Phytochemistry, 1974, Vol. 13, pp. 1719 to 1725. Pergamon Press. Printed in England.

CATECHOL *O*-METHYLTRANSFERASES IN PAMPAS GRASS: DIFFERENTIATION OF *m*- AND *p*-METHYLATING ACTIVITIES*

BERNARD J. FINKLE and STANLEY H. KELLY

Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, CA 94710, U.S.A.

(*Revised Received* 6 *February* 1974)

Key Word Index—*Cortaderia selloana*; Gramineae; pampas grass; catechol *O*-methyltransferases; hydroxycinnamic acids; hydroxybenzoic acids.

Abstract—Partially purified catechol *O*-methyltransferase from pampas grass (*Cortaderia selloana*) catalyzes the methylations of substrates at both their *meta* and *para* positions. This capability was shown, by heat treatments, to arise from a less stable *m*-*O*-methyl-transferring activity and a more stable *p*-*O*-methyltransferring activity, tested against protocatechuic acid. When acting upon caffeic acid, the preparation catalyzes a reaction of solely *m*-*O*-methyltransfer (in contrast to the mixed methylation of this substrate exhibited by rat liver catechol *O*-methyltransferase). A small degree of *m*-*O*-methylation of monophenolic substrates also occurs.

INTRODUCTION

CONSTITUENTS as diverse as the lignin of wood, the anthocyanin flower pigments, and *O*-methylated catechol acids, that may be involved in metabolic regulation,[1] are methylated by catechol *O*-methyltransferase.[2,3] Some degree of characterization of the enzyme was early reported,[2,4] and the importance of its purification became evident when, as reported here, two distinguishable enzyme activities—namely *meta*- and *p*-*O*-methylation—were found in enzyme preparations from pampas grass (*Cortaderia selloana*).

With caffeic acid (3,4-dihydroxycinnamic acid) as substrate, methylation occurred where it most usually occurs in natural constituents at the hydroxyl group *meta* to the side chain. This *m*-*O*-methyltransferase activity has since been found (or in some cases assumed, without positive distinction being made between ferulic and isoferulic as product) in several organs of many plants[4–9] and in cell cultures of parsley.[10]

* Part VIII in the series, Enzyme Reactions with Phenolic Compounds. For Part VII see Finkle, B. J., Nozaki, M. and Fujisawa, H. (1971) *Phytochemistry* **10**, 235. Preliminary reports of portions of this work have appeared.[3]

[1] FINKLE, B. J. (1967) *Phenolic Compounds and Metabolic Regulation* (FINKLE, B. J. and RUNECKLES, V. C., eds.), p. 9, Appleton-Century-Crofts, New York.
[2] FINKLE, B. J. and NELSON, R. F. (1963) *Biochim. Biophys. Acta* **78**, 747.
[3] FINKLE, B. J. and KELLY, S. H. (1970) *Plant Physiol.* **46**, 43; (1971) *Fed. Proc.* **30**, 1305.
[4] FINKLE, B. J. and MASRI, M. S. (1964) *Biochim. Biophys. Acta* **85**, 167.
[5] HESS, D. (1964) *Z. Naturforsch.* **19b**, 447.
[6] GLASS, A. D. M. and BOHM, B. A. (1972) *Phytochemistry* **11**, 2195.
[7] SHIMADA, M., OHASHI, H. and HIGUCHI, T. (1970) *Phytochemistry* **9**, 2463.
[8] SHIMADA, M., FUSHIKI, H. and HIGUCHI, T. (1972) *J. Japan Wood Res. Soc.* **18**, 43.
[9] MANSELL, R. L. and SEDER, J. A. (1971) *Phytochemistry* **10**, 2043.
[10] EBEL, J., HAHLBROCK, K. and GRISEBACH, H. (1972) *Biochim. Biophys. Acta* **268**, 313.

Yet an additional action, *p*-*O*-methylation of catecholic substrates (other than caffeic acid), has been reported in pampas grass,[4] in *Nerine bowdenii*,[11] and in peyote,[12] an activity required for the synthesis of compounds such as hesperidin and some alkaloids. When the pampas grass enzyme was tested against several substrates, the *m*:*p* ratio of methylation was found to vary among them.

This paper describes a study of the *O*-methyltransferases in pampas grass to resolve whether the activities arise from a single enzyme having both *m* and *p* capabilities, or from a mixture of *O*-methyltransferases, each one specific for a given ring position. However, the usual procedure of separation and purification carried out on the initially stable preparation led to enzyme instability after only a few steps. Data are presented that, while they cannot be considered as proof of the physical presence of separate enzymes, do suggest this, particularly in showing a greater stability of the *p*-*O*-methylating activity.

TABLE 1. SPECIFIC ACTIVITIES OF RECRYSTALLIZED FERULIC AND ISOFERULIC ACIDS*

Number of recrystallizations	Pampas grass enzyme		Rat liver enzyme	
	Ferulic (cpm/mg)	Isoferulic (cpm/mg)	Ferulic (cpm/mg)	Isoferulic (cpm/mg)
0	2760	2770	3090	3270
1		945	2740	1650
2	2640	401	2490	1390
3	3180	171	2420	1125
4	3040	69	2220	1130
5		32		1225
8		12		
9		9		
Sp. act. comparison (%)				
Terminal/original	110	0·3	72	38

* Conditions of enzymic formation of the [*Me*-^{14}C] products and of determination of radioactivity are described in the text. A portion of the radioactive product was mixed with 100 mg of ferulic or isoferulic and recrystallized repeatedly from hot water. After each recrystallization a weighed portion of the dried product was removed for scintillation counting.

RESULTS

Ferulic acid as methylation product from caffeic acid

In order to define the main products from caffeic acid methylation, two 4·0 ml portions of the ether-extracted pampas grass [*Me*-^{14}C] reaction product were dissolved in 10·0 ml ethanol, as described in the Experimental. In one portion 100 mg ferulic were dissolved, in the other portion 100 mg isoferulic. Each portion was evaporated to dryness in N_2, recrystallized, washed, dried and a few milligrams weighed out for scintillation counting. The effects on specific activity of serial recrystallizations of each portion of pampas grass product are shown in Table 1. Methylation took place entirely at the *m* position (>99·7% of the *O*-methylating reaction) to yield ferulic, as had been similarly indicated for the enzyme obtained from apple.[2] This same methylating specificity was confirmed by rechromatographing extracts of each of the ferulic and isoferulic areas of a thin-layer chromatogram of the original extracted reaction product, with the addition of both acids as markers.

[11] MANN, J. D., FALES, H. M. and MUDD, S. H. (1963) *J. Biol. Chem.* **238**, 3820.
[12] BASMADJIAN, G. P. and PAUL, A. G. (1971) *Lloydia* **34**, 91.

Scans showed no trace of radioactive isoferulic from either rechromatographed area. Activity in the combined extracts of the isoferulic spots was, by scintillation counting, less than 0·1% of that found for ferulic.

Results from the action on caffeic acid of catechol *O*-methyltransferase obtained from rat liver, reacting under similar conditions are also shown in Table 1. The rat liver preparation was carried through the 0·5 saturated ammonium sulfate step (dialyzed) of Axelrod and Tomchick.[13] The liver enzyme products, recrystallized to constant specific activity, were ferulic and isoferulic acids, with the *m*:*p* ratio of about 13:7.

Products from protocatechuic acid

O-methylation of protocatechuic acid by pampas grass preparations was confirmed[4] and found to proceed at about 1/10 the rate for caffeic acid (see below). It was also confirmed that, unlike caffeic, protocatechuic acid is methylated at both its hydroxyls. Separation of the two products vanillic and isovanillic acids by long-bed chromatography was demonstrated in a previous publication.[14]

TABLE 2. DIFFERENTIATION OF *O*-METHYLATING ACTIVITIES*

Time at 45° (min)	Activity in vanillic (cpm)	Activity in isovanillic (cpm)	Fraction of total in vanillic
0	2350	5489	0·30
	3711	4926	0·43
10	1334	4574	0·23
	1683	4634	0·27
20	805	4491	0·15
	921	5801	0·14

* Portions of enzyme were heated at 45° for the time intervals shown, then incubated 6 hr with protocatechuic acid and [^{14}C]*S*-adenosyl-methionine. Radioactivity of the extracted and chromatographed products was determined by scintillation counting as described in the text.

Detection of m- *and* p-O-*methyltransferases*

During assays of the products from protocatechuic acid, the ratio of vanillic to isovanillic varied with incubation time at 37° (see ref. 14 Fig. 2, where 6 and 24 hr incubations are represented by the upper and lower curves, respectively), indicating that two methylating enzymes might be involved. The same conclusion was also suggested by purification steps (unpublished) which resulted in a preferential loss of *m*-methylating activity.

Heat stability trials on a dialyzed enzyme preparation (0·3–0·5 saturated ammonium sulfate fraction, in 0·001 M phosphate buffer, pH 7·4) indicated 50° for 10 min as giving about half-activity. When portions of enzyme were heated in a 45° bath for intervals as shown in Table 2, then incubated with protocatechuic acid in the presence of [^{14}C]*S*-adenosyl-methionine as methyl donor, the results on product formation showed that heat treatment produces a large differential decrease in *m*-methylating activity. In another experiment, heating at 50° for 20 min reduced the amount of vanillic formed to a trace, while the amount of isovanillic was reduced by less than 40%. These findings thus indicate the presence of a less stable *m*-*O*-methyltransferring activity in conjunction with a more stable *p*-*O*-methyltransferring activity.

[13] AXELROD, J. and TOMCHICK, R. (1958) *J. Biol. Chem.* **233**, 702.
[14] KELLY, S. H. and FINKLE, B. J. (1971) *J. Chromatog.* **63**, 438.

Stability of [Me-^{14}C] *vanillic acid and* [Me-^{14}C] *isovanillic acid*

Although the presence of two methylating enzymes is very likely, the ratio of products found could, however, also have been influenced by any further reactions, enzymic or nonenzymic, that would degrade the products formed. Such reactions have been observed in crude animal tissue preparations.[15] We designed an experiment to determine if the above products, once formed, are subject to degradation, transmethylation, or other changes that might affect the *m:p* ratio. Particularly in question was the stability of vanillic in the system, since there always appeared a decrease in the vanillic–isovanillic ratio during extended incubation periods.

The stabilities of vanillic and isovanillic in the incubation system (enzyme: 0·3–0·5 saturated ammonium sulfate fraction) were tested. [*Me*-^{14}C]vanillic at a concentration of 1·3 μM but of 15·4-fold greater specific activity than that of other radioactive compounds present in routine assays was used so as to augment radiochemical detection of degradation products that might be formed in small amount. Incubation was with the usual combination of assay reagents, except that nonradioactive *S*-adenosylmethionine and no protocatechuic acid was used. After incubation periods of 2 and 6 hr, the mixtures were acidified, extracted with ether, and chromatographed with added known acids added to each sample, after which the appropriate regions were scraped off, the scrapings were extracted, and then counted in the scintillation spectrometer. In spite of inaccuracies introduced by an involved methodology used on material of low total activity, the results of two separate, identical experiments clearly showed no significant loss of radioactivity from vanillic after incubation with enzyme for 2 hr (786 and 570 cpm in the two experiments, respectively) or 6 hr (769 and 605 cpm, respectively). Omission of enzyme gave the same results. No other radioactive material separated on the chromatograms. Counts scraped from the isovanillic region of these chromatograms yielded a total of only 7–14 cpm (enzyme present). After 2 hr or 6 hr of incubation vanillic remained neither degraded nor isomerized by exposure to the enzyme extract. Likewise, tests with high specific activity [*Me*-^{14}C]isovanillic in the mixture showed no significant degradation or transmethylation. These findings confirm that the *O*-methylated products are stable.

Reaction with monophenols

In order to see if di-*O*-methylation of a catechol could occur with these enzymes, methylation of several monophenolic compounds was tested using a 0·3–0·6 saturated ammonium sulfate enzyme fraction. Table 3 shows that some of the monophenols are active and indicates the relative rates of activity of the enzyme preparation against such substrates and against caffeic (designated as 100% activity) and protocatechuic acids. *m*-Coumaric acid (3-hydroxycinnamic acid) and isoferulic, contrary to findings on a parsley enzyme,[10] were methylated (although at a lower rate than caffeic acid). However, no methylation of coumaric acid and ferulic was observed. A confirmatory experiment was run in which [α-^{14}C]ferulic (0·061 mCi/mmol) and [α-^{14}C]isoferulic (0·116 mCi/mmol), labeled in the side-chain to avoid loss of label in case the methoxyl group was exchanged out, were used as substrates (0·06 mM in these experiments). On chromatography of the samples containing [α-^{14}C]ferulic, the well-separated region of the possible reaction product dimethoxycinnamic acid showed no radioactivity. By contrast, under the same conditions, *O*-methylation of [α-^{14}C]isoferulic at its free *meta*-hydroxyl group yielded over 600 cpm

[15] FRÉRE, J. M. and VERLY, W. G. (1971) *Biochim. Biophys. Acta* **235**, 73, 85.

of dimethoxycinnamic acid, the formation of this product being confirmed by co-chromatography.

During determinations of the blank samples in Table 3 (enzyme omitted; also substrate omitted), it was found that the particular substrate affected the degree of extraction of radioactive compounds during assay. This resulted in some variation in blanks that was taken into account as footnoted in the table. Other monophenolic compounds tested but not shown in the table (namely, *m*- and *p*-hydroxybenzoic, vanillic and isovanillic) were also tested as substrates. These compounds yielded insufficient radioactivity of samples (coupled with the described variation in blanks) to derive conclusions as to their methylation. A lowered degree of activity of these substrates might, indeed, have been expected by analogy (based on the lesser activity of protocatechuic acid as compared to that of caffeic acid, see Table 3).

TABLE 3. RELATIVE ACTIVITIES OF *O*-METHYLTRANSFER TO PHENOLIC SUBSTRATES*

	Net activity			
	2 hr		6 hr	
Compound	(cpm)	(%)	(cpm)	(%)
Caffeic acid	8354	100·0	19009	100·0
Ferulic acid	—†		—	
p-Coumaric acid	—		—	
Isoferulic acid	1474	18	3805	20
m-Coumaric acid	937	11	2439	13
Protocatechuic acid	1156	14	2965	16

* Conditions of determination of activity are described in the text. Blanks were run both with enzyme omitted and substrate omitted, to reconcile a substrate-related variability in the ether extractions.

† Activities of less than 3% not included because of substrate-related variability of blanks.

DISCUSSION

Although catechol *O*-methyltransferase preparations from pampas grass or other whole plant tissues[7] have not lent themselves to extensive purification, our results support the presence of two types of catechol *O*-methyltransferase activity in pampas grass, of different specificities toward the position of *O*-methylation. One of these, more labile, attacks substrates at the *m* position with respect to the side chain and is particularly active toward caffeic acid. The other, more stable, attacks the *p* position of many of the same substrates (e.g. protocatechuic acid), thus offering control of the alternative possibilities of either *m*- or *p*-*O*-methylated products; but no *p*-methylating activity takes place when caffeic acid is the substrate. Although attempts to separate and purify the *m* and *p* activities failed due to lability of the *m* enzyme activity, a distinction was achieved through their differential inactivation even at near-ambient temperatures. The observed distinction is most directly interpreted as the operation of two separate enzymes, catechol *m*-*O*-methyltransferase and catechol *p*-*O*-methyltransferase. However, the possibility must also be entertained that some temperature modification of a single enzyme, such as a conformational change or the degradation of a position-orienting co-factor could give the observed effect. The apparent presence of two specialized activities here appears as a different situation from that of the animal liver catechol *O*-methyltransferase (*S*-adenoxylmethionine:catechol *O*-methyltransferase, E.C. 2.1.1.6). Liver enzyme has been taken through several treatments beyond the crude extract, with unvarying *m*:*p* methylation ratio after successive steps, for given sub-

strate conditions.[15,16] Before purification, however, assay mixtures containing crude liver extract did show a varying ratio of *m* and *p* products which was demonstrated as being due to degradation of methylation products.[15] Examinations of pampas grass preparations for degradation of *m*- or *p*-*O*-methylated products proved negative, thus further supporting the suggestion that separate, specific enzymes are involved. Again, unlike the liver enzyme, pampas grass *O*-methyltransferase preparations show no activity toward the *p* position when caffeic acid is the substrate (see Table 1).

The exclusively *m*-methylation of caffeic acid by pampas grass enzyme preparations is consistent with the major role of this substrate in lignin biosynthesis.[17,21] *m*-*O*-Methylated catechol units also appear widely in plant constituents other than lignin, thus offering a widespread function for a catechol *m*-*O*-methyltransferase. The *in vitro* participation of such an enzyme in forming flavonoids has been described.[4,10,22]

At the same time the presence of a *p*-*O*-methyltransferase active on some catechol substrates would make possible the alternative, if rarer, controlled formation of *p*-*O*-methylated compounds in the same plant, hesperidin (in citrus fruits) for example, and several alkaloids. A catechol *p*-*O*-methylating enzyme active in *Nerine bowdenii* conforms to the presence there of *p*-*O*-methylated alkaloids.[11]

The finding of phenol *O*-methyltransferase activity in the preparation could account for the biosynthesis both of mono-*O*-methylated phenols and di-*O*-methylated catechols. The solely *m*-methylation toward monohydroxycinnamic acids observed in pampas grass preparations may be a result of these substrates being specifically hydroxycinnamic acids. The observed attack on *m*-monophenols may be by the same enzyme that, at a greater rate, attacks catechols, or alternatively, may represent the activity of a separate monophenol *m*-*O*-methyltransferase. Heating experiments on pampas grass preparations suggest that a single enzyme acts both upon *m*-monophenols and catechols, but resolution of the question will have to await further purification steps.

EXPERIMENTAL

Extraction of enzyme. Vegetative stalks of pampas grass were gathered during March to August by cutting just above the base. The mature, dark green portions of leaves were trimmed away and the light-colored, more succulent portions of stalk were stored in plastic bags at −34°.

The highly fibrous grass is best cut and ground in the frozen state. Extraction was accomplished with a Servall Omnimixer, an Acme Juicer, or, for multi-kilogram extractions, a stainless steel Fitz mill with blades set in the shearing position. For the latter, stalks of grass were embedded in buffer and frozen to −34° before being ground in the prechilled mill. Several passes of the fibrous, icy mush through the mill gave adequate recovery of activity. The wt 0·05 M $NaHCO_3$–0·1% ascorbic acid buffer used for extraction was about 1·5× the fr. wt of pampas grass. $(NH_4)_2SO_4$ was added to the strained extract to give a 0·3 saturated soln. This mixture, or the supernatant after removal of the ppt, is stable in the refrigerator or for many months in the frozen state. The enzyme was also stable when stored frozen as a filtered ppt after further precipitation with 0·6 saturated $(NH_4)_2SO_4$. In some experiments the enzyme was carried through a second (0·3–0·5 saturated) $(NH_4)_2SO_4$ precipitation step.

Measurement of enzyme activity. The reaction mixture consisted of 6·25 mM substrate, 2·5 mM $MgCl_2$, 14 mM ascorbic acid, 0·2 mM EDTA, 250 mM potassium phosphate buffer, and an aliquot of enzyme of rate limiting

[16] BALL, P., KNUPPEN, R. and BREUER, H. (1971) *European J. Biochem.* **21**, 517.
[17] BROWN, S. A. (1964) *Biochemistry of Phenolic Compounds* (HARBORNE, J. B., ed.), pp. 368–371 Academic Press, London.
[18] BYERRUM, R. U., FLOKSTRA, J. H., DEWEY, L. J. and BALL, C. D. (1954) *J. Biol. Chem.* **210**, 633.
[19] SHIMADA, M., FUSHIKI, H. and HIGUCHI, T. (1972) *Phytochemistry* **11**, 2247.
[20] BRAUNS, F. E. and BRAUNS, D. A. (1960) *The Chemistry of Lignin*, Suppl. Vol., p. 157 (Table 11), Academic Press, New York.
[21] BRAUNS, F. E. and BRAUNS, D. A. (1960) *The Chemistry of Lignin*, Suppl. Vol., p. 14 (Table 6), Academic Press, New York.
[22] HESS, D. (1966) *Z. Pflanzenphysiol.* **55**, 374.

amount (unpublished data), to which was added [*Me*-^{14}C]*S*-adenosylmethionine (as the iodide) (0·125 mCi/mmol, 2·0 mM final concentration) to start the reaction (final vol., 0·5 ml; pH 7·6). After an incubation period at 37°, 0·2 ml 1 M HCl was added and the mixture was extracted with ether. A 5·0 ml aliquot of ether was added to the sample, the mixture was swirled for 10 sec in a Vortex mixer, then after 15 min 4·0 ml of extract was withdrawn into a counting vial. With substrates other than caffeic acid (notably protocatechuic acid and monophenols) three additional extractions with 5·0 ml ether were made. The ether extract was evaporated to dryness, after which its radioactive components could be determined directly by scintillation counting[23] or transferred onto TLC strips (see below) for separation of components. Radioactivity on the chromatogram was determined with a Packard Chromatogram Scanner, with platform modified for handling long strips. More precise data were obtained by scraping off the separated radioactive areas into counting vials for scintillation counting. Blanks on the assay were ordinarily run with enzyme omitted.

Chromatography. For the difficult separation of methylated isomers, a method of near-horizontal long-bed TLC was developed.[14] The extracted sample was transferred onto a glass strip coated with micro-crystalline cellulose and developed in toluene–HOAc–H_2O (125:72:3) for 8–48 hr.

Side-chain labeled ferulic and isoferulic acids. [α-^{14}C]Ferulic and [α-^{14}C]isoferulic were synthesized from vanillin and isovanillin, respectively, and [2-^{14}C]malonic acid following the method of Vorsatz.[24]

Enzymatic ^{14}C-O-methylation of caffeic acid for product identification. A portion of $(NH_4)_2SO_4$ (0·03–0·6 saturated) precipitated enzyme was dissolved in 0·2 M potassium phosphate buffer (pH 7·3) and dialyzed in several changes of 0·1 M buffer. To a soln containing 2·0 mg caffeic acid in 0·5 ml of 1 M buffer, and 0·1 ml of 0·1 M $NaHCO_3$, 0·5 ml 0·1 M $MgCl_2$, 0·2 ml 1% ascorbic acid, and 0·6 ml H_2O was added 0·9 ml dialyzed enzyme and finally 50 μl of 0·08 M [*Me*-^{14}C]*S*-adenosyl-methionine (1·8 μc). After holding at 37° overnight, the mixture containing radioactive methylated products was made acid with HCl and extracted repeatedly with ether, after which the ether extractables were dried and dissolved in 10·0 ml ethanol.

Formation and purification of [Me-^{14}C]*vanillic and isovanillic acids.* [*Me*-^{14}C]Vanillic and [*Me*-^{14}C]isovanillic were prepared by enzymatic ^{14}C-*O*-methylation of protocatechuic acid using pampas grass catechol *O*-methyltransferase (0·3–0·5 satd. $(NH_4)_2SO_4$ fraction, dialyzed) under conditions similar to those of enzyme assay but using [^{14}C]*S*-adenosylmethionine of specific activity 1·93 mCi/mmol, 15·4-fold greater than that of a routine assay. In this way, radioctive vanillic and isovanillic were produced of high enough activity to allow their later use in detecting degradation products under conditions simulating normal assay runs. Reaction mixtures were incubated for 6 hr, then acidified and extracted. The combined extracts were chromatographed on several TLC strips with no added vanillic or isovanillic carrier. The separated products, detected with the Packard Scanner, were scraped off, extracted with 0·5 ml of ethanol followed by 2 ml of ether repeated several times, the extracts were evaporated to dryness, and stored at −20°. Chromatograms of aliquots of either purified product (300–700 cpm) yielded no significant activity at the position of the other isomer (<5%, by scintillation counts of extracts of scrapings).

Synthetic nonradioactive phenolic acids used as substrates were kindly supplied by Dr. J. Corse of this laboratory. Other compounds were from commercial suppliers.

Acknowledgement—We thank Dr. M. S. Masri for a preparation of catechol *O*-methyltransferase from rat liver.

[23] Loewus, F. A. (1961) *Intern. J. Appl. Radioat. Isot.* **12**, 6.
[24] Vorstaz, F. (1936) *J. Prakt. Chem.* **145** (n.f.), 265.