Purification and Characterization of an *S*-adenosyl-L-methionine:flavonoid 3′-*O*-methyltransferase from Leaves of *Trillium apetalon* Makino

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Z. Naturforsch. **54c,** 501–507 (1999); received February 1/April 22, 1999

Trillium apetalon, *S*-adenosyl-L-methionine:flavonoid 3′-*O*-methyltransferase, Quercetin, Isorhamnetin, Flavonoids

In the leaf extract of *Trillium apetalon* (Liliaceae) distributed in Japan, an enzyme was demonstrated which catalyzes a methyl group transfer from *S*-adenosyl-L-methionine (SAM) to the 3′ position of quercetin and its glycosides. The enzyme (*Trillium* F3′OMT) was purified 433-fold with a yield of 0.2% by $(NH_4)_2SO_4$ precipitation and chromatographies of DEAE-cellulose, SAH-EAH-Sepharose 4B , Sephacryl S-200 and additional chromatofocusing. *Trillium* F3′OMT has a pH optimum of 7.0 and a pI of 5.3. The apparent molecular weight was estimated by Sephacryl S-200 to be about 78 kDa; SDS-PAGE profile showed that the enzyme was a dimer composed of MW 38 kDa 2 subunits. The enzyme activity was stimulated by EDTA and dithiothreitol (DTT), but strongly inhibited by *p*-chloromercuribenzoate (PCMB) and iodoacetate. The activity was moderately inhibited by Mg^{2+} and Zn^{2+}, and strongly inhibited by Co^{2+}, Mn^{2+} and Hg^{2+}. The apparent K_m values for quercetin and SAM were 10 μM and 3.6 μM, respectively. Lower substrate specificity of the glycosides compared with quercetin indicates that methylation precedes glycosylation in flavonoid biosynthesis of *T. apetalon*.

Introduction

We studied the distribution patterns of flavonoid in *Trillium* species in Japan (Yoshitama *et al.* 1992 and 1997), and showed that quercetin and kaempferol arabinosylgalactosides and their acetylated derivatives are widely distributed in their leaves. In addition, the findings demonstrated that isorhamnetin (3′-*O*-methylquercetin) glycosides with the same glycosidic patterns were characteristically found as major constituents with the above quercetin glycosides in leaves of *T. apetalon*. The characteristic distribution of isorhamnetin in the species are of special interest from chemotaxonomical and biosynthetic viewpoints of flavonoids. Now we detected and characterized a flavonoid 3′-*O*-methyltransferase which catalyzes the synthesis of isorhamnetin from quercetin in the leaves of *T. apetalon*. In this paper, we describe for the first time purification and some characteristics of F3′OMT from *Trillium* species, and also discuss the biosynthetic pathway of flavonoids in *Trillium* species.

Material and Methods

Plant materials

Fresh leaves of *T. apetalon* were collected during the flower season (mid-April to early May) in some locations of Niigata prefecture in Japan, frozen by liquid N_2 and stored at −80 °C until use. Fresh leaves of *T. kamtschaticum* and *T. tschonoskii* were collected during the same season at Hokkaido and Kumamoto prefecture in Japan, respectively.

Chemicals

S-adenosyl-L-[methyl-^{14}C] methionine (1.85 GBq/mmol) were purchased from Amersham International (Great Britain); Dowex 1–2X from Muromachi Kagaku Kogyo (Japan); Polyclar AT, *S*-adenosyl-L-homocysteine (SAH) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) from Sigma; Polybuffer-74, Electrophoresis calibration Kit and LMW Gel Filtration Calibration Kit from Pharmacia; ethylendiaminetetraacetic acid (EDTA), dimethylsulfoxide (DMSO) and

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CBB color solution (5×) from Nakalai Tesque (Japan). All flavonoid substrates and reference compounds were from our laboratory collection.

Buffers

The following buffers were used: A, 0.2 M K-phosphate buffer (pH 7.0, containing 5 mM EDTA, 10 mM diethylammonium *N,N*-diethyldithiocarbamate and 0.5 mM dithiothreitol (DTT)); B, 10 mM K-phosphate buffer (pH 7.0, containing 0.5 mM DTT and 10% glycerol); C, 10 mM K-phosphate buffer (pH 7.0); D, 25 mM histidine-HCl buffer (pH 7.4).

Enzyme preparation

All procedures were carried out at below 4 °C. Leaves (200 g) frozen in liquid N_2 were mixed with PolyclarAT (25%, w/w), and homogenized with 1000 ml of buffer A, followed by filtration through nylon mesh. After centrifugation of the filtrate for 15 min at 13,000×*g*, the supernatant was stirred for 20 min with Dowex 1–2X (20%, w/v) which had previously been equilibrated with buffer A, then it was filtered through glass wool. The filtrate was brought to 20% saturation by addition of solid $(NH_4)_2SO_4$, stirred for 60 min and centrifuged for 20 min at 13,000×*g*. The supernatant was brought to 80% saturation by the further addition of solid $(NH_4)_2SO_4$, and centrifuged for 20 min at 13,000×*g*. The precipitate dissolved in a small volume of buffer C was applied to a Sephadex G-25 column (3×15cm) previously equilibrated with buffer C. The eluate was applied to a DEAE-cellulose column (2×35cm) equilibrated with buffer C. After washing the column with buffer C, protein was eluted with a linear gradient of 0–500 mM NaCl. The active fractions were combined, brought to 80% saturation by addition of solid $(NH_4)_2SO_4$, centrifuged for 20 min at 13,000×*g* and passed through a Sephadex G-25 column (3×15cm). The resultant was applied to affinity column chromatography (2×5cm) of SAH-EAH Sepharose 4B prepared by the method of Sharma and Brown (1978). The elution from the affinity column (linear gradient of 0–400 mM NaCl), a followed $(NH_4)_2SO_4$ precipitation and the desalting are the same as the case of DEAE-cellulose column. The active fractions after addition of sucrose (to 1.2%) was applied to a Sephacryl S-200 column (3×90 cm) equilibrated with buffer C, and eluted with buffer C. Fractions with high activity were concentrated by ultrafiltration (Centricell 20, Polysciences). The concentrate was applied to a chromatofocusing column of Mono P HR 5/20 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with buffer D. The protein was eluted by 10% Polybuffer 74-HCl (pH 4.0, Pharmacia) and active fractions were ultrafiltrated by UFP2-TTK-BK (Nihon Millipore Kogyo, Japan).

Enzyme assay

The standard assay mixture consisted of 1.95 nmol of flavonoid substrate (in 10 µl of 50% DMSO), 2.7 nmol *S*-adenosyl-L-[methyl-^{14}C]methionine (250Bq in 10 µl H_2O), 40 µl of enzyme solution and 70 µl buffer B in a total volume of 130 µl. The reaction was started by addition of flavonoid substrate, and the mixture was incubated for 30 min at 30 °C. The reaction was terminated by the addition of 10 µl of 6 N acetic acid. The reaction products were extracted with 400 µl of ethyl acetate and aliquots (200 µl) of the organic phase were transferred to a scintillation vial and radioactivity was measured in a toluene-based scintillation fluid.

To identify the reaction products, the extracts of several assays were pooled, evaporated to dryness, and then chromatographed on TLC plates as described earlier (Ishikura and Yamamoto, 1990), but the solvent system used for flavonols was *t*-butanol–acetic acid–H_2O (3:1:1,v/v).

Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as reference protein.

Polyacrylamide gel electrophoresis

The purity of active fractions from column eluates was monitored by SDS- polyacrylamide gels electrophoresis according to Laemmli (1970). The apparent molecular weight of the denaturated proteins were calculated using standard protein kit (Pharmacia): phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and lactalbumin (14.4 kDa). Electrophoresis was conducted in 12.5% poly-

acrylamide at 40mA/gel, 150V for 45–60 min and the gel was silver-stained.

Molecular weight (MW) determination

Native MW was determined by calculating the elution volume from a calibrated Sephacryl S-200 column using buffer C (pH 7.0) and were confirmed by using a standard calibration kit (Pharmacia): ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa) and bovine serum albumin (67 kDa).

pH optimum and pI

The buffers used to determine the optimum pH for F3′OMT were as follows: 0.01 M sodium acetate buffer (4.0 ~ 5.5), 0.01 M potassium phosphate buffer (5.5 ~ 7.5) and 0.01 M Tris(hydroxymethyl)-aminomethane-HCl buffer (7.5 ~ 10.0). Active fractions from Sephacryl S-200 was used for the determination. pI value was determined by chromatofocusing (Mono-P HR5/20 , Pharmacia).

Kinetics and other enzyme characterization

Kinetic constants were calculated from Lineweaver-Burk plots.

Substrate specificities and the effects of divalent cations and enzymatic reagents were assayed using the active fractions from Sephacryl S-200.

Results

Purification of enzyme F3′OMT

A crude enzyme preparation from the young leaves of *Trillium apetalon* catalyzed the methylation of the hydroxyl group at the 3′ position of quercetin in the presence of methyl-^{14}C SAM as methyl donor. The reaction product of 3′-*O*-methylation was identified as isorhamnetin by TLC and autoradiography .

The results of *Trillium* F3′OMT purification are summarized in Table I. After $(NH_4)_2SO_4$ fractionation and an anion exchange chromatography on DEAE-Cellulose, the pooled F3′OMT active fractions was further purified by affinity chromatography, gel filtration chromatography and chromatofocusing. From each column, a portion of the fractions containing the enzyme activity was pooled and electrophoresed on a SDS-PAGE (Fig. 1). The elution profile on an affinity chromatography (linked SAH as a ligand) showed a broad

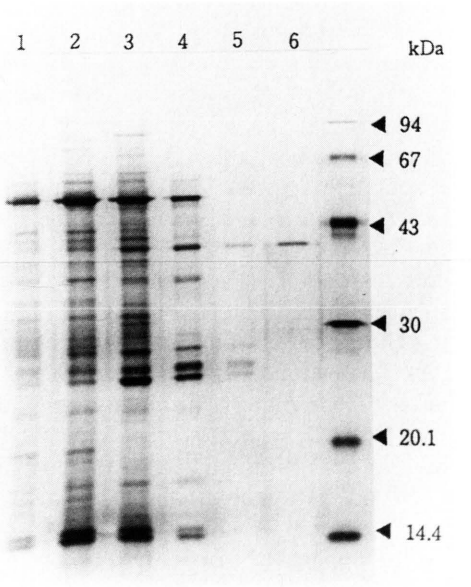

Fig. 1. SDS-Polyacrylamide gel protein patterns of *Trillium O*-methyltransferase during purification. 1, Crude extract; 2, 20 ~ 80% $(NH_4)_2SO_4$ fraction after desalting on Sephadex G-25; 3, DEAE-Cellulose eluate; 4, SAH-EAH Sepharose 4B eluate; 5, Sephacryl S-200 eluate; 6, MonoP HR 5/20 eluate. MW markers are shown by the right lane. Gel was stained with silver nitrate.

Table I. Purification of F3′OMT from leaves of *T. apetalon*.

Purification step	Total protein [mg]	Total activity [pkat]	Specific activity [pkat × mg protein^{-1}]	Purification [-fold]	Recovery [%]
Crude extract	10921.6	109.3	0.01	1.0	100
$(NH_4)_2SO_4$ (20 ~ 80%)	1502.1	67.6	0.045	4.5	61.9
DEAE-Cellulose	74.7	64.9	0.87	87.0	59.4
SAH-EAH Sepharose	12.1	23.9	1.98	198.0	21.9
Sephacryl S-200	0.8	2.6	3.25	325.0	2.4
Mono P	0.06	0.26	4.33	433.0	0.2

peak with a shoulder; however, SDS-PAGE profile of the active fraction exhibited substantial decrease of protein bands compared with the profile on an anion-exchange chromatography (lane 4 in Fig. 1). Chromatofocusing after gel filtration yielded F3′OMT activity as a single peak fraction coincident with the main peak of protein; SDS-PAGE of the ultrafiltrated active fraction showed a major protein band whose apparent MW value was approximately 38 kDa (lane 6 in Fig. 1).

Substrate specificity, kinetic data and molecular mass

In various flavonoids tested for their ability to accept the methyl residue of SAM, quercetin and dihydroquercetin were good substrates, but substrate specificities of quercetin glycosides contained in *Trillium* leaves were lower compared with their aglycone (Table II).

The apparent K_m values were determined in the standard incubation mixture according to Lineweaver-Burk plots (Fig. 2). The values of *Trillium* F3′OMT for quercetin and SAM were 10 μM and 3.6 μM, respectively, with quercetin as methyl acceptor. The apparent molecular mass of native F3′OMT was estimated to be about 78 kDa by a Sephacryl S-200 column chromatography, and 38 kDa by SDS-PAGE (Fig. 1). The typical pH profile showing an optimum pH of 7.0 was exhibited by the enzyme. The peak of F3′OMT activity appeared at pH 5.3 in chromatofocusing column showed the apparent pI of F3′OMT.

Table II. Substrate specificities of F3′OMT[1] from leaves of *T. apetalon*.

Substrate[2]	Relative activity [%]
Quercetin (Q)	100
Dihydroquercetin	97
Q3-galactoside	23
Q3-arabinosylgalactoside	14
Q3-acetylarabinosylgalactoside	35

[1] The enzyme preparation from Sephacryl S-200 was used. The activity of 100%-control was 2.5pkat × mg protein^{-1} using quercetin as substrate.

[2] Each flavonoid (1.95 nmol) was contained as substrate in the reaction mixture. Enzyme assay condition was the same as the procedure described in Material and Methods.

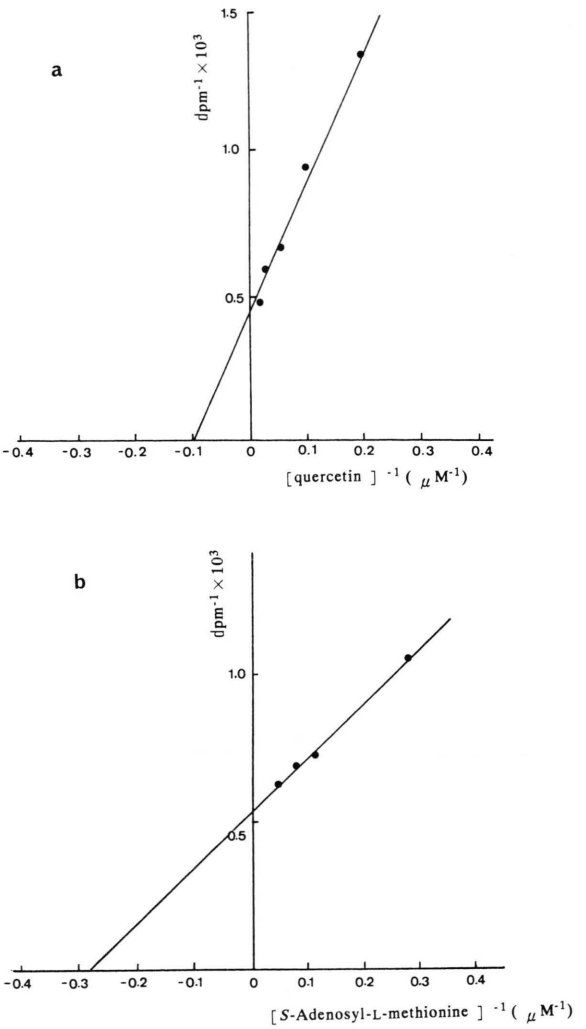

Fig. 2. a and b. Lineweaver-Burk plots of the reciprocal of initial reaction velocity of isorhamnetin formation versus reciprocal of different concentration of quercetin (5 ~ 50 μM) (2a) and *S*-adenosyl-L-(methyl-^{14}C)-methionine (5.5 ~ 20.5 μM) (2b). The enzyme preparation from chromatofocusing (Mono P column) was used. Enzyme assay condition was the same as the procedure described in Material and Methods.

Effect of inorganic ions, EDTA and SH reagents

Addition of heavy metal ions Mn^{2+}, Co^{2+} and Hg^{2+} at 1 mM strongly inhibited the activity and EDTA at 1 mM concentration had only a slight stimulating effect on F3′OMT activity (Table III). F3′OMT activity was strongly inhibited by SH-reagents such as 1 mM *N*-ethylmaleimide (NEM) and 1 mM *p*-chloromercuribenzoate (PCMB), and

Table III. Effect of divalent cations and SH-group inhibitors on *O*-methyltransferase activity[1]

Reagent and concentration	Relative activity
None[2]	100
$MgCl_2$ (1 mM)	66
(5 mM)	70
$ZnCl_2$ (1 mM)	72
(5 mM)	34
$MnCl_2$ (1 mM)	23
(5 mM)	10
$CoCl_2$ (1 mM)	6
(5 mM)	5
$HgCl_2$ (1 mM)	4
(5 mM)	4
EDTA (1 mM)	115
PCMB (1 mM)	7
PCMB + DTT (0.5 mM)	54
IAA (1 mM)	21
NEM (1 mM)	8
NEM + DTT (0.5 mM)	68

[1] The enzyme preparation from Sephacryl S-200 was used. The activity of 100%-control was 3pkat × mg protein^{-1} using quercetin as substrate. Enzyme assay condition was the same as the procedure described in Material and Methods.
[2] 10 µl H_2O was added.
PCMB: *p*-chloromercuribenzoate; DTT: dithiothreitol; IAA: iodoacetamide; NEM: *N*-ethylmaleimide; EDTA: ethylenediaminetetraacetate.

simultaneous addition of 0.5 mM dithiothreitol (DTT) resulted in more than 50% recovery from inhibition in the presence of these inhibitors (Table III).

Discussion

Various flavonoid *O*-methyltransferases (FOMTs), which transfer a methyl residue of *S*-adenosylmethionine to the hydroxyl(s) of flavonoids, have been described (Ebel *et al.*, 1972; Poulton *et al.*, 1976; Tsang and Ibrahim, 1979; Khouri and Ibrahim, 1987; Ishikura *et al.*, 1992). Among these F3′OMT which catalyzes the methylation of the 3′-hydroxyl group of the flavonoid B-ring was partially purified from a cell suspension culture of parsley (Ebel *et al.*, 1972) *Lotus corniculatus* (Jay *et al.*, 1983) and *Zea mays* (Larson, 1989; Tobias and Larson, 1991). In addition, a cDNA clone encoding the gene for 3′/5′-*O*-methylation of partially methylated flavonol was recently isolated from *Chrysosplenium americanum* by Gauthier *et al.* (1996). During the purification step of *Trillium* F3′OMT, the combination of affinity chromatography (SAH-EAH Sepharose 4B) and chromatofocusing (Mono P HR 5/20) was effective although the decrease of total activity after chromatofocusing was caused by the instability of the enzyme in acidic elution buffer (Polybuffer, pH 4.0), and on SDS-PAGE the final preparation showed homogeneous band of 38 kDa (Fig 1). Since no other methylated compounds except isorhamnetin were identified as the reaction products when quercetin was used as the substrate, the enzyme from *Trillium* appears to be specific for the 3′-position. This can be explained by the finding that isorhamnetin glycosides were detected in *Trillium apetalon* as methylated flavonoids only.

With quercetin as the substrate, the pH optimum (7.0) of *Trillium* F3′OMT was found lower than for F3′OMTs from *Lotus corniculatus* (pH 7.7; Jay *et al.* 1983) , soybean cell suspension cultures (pH 8.6 ~ 8.9; Poulton *et al.*, 1976), *Zea mays* (pH 8.0 ~ 8.5; Larson, 1989) and pollens of *Zea mays* (pH. 8.5; Tobias and Larson, 1991). The pI value (5.5) was similar to that (5.1) of *Lotus corniculatus* (Jay *et al.*, 1983) .

SH reagents such as NEM and PCMB, strongly inhibited the F3′OMT activity. Simultaneous addition of 0.5 mM DTT prevented the inhibitory effects of SH reagents, indicating the presence of SH groups at the active site of the enzyme. These enzymatic properties were different from those of methyltransferase from parsley cell cultures, whose enzyme was not effected by the addition of thiol group inhibitors (Ebel *et al.*, 1972).

Concerning some divalent cations on enzyme activity, it is of interest that *Trillium* F3′OMT was inhibited by the addition of Mg^{2+} (1 mM) unlike F3′OMTs from tulip anthers (Sütfeld and Wiermann, 1978), *Lotus corniculatus* (Jay *et al.*, 1983) and pollen of *Zea mays* (Tobias and Larson,1991) which require Mg^{2+} for full activity. The inhibition of Mn^{2+} was similar to the result reported in tobacoo cell cultures (Tsang and Ibrahim, 1979). Interestingly, *Trillium* F3′OMT was strongly inhibited by Co^{2+} and Mn^{2+} contrary to the activation in F3′OMT from *Silene* leaves (Brederode *et al.*, 1981 and 1982).

A lower rate of substrate specificity to quercetin 3-*O*-galactoside (hyperin), quercetin 3-*O*-arabinosylgalactoside and quercetin 3-*O*-acetylarabinosylgalactoside (Table II), which are intermediates and

main flavonoids in the leaves of *T. apetalon*, indicates that methylation precedes glycosylation in the biosynthesis of *Trillium* flavonoids like the results described in soybean cell cultures (Poulton *et al.*, 1976) and in the leaves of *Nicotiana tabacum* (Legrand *et al.*, 1978). No product inhibition was observed in *Trillium* F3′OMT. A difference between the 3′-*O*-methylation step in flavonol- and anthocyanin-biosyntheses is of interest, since aglycones are commonly the best substrates for methyltransferase in flavonol biosynthesis but their glycosides are optimal for anthocyanins (Jonsson *et al.*, 1982 and 1984).

The significant inhibition of kaempferol (data not shown) is of interest because isorhamnetin glycoside was hardly detected in the leaves of *T. tschonoskii* which contained kaempferol glycosides as main constituents. The apparent molecular weight (78 kDa) of native *Trillium* F3′OMT was larger than that from maize pollen (47 kDa; Tobias and Larson, 1991), although the value estimated by SDS-PAGE was 38 kDa (Fig. 1). A discrepancy between MWs estimated by nondenaturing gel filtration *versus* SDS-PAGE suggested that the native enzyme is a dimer. Excluding *Trillium* F3′OMT, a study reported that FOMT from *Pisum sativum* is composed of subunits (Preisig *et al.*, 1989). Recently, Gauthier *et al.* calculated molecular mass 37.6 kDa for the F3′OMT produced by a cDNA clone from *Chrysosplenium americanum*. However that of the active fraction determined by FPLC gel filtration was 78.3 kDa, indicating a tendency to dimerize *in vitro* (Gauthier *et al.*,1996). MWs of native F3′OMT and its subunits from *T. apetalon* are very similar to those of *C. americanum*.

All *Trillium* species distributed in Japan are derived their origins from the hybrids among the 3 elementary species (*T. apetalon, T.tschonoskii* and *T. kamtschaticum*). F3′OMT activity was not detected in the leaves of *T. tschonoskii*, and isorhamnetin glycosides were not detected in its leaves. However, F3′OMT activity was detected in leaves of *T. kamtschaticum*, but isorhamnetin glycosides were minor components. Moreover, its chromatographically and kinetic properties were the same as F3′OMT from *T. apetalon* (data not shown). Examination of a suppression mechanism of F3′OMT in *T. kamtschaticum* is currently in progress.

Acknowledgement

The authors sincerely thank Mr. N. Kato (Niigata Nishi High School) for providing plant materials.

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