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FERULIC ACID: A SUBSTRATE FOR TWO ISOPEROXIDASES FROM *NICOTIANA TABACUM* TISSUE CULTURES*

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Abstract—An anodic isoperoxidase (A_2) from tobacco tissue culture W-38 and a cathodic isoperoxidase (C_4) from tobacco tissue suspension culture WR-132 have been separated and characterized. Both isoperoxidases catalyzed oxidation of ferulic acid in the presence of H_2O_2. When the reaction mixture was subjected to TLC, ferulic acid was found to have been converted to an unknown compound which, after treatment with ammonia, fluoresces green in UV light. Both the isoperoxidases A_2 and C_4 appear to follow simple Michaelis-Menten kinetics with respect to guaiacol as the substrate. The K_ms for guaiacol are 4 and 4·5 mM for isoperoxidases C_4 and A_2, respectively. The pH optimum for both enzymes is about 6·0. The effect of various phenolic and related compounds on the activity of each isoperoxidase is reported and discussed.

INTRODUCTION

NUMEROUS catalytic functions have been reported for peroxidases in higher plants. Among these are peroxidases involved in the oxidation of indole-3-acetic acid (IAA);[1] in lignification;[2] and in the oxidation of reduced pyridine nucleotides[3] and in the conversion of methional to ethylene.[4] The physiological functions of plant peroxidases are further confused by the existence of multiple forms of the enzyme in many plants,[5,6] including tobacco.[7] We have, therefore, decided to isolate the individual isoperoxidases in tobacco tissue cultures and to determine the possible physiological role of each isoperoxidase. Schafer *et al.*[8] in our laboratory have shown that scopoletin (7-hydroxy-6-methoxycoumarin) activates the guaiacol assay of an isoperoxidase (A_3) isolated from tobacco tissue culture W-38, but it has no effect on the guaiacol assay of another isoperoxidase (A_1) isolated from the same source. More recently, Reigh *et al.*[9] have found that A_3 actually catalyzes the oxidation of scopoletin, with scopoletin behaving as a substrate for this isoperoxidase.

This present report describes the separation and characterization of another isoperoxidase (A_2) from tobacco tissue culture W-38 and of a cathodic isoperoxidase (C_4) from tobacco tissue suspension culture WR-132. Our results show that ferulic acid (4-hydroxy-3-methoxycinnamic acid) not only stimulates the guaiacol assay but also may be utilized as a substrate by both these isoperoxidases.

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RESULTS AND DISCUSSION

When subjected to starch gel electrophoresis at pH 7·0, crude extracts of tobacco WR-132 suspension culture tissue are found to contain little, if any, anodic peroxidase bands under the growth conditions outlined in the Experimental, although small amounts of anodics appear in the medium. However, four major cathodic bands from tissue appear upon staining with guaiacol and H_2O_2. These cathodics are similar in mobilities to those reported by Stafford and Galston.[10] Therefore, we will use their nomenclature to distinguish the isoperoxidases. In some runs an additional band appears between C_2 and C_3. The isoperoxidase designated C_4 is by far the most active band (deeply staining) with respect to guaiacol. The separation of C_4 free from other isoperoxidases can be accomplished using CM-cellulose chromatography.

On the other hand, crude extracts of tobacco W-38 callus cultures when subjected to starch gel electrophoresis at pH 7·0 exhibit the whole complement of isoperoxidases (anodic and cathodic) as previously shown in W-38 tobacco tissue by Stafford and Galston.[10] Attempts to isolate individual cathodic isoperoxidases from W-38 have been unsuccessful, thus precipitating the use of WR-132 as our source of individual cathodic isoperoxidases. We have, however, been able to isolate from W-38 another individual isoperoxidase in addition to the previously reported isoperoxidase A_3.[9] Based on electrophoretic mobility this newly isolated anodic isoperoxidase appears to be equivalent to the isoperoxidase termed A_2 by Stafford and Galston.[10] The separation of A_2 from all the other isoperoxidases can be accomplished using CM-cellulose chromatography followed by DEAE-cellulose chromatography.

Both the isoperoxidases A_2 and C_4 appear to follow simple Michaelis–Menten kinetics with respect to guaiacol as the substrate. The K_ms for guaiacol determined by the method of Lineweaver–Burk[11] are 4 and 4·5 mM for isoperoxidases C_4 and A_2, respectively. The pH optimum for both enzymes is about 6·0.

It was reported some time ago[12] that IAA oxidase activity of crude peroxidase preparations is stimulated by monophenols and inhibited by *o*- and *p*-diphenols. Since then many phenolic compounds have been reported to affect the activity of peroxidase preparations, including ferulic and *p*-coumaric acids.[13] In an effort to determine the possible effect of phenolic and related compounds on the physiological role of isoperoxidases C_4 and A_2, scopoletin, caffeic acid and other phenolic compounds were investigated for their effects on the guaiacol oxidizing capabilities of these two enzymes (Table 1). The only significant difference in the pattern of stimulation or inhibition by the phenolics is the stimulation of isoperoxidase A_2 by vanillic acid. Both enzymes are stimulated by ferulic acid, caffeic acid and *p*-coumaric acid, with ferulic acid exerting the most pronounced effect. The stimulation by ferulic acid disappears on preincubation of the enzyme with ferulic acid and H_2O_2 for 10 min, suggesting that the enzyme is capable of the oxidative destruction of ferulic acid in the presence of peroxide. Incubation of the enzyme with ferulic acid alone or with H_2O_2 alone does not have any effect on the observed stimulation.

Chlorogenic acid and other compounds having an *o*-diphenol configuration (esculetin, quercetin, 3-4-dihydroxybenzoic acid, pyrocatechol, and the ethyl ester of caffeic acid, but excluding caffeic acid) inhibit in varying degrees the ability of the two isolated isoperoxidases

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to oxidize guaiacol. Chlorogenic acid, however, is by far the best inhibitor of those tried. In contrast to the inhibition exhibited by the other *o*-diphenol compounds studied, caffeic acid at 0·4 mM concentration strongly stimulated the activity of the two isoperoxidases in their ability to oxidize guaiacol. Ethylation of the carboxyl group of caffeic acid, however, resulted in the usual inhibition by *o*-diphenols.

TABLE 1. EFFECT OF VARIOUS COMPOUNDS ON THE ACTIVITY (GUAIACOL ASSAY) OF ISOPEROXIDASE C_4 AND A_2

Compound added	Concn (mM)	Activity (%) Isoperoxidase C_4	Isoperoxidase A_2
None	—	100	100
Caffeic acid	0 05	108	—
Caffeic acid	0·4	170	160
Hydrocaffeic acid	0·4	—	100
p-Coumaric acid	0·4	133	134
Ferulic acid	0·01	125	—
Ferulic acid	0·05	160	—
Ferulic acid	0·1	252	260
Ferulic acid	0·4	290	300
Glucosidoferulic acid	0·4	100	100
3-*O*-feruloylquinic acid	0·4	40	—
Hydroferulic acid	0·4	58	57
Isoferulic acid	0·4	82	93
Chlorogenic acid	0·025	57	—
Chlorogenic acid	0·05	34	—
Chlorogenic acid	0·4	0	0
3,4-Dihydroxybenzoic acid	0·05	81	—
3,4-Dihydroxybenzoic acid	0·4	15	22
Pyrocatechol	0·4	15	—
Quercetin	0·05	43	—
Caffeic acid, ethyl ester	0·05	18	—
Caffeic acid, ethyl ester	0·4	—	7
Esculetin	0·4	15	14
3,4-Dimethoxybenzoic acid	0·4	—	100
3,4-Dimethoxycinnamic acid	0 4	100	—
Scopolin	0·4	100	—
Scopoletin	0·4	100	100
Vanillic acid	0·4	100	131
Isovanillic acid	0·4	—	90
p-Hydroxybenzoic acid	0·4	90	—
Indole-3-acetic acid	0·2	100	—

Assay conditions are 6 mM guaiacol, 5 mM H_2O_2 in 0·05 M phosphate buffer (pH 6·0).

Of the two unsubstituted monophenols studied, *p*-coumaric acid stimulated enzymic activity as expected, whereas *p*-hydroxybenzoic acid actually exhibited a very slight inhibition. Variations in effect on activities of isoperoxidases A_2 and C_4 were also found among the 4-hydroxy-3-methoxy compounds investigated. Of these, ferulic acid was very highly stimulatory in the guaiacol assay and could behave as a substrate; 3-*O*-feruloylquinic and hydroferulic acids were inhibitory, and isoferulic acid was slightly inhibitory. Glucosidoferulic acid, scopolin, and scopoletin showed neither stimulatory nor inhibitory action. Thus, although many 4-hydroxy-3-methoxyphenyl and unsubstituted monophenolic and diphenolic compounds exert substantial influence in varying degrees on inhibition or

stimulation of the activities of isoperoxidases A_2 and C_4 in the guaiacol assay, it is evident that these phenolic features alone are not the sole determinants of stimulation or inhibition for these two isoperoxidases.

The addition of either isoperoxidase C_4 or A_2 to a solution containing 0·8 mM ferulic acid and H_2O_2 causes rapid conversion of ferulic acid to a new product which is salmon in color at first, and which exhibits a small, transient, broad spectral maximum not shown by ferulic acid, in the 470–540 nm range. The reaction mixture soon becomes yellow in color. When this reaction mixture was subjected to TLC in benzene–methanol (3:1), ferulic acid was found to have been converted to one or more unknown products. A major product, with R_f 0·94, fluoresces green under UV after treatment with ammonia, whereas ferulic acid has R_f 0·88 and fluoresces blue. These data confirm that ferulic acid may act as a substrate for isoperoxidases C_4 and A_2. Under the same experimental conditions, caffeic and *p*-hydroxycinnamic acid, which also stimulate the guaiacol assay, do not appear to be oxidized.

Attempts to develop an assay based on the ability of the enzyme to oxidize ferulic acid have as yet not been completely successful. Therefore, studies were initiated to determine what effect different concentrations of ferulic acid have on the enzymes' ability to oxidize guaiacol. Variation of the ferulic acid concentration at constant guaiacol concentration (6 mM) produces a Michaelis–Menten type saturation curve for isoperoxidase C_4. The same results are obtained with isoperoxidase A_2. The ferulic acid concentration which yields one half of maximal stimulation is $2{\cdot}5 \times 10^{-5}$ M. This value should be related to the K_m of the enzyme for ferulic acid as a substrate. The optimum pH for guaiacol in the presence of ferulic acid is 6·0, which is identical to that in the absence of ferulic acid. The stimulation effect of ferulic acid upon the guaiacol oxidation is overcome by high guaiacol concentrations. This is indicative of a competition for the substrate site by both ferulic acid and guaiacol.

EXPERIMENTAL

Growth of tobacco cultures. Tobacco tissue WR-132 (*Nicotiana tabacum* L. var. Xanthi), obtained from Dr. A. C. Olson of USDA, Albany, California, was grown in suspension on the medium of Murashige and Skoog,[14] in which auxin was provided in the form of 2,4-dichlorophenoxyacetic acid (2,4-D) instead of IAA, and kinetin was deleted. *Ca.* 2 g cells, used as an inoculum, were transferred to 50 ml medium in a 125 ml flask and grown in constant agitation on a rotating shaker (180 rpm). After a 10-day growth period the cells were collected by suction filtration and washed with 0·050 M phosphate buffer (pH 7·0). Tobacco callus tissue W-38 was grown on the revised medium (RM-1964) of Linsmaier and Skoog[15] with 2 mg/l. IAA, 200 μg/l. kinetin and an increased amount of thiamine hydrochloride (1 mg/l.). Portions of callus tissue approximately 5 × 5 × 3 mm cut from 5-week-old stock culture were used as the inoculum. The inoculum was allowed to grow about 37 days. All transfers were accomplished in a laminar flow hood. Growth was carried out at room temp. in continuous subdued light.

Preparation of the enzymes. 100 g washed cells (WR-132), 100 g glass beads, 70 g washed hydrated polyclar AT, 2 g sodium lauryl sulfate (SLS) and 200 ml 0 05 M phosphate buffer (pH 7·0) were homogenized in a blender at 6000–7000 rpm for 5 min. Breakage has also been performed in 0·05 M imidazole buffer (pH 7·0) containing β-mercaptoethanol. The resulting homogenate was centrifuged at 30 000 *g* for 20 min and the supernatant was decanted through glass wool. The supernatant from the imidazole β-mercaptoethanol breakage was not as dark brown as the supernatant from the phosphate breakage. This preparation was made 40% saturated with solid $(NH_4)_2SO_4$. The resulting precipitate was removed by centrifugation at 17 000 *g* for 15 min and discarded. The supernatant was brought to 80% saturation by the addition of solid $(NH_4)_2SO_4$. The resulting precipitate was collected by centrifugation and dissolved in 10 ml 0·05 M phosphate buffer (pH 6·0). After overnight dialysis the enzyme solution was applied to a pre-equilibrated (0 02 M phosphate buffer, pH 6 0) CM-cellulose) column and eluted with 0 02 M phosphate buffer (pH 6 0) The eluate was concentrated and then subjected to electrophoresis The crude enzyme preparation from W-38

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was prepared as outlined above except 60 g cells, 10 g glass beads, 25 g polyclar AT and 0·5 g SLS in 100 ml 0·04 M phosphate buffer (pH 6·0) were used. Also cell breakage time of 12 min was required. The crude enzyme preparation was concentrated and dialyzed for 24 hr. The dialyzed sample was then applied to a pre-equilibrated CM-cellulose column and eluted with the equilibrating buffer, 0 005 M phosphate buffer (pH 6 0). Peaks of activity were concentrated and applied to a pre-equilibrated DEAE cellulose column and eluted with 0·005 M phosphate (pH 6·0). Active fractions were analyzed by electrophoresis.

Electrophoresis. Anodic polyacrylamide gel electrophoresis using a Buchler Polyanalyst Disc Electrophoresis apparatus[16] and/or starch gel electrophoresis at pH 7·0 using a modified Smithies apparatus[17] were performed on active column fractions. Peroxidase bands were visualized by placing the gels in a mixture of 2 parts 1% guaiacol in 0·05 M phosphate buffer (pH 6·0), 2 parts 0·05 M phosphate buffer and 1 part 0·5% H_2O_2.

Enzyme assays. The peroxidase activity was measured based on the procedure of Lance.[18] Linear rates were observed. The reaction was initiated by the addition of 0·1 ml of the enzyme preparation. Solutions of effector compounds were prepared in 0·05 M phosphate buffer (pH 6·0) and added at various concentrations to the reaction mixture. IAA and quercetin were prepared in 95% EtOH. When these two effectors were used in the assay, the concentration of ethanol did not exceed 4% (v/v).

TLC. Thin layer plates of Avicel SF were developed with C_6H_6–MeOH (3:1) and spots were visualized in UV light (366 nm).

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