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PURIFICATION AND PROPERTIES OF AN *o*-DIHYDRICPHENOL *meta*-*O*-METHYLTRANSFERASE FROM CELL SUSPENSION CULTURES OF PARSLEY AND ITS RELATION TO FLAVONOID BIOSYNTHESIS

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SUMMARY

1. An *O*-methyltransferase directly related to flavone glycoside biosynthesis has been isolated from cell suspension cultures of parsley. The enzyme has been purified 82-fold by $MnCl_2$ and $(NH_4)_2SO_4$ precipitation and chromatography on DEAE-cellulose, Sephadex G-100 and hydroxylapatite. The enzyme catalyses the transfer of the methyl group of *S*-adenosyl-L-methionine to the *meta* position of *o*-dihydric phenols.

2. Highest enzyme activities were obtained after illumination of the cells for 24 h with high intensities of white light from fluorescent lamps.

3. The enzyme has a pH optimum around pH 9.7. It requires Mg^{2+} and is not inhibited by *p*-chloromercuribenzoate or iodoacetamide. A molecular weight of about 48 000 was estimated from the elution volume after chromatography on a Sephadex G-100 column.

4. Only *o*-dihydric phenols can function as substrates for the enzyme and only the *meta*-hydroxyl group is methylated. Luteolin (5,7,3′,4′-tetrahydroxyflavone) and its 7-*O*-glucoside with apparent K_m values of $4.6 \cdot 10^{-5}$ and $3.1 \cdot 10^{-5}$ M, respectively, are the best substrates tested, whereas eriodictyol (5,7,3′,4′-tetrahydroxyflavanone) ($K_m = 1.2 \cdot 10^{-3}$ M) and caffeic acid ($K_m = 1.6 \cdot 10^{-3}$ M) had much lower affinity for the enzyme. With luteolin as substrate the apparent K_m value for *S*-adenosyl-L-methionine is $1.5 \cdot 10^{-4}$ M.

5. Cell cultures also contain enzymatic activity for the synthesis of *S*-adenosyl-L-methionine from L-methionine and ATP. In contrast to the *O*-methyltransferase the activity of this enzyme is not influenced by illumination of the cultures.

INTRODUCTION

In the course of our work on the regulation of enzyme activities related to the biosynthesis of apiin [7-*O*-(β-D-apiofuranosyl-(1 → 2)-β-D-glucosyl)-5,7,4′-trihydroxyflavone] and graveobiosid B (3′-methoxyapiin) in cell suspension cultures of parsley *(Petroselinum hortense)*, we have discovered an *O*-methyltransferase in these cultures

which catalyses the transfer of the methyl group of *S*-adenosyl-L-methionine to the 3′-hydroxyl group of luteolin (5,7,3′,4′-tetrahydroxyflavone) to form chrysoeriol[1]. According to observed changes of enzyme activity with time after illumination of the cultures we proposed that this methyltransferase is directly involved in the biosynthesis of 3′-methoxyflavonoids in parsley[1].

A number of enzyme preparations from higher plants have been described which catalyse the transfer of the methyl group of *S*-adenosyl-L-methionine to the *para*[2] and/or *meta* position[3–9] of vicinal polyphenolic compounds. Only in one case was the *O*-methylation discussed with regard to the biosynthesis of flavonoids. Cell free extracts from flower petals of *Petunia hybrida* catalyse the methylation of anthocyanins (probably at the 3′-hydroxyl group), but cinnamic acids were about 100 times more effective as substrates[7].

None of the methyltransferases from higher plants have been purified to any extent. K_m and V values with different substrates have not been determined.

In this paper we describe the partial purification of the methyltransferase from parsley cell cultures and report some of its properties. An enzyme catalysing the synthesis of *S*-adenosyl-L-methionine from ATP and L-methionine has also been detected in these cultures.

MATERIALS AND METHODS

Materials

L-[*Me*-^{14}C]Methionine and [*Me*-^{14}C]*S*-adenosyl-L-methionine were obtained from the Radiochemical Centre, Amersham; ampholytes for isoelectric focusing from LKB, Stockholm. Chrysoeriol was isolated from parsley seeds[21]. Diosmetin was a gift from Prof. R. Pohl, Freiburg. Hydroxylapatite was prepared by the method of Tiselius *et al.*[22].

All of the other chemicals were purchased from various companies and purified by paper or thin-layer chromatography when products of reagent grade purity were not available.

Chromatographic methods

Thin-layer chromatography was performed on silica gel G (Merck, Darmstadt) with: (1) toluene–ethyl formate–formic acid (5:4:1, by vol.); (2) benzene–dioxane–acetic acid (90:25:4, by vol.); (3) ethylacetate–methyl ethyl ketone–formic acid–water (5:3:1:1, by vol.), on polyamide (Macherey and Nagel) with: (4) benzene–methanol–methyl ethyl ketone–acetyl acetone (15:10:5:1, by vol.) and on cellulose (Macherey and Nagel) with: (5) *n*-butanol–acetic acid–water (4:1:1, by vol.).

For descending paper chromatography on Whatman 3 MM, pre-washed with 0.01 M EDTA, water and methanol the following solvent systems were used: (6) benzene–acetic acid–water (125:72:3, by vol.); (7) 50% acetic acid; (8) *n*-propanol–ammonia (25%)–water (6:3:1, by vol.); (9) *n*-butanol–ammonia (25%)–ethanol–benzene (5:3:2:1, by vol.); (10) *n*-butanol–ethyleneglycol–water–acetic acid (5:1:5:2, by vol.).

The R_F values of reaction products of the *O*-methyltransferase are recorded in Table I.

TABLE I

R_F VALUES OF REACTION PRODUCTS OF THE O-METHYLTRANSFERASE

Details of the solvent systems can be found under Chromatographic methods. R_D, migration distance relative to diosmetin; R_J, migration distance relative to isoferulic acid.

	Solvent system								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Chrysoeriol	0.55	0.43	0.66	0.48	0.80	0.68	0.50	0.49	0.65 (R_D)
Chrysoeriol-7-glucoside			0.97	0.57	0.44				
Ferulic acid	0.59	0.60				0.84	0.78 (0.85)*	0.53 (0.60)*	0.70 (R_J)

* In Solvent systems 7 and 8 the *cis* and *trans* isomers of ferulic acid were separated. The upper values correspond to the *trans* isomer.

Plant material

Cell suspension cultures of *Petroselinum hortense* were grown, illuminated, and harvested as described previously[1]. In order to obtain quantities sufficient for purification of the methyltransferase, 15 2-l Erlenmeyer flasks containing 400 ml each of the culture medium were inoculated with 10-day-old cultures of 40 ml (approx. 8 g fresh weight of cells)[1]. The cells were harvested after 9 days of growth in the dark and subsequent continuous illumination for 24 h.

Assay for O-methyltransferase

The incubation mixture consisted of 0.1 μmole of luteolin (dissolved in 10 μl of ethyleneglycol monomethyl ether), 0.13 μmole of $MgCl_2$, 0.05 μmole of [*Me*-^{14}C]-*S*-adenosyl-L-methionine ($5 \cdot 10^4$ dpm), enzyme, and 50 μmoles of glycine–NaOH (pH 9.3) in a total volume of 130 μl. The reaction was started by addition of *S*-adenosyl-L-methionine and incubated for 30 min at 30 °C. At the end of this period 0.1 μmole of chrysoeriol (or with substrates other than luteolin the expected product) in 100 μl of ethyleneglycol monomethyl ether were added. The reaction mixture was then applied in an 8-cm-long band to a silica gel thin-layer plate (0.3 mm) and developed with Solvent system 1 or when luteolin-7-glucoside was the substrate with Solvent system 3 (see Table I). Methylated compounds were detected under ultraviolet light or by means of a chromatogram scanner, scraped off and counted in a dioxane cocktail (1 l dioxane, 100 g naphthalene, 5 g PPO) with a Beckman LS 100 liquid scintillation spectrometer (counting efficiency approx. 70%).

Purification of O-methyltransferase

All steps were carried out at 4 °C.

(a) Acetone powder. About 1000 g (fresh wt) of cells were extracted in a Waring blendor with a total of 6 l of acetone (−20 °C). The moist powder was first kept for 1 day in a dessicator connected to a water jet vacuum pump and was then dried *in vacuo* over phosphorous pentoxide. About 25 g of the dry powder were obtained.

(b) Extraction and purification. 25 g of the acetone powder were stirred for 45 min with 1250 ml of 0.2 M Tris–HCl (pH 7.5) containing $6 \cdot 10^{-3}$ M mercaptoethanol. The suspension was centrifuged for 10 min at 23 000 × *g*. The supernatant

(1150 ml) was stirred for 30 min with 30 g of Dowex 1-X2 (Cl^- form equilibrated with 0.2 M Tris–HCl buffer, pH 7.5) and the mixture filtered through glass wool. 56 ml of 1 M $MnCl_2$ (pH 6.9) were added to 1120 ml of this extract over a period of 20 min. After stirring for another 30 min the solution was centrifuged for 10 min at 23 000 × *g*. The supernatant (1170 ml) was adjusted to 40% saturation by the addition of solid $(NH_4)_2SO_4$, stirred for 30 min, and then centrifuged at 23 000 × *g* for 10 min. A second fraction of 40–80% saturation was obtained from the supernatant in the same manner. It was dissolved in 42 ml of 0.2 M Tris–HCl (pH 7.5) and chromatographed with 0.02 M Tris–HCl on a column (2.8 cm × 30 cm) of Sephadex G-25 equilibrated with the same buffer.

The filtrate from the G-25 column (64 ml) was then chromatographed with a linear gradient of Tris–HCl (pH 7.5), prepared by mixing 300 g of 0.02 M Tris–HCl and 300 g of 0.5 M Tris–HCl, on a column of DEAE cellulose (3 cm × 28 cm) equilibrated with 0.02 M of the same buffer. Fractions of 10 ml were collected with a flow rate of 35 ml/h. The protein of Fractions 36–42 (0.16–0.23 M Tris–HCl), which contained most of the enzymatic activity, was precipitated by addition of $(NH_4)_2SO_4$ to 80% saturation, collected by centrifugation for 10 min at 23 000 × *g*, and redissolved in 4 ml of 0.2 M Tris–HCl.

The above solution was chromatographed with 0.02 M Tris–HCl buffer (pH 7.5) on a column (3 cm × 52 cm) of Sephadex G-100 with a flow rate of 14 ml/h. The protein of Fractions 35–39 (6 ml each) was precipitated with $(NH_4)_2SO_4$ (80% saturation), collected by centrifugation at 20 000 × *g*, and redissolved in 1.5 ml 0.2 M potassium phosphate (pH 6.8). This solution was chromatographed on a column of Sephadex G-25 (1 cm × 15 cm) with 0.01 M potassium phosphate (pH 6.8). The resulting protein solution was then passed through a column (2.2 cm × 2.3 cm) of hydroxylapatite equilibrated with 0.01 M potassium phosphate (pH 6.8). The protein was eluted batchwise with 40 ml 0.01 M, 37 ml 0.05 M, 25 ml 0.1 M and 24 ml 0.2 M potassium phosphate (pH 6.8). The fractions of 0.05–0.1 M buffer contained the bulk of the enzymatic activity. Protein of these fractions was precipitated with $(NH_4)_2SO_4$ (80% saturation), collected by centrifugation, and redissolved in 1.5 ml of 0.02 M Tris–HCl (pH 7.5). This solution was chromatographed on a column of Sephadex G-25 with the same buffer.

Analytical disc electrophoresis

Before electrophoresis the protein solution was dialysed for 12 h against 1 l of 0.04 M Tris–glycine (pH 8.9). Disc electrophoresis was carried out in a polyacrylamide gel (gel No. 1 according to Maurer[20]). After electrophoresis for 2.5–4 h (2 mA/tube) the gel was cut into 2-mm thick discs which were each crushed in 150 µl 0.2 M Tris–HCl (pH 7.5) and eluted for 12 h.

Isoelectric focusing

The electrofocusing was carried out on Sephadex G-75 thin-layer plates (20 cm × 20 cm) in the pH ranges of 3–10 and 4–6 (ref. 17). Electrode solutions were 0.2 M H_2SO_4 and 0.4 M ethylenediamine, respectively. Separation of proteins was achieved by electrofocusing in a cooled apparatus for thin-layer electrophoresis (Desaga, Heidelberg) at 4 °C with increasing voltage from 15 V/cm to 25 V/cm

during 6–7 h. pH was measured directly on the plate (electrode type 403-30 from Ingold, Frankfurt). Protein was determined by the print technique[19] with Coomassie blue. For determination of enzymatic activity zones of 0.5 cm each were eluted with 1 ml of 0.2 M Tris–HCl (pH 7.5).

Hydrolysis of chrysoeriol-7-O-glucoside with emulsin

3 mg of emulsin dissolved in 0.4 ml of 0.2 M sodium phosphate–acetic acid buffer (pH 6.2) were added to a solution of 200 μg of chrysoeriol-7-*O*-glucoside (10^5 dpm of the enzymatic product diluted with inactive material) in 50 μl of ethyleneglycol monomethyl ether. After incubations for 15 h at 30 °C 4 ml of ethanol were added, the precipitated protein was collected by centrifugation, and the solvent of the supernatant was removed *in vacuo*. The residue was dissolved in 0.2 ml of a mixture of ethyleneglycol monomethyl ether and water (1:1) and chromatographed on a silica gel thin-layer plate with Solvent system 3.

Indirect assay for S-adenosyl-L-methionine synthetase

The incubation mixture consisted of 0.1 μmole of luteolin, 5 μmoles of $MgCl_2$, 5 μmoles of ATP (aqueous solution adjusted to pH 7.5 with 0.5 M NaOH), 0.005 μmole of L-[*Me*-^{14}C]methionine ($4 \cdot 10^5$ dpm), 100–500 μg of protein and 50 μmoles of Tris–HCl (pH 7.5) in a total volume of 300 μl. The reaction mixture was incubated for 60 min at 30 °C. Identification of the reaction products was carried out as described for the assay for *O*-methyltransferase.

Direct assay for S-adenosyl-L-methionine synthetase

The incubation mixture consisted of 3 μmoles of ATP (see above), 5 μmoles of $MgCl_2$, 0.1 μmole of L-[*Me*-^{14}C]methionine ($1.5 \cdot 10^5$ dpm), protein up to 250 μg and 30 μmoles of Tris–HCl (pH 7.5) in a total volume of 150 μl. The reaction mixture was incubated for 60 min at 30 °C. At the end of this period 10 μg of unlabelled *S*-adenosyl-L-methionine (dissolved in 20 μl water) was added. The reaction mixture was then applied to Whatman 3 MM paper in a 4 cm wide band and the chromatogram developed with solvent system 10 (*S*-adenosyl-L-methionine $R_F = 0.34$; L-methionine $R_F = 0.75$). The zone corresponding to *S*-adenosyl-L-methionine was detected under ultraviolet light (254 nm) or by means of a chromatogram scanner. The zone was cut out and the radioactivity determined by liquid scintillation counting (counting efficiency about 49%). Blank values were obtained in incubations without ATP and corresponded to about 0.1% of the radioactivity recorded in the presence of ATP.

Preparation of S-adenosyl-L-methionine synthetase from cell cultures

All steps were carried out at 4 °C. About 4 g (fresh wt) of cells were suspended in 2 ml of 0.2 M Tris–HCl (pH 7.5) containing $15 \cdot 10^{-3}$ M mercaptoethanol and homogenized for 3 min with 2 g of quartz sand in a precooled mortar. The homogenate was centrifuged for 10 min at 20 000 × *g* (A1). The supernatant (about 4.5 ml) was stirred for 30 min with 0.25 g of Dowex 1-X2 (Cl^- form, equilibrated with the extraction buffer) and the Dowex filtered off. The filtrate was centrifuged for 10 min at 20 000 × *g*. The protein was then precipitated by addition of $(NH_4)_2SO_4$ to 80% saturation. The precipitate was collected by centrifugation for 10 min at 20 000 × *g*

and the protein redissolved in the extraction buffer. Desalting was effected on a Sephadex G-25 column with 0.02 M Tris–HCl buffer (pH 7.5) (A2).

RESULTS

Purification of the methyltransferase

We have shown earlier that the extractable activity of the methyltransferase is drastically increased by illuminating the cells with white light and that maximum activity is reached 24 h after the onset of illumination[1]. Therefore the cell cultures were illuminated for 24 h before extraction of the enzyme.

An acetone powder of the fresh cells proved to be the best source of the enzyme. This powder could be kept at 4 °C without significant loss of enzyme activity. The purification procedure for the methyltransferase is summarized in Table II. In the

TABLE II

PURIFICATION OF THE *O*-METHYLTRANSFERASE FROM CELL CULTURES OF PARSLEY

One unit of enzyme activity is defined as amount of protein which catalyses the formation of 1 μmole of chrysoeriol per min at 30 °C in the standard assay.

Purification step	*Protein (mg)*	*Specific activity (munits/mg protein)*	*Enzyme purification*	*Yield (%)*
Crude extract	1800	—	—	—
Dowex 1-X2	1600	0.33	1	100
$MnCl_2$ and $(NH_4)_2SO_4$* fractionation	720	0.5	1.5	69
DEAE-cellulose column	150	1.5	4.5	42
Sephadex G-100 column	19	5.8	18	21
Hydroxylapatite column	2.6	27	82	14

* After removal of salts with Sephadex G-25.

crude extract the enzyme is partially inhibited. Enzyme activity could also not be determined in the presence of Mn^{2+}. The $(NH_4)_2SO_4$ fractionation was difficult to standardize, especially with larger quantities of protein. A fractionation within narrower limits of $(NH_4)_2SO_4$ concentration than 40–80% saturation was unsuccessful. Chromatography of the enzyme on DEAE-cellulose after $(NH_4)_2SO_4$ precipitation is shown in Fig. 1. Fractions 36–42 were used for gel filtration on Sephadex G-100 (Fig. 2).

As can be seen from Fig. 2a an unsymmetrical peak for enzyme activity was obtained on this column. When Fractions 23–29 and 32–41 were separately rechromatographed on the same column they appeared as almost symmetrical peaks at about the same elution volumes as the original fractions (Figs 2b and 2c).

Fractions 35–39 from chromatography on Sephadex G-100 were pooled and the protein precipitated by $(NH_4)_2SO_4$. After removal of salts with Sephadex G-25 the protein was absorbed on a column of hydroxylapatite and eluted by increasing concentrations of phosphate buffer. The bulk of the enzymatic activity was eluted with 0.1 M buffer (Fig. 3).

An 82-fold purification of the enzyme was obtained by this procedure. The

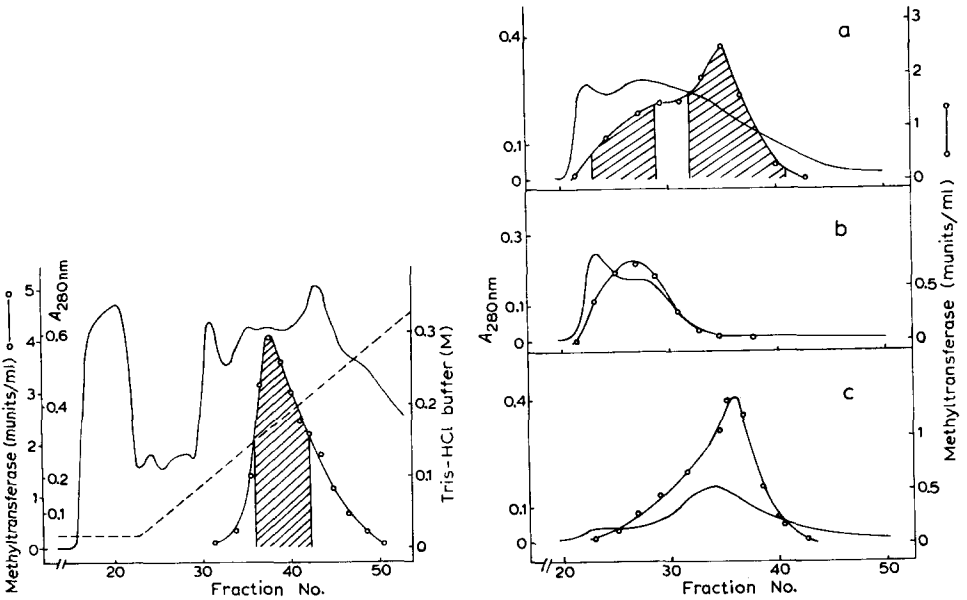


Fig. 1. Chromatography of methyltransferase on DEAE-cellulose. ———, protein; – – – –, buffer gradient.

Fig. 2. (a) Chromatography of Fractions 36–42 from DEAE-cellulose on Sephadex G-100. ———, protein; ○——○, methyltransferase. (b) Rechromatography of Fractions 23–29 from (a). (c) Rechromatography of Fractions 32–41 from (a).

question whether there is only one *O*-methyltransferase present in the partially purified enzyme was further investigated by analytical disc electrophoresis and electrofocusing. When the separately pooled proteins of the two fractions of Sephadex G-100 chromatography were subjected to isoelectric focusing only one symmetrical peak of enzymatic activity at pH 4.8–5.0 was obtained in both cases (Fig. 4). In addition, analytical disc gel electrophoresis with the enzyme fractions after DEAE-cellulose,

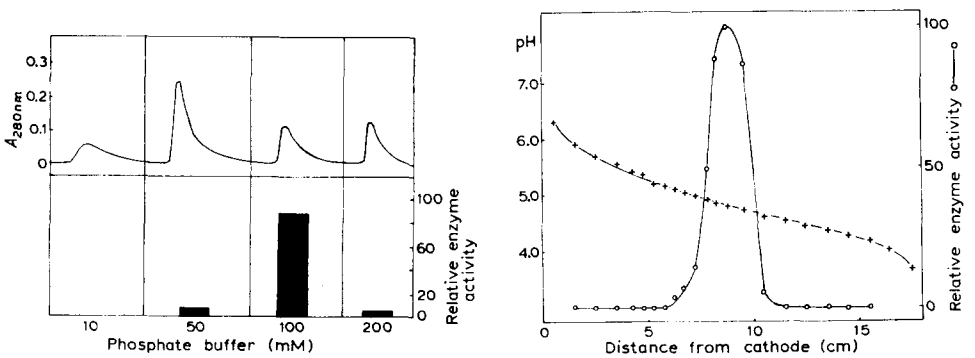
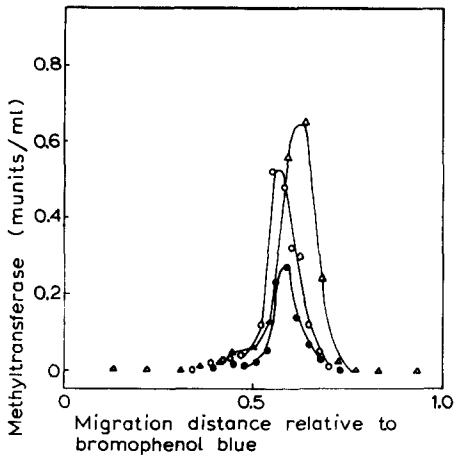


Fig. 3. Chromatography of Fractions 35–39 from Sephadex G-100 (Fig. 2a) on hydroxylapatite. ———, protein.

Fig. 4. Isoelectric focusing of Fractions 32–41 from Sephadex G-100. +——+, pH; ○——○, methyltransferase.



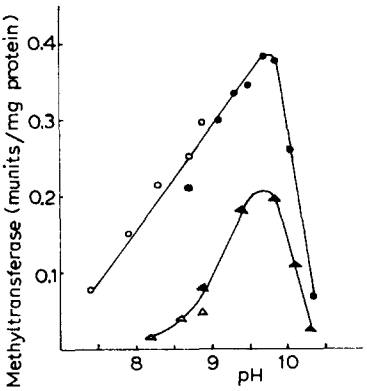


Fig. 5. Separation of enzyme from Fractions 36–42 of DEAE-cellulose (●——●), from Fractions 35–39 of Sephadex G-100 (○——○) and from hydroxylapatite (△——△) by analytical disc electrophoresis.

Fig. 6. Dependence of activity on pH. Substrate luteolin: ○——○, Tris–HCl; ●——●, glycine–NaOH. Substrate caffeic acid: △——△, Tris–HCl; ▲——▲, glycine–NaOH.

Sephadex G-100 (Fractions 35–39), and hydroxylapatite chromatography, respectively, showed only one band of methyltransferase activity (Fig. 5).

Since at best only 20% of the enzymatic activity could be recovered after the electrofocusing this method was not used as a further purification step.

Properties of the O-methyltransferase

If not stated otherwise the enzyme obtained from the hydroxylapatite column (Table II) was used for the experiments.

Stability of the enzyme

At −20 °C and in the presence of 0.02 M Tris–HCl buffer the enzyme (1–3 mg protein/ml) had lost about 30% of its activity after 1 month.

Protein and time linearity

The O-methylation of luteolin was linear with protein concentration up to 1 mg protein/ml and with time up to 60 min.

pH optimum

The pH optimum for O-methylation in glycine–NaOH buffer is 9.6–9.8 with caffeic acid and luteolin as substrates (Fig. 6). At higher pH values enzyme activity rapidly declines.

Influence of inorganic ions and other reagents

The enzyme has a requirement for Mg^{2+} (Table III). Optimal enzymatic activity in the standard assay was obtained at about $1 \cdot 10^{-3}$ M Mg^{2+}. When Mg^{2+} was omitted 10–20% of the activity remained, but when EDTA was added the

TABLE III

INFLUENCE OF INORGANIC SALTS AND SH REAGENTS ON THE ACTIVITY OF THE O-METHYLTRANSFERASE

Incubations were carried out with the desalted enzyme from the hydroxylapatite column in the enzyme assay.

Addition	*Concn (M)*	*Relative activity (%)*
Mg^{2+}	$1 \cdot 10^{-3}$	100
$-Mg^{2+}$		20
Mg^{2+} + EDTA	$1 \cdot 10^{-3}$*	2
$-Mg^{2+}$ + EDTA	$1 \cdot 10^{-3}$	0
$-Mg^{2+}$ + Ca^{2+}	$1 \cdot 10^{-3}$	7
$-Mg^{2+}$ + Zn^{2+}	$1 \cdot 10^{-3}$	21
Mg^{2+} + NH_4^+	$3 \cdot 10^{-3}$	100
Mg^{2+} + KCN	$1 \cdot 10^{-3}$	87
$-Mg^{2+}$ + KCN	$1 \cdot 10^{-3}$	13
p-Chloromercuribenzoate	$1 \cdot 10^{-5}$	100
	$1 \cdot 10^{-4}$	100
	$1 \cdot 10^{-3}$	84
Iodoacetamide	$1 \cdot 10^{-4}$	100
	$1 \cdot 10^{-3}$	100
	$1 \cdot 10^{-2}$	75

* Concentration of EDTA or other additions. When Mg^{2+} was present its concentration was kept at $1 \cdot 10^{-3}$ M.

activity was completely lost. Ca^{2+} and Zn^{2+} could not replace Mg^{2+}, and NH_4^+ or CN^- had also no influence on enzymatic activity. The enzyme is not inhibited by *p*-chloromercuribenzoate or iodoacetamide.

Ethyleneglycol monomethyl ether, which was added up to 10 vol./100 ml to the assay for solubilisation of substrates, did not inhibit the reaction at this concentration.

Molecular weight

An estimation of the molecular weight of the O-methyltransferase was obtained by determination of the elution volume on a calibrated Sephadex G-100 column[18]. The molecular weight is about 48 000 (Fig. 7).

Identification of reaction products

With caffeic acid, luteolin and luteolin-7-glucoside as substrates for the O-methyltransferase the reaction products were identified as ferulic acid (3-methoxy-4-hydroxycinnamic acid), chrysoeriol (5,7,4'-trihydroxy-3'-methoxyflavone), and chrysoeriol-7-glucoside, respectively, by comparison with authentic reference samples, by paper chromatography and by thin-layer chromatography on silica gel, polyamide and cellulose in several solvent systems (Table I).

With Solvent system 9 it was possible to separate the isomeric substances ferulic acid/isoferulic acid and chrysoeriol/diosmetin (5,7,3'-trihydroxy-4'-methoxyflavone) (Table I). No radioactivity could be detected in the zones corresponding to isoferulic acid and diosmetin. 5% of the total radioactivity would easily have been detected in the *para*-O-methyl product by this method. Chrysoeriol-7-O-glucoside was hydrolysed by emulsin to chrysoeriol and glucose. Radioactivity was present only in the aglycone.

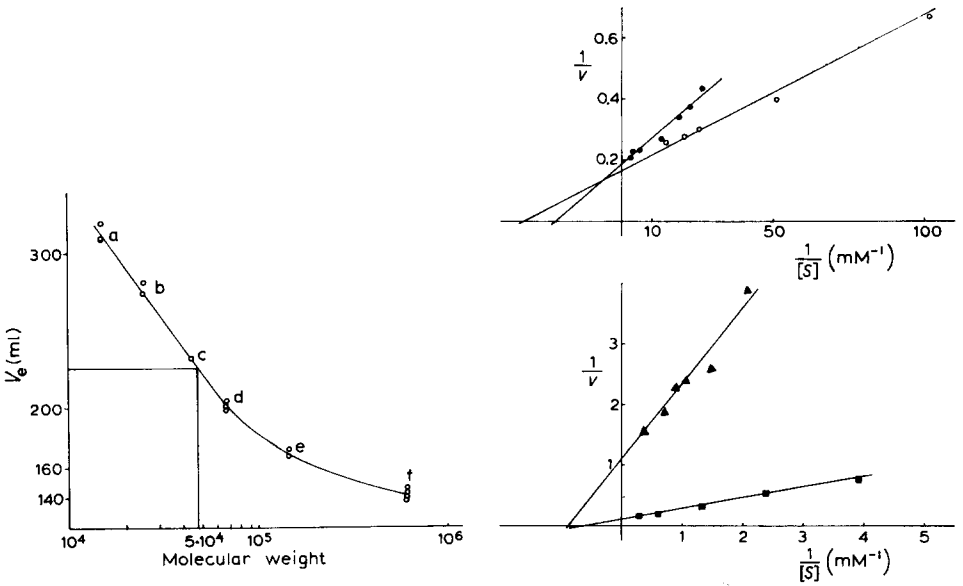


Fig. 7. Molecular weight determination on Sephadex G-100. a, Cytochrome *c*; b, chymotrypsinogen; c, ovalbumin; d, albumin (bovine); e, aldolase; f, blue dextran.

Fig. 8. Effect of different substrates on reaction rate. ○——○, luteolin-7-glucoside; ●——●, luteolin; ▲——▲, eriodictyol; ■——■, caffeic acid.

Substrate specificity

The apparent K_m values for luteolin, luteolin-7-glucoside, eriodictyol (5,7,3′,4′-tetrahydroxyflavanone) and caffeic acid were determined according to the method of Lineweaver and Burk on the basis of the effect of substrate concentration on reaction rate (Fig. 8). The values recorded in Table IV show that the enzyme has a 30–50 times higher affinity for luteolin and its 7-*O*-glucoside than for eriodictyol and caffeic acid. With luteolin as substrate the apparent K_m value for *S*-adenosyl-L-methionine was $1.5 \cdot 10^{-4}$ M.

A number of other flavonoids, cinnamic acids, and protocatechuic acid were compared with luteolin for their relative rates of conversion. The results are summarized in Table V. According to these results, only *o*-dihydric phenols can function

TABLE IV

K_m VALUES AND RELATIVE V VALUES FOR SUBSTRATES OF THE *O*-METHYLTRANSFERASE

The values were determined in the standard assay at pH 9.3.

Substrate	K_m *(M)*	*Relative V*
Luteolin-7-glucoside	$3.1 \cdot 10^{-5}$	100
Luteolin	$4.6 \cdot 10^{-5}$	88
Eriodictyol	$1.2 \cdot 10^{-3}$	15
Caffeic acid	$1.6 \cdot 10^{-3}$	148
S-Adenosylmethionine	$1.5 \cdot 10^{-4}$	

TABLE V

COMPARISON OF VARIOUS PHENOLS AS ACCEPTORS FOR THE *O*-METHYLTRANSFERASE

Incubations were carried out for 60 min in the standard assay.
The reaction product of quercetin was identified as isorhamnetin (3′-*O*-methylquercetin) in Solvent system 1 (R_F = 0.65) and the reaction product of protocatechuic acid as 3-methoxy-4-hydroxybenzoic acid in the same solvent system (R_F = 0.58).

Substrate	*Concn in assay (M)*	*Relative activity (%)*
Luteolin	$1 \cdot 10^{-3}$	100
Quercetin	$5 \cdot 10^{-4}$	13
	$1 \cdot 10^{-3}$	40
Diosmetin*	$1 \cdot 10^{-3}$	0
Chrysoeriol	$1 \cdot 10^{-3}$	0
Apigenin	$1 \cdot 10^{-3}$	0
Ferulic acid	$1 \cdot 10^{-3}$	0
Isoferulic acid	$1 \cdot 10^{-3}$	0
p-Cumaric acid	$1 \cdot 10^{-3}$	0
Protocatechuic acid	$1 \cdot 10^{-3}$	8
	$5 \cdot 10^{-3}$	24
	$1 \cdot 10^{-2}$	40

* 5,7,3′-Trihydroxy-4′-methoxyflavone.

as substrates for the enzyme. No reaction takes place if one of the two phenolic hydroxyls is methylated or if only one hydroxyl group is present.

Presence of S-adenosyl-L-methionine synthetase in cell cultures

To prove the presence of *S*-adenosyl-L-methionine synthetase (ATP: L-methionine-*S*-adenosyltransferase, EC 2.5.1.6) in cell cultures of parsley, a protein preparation from fresh cells (see Materials and Methods) was incubated with luteolin, ATP, $MgCl_2$ and L-[*Me*-^{14}C]methionine in Tris–HCl buffer of pH 7.5. The reaction products were separated on silica gel thin-layer plates with Solvent system 1. The results in Table VI show that radioactive chrysoeriol is formed and that the radioactivity in chrysoeriol can be decreased by addition of unlabelled L-methionine. No reaction was observed in the absence of ATP with the purified protein A2.

A direct proof for the formation of *S*-adenosyl-L-methionine was obtained by

TABLE VI

INDIRECT PROOF FOR THE FORMATION OF *S*-ADENOSYL-L-METHIONINE WITH PROTEIN FROM CELL CULTURES

For details see Materials and Methods.

	Chrysoeriol (cpm)	
	Protein A1	*Protein A2*
Complete	39 000	182 000
+ 500 nmoles L-methionine	7 000	35 000
− ATP	2 000	0
− Protein	0	0
+ Heat denatured protein	0	0

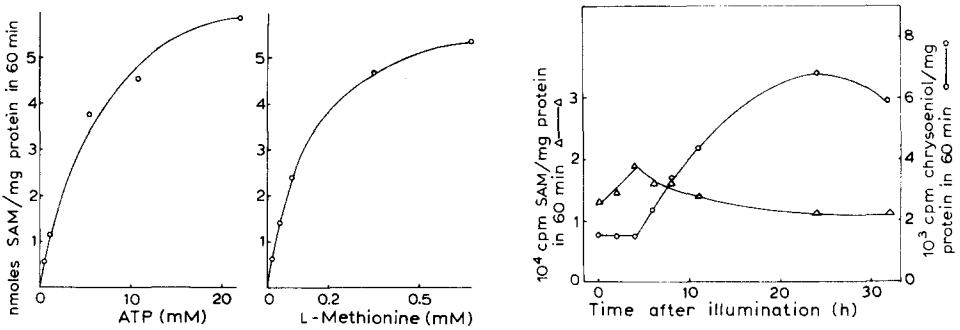


Fig. 9. Dependence of *S*-adenosyl-L-methionine (SAM) formation on ATP and methionine concentration.

Fig. 10. Changes in the activities of *O*-methyltransferase (○——○) and *S*-adenosyl-L-methionine (SAM) synthetase (△——△) during 32 h of continuous illumination of cell suspension cultures from parsley.

omitting luteolin from the incubation mixture. The reaction products were separated by paper chromatography in Solvent system 10. Under the incubation conditions employed the formation of *S*-adenosyl-L-methionine was linear with protein concentration up to 1.8 mg/ml and with time up to 100 min. The dependence of *S*-adenosyl-L-methionine formation on ATP and methionine concentration is shown in Fig. 9.

The influence of illumination of the cell cultures on the activity of the *S*-adenosyl-L-methionine synthetase was compared with that of the *O*-methyltransferase. Whereas *O*-methyltransferase activity was strongly dependent on light treatment[1], no significant change of the activity of *S*-adenosyl-L-methionine synthetase was observed for 30 h after onset of illumination (Fig. 10).

DISCUSSION

Results obtained from experiments with the partially purified methyltransferase show that only *ortho*-dihydric phenols can serve as substrates of this enzyme. Moreover, methylation took place exclusively in the *meta* position of 1-substituted 3,4-dihydric phenols. The enzyme must therefore be classified as *S*-adenosyl-L-methionine: *ortho*-dihydric phenol *meta*-*O*-methyltransferase. Among the compounds tested, luteolin and its 7-*O*-glucoside had the highest affinity for the enzyme. This observation is in agreement with the assumption that one or both of these substrates are the natural precursors for 3′-*O*-methylated flavone glycosides in cell suspension cultures of parsley. With respect to luteolin-7-*O*-glucoside as a possible endogenous substrate of the methyltransferase, it is interesting to note that this compound has recently been detected in extracts from illuminated cell cultures from this plant[14].

In a previous publication[1] we were able to demonstrate that changes in the methyltransferase activity after illumination of the cell cultures are directly related to changes in the activities of all other enzymes of flavonoid biosynthesis so far isolated. According to these observations and to the results presented in this paper

it is suggested that the partially purified methyltransferase is an enzyme specifically involved in the biosynthesis of flavonoid compounds in parsley.

The enzyme activity appears as one symmetrical peak after disc electrophoresis (Fig. 5) and electrofocusing (Fig. 4). However, the question whether only one molecular form of the enzyme is present in this preparation cannot be answered definitely at this stage of purification. The occurrence of two peaks of enzymatic activity with different elution volumes from Sephadex G-100 (Fig. 2) might be explained either by a formation of an aggregated form of higher molecular weight or by a contamination of the original crude enzyme preparation with other methyltransferase(s). This question has not been further investigated.

In contrast to a catechol *O*-methyltransferase (*S*-adenosylmethionine:catechol *O*-methyltransferase, EC 2.1.1.6) from animal tissues[15,16], the enzyme from parsley cell cultures is not affected by the addition of inhibitors of thiol groups such as *p*-chloromercuribenzoate and iodoacetamide (Table III). Like the transferase from rat liver the parsley enzyme requires Mg^{2+} for optimal enzymatic activity.

The enzyme which catalyses the synthesis of *S*-adenosyl-L-methionine from ATP and L-methionine (ATP: L-methionine *S*-adenosyltransferase, EC 2.5.1.6) has been found in mammalian liver[10], yeast[11], *Escherichia coli*[12], and extracts from barley[13]. The presence of this enzyme in parsley cultures has also been demonstrated. These cultures are a good source for the enzyme, which has not yet been studied more closely in higher plants.

In contrast to the enzymes involved exclusively in flavonoid biosynthesis in parsley the activity of *S*-adenosyl-L-methionine synthetase is not significantly altered after illumination of the cells (Fig. 10). It can therefore be assumed that this enzyme plays a more general role in this plant and is not confined to flavonoid biosynthesis.

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