An Oxidation Product of Chlorogenic Acid in Tobacco Leaves Infected With Tobacco Streak Virus

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ABSTRACT

A method for the isolation and identification of an oxidation product of chlorogenic acid formed in vivo in tobacco leaves infected with tobacco streak virus is described. Caffeic acid, a hydrolysis product of chlorogenic acid, was associated with an acetone-insoluble cell fraction, presumably protein, extracted from tobacco leaves infected with tobacco streak virus but not from healthy leaves. In vitro experiments demonstrated that the quinone of chlorogenic acid binds to protein to form a complex which yields caffeic acid upon hydrolysis. Chlorogenic acid in its reduced state did not bind to protein. It is proposed that chlorogenic acid is oxidized in vivo in streak-infected tobacco leaves, and forms a protein-quinone complex. Phytopathology 60:1677-1681.

Plant phenolic compounds and their associated oxidases have been implicated in various aspects of pathogenesis in a number of plant diseases. The drastic modifications in the levels of phenolic compounds and in the activity of phenol oxidase (o-diphenol: o-quinone oxidoreductase) and peroxidase in diseased and injured plant tissues have led to the assumption that oxidation products of phenols accumulate under these conditions (3, 9, 10). In some cases, as for example when tissue disintegration is involved, this assumption seems justified. When tissue damage is less severe, however, the assumption that an increased activity of phenol oxidizing enzymes results in an in vivo oxidation of phenolic compounds is open to question for several reasons. Phenol oxidases that have been studied have a low affinity for oxygen (2) and, as shown below, the enzyme from tobacco is no exception. Secondly, some plants, including tobacco, have a high level of extractable phenol oxidase activity and a high level of substrates. Thus, neither enzyme nor substrate is limiting. Thirdly, as Beckman & Mueller (1) recently showed, phenolic compounds may be localized in the plant cell and thus spatially separated from their oxidases.

The evidence for or against the in vivo oxidation of phenolic compounds in diseased plants is often convincing but rarely conclusive. One of the major obstacles to making an accurate evaluation of the in vivo oxidation of phenolic compounds and the possible function of quinones in pathogenesis is that a product of such a reaction has not been unequivocally demonstrated to be formed in vivo. Wright et al. (11, 12) investigated the brown pigments of aged burley tobacco and found them to consist of protein-phenol complexes. Caffeic acid and quinic acid, both constituents of chlorogenic acid, were recovered from acid hydrolysates of the pigments. The formation of these pigments probably resulted from rapid oxidation of chlorogenic acid during the curing process. If phenol oxidation occurs in vivo in plant tissues, it should be possible to isolate similar protein-phenol complexes from living tissue under conditions which would prevent their formation during extraction. The purpose of this investigation was to isolate and identify a product of the in vivo oxidation of chlorogenic acid if present. Tobacco was selected because of its relatively high level of both chlorogenic acid and phenol oxidase, and because of the absence of free caffeic acid. Tobacco streak virus was chosen because it produces two distinctly different symptoms in tobacco, an initial necrosis followed by an almost symptomless condition in leaves which are formed later (4). Thus, using one host-pathogen combination, the possible relationship between symptom type and quinone accumulation could be observed.

MATERIALS AND METHODS.—*Nicotiana tabacum* L. 'Ky 12' was grown in vermiculite subirrigated with Hoagland's solution as previously described (6). Five to 6 weeks after planting, when the plants had four to six leaves, the two lower leaves were inoculated with tobacco streak virus. Inoculum was prepared by grinding tissue from infected plants in 0.05 M phosphate buffer, pH 7.0, containing 0.01 M Na_2SO_3 at approx 10 ml/g of tissue. Celite-dusted leaves were rubbed with a cheesecloth pad saturated with inoculum. Four days after inoculation, the systemically infected leaves above the inoculated leaves were harvested. Symptomless leaves were obtained from plants inoculated 3-4 weeks prior to harvesting.

Tissue was homogenized in the various media described below using either a Waring Blendor or a Sorvall Omni-Mixer at a temp of near 0 C when aq media were used and at −10 to −15 C when acetone was used.

Samples to be assayed for the presence of protein-quinone complex were hydrolyzed for 18-20 hr with 2 N HCl under reflux. When small amounts of material were involved, hydrolysis was carried out in ampules sealed under reduced pressure followed by incubation at 110 C for 18-20 hr. The hydrolysates were then extracted 3 times with anhydrous diethyl ether, the combined ether fractions were evaporated to dryness under reduced pressure, and the residues were redissolved in a small volume of 95% ethyl alcohol for paper chromatography. This is essentially the method used by Wright et al. (11).

Descending paper chromatography was carried out

using acid-washed Whatman No. 1 and No. 3 MM paper. Samples were applied as bands 3 inches from the top of 6- × 22-inch or 18- × 22-inch sheets and developed without equilibration using 4:1:1 *n*-butanol-acetic acid-water (B-A-W), 1% acetic acid, or 20% KCl as solvents. Sequential paper chromatography in all three solvents was accomplished by eluting bands of interest with redistilled 95% ethanol, streaking the eluate on a fresh sheet of paper, and developing in the next solvent. Known caffeic acid, obtained from Sigma Chemical Co. and recrystallized twice from deionized water, was included as a standard and chromatographed on separate sheets.

Fluorescence excitation and emission spectra of materials eluted from paper chromatograms were obtained using an Aminco-Bowman spectrophotofluorimeter. Acid-washed paper and redistilled 95% ethanol were used to minimize fluorescent impurities which otherwise were present.

Partially purified phenol oxidase was prepared from tobacco leaf acetone powders by the procedure outlined in Fig. 1. Enzyme assays were conducted using a Clark oxygen electrode (Yellow Springs Instruments). The reaction mixture, consisting of 3 ml of 5×10^{-3} M chlorogenic acid in 0.1 M phosphate buffer, pH 5.6, was equilibrated to air saturation at 30 C for routine assays. The reaction was started by addition of 0.1 ml-0.3 ml of enzyme preparation diluted, if necessary, to give usable reaction rates. Specific activity is expressed as

µliters O_2/mg protein/min. Assay conditions for determining oxygen affinity are given in Fig. 2, below.

RESULTS.—*Reaction of quinones with protein in vitro.* —Prior to attempting the isolation of a product of phenol oxidation from plant tissues, in vitro reaction products of chlorogenic acid and its quinone with protein were investigated. According to Loomis (5), phenols may form reversible complexes with protein by hydrogen bonding, whereas quinones form stable covalent linkages. Such protein-quinone complexes are brown in color. Reaction mixtures consisting of 10 mg/ml bovine albumin fraction V (Nutritional Biochemicals Corp.) and 0.01 M chlorogenic acid in 5 ml of 0.1 M phosphate buffer, pH 5.6, were prepared in two 30-ml Corex centrifuge tubes. To one tube was added a few drops of partially purified tobacco phenol oxidase. The tubes were incubated for 30 min at room temp with frequent agitation. Rapid browning occurred in the mixture with enzyme, while the mixture without enzyme remained colorless. At the end of the incubation period, 10 ml acetone were added to each tube and the tubes were centrifuged at 12,000 g for 10 min. The precipitates were washed 3 times by resuspending in acetone and recentrifuging. The precipitate from the enzyme-treated sample remained brown throughout, while that from the mixture without enzyme was white. The precipitates were hydrolyzed in 2 N HCl in sealed ampules, extracted with ether, and separated by chromatography as described above. Recrystallized caffeic

Tobacco leaf acetone powder (40-50 g) extracted in 1000 ml 0.1 M Tris [tris(hydroxymethyl) amino methane]-HCl buffer, pH 8.0 for 1 hr at 0 C with constant stirring. Filtered through cheesecloth and centrifuged 10,000 rev/min for 10 min. Supernatant made to 30% saturation with $(NH_4)_2SO_4$ and centrifuged 10,000 rev/min for 10 min

Pellet — discarded ⟵——————⟶ Supernatant. Made to 50% saturation with $(NH_4)_2SO_4$ and centrifuged 10,000 rev/min for 10 min

Supernatant — discarded ⟵——————⟶ Pellet. Resuspended in 100 ml 0.1 M Tris-HCl, pH 8.0 and adjusted to pH 4.5 with 6 N HCl. Centrifuged 10,000 rev/min for 10 min

Pellet — discarded ⟵——————⟶ Supernatant. Dialyzed overnight against deionized H_2O. Centrifuged 10,000 rev/min for 10 min

Pellet — discarded ⟵——————⟶ Supernatant. Freeze-dried and resuspended in 10 ml Tris-HCl as above and passed through a 5 cm × 75 cm column of water-equilibrated Sephadex-G-200. Ten-ml fractions were collected. Active fractions were freeze-dried and stored at −15 C until used. Samples were prepared for use by resuspension of 0.1 M phosphate buffer 5.6.
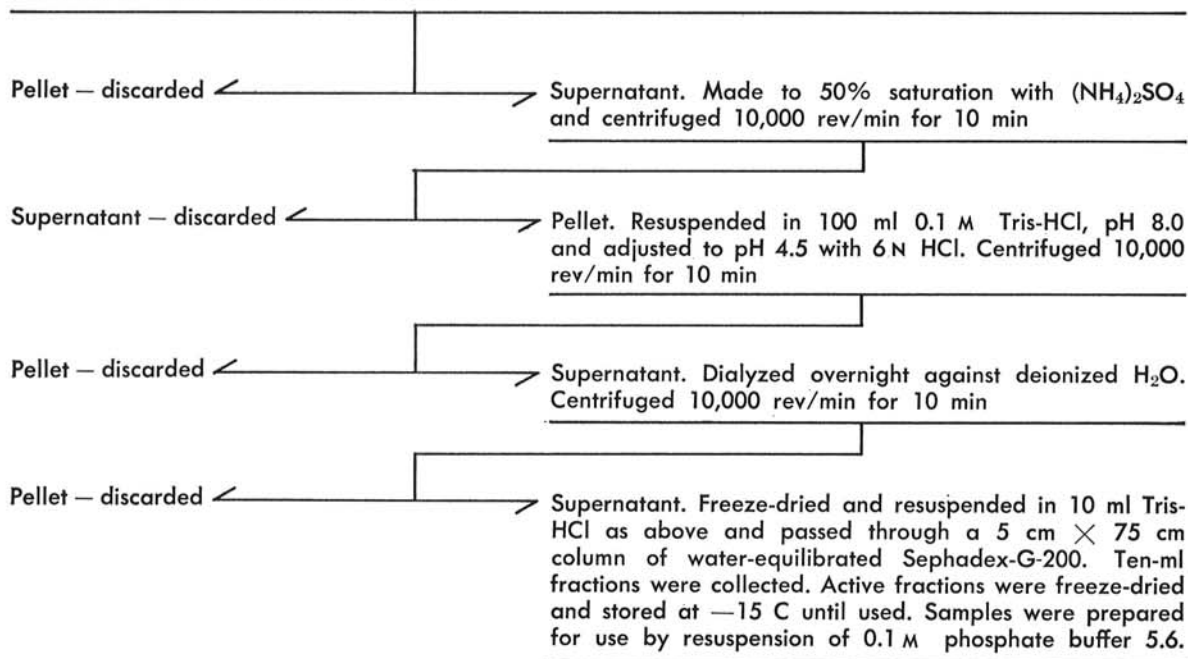
Fig. 1. Procedure for the partial purification of tobacco leaf phenol oxidase. Specific activity of the final preparation was 6,250 µliters O_2/mg protein per min. This represents an approx 10-fold concn of crude preparations.
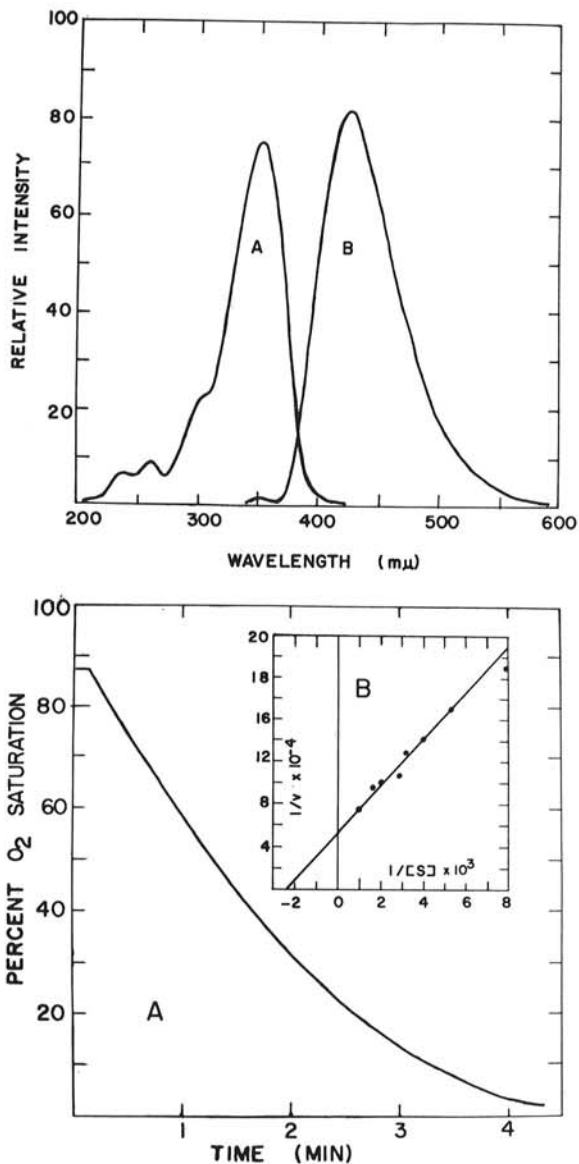
Fig. 2. (Above) Fluorescence excitation (A) and emission (B) spectra of caffeic acid isolated from diseased tissue as described in the text. Known caffeic acid eluted from paper chromatograms had identical spectra. (Below) Oxygen affinity of tobacco leaf phenol oxidase. Curve A is a plot of oxygen concn vs. time (100% O_2 saturation = 1.24 μM/ml). Curve B is a plot of $1/v$ vs. $1/(S)$, with v expressed as μliters O_2/mg protein per min at any given O_2 concn.

acid, which had been alternately oxidized at high pH and reduced by adjustment to pH 2.0 and extracted into ether, was chromatographed simultaneously.

A blue fluorescent compound with R_F values identical to the caffeic acid standard was present in the hydrolysate from the enzyme-treated preparation but not in the control hydrolysate. The R_F values were 0.84 in B-A-W, 0.25 in 1% acetic acid, and 0.16 in 20% KCl. Fluorescence excitation and emission spectra of

the known and unknown were identical (Fig. 2, above).

Several conclusions can be drawn from this experiment, which was repeated several times. Firstly, under these conditions, chlorogenic acid reacts to form a stable complex with protein only if it is oxidized. Secondly, this complex yields caffeic acid upon hydrolysis. Thirdly, the reduced chlorogenic acid present in the original mixture can be separated from the protein-quinone complex by precipitation of the latter with acetone followed by an acetone wash.

In order to insure that any protein-phenol complex that might be detected in plant tissues was not formed during homogenization of the tissue and subsequent treatments, various extracting media were tested using healthy leaf tissue. Tissues were homogenized in 0.1 M phosphate buffer containing 5×10^{-3} M KCN, 0.1% cysteine HCl, and 4 g insoluble polyvinylpyrrolidone (PVP) per 100 ml (5) in various combinations, followed by clarification of the homogenate by centrifugation. The protein fraction was then separated from the remaining free phenolic compounds by dialysis against KCN and PVP, by precipitation with acetone or $(NH_4)_2SO_4$, or by gel filtration with Sephadex G-50. All steps were carried out at near 0 C. A definite browning occurred in all cases before the protein fraction could be separated from the low mol wt fraction, indicating that some oxidation of phenols had occurred during the extraction procedure.

In an effort to shorten the time between homogenization and separation of the protein fraction from low mol wt materials, the homogenate was filtered through a celite bed into a flask containing 10 volumes of cold acetone. This mixture was then rapidly filtered through Whatman No. 1 paper and washed several times with cold acetone. Again, the acetone-insoluble fraction had a definite brown color.

Experiments in which partially purified phenol oxidase was added to chlorogenic acid in acetone at 0 to −15 C followed by agitation of the mixture indicated that oxidation of chlorogenic acid did not occur under these conditions. Therefore, prechilled leaf tissue was homogenized in acetone at −15 C and filtered, and the residue washed with additional cold acetone. The residue was then transferred to a cellulose extraction thimble and extracted for 2 to 3 hr with acetone by means of a Soxhlet extractor. This was followed by three successive washes with anhydrous diethyl ether. The resultant acetone powders were white when healthy plants were used and brownish when diseased plants were used. When extraction with the Soxhlet apparatus was omitted and the powders were dried after filtration and washing with cold acetone, a brown color developed even in extracts of healthy plants after resuspension in aq media. Therefore, exhaustive extraction was apparently required to remove all traces of unbound reduced chlorogenic acid. This rather drastic treatment rendered the protein fraction insoluble, probably because of denaturation.

Acetone powders from diseased and healthy plants were hydrolyzed either by refluxing in 2 N HCl for 20 hr or by incubating in 0.1 M phosphate buffer contain-

ing 0.33 mg pronase/100 ml for 18-20 hr at 24 C. The pronase hydrolysates were then made to 2 N HCl and refluxed 2-3 hr after insoluble materials were removed by centrifugation. The acid and pronase hydrolysates were then extracted with ether as described above, and the combined ether fractions were evaporated to near dryness using a rotary flash evaporator. Drying of the residue was completed by lyophilization. The samples were then taken up in a small volume of 95% ethyl alcohol and chromatographed on Whatman No. 3 MM as described. Caffeic acid was chromatographed simultaneously on a separate sheet. A blue fluorescent band corresponding to caffeic acid in R_F value was present in extracts from diseased but not from healthy plants. This band and the band on the standard chromatogram were cut out and eluted overnight with three changes of redistilled 95% ethanol. The eluates were diluted with redistilled 95% ethanol to give a concn equivalent to 1 g of acetone powder/100 ml, and their fluorescence excitation and emission spectra were determined. The compound isolated from diseased plants was identical to the caffeic acid standard in R_F values and fluorescence spectra.

Quinones in necrotic and symptomless leaves.—Acetone powders were prepared from systemically infected leaves exhibiting extensive necrosis (4 days after inoculation) and from symptomless leaves (3 weeks after inoculation) and subjected to acid hydrolysis and paper chromatography as described above. The final ethanol eluates from the paper chromatograms were made to a concn equivalent of 0.8 g acetone powder/100 ml. Relative fluorescence of the eluates was determined to be 48 from necrotic leaves and 0.96 from symptomless leaves, using an excitation wavelength of 350 mμ and an emission wavelength of 425 mμ. There was, therefore, a much higher concn of the protein-quinone complex in the necrotic tissue than in the symptomless tissue. A quantitative estimation could not be obtained from these data, however.

Oxygen affinity of tobacco phenol oxidase.—Plant phenol oxidases generally have a rather low affinity for oxygen. The oxygen affinity of partially purified tobacco phenol oxidase was determined using a Clark oxygen electrode (Yellow Springs Instruments). The reaction mixture, consisting of 2.5×10^{-2} M chlorogenic acid in 3 ml of 0.1 M phosphate buffer, was saturated with oxygen at 20 C. The barometric pressure was noted and the reaction was started by addition of 0.1 ml of partially purified tobacco phenol oxidase (0.023 mg protein). A continuous plot of oxygen concn vs. time was obtained from which the instantaneous reaction rate (v) at any oxygen concn (S) could be obtained by taking tangents to the curve (2). A plot of $1/v$ vs. $1/(S)$ gave a Km value of 4.6×10^{-4} M (Fig. 2, below) which compares favorably with the value of 1.4×10^{-4} M for the enzyme from tea (2).

DISCUSSION.—The characteristic browning of plant tissues which occurs as a response to various types of injury and stress has long been thought to result from the oxidation of phenolic compounds. This assumption is apparently valid. Although oxidation of phenolic compounds under less severe conditions is assumed to occur, the evidence is less clear-cut. For example, in numerous systemic virus infections, visible browning of tissue does not occur even though the activity of phenol oxidizing enzymes may be much higher than in healthy plants. Using the methods reported here, it was possible to obtain evidence for the in vivo oxidation of chlorogenic acid in tobacco plants infected with tobacco streak virus. Symptomless leaves contained relatively much less of this product than did leaves exhibiting severe necrosis. The product was not detected in healthy leaves, indicating that it was not present or present only in small quantities. Therefore, it may be concluded that oxidation of phenolic compounds is either initiated or accelerated in tobacco by infection with tobacco streak virus.

The in vivo oxidation of chlorogenic acid to a quinone results in the formation of a complex between the quinone and an acetone-insoluble cell component. Since this complex is degraded by ether acid hydrolysis or by pronase, it is probably a protein-quinone complex. This conclusion is supported by the in vitro experiments described above.

It was not the purpose of the present investigation to explore the possible function of phenolic oxidation in pathogenesis. However, the concn of the protein-quinone complex was much greater in leaves in which considerable necrosis had occurred than in symptomless leaves. It has also been observed (*unpublished data*) that the activity of phenol oxidase in the necrotic leaves is as much as 10 times higher than in healthy leaves, while in symptomless leaves the enzyme activity is only 2-3 times that of healthy leaves. Thus, a relationship between enzyme activity and accumulation of quinones is indicated.

Whether or not the relatively high levels of quinones which accumulated in the necrotic leaves were responsible for the necrosis as has been suggested (7, 8, 9) or were simply a result of the necrosis cannot be concluded from the data presented here. Using the methods described, however, it should be possible to obtain definitive data on this and other aspects of the role of phenolic oxidation in diseased plants.

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