F3′OMT, OMT1 and OMT2 is underlined by their remarkably similar hydropathy profiles (Fig. 5), including the AdoMet binding region [25], in spite of the fact that these three proteins exhibit distinct substrate specificities. This, together with the fact that the anti-F3′OMT antibody cross reacts with other OMTs, suggests that differences in the substrate specificity of these three distinct OMTs are the result

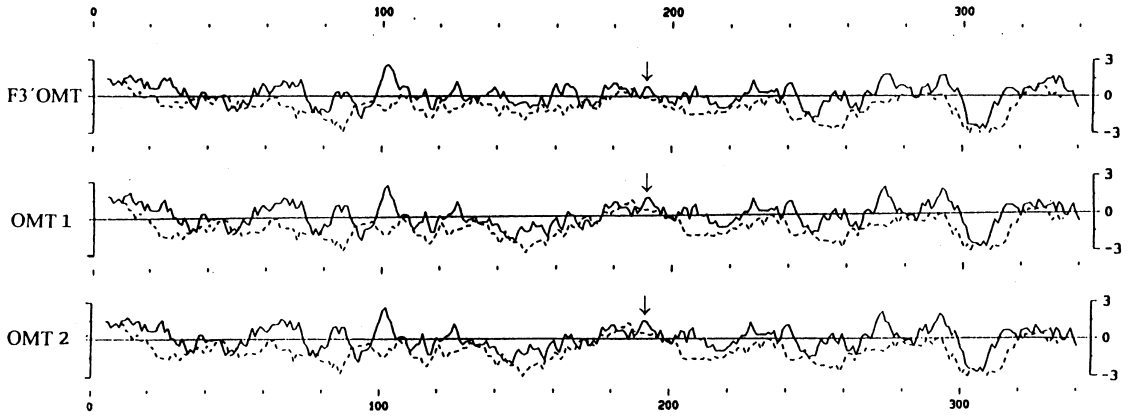

Fig. 5. Hydropathy profiles generated for F3′OMT, OMT1 and OMT2 amino acid sequences according to [29]. Positive and negative values indicate hydrophobic (———) and hydrophilic (· · ·) regions of the proteins, respectively. The location of the putative AdoMet binding site is indicated with an arrow on each protein.

of relatively minor amino acid substitutions, rather than alteration to major protein domains.

EXPERIMENTAL

Plant material

Chrysosplenium americanum Schwein ex Hooker (Saxifragaceae), a semi-aquatic weed, was collected from St. Anicet (Quebec, Canada). It was maintained in the greenhouse under conditions simulating its natural habitat, with respect to light intensity (*ca.* $250\,E \cdot m^{-2} \cdot s^{-1}$), temperature (15–17°C) and humidity (constant running water).

Expression of the recombinant protein

Luria-Bertani (LB) medium containing $100\,\mu g \cdot ml^{-1}$ ampicillin was inoculated with *E. coli* Top 10 carrying p*FOMT*3′ after cloning as was previously described [13]. The bacterial cells were grown to a density of $A = 0.6$–0.7 and were induced by the addition of IPTG at a final concentration of 1 mM and then harvested 2 h later. The cells were collected by centrifugation at 8,000*g* for 10 min and the pellets were suspended in buffer A (50 mM sodium phosphate, pH 7.8, 300 mM NaCl) containing $1\,mg \cdot ml^{-1}$ lysozyme and allowed to stand on ice for 30 min. The mixture was centrifuged at 10,000*g* for 20 min and the clear supernatant was collected for further purification.

Purification of the fusion protein

The fusion protein was first purified by affinity chromatography using Ni–nitrilo triacetic acid resin (Ni–NTA, Qiagen, U.S.A.) in a batch procedure according to manufacturer's specifications. Briefly, the clear lysate was incubated overnight at 4°C with the resin that was previously equilibrated with buffer A and the mixture was loaded onto a 1.6 cm diameter column. After flowthrough, the column was thoroughly washed with buffer A, followed by a second wash with the same buffer containing 25 mM imidazole. The recombinant protein was eluted with 250 mM imidazole in buffer A and concentrated on an Amicon stirred cell (YM 10 membrane), changing to buffer A containing 10 mM EDTA, 10% glycerol and 14 mM β-mercaptoethanol. The concentrated sample was then chromatographed on a Superose 12 column using an FPLC facility (Pharmacia). Protein purification was monitored by enzyme assays and SDS-PAGE.

Protein extraction

Protein was routinely extracted from the plant material at 4°C using 0.1 M sodium phosphate buffer pH 7.8, containing 10% PVPP (w/w), 1 ppm diethylammonium diethyldithiocarbamate (w/v), 5 mM EDTA and 14 mM β-mercaptoethanol. After filtration through miracloth and centrifugation at 10,000*g*, the supernatant was desalted on a PD-10 column (Pharmacia) before being used.

Enzyme assay

The OMT assay was performed as previously described [16] using various flavonoids as substrates and [^{14}C]AdoMet as the cosubstrate. The reaction products were extracted with a mixture of benzene–ethyl acetate (1:1, v/v) and the organic phase was analyzed for radioactivity by liquid scintillation spectrometry. Product identification was carried out by co-chromatography with an authentic sample of 3,7,3′,4′-tetramethylquercetin [16] and by HPLC analysis on an RP C_{18} semi-prep silica column using 85% MeOH, 14% H_2O, 1% HOAc as solvent in isocratic mode.

Protein determination

Protein concentration was determined by the method of Bradford [26], using the Bio-Rad reagent and bovine serum albumin as the standard protein.

Molecular mass determination

The molecular mass of the native recombinant protein was estimated by gel filtration on a calibrated Superose 12 column. The reference proteins used were bovine serum albumin (67.0 kDa), carbonic anhydrase (31.0 kDa) and ribonuclease A (13.7 kDa).

Antibody production

New Zealand rabbits were immunized with 50 μg of the Superose 12-purified protein in complete Freund's adjuvant, followed by two booster injections of 25 μg each, at 2-week intervals, according to an established protocol [27]. The immune reaction was monitored by ELISA and Western blotting before collection of the sera.

SDS-PAGE and Western blot analysis

Denaturing electrophoresis was performed according to a standard procedure [28] using 12 or 15% acrylamide separating gels and 4% acrylamide stacking gels. Electroblotting on nitrocellulose membranes was carried out using a Bio-Rad Transblot Semi-Dry Blotter according to the manufacturer's instructions. After blocking the membranes with 3% non-fat dry milk, the blots were incubated with non-immune or immune serum (1:1000 dilution), followed by the secondary antibody labeled with alkaline phosphatase (1:3000 dilution). The immune reaction was revealed using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates.

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