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PURIFICATION AND PROPERTIES OF CAFFEIC ACID *O*-METHYLTRANSFERASE FROM ALFALFA ROOT NODULES*

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Abstract—An *O*-methyltransferase which catalyses the methylation of caffeic acid to ferulic acid using *S*-adenosyl-L-methionine as methyl donor has been isolated and purified *ca* 70-fold from root nodules of alfalfa. The enzyme also catalysed the methylation of 5-hydroxyferulic acid. Chromatography on 1,6-diaminohexane agarose (AH-Sepharose-4B) linked with *S*-adenosyl-L-homocysteine (SAH) gave 35% recovery of enzyme activity. The K_m values for caffeic acid and *S*-adenosyl-L-methionine were 58 and 4.1 μM, respectively. *S*-Adenosyl-L-homocysteine was a potent competitive inhibitor of *S*-adenosyl-L-methionine with a K_i of 0.44 μM. The MW of the enzyme was *ca* 103 000 determined by gel filtration chromatography.

INTRODUCTION

Several studies have reported the role and regulation of *O*-methyltransferase (OMT) in the biosynthesis of phenolic compounds in plants [1–4], particularly with respect to the importance of caffeic acid OMT (COMT) in ferulic acid biosynthesis [5–7]. It has been shown that COMT catalyses the formation of ferulic acid from caffeic acid in both angiosperms and gymnosperms [3, 5–8].

Ferulic acid in plants can function in many physiological processes, the best documented being the involvement of ferulic acid in lignin biosynthesis [2, 5, 9]. Fausch *et al.* [10] suggested that feruloyl side-chains endow some soluble polysaccharides with the property of forming gels upon oxidation with peroxidase/H_2O_2. Ferulic acid has been implicated in IAA turnover rates via regulation of IAA oxidase activity [11]. Fry [12] implicated ferulic acid cross-linking with cell wall matrix polysaccharides in regulating cell wall growth.

A previous study of alfalfa [13] showed COMT activity to be much higher in nodules than in roots. Ferulic acid was suggested to be involved in root nodule morphogenesis through regulation of IAA oxidase. The present investigation describes the purification and some properties of the COMT found in alfalfa root nodules.

RESULTS AND DISCUSSION

Alfalfa (*Medicago sativa*) root nodule COMT was purified 68-fold with a yield of 35% with respect to the crude homogenate (Table 1). Affinity chromatography on AH-Sepharose-4B linked with SAH was effective in purifying the enzyme. Only one peak of enzyme activity

Table 1. Purification of *O*-methyltransferase from *Medicago sativa* nodules

Purification step	Protein (mg)	Specific* activity (pkat/mg protein)	Purification (-fold)	Recovery (%)
1. Crude	93	0.43	0	100
2. Dowex-1 extract	84	0.47	1.1	98
3. $(NH_4)_2SO_4$, 45–65%	21	1.54	3.7	81
4. DEAE-cellulose	2.7	6.01	14.0	41
5. *S*-Adenosylhomocysteine affinity	0.5	28.4	68.0	35

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was found after DEAE-cellulose and affinity chromatography. Polyacrylamide disc gel electrophoresis of the concentrated preparation from affinity chromatography gave only one band of COMT activity. A single major and one faint minor band of protein was resolved when the affinity chromatography preparation was subjected to SDS polyacrylamide electrophoresis.

The MW of alfalfa nodule COMT was *ca* 103 000 as estimated from the elution volume on a calibrated P-150 column. This coincides closely with the MW reported for *Cicer arietinum* isoflavone OMT [8] but is greater than the value obtained for OMT from *Pinus thunbergii* [5].

The apparent K_m of the purified enzyme for *S*-adenosyl-L-methionine (SAM) was 4.1 μM when assayed at pH 7 in the presence of saturating conditions of caffeic acid (4 mM). The K_m for the methylation of caffeic acid with the purified enzyme was 58 μM under optimum conditions. The V_{max} value for caffeic acid was 0.33 pkat while that for *S*-adenosyl-L-methionine was 0.14 pkat. Lineweaver–Burk plots of data, obtained when caffeic acid methylation was allowed to proceed at three different concentrations *S*-adenosylhomocysteine (SAH), showed that this SAM analogue is a potent competitive inhibitor of SAM, with an apparent K_i of 0.44 μM.

The kinetic constants for alfalfa nodule COMT are in general agreement with those reported for other plants [5–7]. However, the K_i for SAH which we obtained (0.44 μM) is an order of magnitude less than that reported for spinach (4.4 μM) [6] and soybean (6–9 μM) [7]. The K_i data show that COMT from alfalfa nodules is extremely sensitive to the presence of SAH. This suggests that COMT activity within the nodule may be regulated by SAM/SAH ratios and that the nodule may have a mechanism to convert SAH to other products. The enzymic hydrolysis of SAH has been implicated in the regulation of COMT in spinach [6].

The pH optimum for alfalfa nodule COMT activity was *ca* 7 with over half maximum activity retained between pH 6.5–8.

Relative rates of methylation of various phenolic substrates were caffeic acid (100%), 5-hydroxyferulic acid (70%) protocatechuic acid (5%) daidzein (3%), pyrogallol (2%), genistein (2%), *p*-hydroxybenzoic acid (0%) catechol (0%) and *p*-coumaric acid (0%). These data show that the enzyme preferentially methylates caffeic and 5-hydroxyferulic acid, like the COMT in other angiosperms [3, 6, 7], and that the enzyme is probably involved in lignin biosynthesis in the nodule.

EXPERIMENTAL

Plant material. Field and greenhouse-grown alfalfa (*M. sativa* L. Saranac) plants were dug 65–75 days after emerging from the soil. Nodules and roots were separated by hand and either stored on ice for immediate use or frozen and stored at −20° for later use.

Purification was conducted at 4° and all buffers contained 28 mM mercaptoethanol. Nodules (10–12 g) were homogenized in the presence of Polyclar AT (0.1 g/g tissue) and extracted with 0.1 M Tris–HCl buffer (3 ml/g tissue), pH 7.5. The resultant slurry was centrifuged at 12 000 *g* for 15 min and the pellet discarded. The supernatant (30–35 ml) was stirred for 30 min with 3 g Dowex 1 − × 2 (Cl^- form equilibrated with extraction buffer) and the resin removed by filtration through glass wool. Solid $(NH_4)_2SO_4$ was added to the supernatant (26–30 ml) and the protein fraction that pptd between 45 and 65% satn was collected by centrifugation at 12 000 *g* for 20 min. The ppt. was dissolved in 8–10 ml 0.1 M Tris–HCl, pH 7.5 and the prepn was desalted by dialysis overnight against 3 changes of buffer. Desalted enzyme soln was applied to a DEAE-cellulose column (2 × 10 cm) and eluted by a discontinuous gradient of Tris–HCl buffer, pH 7.5, from 0.15 to 0.25 M in 0.5 M increments. COMT assays of fractions showed peak activity between 0.22 and 0.25 M. Fractions with highest activity were pooled and dialysed overnight in 0.1 M Tris–HCl buffer, pH 7.5, containing 15% ethylene glycol, added to stabilize the purified enzyme. The dialysed prepn was further purified by ligand affinity chromatography on AH-Sepharose-4B linked with SAH. Ligand affinity gel was prepared according to ref. [14] and the active enzyme was eluted with a discontinuous pH gradient from pH 7.5 to 5.5 in 0.5 pH unit increments of 0.1 M K-Pi buffer containing 0.2 M NaCl and 15% ethylene glycol. Assays of fractions for COMT activity showed one major peak of activity eluting between pH 6.0 and 6.5.

COMT assays and electrophoresis. The reaction mixture (total vol. 1 ml) contained 0.2 ml enzyme soln, 1 μmol $MgCl_2$, 2 μmol caffeic acid, 4 μmol ascorbic acid, 0.1 μCi [Me-^{14}C]SAM (50 mCi/mM) in 0.1 M Tris–HCl buffer, pH 7.5. Assays were incubated for 0.5 hr at 30° and extraction and measurement of radioactive product were according to refs. [2, 8]. Relative rates of methylation were measured by incubating the DEAE-cellulose enzyme fraction in the presence of phenolic substrates under optimum conditions. The rate obtained with caffeic acid was designated 100%. Disc gel electrophoresis was performed in polyacrylamide gels according to ref. [15]. SDS polyacrylamide electrophoresis was carried out according to ref. [16].

MW of COMT. The MW was estimated by determination of eultion vol. on a calibrated P-150 column (2 × 60 cm; flow rate 7 ml/hr; 0.1 M Tris–HCl buffer, pH 7.5), using the enzyme prepn from step 4 in Table 1.

General methods. Protein was measured by the method of refs. [17, 18]. The radioactive product from alfalfa nodule COMT was identified as ferulic acid according to ref. [13].

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