Detergent Extraction of Enzymes from Tobacco Leaves Varying in Maturity

DONALD W. DE JONG
Plant Science Research Division, Agricultural Research Service, United States Department of Agriculture, Oxford, North Carolina 27565

ABSTRACT
Enzyme activities of tobacco leaves were compared in detergent extracts. Highest levels of chlorogenic acid oxidase, malate-NAD oxidoreductase, and acid phosphatase were obtained from bud tissues. Peroxidase activity was least in young leaves and highest in senescent leaves yellowed with Ethrel. Peroxidase zymograms obtained by means of disc electrophoresis showed differences in isozyme composition among all five samples examined. Although protease was found in material extracted from buds, upper, middle, and lower leaf positions, none could be demonstrated in Ethrel-treated mature leaves.

Steinberg and Tso (32) summarized a number of enzymatic changes reported to occur during the maturation of tobacco leaves. These authors state that peroxidase activity increases whereas polyphenoloxidase activity decreases during leaf development. However, Weston (37) reported that peroxidase and polyphenoloxidase activities fluctuate similarly during growth and senescence of tobacco leaves. The discrepancy resulting from similar banding patterns for peroxidase and polyphenoloxidase isozymes after electrophoresis of tobacco leaf extracts (28) has since been resolved by Van Loon (34) using improved staining techniques. Changes in activity and isozyme composition of peroxidase (4) and of polyphenol oxidase (31) are subject to hormonal control. Ethylene has been shown to increase peroxidase activity without regard to the stage of maturity in pea plants (24) whereas GA, has the opposite effect on peroxidase activity in the elongating internodes of dwarf pea (4). The decline in alkaline pyrophosphatase which normally accompanies maturation of tobacco leaves can be prevented with benzyladenine (22). On the other hand, kinetin prevented an increase of protease activity in senescing oat leaves (16). Daily rhythmic changes in dehydrogenase activities, particularly that of malate-NAD oxidoreductase, have been demonstrated in tobacco cells grown in liquid culture (8). In the present work, the observations of previous investigators were extended by including a study of enzymatic differences in immature tobacco leaves at the bud stage and in mature leaves induced to senesce by treatment with Ethrel, an ethylene precursor (36). The use of Ethrel as a yellowing agent for tobacco leaves has been described by Cutler and Gaines (6). One of the objectives of the study was to maximize extraction of particle-bound enzymes by solubilizing subcellular organelles and other membrane components using the anionic detergent, DOC.

MATERIALS AND METHODS
Tobacco plants, Nicotiana tabacum, L., cultivar NC 95, potted in a 1:1 mixture of sand and vermiculite, were grown in the greenhouse during the winter months of 1970-1971. After topping (removal of inflorescence), some plants were sprayed with a dilute solution (3 mg/ml) of Ethrel (2-chloroethyl phosphonic acid) to hasten yellowing. Two 20-ml applications were given to each plant 3 days apart. One week after the first treatment, the lower leaves were harvested for analyses. Bud tissue was obtained from axillary suckers of untreated plants.

Generally, 10 g, fresh weight, of leaf tissue were homogenized for 2 min with a Virtis blender (20,000 rpm) in 40 ml of 0.5 mM HEPES buffer, pH 8.0, containing 0.5 mM cysteine-HCl. For detergent extraction, either 0.5% (w/v) CTAB or 0.5% (w/v) DOC was used. Foaming was suppressed by spraying the surface with a short burst of Anti-Foam aerosol (Sigma). Particulate matter was removed by centrifugation at 1,500g for 10 min.

Protein was precipitated from supernatant solution at 4°C with 20% (w/v) trichloroacetic acid added to the enzyme extract in a ratio of 1:1 (v/v). The protein pellet was redissolved in 0.1 M NaOH and calculated as bovine serum albumin equivalents by the method of Lowry et al. (15). The difference between Folin values obtained from the original extract and from the trichloroacetic acid pellet was used to estimate soluble phenol concentration in terms of tyrosine.

Six enzymes were selected for study. Besides peroxidase (donor: H2O2 oxidoreductase; E.C. 1.11.1.7) and polyphenoloxidase (as chlorogenic acid oxidase (o-diphenol:O2 oxidoreductase; E.C. 1.10.3.1)); NADH oxidase (NADH:lipoxidase oxidoreductase; E.C. 1.6.4.3), malate-NAD oxidoreductase (E.C. 1.1.1.37), acid phosphatase (orthophosphoric monooester phosphohydrolase; E.C. 3.1.3.2), and protease (peptide peptidase; E.C. 3.4.4.-) were examined in leaf extracts sampled from five different stalk positions.

Chlorogenic acid oxidase was determined polarographically with the Clark electrode using a Yellow Springs Instrument.

1 North Carolina Agricultural Experiment Station Journal Series 3762.
apparatus. The assay mixture consisted of 2.0 ml of 10 mM HEPES buffer, pH 6.0, and 1.0 ml of enzyme preparation; 0.2 ml of 1 mM chlorogenic acid was added after measuring endogenous O₂ uptake. Peroxidase was assayed spectrophotometrically with the Gilford model 2400 instrument at 460 nm with o-dianisidine following the Worthington procedure (38). A 0.05-ml enzyme aliquot was added to 2 ml of buffer (10 mM potassium phosphate at pH 5.0), 0.2 ml of 0.5% H₂O₂, and 0.2 ml of 0.5% o-dianisidine, the latter dissolved in 50% methanol.

NADH oxidase was assayed according to Worthington (38) with 0.8 ml of 10 mM HEPES, pH 8.0; 0.2 ml of 1 mM NADH; 0.1 ml of 1 mM DCIP; and 0.1 ml of enzyme preparation. The enzyme extract was dialyzed overnight to remove soluble reducing substances prior to assaying. Reduction of DCIP was monitored by measuring the rate of color loss at 600 nm. Malate-NAD oxoreductase was measured in the reverse direction with 0.1 ml of 10 mM oxalacetate and 0.1 ml of 1 mM NADH in 0.8 ml of HEPES buffer, pH 8.0. After adding 0.1 ml of enzyme, the activity was followed by decrease of absorbency at 340 nm. Acid phosphatase was assayed with p-nitrophenyl phosphate; 0.1 ml of enzyme solution was incubated at 37° C with 1.0 ml of 0.2 M sodium succinate, pH 4.5; 1.0 ml of 20 mM substrate; and 0.5 ml of 0.1 M MgCl₂, for specific time intervals over a 30-min period. After 5, 10, 15, and 30 min, a 0.5-ml aliquot of the incubation mixture was pipetted into 2.5 ml of 0.1 M Na₂CO₃ and the increase in color absorbancy at 415 nm was recorded. Proteolytic activity was assayed with Congocoll, a conjugate of Congo Red and hide powder (Calbiochem). For the protease assay, the enzyme preparation was diluted 1:1 with water, and 0.5 ml of this solution was combined with 0.5 ml of 0.1 M phosphate buffer, pH 5.5, and 50 mg of Congocoll. After incubating for 2 hr at 37° C, 1.0 ml of phosphate buffer was added. The resultant mixture was filtered through Whatman No. 1 paper, and absorbancy of the filtrate was measured at 495 nm.

Anodic isoperoxidases were analyzed by disc electrophoresis at pH 9.5 using the standard Canalco system (5). Samples were mixed with 7% (w/v) acrylamide monomer solution in a ratio of 1:1 (v/v) and following light polymerization of the gel were electrophoresed at 3 mA per tube. Enzyme localization was performed by incubating the gels in 2 ml of the o-dianisidine-H₂O₂ assay mixture described previously. Isoenzyme profiles were obtained by scanning the gels at 460 nm with a Gilford linear transport module.

RESULTS

Comparison of the Extracting Efficiencies of Different Detergents. Complex extracting media have been designed to prevent inactivation of plant enzymes during the initial homogenization step (2). In addition, many enzymes which are particulate in the native state or which bind readily to membrane or cell wall material cannot be extracted completely by means of buffered aqueous media alone. Acid phosphatase and protease have been demonstrated in association with spheromes in plants and might be expected to exhibit latency properties (3). Many investigators have reported difficulties in achieving total extraction of plant peroxidases (1). Malate-NAD oxoreductases as well as o-diphenol oxidases occur in soluble and in particulate form in plant tissue (20, 25). Consequently, a preliminary study was conducted to test the extraction efficiency of two detergents with different ionic properties. Tobacco leaf samples taken from near the upper portion of mature plants were extracted with DOC, an anionic detergent, and CTAB, a cationic detergent. Of the two detergents examined, DOC was found to be superior for extracting more protein (1.85 mg/ml compared to 1.30 mg/ml) and less phenolic material. Chlorophyll was precipitated by low speed centrifugation in DOC extracts, but CTAB extraction resulted in a transparent green solution with a relatively small amount of pelleted material after centrifugation.

In efforts to remove chlorophyll from CTAB extracts, these preparations were further subjected to equilibrium dialysis or solvent extraction with tert-amyl alcohol. Very little improvement was gained by dialyzing since the chlorophyll and phenols as well as the protein were almost completely retained after overnight dialysis at 5 C. Partitioning with tert-amyl alcohol initially showed some promise because the chlorophyll was quantitatively extracted into the solvent phase. Unfortunately, 75% of the protein was also lost by this treatment.

Enzyme analyses revealed that twice as much peroxidase was recovered by DOC extraction than when no detergent was used while compared to CTAB extraction DOC was four times more effective with respect to peroxidase extraction (Table I). Interestingly, extraction of CTAB preparations with tert-amyl alcohol restored peroxidase activity to levels which were higher than in buffer extracts. Whether the low peroxidase activity in CTAB extracts was due to the detergent inhibition of CTAB or to the presence of a naturally occurring inhibitor was not determined. In contrast to the results with peroxidase, preservation of chlorogenic acid oxidase was better in CTAB preparations than in comparable DOC extracts, but extracting CTAB preparations with tert-amyl alcohol caused a minor loss of the original chlorogenic acid oxidase activity.

Although DOC extraction appeared to result in slightly lower chlorogenic acid oxidase activity than did CTAB, the higher protein and peroxidase values obtained with DOC extraction prompted adoption of the latter detergent for the subsequent enzyme studies reported in this paper.

Fractionation of DOC Extracts. Leaf samples from five stages of development were harvested from mature tobacco plants 1 to 2 weeks after removal of flower heads. After homogenization and low speed centrifugation, the DOC extract

Table I. Comparison of Enzyme Activities Extracted from Tobacco Leaves with or without a Detergent in the Homogenizing Medium

<table>
<thead>
<tr>
<th>Extraction Conditions</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chlorogenic acid oxide</td>
</tr>
<tr>
<td></td>
<td>μmoles O₂ consumed/min·g fresh wt</td>
</tr>
<tr>
<td>Buffer without detergent</td>
<td>4.60</td>
</tr>
<tr>
<td>Buffer with DOC</td>
<td>2.54</td>
</tr>
<tr>
<td>Buffer with CTAB</td>
<td>3.84</td>
</tr>
<tr>
<td>Original extract</td>
<td>3.53</td>
</tr>
<tr>
<td>Aqueous phase (after extraction with tert-amyl alcohol)</td>
<td></td>
</tr>
</tbody>
</table>

¹ Based on relationship between oxidized dye and molar absorbancy of 1.13 × 10⁴ cm⁻¹ for H₂O₂ at 460 nm (38).
was optically clear. Chlorophyll in the form of a non-emulsifiable aggregate was pelleted by centrifugation at 1500g. The light brown or yellow solution was used as the enzyme source for these studies. The relative amounts of particulate and soluble material varied with each preparation. The lowermost layer, composed primarily of starch, was largest in leaf extracts from Ethrel-treated plants whereas extracts from upper leaf material contained the largest chlorophyll pellet. Protein levels in the soluble extracts varied from 0.275 mg/ml in the bud extract to 1.850 mg/ml in the upper leaf extract. In general there was a decline in extractable protein with increasing leaf maturity. Phenol concentration, based on tyrosine as standard, averaged 0.5 mg/ml extract, but no significant differences in phenol content from one extract to another were observed.

**Enzyme Activities in DOC Extracts of Tobacco Leaves as a Function of Leaf Maturity.** Because of the wide variation in fluid levels separated from DOC homogenates by centrifugation, enzyme activities have been expressed in terms of protein concentration in each extract.

In agreement with data of previous investigators, total peroxidase activity increased with leaf maturity whereas chlorogenic acid oxidase activity decreased (Table II). The decline in chlorogenic acid oxidase activity was most noticeable between the bud stage and the upper leaf stage. Thereafter, the decrease in chlorogenic acid oxidase was more gradual, persisting into the senescent stage. By contrast, peroxidase activity was highest in senescent leaf extracts but lowest in immature leaf samples (Table II). Other differences in peroxidase relating to isozyme patterns will be described in a later section.

The enzyme which catalyzes the oxidation of NADH with DCIP as acceptor is referred to as NADH oxidase or diaphorase. In animal systems it has been identified as lipoamide dehydrogenase (17). If this characterization is also true for such enzymatic activity in plants, the assay for NADH oxidase in tobacco leaf extracts should relate to one portion of the enzyme complex responsible for converting pyruvate or α-ketoglu tarate to acetyl-CoA or to succinyl-CoA, respectively. A slight decrease in enzyme activity occurred from the bud to the upper leaf stage, but the highest activity was associated with the untreated lower leaves (Table II).

Malate-NAD oxidoreductase followed a pattern similar to that found for chlorogenic acid oxidase, except for a more precipitous decline in activity at the upper leaf stage (Table II). The difference in specific activity from the bud stage to the senescent stage encompassed a 45-fold range, which was the greatest variation observed in these studies. Since it is assumed that mitochondria would be solubilized by DOC, malate-NAD oxidoreductase activity in these extracts is probably a composite of membrane-associated and soluble phase enzymes. Malate and citrate are interconvertible as a consequence of the tricarboxylic acid cycle, but the curing process appears to favor the accumulation of citrate over that of malate in tobacco leaves (35), suggesting that malate-NAD oxidoreductase is more stable than other enzymes in the sequence associated with senescence.

Acid phosphatase is the term applied to a class of enzymes having phosphohydrolytic activity but without a clearly defined role in metabolism. Their primary function in juvenile tissue is probably not degradative. The high content of acid phosphatase in bud extracts may indicate enhanced requirement for phosphate transfer during meristematic activity. Acid phosphatase fell to lowest levels in the middle leaf stage, thereafter rising to slightly higher levels in mature and senescent leaves harvested from the bottom of the plant (Table II).

Although the absolute values were dissimilar, particularly at the bud stage, the pattern for protease resembled that for acid phosphatase in the first four stages of leaf development (Table II). No protease activity could be detected in lower leaves treated with Ethrel. Since senescence is associated with degradative processes, the lack of protease in Ethrel-treated material is difficult to explain. If the use of DOC for extraction somehow interfered with the enzyme activity, one must conclude that the molecular properties of protease from senescent tissue are different from that of the enzyme found in less mature leaf material. Alternatively, it is possible that autolysis destroyed protease activity during the later stages of senescence.

**Isoperoxidase Differences in DOC Extracts from Tobacco Leaves at Different Stages of Maturity.** Peroxidase activity was analyzed by means of disc electrophoresis in order to determine the influence of maturation on isozyme patterns. A total of 10 distinct anodic isoperoxidase bands were localized, but no one extract contained all of the peroxidase forms in equal amounts (Fig. 1). Extracts from bud tissue and upper leaves produced identical bands, but the relative staining intensities of each band were distinctly different. The two bands characterized by lowest relative mobilities were absent in extracts from the mature leaves. Two new bands with intermediate migration properties were found in extracts of senescent leaf material, and the entire spectrum of more rapidly migrating isoperoxidase types appeared to be shifted toward the anode (see bottom scan in Fig. 1). Judging from the increasing levels of peroxidase activity during maturation (see Table II), it is clear that the general deterioration which is assumed to occur during senescence does not inactivate peroxidase per se but has the opposite effect. Whether this change is essential to metabolic processes in the older leaf tissue cannot be resolved since the physiological role of peroxidase is still a matter of conjecture. Individual peroxidases probably function differently (10) and most likely are associated with different intracellular compartments (9) and different tissues (7).

**DISCUSSION**

The aim of these studies was to assess the variability in enzyme levels of leaves differing in maturity. Although only a limited number of enzymes were investigated, the six enzymes studied were representatives of several types of oxidoreductive and hydrolytic systems known to occur in plants. The sharp fall in malate-NAD oxidoreductase activity in extracts of upper

---

**Table II. Enzyme Activities Extracted from Tobacco Leaves as a Function of Maturity of the Leaf**

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Chlorogenic Acid Oxidase</th>
<th>NADH Oxidase (Diaphorase)</th>
<th>Malate-NAD Oxidoreductase</th>
<th>Peroxidase</th>
<th>Acid Phosphatase</th>
<th>Protease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles O₂ taken up/min mg protein</td>
<td>µmoles DCIP reduced/ min mg protein</td>
<td>µmoles NADH oxidized/ min mg protein</td>
<td>µmoles H₂O₂ decomposed/ min mg protein</td>
<td>µmoles p-nitrophenol oxidized/ min mg protein</td>
<td>ΔA₄₉₅/ min mg protein X 10⁻²</td>
</tr>
<tr>
<td>Buds</td>
<td>5.90</td>
<td>0.87</td>
<td>3.62</td>
<td>9.45</td>
<td>3.12</td>
<td>0.68</td>
</tr>
<tr>
<td>Upper leaves</td>
<td>2.70</td>
<td>0.39</td>
<td>0.72</td>
<td>0.09</td>
<td>0.19</td>
<td>0.30</td>
</tr>
<tr>
<td>Middle leaves</td>
<td>1.30</td>
<td>0.75</td>
<td>0.45</td>
<td>32.50</td>
<td>0.14</td>
<td>0.09</td>
</tr>
<tr>
<td>Lower leaves</td>
<td>1.00</td>
<td>1.12</td>
<td>0.40</td>
<td>49.20</td>
<td>0.18</td>
<td>0.54</td>
</tr>
<tr>
<td>Upper leaves</td>
<td>0.90</td>
<td>0.96</td>
<td>0.08</td>
<td>70.60</td>
<td>0.20</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Copyright © 1972 American Society of Plant Biologists. All rights reserved.
The small fluctuations in levels of NADH oxidase among the five leaf samples were in contrast to wide differences in activity for some of the other enzymes investigated. Apparently, the glycolytic pathway continues to function at relatively constant rates throughout leaf development. It would seem that a need for regenerating NADH by this enzyme persists in older senescing leaves since NADH oxidase activity remained fairly high in lower leaves, even after treatment with Ethrel.

Besides having a presumed role in intra- or extracellular digestion, or both, acid phosphatase may function in concert with a phosphate-recycling system since many organic compounds require activation by phosphorylation in order to be utilized. The need for maintaining sufficient levels of phosphate for the formation of high energy intermediates during biosynthetic processes probably explains why leaf buds contain relatively high acid phosphatase activity. Similarly, the rise of acid phosphatase in older leaves probably aids in the translocation of phosphate to younger tissues near the tip of the plant, since phosphate is often in short supply and must be recycled for continued growth and development. Others have shown that extracellular acid phosphatase levels in tobacco cell culture medium increased in response to phosphate depletion (33).

Since protease is essentially degradative in function, one would expect protease activity to increase as the senescent stage is reached. The lack of protease in Ethrel-treated leaf extracts may be a result of inactivation by DOC. However, it is also possible that the substrate (Congocoll) used for these assays is not capable of reacting with the enzyme in senescent tissues although others have used Congocoll with success to detect plant proteases (27). In considering either of these alternatives, one must assume that protease in Ethrel-treated leaves is different in form or function from similar enzyme activity in less mature leaves. The most obvious conclusion is that protease is simply absent in leaves after treatment with Ethrel. The lack of protease in Ethrel-treated tobacco leaves is in conflict with what appears to be a need for such activity during senescence. However, Martin and Thimann (16) showed that in oat leaves the acid protease activity decreased in late senescence in contrast to the neutral protease which continued to increase. In this same connection, Ryan found an endogenous protease inhibitor in a number of plants, including tobacco (26).

Differences in peroxidase levels and isozyme composition in leaves at different stages of maturity raise the question of whether or not these enzymes have a role in maturation. Assuming that green leaves contain adequate catalase for the disposal of excess hydrogen peroxide generated by flavoprotein oxidations during photosynthesis and photorespiration, peroxidase is probably involved in channeling the oxidizing power of hydrogen peroxide into some more useful purpose. IAA oxidation (11) as well as lignification (14) has been attributed to peroxidase activity. However, several lines of research including histochemical localization suggest that peroxidases may be loosely associated with membranes in the natural state (9, 21). Peroxidase H^+ has been solubilized from ribonucleoprotein particles of pea seedlings by chelating agents such as EDTA (18), indicating that the enzyme may be bound to the membrane system by metals. The increased solubilization of tobacco leaf peroxidase with DOC supports this concept (see
Table I). One is reminded that permeability or membrane changes generally precede development changes and could be critical in establishing the course to senescence (19). The peroxidase gradients in tobacco pith were shown by Lavee and Galston (13) to be inversely correlated with growth potential for forming callus under tissue culture conditions.

In conclusion, the major objective of these investigations was to correlate enzymatic activity with leaf maturation in terms of stalk position on a fully developed tobacco plant. The six enzymes chosen for these investigations have some if not all of their activity associated with a particulate compartment in plants. DOC was selected for enzyme extraction in order to solubilize the membrane-bound forms of each of the enzymes studied. Some enzymes exhibit a biphasic response to DOC (23), but usually this effect is reversed by dilution. It would be of interest to supplement the present studies with further research on varying the concentration of DOC used for extraction and comparing DOC with other detergents besides the one tested here, viz. CTAB.

Acknowledgment—The technical assistance of Mr. William G. Woodlief is gratefully acknowledged.

LITERATURE CITED