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EXTRACTION OF NITRATE REDUCTASE FROM LEAVES OF ERICACEAE

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Key Word Index—*Leucothoe catesbaei*; *Rhododendron catawbiense*; Ericaceae; nitrate reductase; enzyme inactivation; tannic acid.

Abstract—The inability to obtain active nitrate reductase (E.C. 1.6.6.2) preparations from leaf extracts of *Leucothoe* and *Rhododendron* (Ericaceae) was due to a galloyl ester-like compound, similar in nature to tannic acid.

INTRODUCTION

THE ABILITY to extract enzymes in the active state from plant tissues depends on the methodology employed.[1–7] Homogenization results in the mixing of cytoplasmic and vacuolar contents, and, may therefore cause enzyme inactivation[8–11] often attributable to phenolic compounds present in the vacuole.[3,8,11,12] Members of the Ericaceae family contain a wide spectrum of simple phenolic compounds and condensed or hydrolyzable tannins[13–15] which could reduce the recovery of active enzymes.

The inability to demonstrate nitrate reductase (E.C. 1.6.6.2.) activity from leaves of lowbush blueberry[16] (*Vaccinium angustifolium*, Ait.) and cranberry[10] (*Vaccinium macrocarpon*, Ait.) has led some investigators to propose that a nitrate reducing system is absent in the leaves of the Ericaceae. Recently,[17] nitrate reductase activity has been shown in the leaves

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and roots of *Leucothoe catesbaei*, Gray, *Rhododendron catawbiense*, Michx., highbush blueberry (*V. corymbosum*, L.), and several other ericaceous plants when an *in vivo* technique[18] was used, but not when *in vitro* assays[19–21] were employed.

The present work is concerned with the characterization of the compounds which inactivate nitrate reductase extracted from leaves of ericaceous plants.

RESULTS AND DISCUSSION

Enzyme Inhibition by Leaf Extracts of Ericaceae

Initial work indicated that nitrate reductase, glutamate dehydrogenase (E.C. 1.4.1.2), and malate dehydrogenase (EC 1.1.1.37) could not be detected in simple extracts of leaves of *Leucothoe* and *Rhododendron*. Modifications of the extraction process, including the use of polyvinylpyrrolidone (PVP, Polyclar-AT), ascorbate, reducing agents, different buffers during homogenization, or dialysis after homogenization, afforded no protection against inactivation of the enzymes. These enzymes were quite active in extracts of corn (*Zea mays indentata*, Bailey) leaves homogenized with no modifications of the extraction procedure (Table 1). Extracts of *Leucothoe* (0·2 ml) and *Rhododendron* (0·05 ml) leaves added to corn extracts (0·5 ml) completely inhibited the three enzymes.

TABLE 1. ENZYMATIC ACTIVITY IN LEAVES OF SELECTED SPECIES ASSAYED FOLLOWING SIMPLE HOMOGENIZATION[19–21]

Enzyme	Species	
	Zea mays	Ericaceous plants*
Nitrate reductase (μmol NO_2^- produced/g fr. wt/hr)	2·24	0
Glutamate dehydrogenase (mmol NADH oxidized/g. fr. wt/hr)	2·34	0
Malate dehydrogenase (mmol NADH oxidized/g fr. wt/hr)	153·6	0

* For nitrate reductase: *Leucothoe catesbaei*, Gray; *Rhododendron catawbiense*, Michx., cv. Roseum Elegans; *R. carolinianum*, Redh.; *Kalmia latifolia*, L ; *Oxydendrum arboreum*, DC , *Pieris japonica*, D. Don; *Arctostaphylos uva-ursi*, Spreng, and *Vaccinium corymbosum*, L. For glutamate and malate dehydrogenase. *Leucothoe catesbaei*, Gray and *Rhododendron catawbiense*, Michx., cv. Roseum Elegans

Since the inhibitors were easily extracted and nonspecific in enzyme inactivation (Table 1), it appeared that they might be phenolic compounds.[3] Three successive treatments of the ericaceous plant extracts with Polycar-AT reduced enzyme inhibition to *ca.* 20%. The phenolic content of the extracts was reduced 25-fold by treatment with Polyclar-AT.[22]

Boiling or dialysis of the extracts for 24 or 48 hr against distilled water did not remove the inhibitory effect from the extracts. These results indicated that the inhibitor was macro-

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molecular but not proteinaceous; thus, if phenolic, the compounds were probably a condensed or hydrolyzable tannin or similar compound.[23]

Acetone treatment of the extracts from *Leucothoe* and *Rhododendron* yielded a soluble fraction and a precipitate. In each case the soluble fraction inhibited nitrate reductase activity totally in corn leaf extracts, but the precipitate resuspended in buffer had no effect. Successive treatments of the soluble fraction with Polyclar-AT lowered the inhibition to 18 and 20 % for each species respectively. This value was similar to that obtained with fresh extracts likewise treated with Polyclar-AT. No protein was present in either the soluble fraction or in the precipitate indicating that during homogenization, the protein in extracts from the Ericaceae was precipitated, or eliminated in the pellet of the first centrifugation.

Enzyme Inhibition by Prepared Phenolic Compounds

Tannic acid at final concentrations of 0·85 or 0·085 g/l was totally inhibitory of nitrate reductase activity in extracts from corn leaves. Solutions of tannic acid dialyzed against distilled water for 24 or 48 hr or boiled gave complete inhibition of nitrate reductase activity in the extracts from corn leaves, but Polyclar-AT added to the tannic acid prevented the inhibition. Caffeic acid, catechin, chlorogenic acid, ferulic acid, hydroquinone, juglone, pyrogallol, quinic acid, rutin and sinapic acid added at 10^{-3} M to equal volumes of extracts from corn leaves were not inhibitory of nitrate reductase activity.

The characteristics of the inhibition of nitrate reductase by tannic acid, that is, its potency, high solubility, heat stability, resistance to dialysis, and complexation by Polyclar-AT, match those of the leaf extracts of the Ericaceae. Hulme and Jones[24] reported that tannic acid inhibited 'succinoxidase' reactions of apple peel mitochondria and that the inhibition was prevented by PVP. Tannic acid at 5×10^{-4} M was 90% inhibitory to the malic enzyme (malate: NAD(P) oxidoreductase, decarboxylating) of apple mitochondria, whereas chlorogenic acid stimulated its activity. Quercitin had little effect on 'succinoxidase' reactions.

Tannic acid is a mixture of gallic acid and various galloyl esters of glucose.[25] PC of tannic acid yielded two spots. R_fs were 0·33 and 0·54 for the *n*-butanol solvent mixture and 0·47 and 0·67 for the *sec*-butanol solvent mixture. Co-chromatography of *Leucothoe* and *Rhododendron* extracts with tannic acid gave spots with the same R_fs as tannic acid. These spots fluoresced in the blue–violet under long-wave UV light and gave positive colorimetric tests with ferric salts, KIO_3, and Gibbs' reagent.[26] After treatment with Polyclar-AT the plant extracts gave no spots which would fluoresce under the UV light or react with the colorimetric reagents.

Strips corresponding to the substances with the higher R_fs on the chromatogram of the extracts untreated with Polyclar-AT were eluted in ethanol. The eluates gave positive tests with the colorimetric reagents, but after evaporation to dryness and dissolving in water, only partially inhibited nitrate reductase activity in corn leaf extracts, perhaps because the concentration of inhibitor was low in the eluate. The inhibition was linear with amount of eluate, for 0·5 ml of eluate gave 20% reduction in the enzyme activity, 1·0 ml gave 40% and 1·5 gave 60%.

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Spectral analyses of eluates of the tannic acid and unknown spots at the higher R_f showed a broad absorption peak at 270–300 nm in each extract.

Corn leaf extracts prepared in the same way as the extracts from the ericaceous leaves and chromatographed in the same solvents showed no spots corresponding to tannic acid. No spots on these chromatograms fluoresced or reacted with the colorimetric reagents.

EXPERIMENTAL

Plant culture and preparation of leaf extracts. Leucothoe catesbaei, Gray, *Rhododendron catawbiense*, Michx., cv. Roseum Elegans, and *Zea mays indentata*, Bailey were grown in 2:2:1 soil, peat, perlite mixture and watered with Hoagland's No. 1 solution, plus minor elements.[27] Nitrate reductase was extracted from homogenized leaves and assayed according to the methods of Hageman and Flesher.[19] Polyclar-AT, and insoluble polyvinylpyrrolidone (GAF Corporation, 140 W. 51 St., New York, U.S.A.) was used according to the directions of Klepper and Hageman.[9] Ascorbate as suggested by Loomis and Battaile,[3] reducing agents according to Anderson and Rowan,[8] buffers as used by King[28] and dialysis as employed by Grasmanis and Nicholas[1] were additional modifications tried in the enzyme extraction and assay procedures. Glutamate dehydrogenase and malate dehydrogenase were extracted and assayed according to Pahlich and Joy[20] and Danner and Ting,[22] respectively.

Inhibition studies. Leaves were extracted at 0° by homogenizing in 4 vol. of a soln containing 0·2 M Tris, 0·01 M L-(+)-cysteine, and 3×10^{-4} M EDTA and which was adjusted to pH 7·5 with HCl. The homogenates were filtered through cheese-cloth and centrifuged (15 min at 10 000 *g*, 0°). The assay mixtures consisted of 0·5 ml corn leaf extract, 0 to 0·5 ml of ericaceous extracts, 1·5 ml 0·1 M K phosphate buffer, pH 7·5, 0·2 ml 0·1 M KNO_3, 0·5 ml 0·00136 M NADH, and dist. H_2O for a total vol. of 3 ml. Incubation time was 15 min, and the reaction was stopped with 1 ml of 1% sulfanilamide in 1·5 N HCl. N-(1-naphthyl)-ethylenediamine hydrochloride (0·02%) in 0·2 N HCl (1 ml) was added for color development. 5 min later absorbancy was measured at 540 nm.

Polyclar-AT, when employed, was added to the extracts of ericaceous leaves in saturating quantities, stirred for 5 min, and centrifuged for 5 min at 5000 *g*. The supernatant fraction was saved, and the treatment with Polyclar-AT was repeated several times. Acetone precipitation involved mixing 4 vol. of cold acetone with 1 vol. of extract. The resultant suspension was centrifuged for 10 min at 10 000 *g*. The supernatant fraction was concentrated *in vacuo* and resuspended in the same vol. of 0·2 M potassium phosphate buffer, pH 7·0, as used in the original extraction. The pellet was washed and resuspended in 0·2 M potassium phosphate buffer or 1 N NaOH in the same vol. used in extraction. Protein in each fraction was estimated according to the Lowry and biuret methods.[29] All commercially prepared phenolic compounds mentioned in the text were tested at 10^{-3} M.

Chromatography. The supernatant fractions concentrated *in vacuo* after acetone precipitation were chromatographed on Whatman No. 3 paper (20 × 55 cm) which had been eluted for 2 days with dist. H_2O. Solvents were *n*-BuOH–HOAc–H_2O (4:1:5) and *sec*-BuOH–HOAc–H_2O (14:1:5) employed in descending, 1-D chromatography. Two spots resulted from the chromatography of pure tannic acid. The spot in the extracts of *Leucothoe* and *Rhododendron* corresponding to the one with the higher R_f of authentic tannic acid was eluted in 70% v/v EtOH. The eluate was concentrated *in vacuo* and resuspended in 10 ml of dist. H_2O. Fluorescent or colorimetric inspections of chromatograms were accomplished under long-wave UV light or with 0·1% w/v $FeCl_3$ and 0·2% w/v $FeNH_4(SO_4)_2$ $12H_2O$, saturated KIO_3, or Gibbs' reagent sprayed onto the chromatograms.[26]

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