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PURIFICATION AND PROPERTIES OF *S*-ADENOSYL-L-METHIONINE: CAFFEIC ACID O-METHYLTRANSFERASE FROM LEAVES OF SPINACH BEET (*BETA VULGARIS* L.)

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Summary

1. An enzyme catalysing the methylation of caffeic acid to ferulic acid, using *S*-adenosyl-L-methionine as methyl donor, has been extracted from leaves of spinach beet and purified 75-fold to obtain a stable preparation.

2. The enzyme showed optimum activity at pH 6.5, and did not require the addition of Mg^{2+} for maximum activity.

3. It was most active with caffeic acid, but showed some activity with catechol, protocatechuic acid and 3,4-dihydroxybenzaldehyde. The K_m for caffeic acid was 68 μM.

4. The K_m for *S*-adenosyl-L-methionine was 12.5 μM. *S*-Adenosyl-L-homocysteine (K_i = 4.4 μM) was a competitive inhibitor of *S*-adenosyl-L-methionine.

5. The synthesis of *S*-adenosyl-L-homocysteine from adenosine and L-homocysteine and its consequent effect on caffeic acid methylation were demonstrated with a partially-purified preparation from spinach-beet leaves, which possessed both *S*-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1) and adenosine nucleosidase (EC 3.2.2.7) activities. This preparation was also able to catalyse the rapid breakdown of *S*-adenosyl-L-homocysteine to adenosine and adenine; the possible significance of this reaction in relieving the inhibition of caffeic acid methylation by *S*-adenosyl-L-homocysteine is discussed.

Introduction

The methylation of caffeic acid (3,4-dihydroxycinnamic acid) to ferulic acid (3-methoxy-4-hydroxycinnamic acid) is an intermediate stage in the biosynthesis of lignin and of some flavonoids (Kreuzaler, F., Hrazdina, G. and Grisebach, H., unpublished results) in plants. This reaction has been successfully demonstrated in whole tissues [1] and in crude enzyme preparations [2—8].

The purification of a caffeic acid O-methyltransferase from bamboo tissue [9] and from parsley cell cultures [10] has been reported. The latter enzyme, which catalysed the methylation of flavonoids more effectively than caffeic acid, was suggested to be specifically involved in the biosynthesis of flavonoid compounds in parsley, since the changes in its activity after illumination of the cell cultures were directly related to changes in the activities of all other enzymes of flavonoid biosynthesis. In contrast, the activity of the caffeic acid O-methyltransferase from soybean cell suspension cultures appeared to be regulated independently of the enzymes of general phenylpropanoid metabolism [11].

This paper describes the purification of a stable *S*-adenosyl-L-methionine: caffeic acid O-methyltransferase from leaves of spinach beet (*Beta vulgaris* L. ssp. *vulgaris*), which appears to be quite distinct from the enzyme isolated from parsley cells. The properties of this enzyme have been investigated in order to determine how its activity may be regulated in vivo.

Many O-methyltransferases [12—14] and N-methyltransferases [12,15, 16] are inhibited by *S*-adenosyl-L-homocysteine produced during the methylation reaction. The effects of *S*-adenosyl-L-homocysteine on the activity of the purified caffeic acid O-methyltransferase and on the reaction course have been studied and are discussed in relation to the regulation of the activity of this enzyme in vivo. In order to sustain caffeic acid methylation in vivo, the intracellular level of *S*-adenosyl-L-homocysteine must probably be kept low relative to that of *S*-adenosyl-L-methionine. Reactions converting *S*-adenosyl-L-homocysteine to other products are therefore important in relation to the methylation reaction and were investigated with crude preparations of spinach-beet leaves. In yeast [17], rat liver [18] and germinating pea seeds [19], it has been shown that *S*-adenosyl-L-homocysteine can be enzymically hydrolysed to adenosine and homocysteine. However, with purified preparations [18,20], the equilibrium constant was found to be heavily in favour of *S*-adenosyl-L-homocysteine synthesis, and it was necessary to add adenosine deaminase (adenosine amino-hydrolase, EC 3.5.4.4) to facilitate the hydrolysis of *S*-adenosyl-L-homocysteine by the conversion of adenosine. Preliminary experiments reported here indicate the presence of both *S*-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1) and adenosine nucleosidase (EC 3.2.2.7) activities within crude extracts of spinach-beet leaves. The reversibility of the *S*-adenosyl-L-homocysteine hydrolase reaction was investigated in order to determine whether the inhibition of caffeic acid methyltransferase by *S*-adenosyl-L-homocysteine could be relieved by the hydrolysis of this compound.

Materials and Methods

Chemicals

Purified *S*-adenosyl-L-[*Me*-^{14}C]methionine (58 Ci/mol) and [8-^{14}C]adenosine (52 Ci/mol) were purchased from the Radiochemical Centre, Amersham, Bucks., Great Britain. The radioactive *S*-adenosyl-L-methionine was diluted with unlabelled *S*-adenosyl-L-methionine, purchased from British Drug Houses, Poole, Dorset, Great Britain, to give a specific activity of 0.45 Ci/mol. The non-radioactive *S*-adenosyl-L-methionine had been freed from contaminating

S-adenosyl-L-homocysteine by the method of Shapiro and Ehninger [21]. *S*-[8-^{14}C]adenosyl-L-homocysteine (47 Ci/mol) was prepared from [8-^{14}C]-adenosine and homocysteine using a purified preparation of *S*-adenosyl-L-homocysteine hydrolase (Poulton, J.E. and Butt, V.S., unpublished). Caffeic acid, obtained from Koch-Light Laboratories, Colnbrook, Bucks., Great Britain, was purified by repeated crystallisation from aqueous solution. All chemicals used were the purest available from British Drug Houses Ltd., Poole, Dorset, Great Britain. Adenosine deaminase was purchased from Sigma Chemical Co., London.

Plant materials

Fresh leaves from mature spinach beet (*Beta vulgaris* L. ssp. *vulgaris*) plants, which had been grown outdoors, were used.

Preparation of the purified enzyme

Preliminary experiments detected caffeic acid O-methyltransferase activity in mature leaves of spinach (*Spinacia oleracea* L.), French beans (*Phaseolus vulgaris* L.), spinach beet (*Beta vulgaris* L.) and peas (*Pisum sativum* L.). Spinach-beet leaves, harvested from plants grown outdoors, were used in the experiments described here.

210 g of the washed laminae of spinach-beet leaves were macerated in an Ato-mix with 250 ml of ice-cold 0.1 M KH_2PO_4/K_2HPO_4 buffer, pH 7.5. The macerate was squeezed through muslin and the liquid centrifuged at 30 000 × *g* for 20 min. Unless otherwise indicated, all stages of purification of this extract were carried out at 4° C.

The supernatant liquid was brought to 45% saturation by addition of solid $(NH_4)_2SO_4$ with continuous addition of KOH solution to maintain the pH at 7.5. The mixture was allowed to stand at 0° C for 25 min and then centrifuged at 10 000 × *g* for 15 min. The supernatant was brought to 75% saturation with solid $(NH_4)_2SO_4$, and the precipitate collected in the same way and dissolved in the minimum volume of 20 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.5. A suitable aliquot was chromatographed on a column (37.5 cm × 3 cm) containing Sephadex G-200 in the same buffer. The active fractions, eluted between 150 and 200 ml were pooled, their pH adjusted to 7.7 and then applied to a DEAE-cellulose column (5.2 cm × 1.25 cm) equilibrated with 20 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.7. Inactive protein was washed from the column with 45 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.7, and elution then continued with a linear gradient up to 200 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.7. The most active fractions (Nos 24—26, each 7 ml vol.) were combined. The enzyme was concentrated by precipitating it by the addition of solid $(NH_4)_2SO_4$ to 80% saturation. It was then dissolved in 10 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.5, and residual $(NH_4)_2SO_4$ removed by gel filtration through a column (45 cm × 1.5 cm) containing Sephadex G-25 in the same buffer.

Preparation of the crude extract containing both caffeic acid O-methyltransferase and S-adenosyl-L-homocysteine hydrolase activities

The fraction, precipitated by ammonium sulphate between 45 and 80% saturation, was collected by the method described above, except that the pre-

cipitation was carried out at pH 7.7. The precipitate was redissolved in the minimum volume of 10 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.7. Residual $(NH_4)_2SO_4$ was removed from a suitable volume (6.0 ml) of this solution by passage through a column (45 cm × 2 cm) of Sephadex G-25, which had been equilibrated with the above buffer. The protein-containing fractions were mixed and stored frozen at −20° C; these were thawed when required.

S-Adenosyl-L-methionine caffeic acid methyltransferase assay

S-Adenosyl-L-methionine: caffeic acid methyltransferase activity was assayed by measuring the rate of formation of [*Me*-^{14}C] ferulic acid which was separated from *S*-adenosyl-L-[*Me*-^{14}C] methionine by extraction into a toluene-based scintillation fluid. The assay mixture contained 10.9 nmol of *S*-adenosyl-L-[*Me*-^{14}C] methionine (110 000 dpm), 100 nmol of caffeic acid, 400 nmol of sodium D-isoascorbate, 1 μmol of $KHCO_3$ and 7 μmol of phosphate (KH_2PO_4/K_2HPO_4 mixture depending upon pH used), to which was added 10—50 μl of the methyltransferase to give a final volume of 0.15 ml. Preliminary experiments had suggested that the enzyme from spinach beet showed greatest activity at pH 7.0—7.5, so that it was assayed at pH 7.5 during the purification stages and, in view of reports that catechol-O-methyltransferases were stimulated by Mg^{2+} [5,8,10,22] with 1.33 mM $MgCl_2$. For reasons given later, the purified enzyme was assayed at pH 6.5 without added Mg^{2+}.

The reaction mixture was incubated at 30° C for 20 min and the reaction terminated by the addition of 20 μl of 50% (v/v) HCl. The precipitated protein was removed by centrifugation, and ferulic acid was then separated from unreacted *S*-adenosyl-L-methionine by shaking 0.1 ml aliquots of the supernatant liquid with 10 ml of scintillation fluid (composition: 0.5% (w/v) butylphenylbiphenylyl-oxadiazole in toluene). The extraction mixtures were centrifuged and the radioactivity of 5-ml aliquots of extract determined by counting in a Tracerlab Corumatic/200 scintillation counter. One unit of methyltransferase activity was defined as the amount of enzyme required to catalyse the formation of 1 μmol of ferulic acid per min under these assay conditions.

The *S*-adenosyl-L-methionine: caffeic acid O-methyltransferase activity of the crude extract, which contained both methyltransferase and *S*-adenosyl-L-homocysteine hydrolase activities, was assayed in the absence of Mg^{2+} by the above method but at pH 7.7, since the optimum pH for *S*-adenosyl-L-homocysteine hydrolase was above 8 (Poulton, J.E. and Butt, V.S., unpublished).

S-Adenosyl-L-homocysteine hydrolase assay

S-Adenosyl-L-homocysteine hydrolase activity was assayed by the formation of *S*-[8-^{14}C] adenosylhomocysteine from [8-^{14}C] adenosine and DL-homocysteine, the product being separated chromatographically from labelled adenosine. 0.2 μmol of [8-^{14}C] adenosine (153 700 cpm) was incubated with 1 μmol of DL-homocysteine, 0.3 μmol of β-mercaptoethanol and 5 μmol of KH_2PO_4/K_2HPO_4 buffer, pH 7.7, to which was added 5 μl of enzyme preparation, in a total volume of 0.09 ml at 30° C. Control vessels, in which either DL-homocysteine or enzyme preparation were omitted or in which a boiled enzyme preparation replaced the active extract, were included. After suitable periods, the reaction was terminated by the addition of 10 μl of 50% (w/v) trichloro-

acetic acid. The mixture was centrifuged to remove protein, and 10 µl aliquots applied to Polygram SIL N-HR/UV254 sheets (Macherey and Nagel). The chromatograms were developed using *iso*-propanol/ethyl acetate/ammonia/water (23 : 27 : 4 : 4, by vol.). Spots corresponding to adenosine (R_F, 0.43) and *S*-adenosyl-L-homocysteine (R_F, 0.13) could be seen under UV light. The areas corresponding to *S*-adenosyl-L-homocysteine were cut out and counted in 2 ml of scintillation fluid containing 0.5% (w/v) butylphenylbiphenylyl-oxadiazole in toluene, using a Tracerlab Corumatic/200 scintillation counter.

Adenosine nucleosidase activity

Adenosine nucleosidase activity was assayed by the formation of [8-^{14}C]-adenine from [8-^{14}C]adenosine, which were separated chromatographically. 0.1 µmol of [8-^{14}C]adenosine (222 000 dpm) and 10 µmol of KH_2PO_4/K_2HPO_4 buffer, pH 7.7, were incubated with up to 50 µl of enzyme preparation in a total volume of 0.1 ml at 30° C for 15 min. Control tubes, in which boiled enzyme preparation replaced the active extract, were included. The reaction was terminated by heating in boiling water for 2 min. The mixture was cooled and centrifuged, and 10 µl aliquots applied to Polygram CEL 300 UV 254 sheets (Macherey and Nagel). The chromatograms were developed with water. Adenosine (R_F, 0.50) and adenine (R_F, 0.30) could be seen in UV light. The area corresponding to adenine was cut out and counted in the scintillation fluid used above.

Enzymic hydrolysis of S-adenosyl-L-homocysteine

0.1 µmol of *S*-[8-^{14}C]adenosyl-L-homocysteine (75 000 dpm) was incubated with 0.01 µmol of dithioglycol, 5 µmol of KH_2PO_4/K_2HPO_4 buffer, pH 7.7 and 50 µl of enzyme preparation in a total volume of 0.09 ml at 30°C. Where indicated, 0.2 units of adenosine deaminase was also included. Control tubes, in which boiled extract or boiled adenosine deaminase replaced the active enzymes, were included. After 60 min, the reaction was terminated by heating in boiling water for 2 min. The mixture was cooled and centrifuged, and 10 µl aliquots applied to Polygram SIL N-HR/UV254 sheets. The chromatograms were developed as above using the *iso*-propanol/ethyl acetate/ammonia/water mixture. The areas corresponding to *S*-adenosyl-L-homocysteine (R_F, 0.05), adenosine (R_F, 0.40), adenine (R_F, 0.55) and inosine (R_F, 0.16) were cut out and counted in the scintillation fluid used above. The distribution of activity between these compounds was checked by separating the reaction products also on Polygram CEL 300 UV 254 sheets, using acetone/water (5 : 2, by vol.) for development, the R_F values for *S*-adenosyl-L-homocysteine, adenosine, adenine and inosine being 0.25, 0.78, 0.67, and 0.77 respectively; this solvent did not however separate adenosine from inosine.

Protein determination

The protein content of crude preparations was estimated by the Lowry method, as described by Leggett Bailey [23], after precipitation from solution by 5% (w/v) trichloroacetic acid; crystalline bovine serum albumin, desiccated before use, was the standard. The protein content of column eluates was determined by the method of Warburg and Christian [24].

Results and Discussion

Proof of reaction product

The reaction product was identified as ferulic acid by descending chromatography of samples of the reaction mixture on Whatman No. 1 paper in benzene/acetic acid/water (6 : 7 : 3, by vol., upper layer); ferulic acid spots (R_F, 0.73) were identified by their blue fluorescence under UV light, cut out and counted in the scintillation counter. The product was further identified as ferulic acid by spraying duplicate spots with diazotised sulphanilic acid [25], which gave reddish-violet colours. The radioactive spots also ran parallel with marker spots of ferulic acid in descending chromatograms on Whatman No. 1 paper in toluene/acetic acid/water (4 : 1 : 5, upper layer) and on thin-layer chromatograms on Polygram SIL G/UV254 sheets (Macherey and Nagel) in toluene/ethyl formate/formic acid (5 : 4 : 1, by vol.).

Ferulic acid was routinely separated from unreacted *S*-adenosyl-L-methionine by extraction into the toluene-based scintillation fluid. Fig. 1 shows that the total quantity of ferulic acid synthesized as measured by this method and by paper chromatography does not differ by more than 2% at each time interval of incubation. Extraction into toluene is considerably simpler and more rapid than paper chromatography, while retaining high sensitivity and precision. Control tubes, in which boiled enzyme preparation replaced the fresh extract or from which caffeic acid had been omitted, were also included.

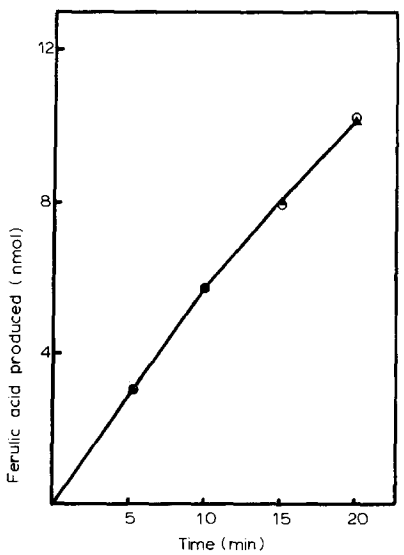

Fig. 1. A comparison of methods of separation of ferulic acid and *S*-adenosyl-L-methionine. An extract of spinach-beet leaves was prepared by $(NH_4)_2SO_4$ precipitation between 45 and 80% saturation as described in the Methods section. An aliquot (0.35 ml) was incubated at 30°C with 92.5 nmol of *S*-adenosyl-L-[*Me*-^{14}C]methionine (containing $1.55 \cdot 10^6$ dpm.), 0.7 μmol of caffeic acid, 2.8 μmol of sodium D-isoascorbate, 7 μmol of $KHCO_3$ and 49 μmol of KH_2PO_4/K_2HPO_4 buffer, pH 6.5, in a total volume of 1.05 ml. At intervals, aliquots (0.2 ml) were withdrawn and added to 20 μl of 66% (v/v) HCl. Precipitated protein was removed by centrifugation. [*Me*-^{14}C] Ferulic acid was separated from unreacted *S*-adenosyl-L-methionine by paper chromatography (o———o) and by extraction into a toluene-based scintillation fluid (▲———▲) as described in the Methods section.

TABLE I

PURIFICATION OF SPINACH-BEET *S*-ADENOSYL-L-METHIONINE: CAFFEIC ACID O-METHYLTRANSFERASE

Stage	Total protein (mg)	Total activity (munits)	Yield (% initial)	Specific activity (munits/mg protein)
Crude homogenate	1060	38.2	100	0.036
Supernatant after 30000 × *g* centrifugation	574	43.9	115	0.076
$(NH_4)_2SO_4$ precipitate (45—75% saturated)	200	—	—	—
Eluate from G-200 fractionation	73	41.1	108	0.561
Eluate from DEAE-cellulose fractionation, after precipitation	5.1	13.8	36	2.70

The purification of caffeic acid O-methyltransferase

In contrast to the phenolase activity from spinach-beet leaves [26], caffeic acid methyltransferase activity was not found to be firmly bound to the particulate fractions when crude homogenates were fractionated by the procedure of Tolbert et al. [27]. The crude homogenate was therefore centrifuged first at 30 000 × *g*, achieving not only a two-fold increase in the specific activity of the enzyme, but also a considerable increase in the total activity of the enzyme, perhaps because of the removal of a sedimentable inhibitor (Table I).

The enzyme was precipitated by ammonium sulphate between 45—75% saturation. Caffeic acid methyltransferase activity was inhibited by $(NH_4)_2SO_4$, since the removal of this salt from a redissolved ammonium sulphate precipitate by passage through Sephadex G-25 resulted in a large increase in the specific activity of the enzyme. Furthermore, when a dialysed fraction from ammonium sulphate precipitation was assayed in the presence of this salt, methyltransferase activity was considerably inhibited. A preparation of the methyltransferase, having a specific activity 75 times greater than that of the crude homogenate, was obtained in nearly 40% yield after further chromatography on Sephadex G-200 and DEAE-cellulose (Fig. 2) columns. This preparation was concentrated as described in the Methods section and used in the kinetic studies reported here; it was found to exhibit no *S*-adenosyl-L-homocysteine hydrolase activity.

The instability of many plant and animal catechol-O-methyltransferases after partial purification has made difficult their further purification and study. However, although dithiothreitol and mercaptoethanol have been reported to prevent the loss of activity of the purified enzymes [8,28,29], enzyme preparations from spinach beet were stable at all stages of purification for at least a month at −20° C when stored in the absence of thiol compounds. Indeed, in the presence of 70 mM mercaptoethanol, 5 mM dithiothreitol or 5 mM reduced glutathione, enzyme activity was rapidly lost during storage at either 4° C or −20° C.

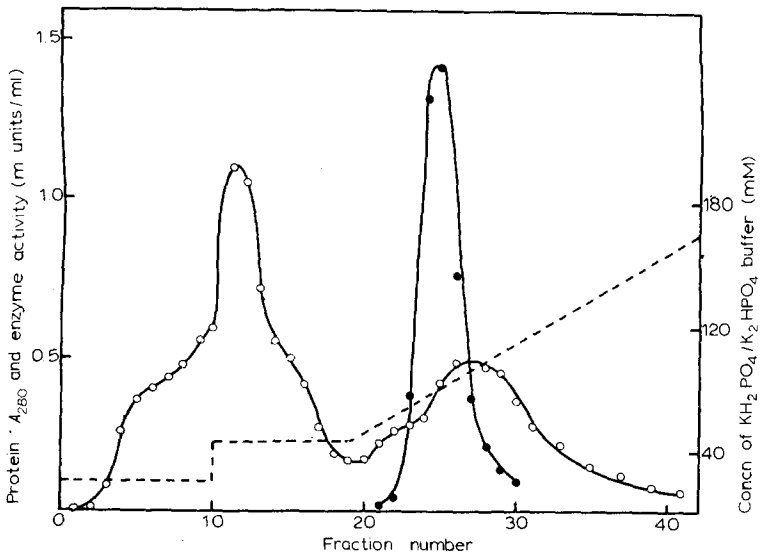

Fig. 2. DEAE-cellulose column chromatography of spinach-beet *S*-adenosyl-L-methionine:caffeic acid O-methyltransferase. The active fractions obtained from gel-filtration on Sephadex G-200 were pooled and applied to a 5.2 cm × 1.25 cm column of DEAE-cellulose, which had equilibrated with 20 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.7. The column was eluted with 20 mM KH_2PO_4/K_2HPO_4 buffer for 10 × 7 ml fractions, then with 45 mM KH_2PO_4/K_2HPO_4 buffer for a further 9 × 7 ml fractions and finally with a linear gradient of 45—200 mM KH_2PO_4/K_2HPO_4 buffer. 34% of the applied methyltransferase was recovered. Protein content of fractions, measured by absorbance at 280 nm (○———○); methyltransferase activity (●———●); and KH_2PO_4/K_2HPO_4 concentration (broken line).

Properties of the purified methyltransferase

As will be discussed in detail subsequently (see Fig. 3), the rate of the reaction at pH 6.5 was almost constant over the first 30 min, and then progressively declined. The reaction course was similar at pH 7.5. At either pH, the extent of the reaction after incubation for 20 min was proportional to the enzyme concentration up to 25 μg of purified protein.

Preparations of catechol-O-methyltransferases from both plant and animal sources show considerable variation in their requirement for Mg^{2+} [5,8,10,22,30]. The addition of $MgCl_2$ did not lead to any significant increase in the activity of the purified enzyme from spinach beet, and, indeed, at concentrations above 4 mM $MgCl_2$, some inhibition was observed. Moreover, EDTA at concentrations up to 10 mM did not inhibit the enzyme.

The optimum pH for the methylation of caffeic acid with the purified enzyme was determined using several different buffers (Fig. 4). The greatest activity was observed at about pH 6.5, which is lower than that reported for other plant catechol O-methyltransferases [5,8,10]. Using a purified preparation, without added $MgCl_2$, 0.67 mM caffeic acid was much more actively methylated than 1.33 mM catechol or 1.33 mM 3,4-dihydroxybenzaldehyde; 1.33 mM 3,4-dihydroxybenzoic acid was also methylated (Table II). In contrast to the enzyme from parsley cell cultures however [10], no evidence for the methylation of luteolin was obtained at concentrations up to 0.77 mM in 2-methoxyethanol or up to 0.47 mM in ethanol in 0.1 M KH_2PO_4/K_2HPO_4 buffer (1 : 1, by vol.) at pH 7.5. Moreover, the methylation of caffeic acid in

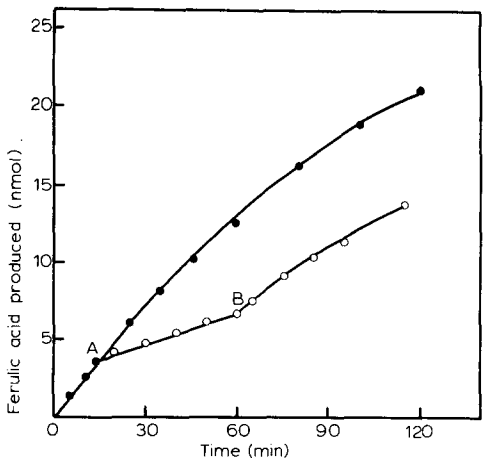

Fig. 3. Effect of added *S*-adenosyl-L-homocysteine and *S*-adenosyl-L-methionine on reaction course. The reaction course (●———●) was followed using purified methyltransferase (100 µl), incubated with 0.8 µmol of caffeic acid, 86 nmol of *S*-adenosyl-L-methionine (734 000 dpm), 56 µmol of potassium phosphate (pH 6.5), 3.2 µmol of sodium D-isoascorbate and 8 µmol of $KHCO_3$ in a total volume of 1.22 ml at 30° C. 0.1 ml samples were removed and assayed for labelled ferulic acid. In a parallel vessel (○———○), *S*-adenosyl-L-homocysteine (50 nmol) was added at A, followed by *S*-adenosyl-L-methionine (0.145 µmol; 458 700 dpm) at B.

the presence of these solvents was partially inhibited. Using a redissolved $(NH_4)_2SO_4$ precipitate, which was active in the methylation of caffeic acid, no evidence for the O-methylation of 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylamine (dopamine), chlorogenic acid or 3,4-dihydroxyphenylacetic acid nor for the O- or N-methylation of tyramine was obtained.

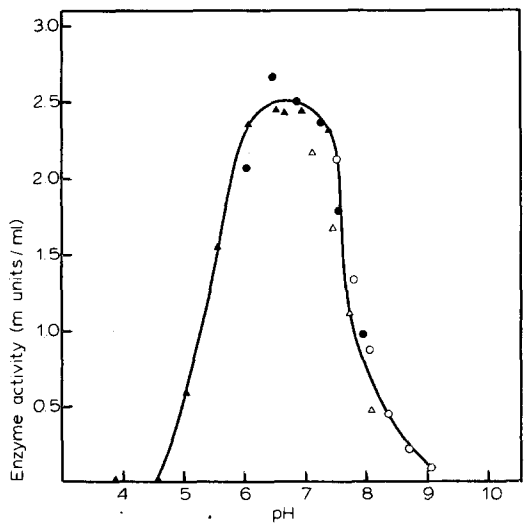

Fig. 4. Effect of pH on the activity of *S*-adenosyl-L-methionine: caffeic acid O-methyltransferase. Caffeic acid and *S*-adenosyl-L-methionine were incubated with enzyme under the conditions given in the Methods section using 47 mM concentrations of the following buffers: KH_2PO_4/K_2HPO_4 (●———●), HEPES/NaOH (△———△), Tris · HCl (○———○), and 47 mM K_2HPO_4 with citric acid added to give the required pH (▲———▲).

TABLE II

SPECIFICITY OF SPINACH-BEET *S*-ADENOSYL-L-METHIONINE: CAFFEIC ACID O-METHYL-TRANSFERASE

Assay conditions were those described in the Methods section, using the purified enzyme, at pH 6.5 and 30°C, without the addition of $MgCl_2$. Luteolin was dissolved in 2-methoxyethanol, and assayed at pH 7.5 against caffeic acid also dissolved in this solvent.

Substrate	Concentration (mM)	Relative activity (%)
Caffeic acid	0.67	100
Catechol	1.33	24
3,4-Dihydroxybenzaldehyde	1.33	37
3,4-Dihydroxybenzoic acid	1.33	6
Luteolin	0.77	0

The K_m for methylation of caffeic acid with the purified enzyme at pH 6.5, using a saturating concentration of *S*-adenosyl-L-methionine (0.11 mM), was 68 μM. The Lineweaver-Burk plot was linear and showed no detectable co-operative or inhibitory effects. The K_m for *S*-adenosyl-L-methionine fell with increasing purity of the enzyme from 106 μM with an $(NH_4)_2SO_4$ precipitate to 28 μM and 21 μM after passing this precipitate through Sephadex G-25 and Sephadex G-200 respectively, and then to 12.5 μM with the purified enzyme under optimum conditions (Fig. 5).

The decline in reaction velocity with time was investigated in greater detail. Control experiments showed that this phenomenon was not the result of

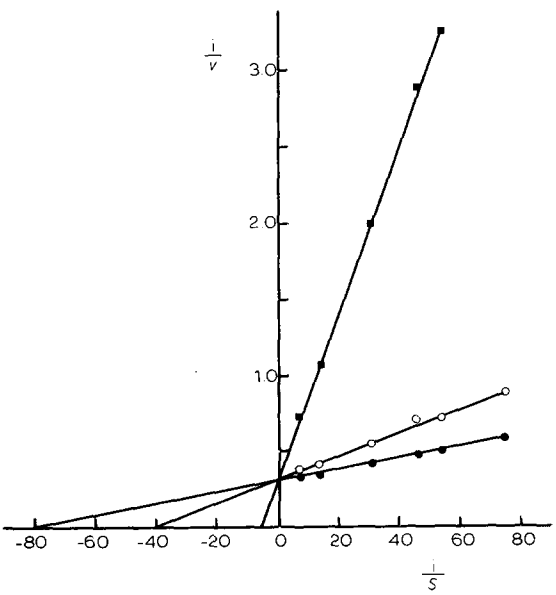

Fig. 5. Lineweaver-Burk plot of enzyme activity (v) and *S*-adenosyl-L-methionine concentration (s). Caffeic acid concentration was 0.67 mM. The reaction rate was measured without added *S*-adenosyl-L-homocysteine (●———●), and in the presence of 5 μM (○———○) *S*-adenosyl-L-homocysteine and 50 μM (■———■) *S*-adenosyl-L-homocysteine. v is expressed as nmol of ferulic acid produced/h; s is expressed in mmol/l.

thermal inactivation of the enzyme during the reaction period nor of the accumulation of the product ferulic acid. Instead, it appears very likely that this decline is caused by the accumulation of the second product *S*-adenosyl-L-homocysteine, since the methylation of caffeic acid was competitively inhibited by *S*-adenosyl-Lhomocysteine, with a K_i of 4.4 μM (Fig. 5). The inhibition of O-, C-, N- and S-methyltransferases by *S*-adenosyl-L-homocysteine is well documented [12,13,15,31,32]; in many of these systems, the K_i for *S*-adenosyl-L-homocysteine was smaller than the K_m for *S*-adenosyl-L-methionine, and such a relationship may well point to a general mechanism for the regulation of the activity of these enzymes.

The addition of *S*-adenosyl-L-homocysteine during the methylation of caffeic acid predictably caused an immediate inhibition of the reaction, which was relieved at once by the further addition of *S*-adenosyl-L-methionine (Fig. 3). These results suggest that the rate of caffeic acid methylation in vivo may be controlled by the relative concentrations of *S*-adenosyl-L-homocysteine and *S*-adenosyl-L-methionine. Caffeic acid methylation could be sustained if the intracellular levels of *S*-adenosyl-L-homocysteine are kept low relative to those of *S*-adenosyl-L-methionine; reactions which convert *S*-adenosyl-L-homocysteine to other products were therefore investigated.

S-Adenosyl-L-homocysteine metabolism in crude extracts of spinach-beet leaves

S-Adenosyl-L-homocysteine hydrolase (EC 3.3.1.1), which catalyses both the hydrolysis of *S*-adenosyl-L-homocysteine to adenosine and L-homocysteine and its synthesis from these compounds, has been extracted and partially purified from rat liver [18] and yeast [20] cells. The equilibrium position for this reaction was found to be far in the direction of *S*-adenosyl-L-homocysteine synthesis [13,18]. This property could be used to determine indirectly the presence of "*S*-adenosyl-L-homocysteine synthetase" activity within crude extracts of spinach-beet leaves, since the caffeic acid methyltransferase from this tissue is severely inhibited by *S*-adenosyl-L-homocysteine. The effect of *S*-adenosyl-L-homocysteine and related compounds on the methylation of caffeic acid was therefore investigated using the crude extract prepared as described in the Methods section. Table III shows the severe inhibitory effect of 0.66 mM and 1.32 mM *S*-adenosyl-L-homocysteine on the methylation of caffeic acid with the partially-purified extract during incubation for 30 min. By comparison, 0.66 mM adenine and 0.66 mM adenosine inhibited the reaction only slightly. 3.3 mM DL-homocysteine had no effect when supplied alone, nor did it further increase the slight inhibition observed with 0.66 mM adenine. However, in combination with 0.66 mM adenosine, it inhibited the reaction by almost the same amount as 0.66 mM *S*-adenosyl-L-homocysteine. When the reaction mixtures, after incubation, were chromatographed as described in the Methods section, *S*-adenosyl-L-homocysteine was detected when it was included in the incubation mixture, and also when adenosine and homocysteine were added in combination; no *S*-adenosyl-L-homocysteine could be detected visually in the reaction mixtures which had been incubated with adenine, adenosine or homocysteine, nor with adenine and homocysteine together.

The enzymic formation of *S*-adenosyl-L-homocysteine by the extract was

TABLE III

EFFECT OF *S*-ADENOSYL-L-HOMOCYSTEINE AND RELATED COMPOUNDS ON METHYLATION OF CAFFEIC ACID

20 μl of the partially-purified preparation (3.6 mg protein/ml) was incubated with caffeic acid and *S*-adenosyl-L-methionine as already described [1].

Addition	Ferulic acid produced (nmol/h/mg protein)	Relative activity (% control)
None	23.8	100
0.66 mM *S*-adenosyl-L-homocysteine	0.7	3
1.32 mM *S*-adenosyl-L-homocysteine	0	0
0.66 mM adenosine	18.2	76
3.33 mM DL-homocysteine	23.9	101
0.66 mM adenosine + 3.33 mM DL-homocysteine	1.4	6
0.66 mM adenine	21.3	90
0.66 mM adenine + 3.33 mM DL-homocysteine	21.1	89

suggested by these results and this was unequivocally demonstrated by following the time-course of *S*-[8-^{14}C]adenosyl-L-homocysteine formation, when [8-^{14}C]adenosine was incubated with homocysteine. *S*-Adenosyl-L-homocysteine production was linear with time up to 40 min, representing a specific activity of 11.4 μmol formed/h/mg protein. If DL-homocysteine or the extract were omitted, or if a boiled extract replaced the active preparation, no *S*-adenosyl-L-homocysteine was formed.

Although the equilibrium constant of the *S*-adenosyl-L-homocysteine hydrolase reaction favours *S*-adenosyl-L-homocysteine synthesis, the reversibility of this reaction in vitro and in vivo has been reported. For instance, the production of adenosine from *S*-adenosyl-L-homocysteine has been demonstrated with cell-free extracts of pea seedlings [19]. However, when *S*-[8-^{14}C]adenosyl-L-homocysteine was here incubated with the crude preparation from spinach-beet leaves, labelled adenosine was detected (Table IV). It was accompanied by much greater quantities of adenine, which were replaced by inosine when adenosine deaminase was added to the reaction mixture. It may well be important that the rate of *S*-adenosyl-L-homocysteine hydrolysis to adenine with this preparation is 4—5 times greater than the rate of its synthesis by the methylation of caffeic acid (Tables III and IV). The large quantities of adenine produced from *S*-adenosyl-L-homocysteine were probably caused by the presence of adenosine hydrolase activity in the extract. When [8-^{14}C]adenosine was incubated with the preparation (see Methods section), labelled adenine was produced at a rate of 0.5 μmol/h/mg protein. This was sufficient to account for its production from *S*-adenosyl-L-homocysteine. Moreover, no inosine formation could be demonstrated, and the hydrolysis did not proceed when the enzyme preparation had been boiled.

It is concluded that the caffeic acid O-methyltransferase described here is quite distinct from the O-methyltransferase from parsley [10], which has been purified and studied in detail. The spinach-beet enzyme is inactive towards luteolin and more active with caffeic acid than with any other substrate, having a much lower K_m for both caffeic acid and *S*-adenosyl-L-methionine than the parsley enzyme. Its optimum at pH 6.5 contrasts with that for the parsley

TABLE IV

PRODUCTS OF *S*-ADENOSYL-L-HOMOCYSTEINE CONVERSION WITH PARTIALLY-PURIFIED PREPARATION

S-Adenosyl-L-homocysteine was incubated as described in the Methods section, with 50 μl of fresh (+) or boiled (B) partially-purified enzyme preparation (3.6 mg protein/ml), in the absence (−) or presence of fresh (+) or boiled (B) adenosine deaminase (1 unit).

Enzymes included		Products formed (nmol/h/mg protein)			Total *S*-adenosyl L-homocysteine converted (nmol/h/mg protein)
Spinach-beet preparation	Adenosine deaminase	Inosine	Adenine	Adenosine	
+	−	4.8	116.7	2.8	124.3
B	−	2.3	0	0.1	2.4
+	+	232.0	7.1	0.9	240.0
B	+	4.2	0	0	4.2
+	B	1.2	113.2	3.9	118.3
B	B	0	0	0	0

enzyme at pH 9.5, and it shows no requirement for Mg^{2+}.

It is suggested that the potential interference of *S*-adenosyl-L-homocysteine in the methylation of caffeic acid, of which it is a highly effective competitive inhibitor, may be avoided in vivo through its hydrolysis by *S*-adenosyl-L-homocysteine hydrolase, whose "synthetase" and "hydrolase" activities have been detected in crude extracts of spinach-beet leaves. The hydrolysis of *S*-adenosyl-L-homocysteine by this enzyme must however be coupled with reactions, which dispose of either adenosine or homocysteine or both. In these experiments, this was achieved by adenosine nucleosidase, which has been partially purified from plant tissues [33], but other reactions in which ATP and *S*-adenosyl-L-methionine are regenerated from adenosine and homocysteine, respectively, may be equally important.

The demonstration and characterization of these enzymes and the further purification of *S*-adenosyl-L-homocysteine hydrolase are currently being investigated in this laboratory in order to determine whether the "synthetase" or the "hydrolase" activity of *S*-adenosyl-L-homocysteine hydrolase predominates in vivo.

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