Biochemical and Model Chemical Reactions for the Basis of Red Pigment in Flue-Cured Tobacco

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Polyphenol oxidase (PPO) preparations from green tobacco leaves oxidized chlorogenic and caffeic acids to *o*-quinones, which reacted in vitro with nornicotine to produce red color at 570 nm. Activity was not obtained when ferulic acid was used as a substrate. Variable temperature data showed PPO was stable for 1 h at 75 °C. Nicotine, myosmine, pyrrolidine, asparagine, alanine, and glutamic acid failed to react with chlorogenic acid and enzyme; nor did these compounds interfere with color development between nornicotine and *o*-quinones. Aqueous ethanolic extracts (80%) gave similar red color from tobacco grade FR and cherry red tobacco (CR) but a brown color from orange tobacco grade F. Alkaloid analysis showed CR and FR grade tobaccos contained higher levels of nornicotine than F grade tobacco. Model chemical reactions with nornicotine showed coupling of nornicotine with *o*- and *p*-quinones by Schiff base and Michael addition reaction.

INTRODUCTION

Flue-cured tobacco is an important commodity to the tobacco industry. Recently, there has been a change in demand from lemon (L) and bright tobacco grades to orange (F) and orange-red (FR) flue-cured grades. FR grade tobacco is easily confused with "Cherry Red" (CR) tobacco, a dappled red color produced from enzymatic conversion of nicotine to nornicotine, which reacts with oxidized *o*-diphenols producing red color (Penn and Weybrew, 1958). Although tobacco growers are not permitted to produce nicotine-converting cultivars, CR tobacco continues to occur in each flue-cured crop (Bowman and Rawlings, 1985). CR tobacco is graded as FR tobacco (U.S. Department of Agriculture, 1989). The production of CR tobacco has been an ongoing problem since the 1950s; however, the structure and chemistry of the CR pigment have only been studied enzymatically in vitro.

Wernsman and Matzinger (1968) concluded that nicotine conversion precedes yellowing in some flue-cured cultivars; however, most flue-cured cultivars convert during yellowing. Wada (1956) reported that the CR color was formed in the latter stages of curing and was enhanced by heat. The formation of the CR pigment in vitro was studied by Penn and Weybrew (1958), Wada (1956), and Wada and Ihida (1957). These studies concluded that nornicotine and oxidized *o*-diphenols react to produce a CR pigment. Penn and Weybrew (1958) defined two active enzymes, polyphenol oxidase (PPO) and peroxidase, responsible for oxidation of chlorogenic acid and caffeic acid to *o*-quinones. Penn and Weybrew and Wada suggested that red color was formed more rapidly in vitro if both enzymes were active, and to activate peroxidase, H_2O_2 must be added as a H^+ acceptor to obtain oxidation of the *o*-diphenol. Wada (1956) showed PPO could withstand temperatures up to 80 °C for 5 min and still have activity. Wada also tested the enzymes for competition with proline and glycine as substrates and found no interference with nornicotine and its ability to react with *o*-quinones produced from chlorogenic and caffeic acids.

During the early phases of curing, yellowing, chlorophyll degrades and enzymatic activity increases rapidly. Proline and hydroxyproline increase simultaneously with chlorophyll degradation during yellowing, proteins hydrolyze to amino acids (Weybrew et al., 1984), starch hydrolyzes to reducing sugars (Frankenburg, 1946), and other secondary products are formed in the leaf. Amino acids react with reducing sugars to form nonenzymatic browning products, and polyphenols, amino acids, alkaloids, and iron react to form a brown pigment in the cured leaf (Chortyk, 1967; Dymicky et al., 1967). When temperature is increased too rapidly after yellowing, the cured leaf becomes dark and exhibits red color (W. H. Johnson, North Carolina State University at Raleigh, personal communication, 1991). Weybrew et al. (1984) suggested that overripe tobacco that yellowed too long was likely to be brown or reddish.

A broad study was undertaken to evaluate (1) polyphenol, amino acid, nicotine, and nornicotine concentrations of F, FR, and CR tobaccos; (2) in vitro parameters, which influence the reactions of nornicotine with oxidative products of polyphenols; (3) model compounds synthesized to understand the reactivity of nornicotine during senescence and curing; and (4) the red pigment from CR and FR tobaccos by isolation and characterization.

MATERIALS AND METHODS

Flue-cured F and FR grade samples were collected from Georgia, Florida, and eastern North Carolina markets. CR tobacco was taken from the Tobacco Research Station, Oxford, NC. Each tobacco was selectively combined into a homogeneous sample by the USDA Grading Service. Three 100-g samples of tobacco were taken from the larger samples for analyses to compare chemistry among the three tobaccos. The samples were analyzed for total alkaloids as nicotine using the Technicon Autoanalyzer (Harvey et al., 1969); nicotine and nornicotine were analyzed according to the Cundiff and Markunas (1964) procedure and by capillary GC (Severson et al., 1981). The tobaccos were analyzed for free amino acids by HPLC (Lauterbach and Moldoveanu, 1988) and for individual polyphenols by HPLC (Snook and Chortyk, 1982). Ten grams of ground lamina (200 mesh) was extracted with 100 mL of aqueous 80% ethanol on a

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shaker for 1 h to determine if red color associated with FR and CR could be solubilized and was present in all three tobaccos. The 80% ethanol extracts were diluted to 60% ethanol, the pH was adjusted to 10 with 5 N NaOH, and the extracts were partitioned with chloroform. The pH of the solution was readjusted to pH 2 with concentrated H_2SO_4 and reextracted with chloroform. The solutions were extracted at pH 2 and 10 and prior to pH adjustment with *n*-hexane, diethyl ether, and 1-butanol. Pretreated poly(vinylpyrrolidone) was added to the red solutions at pH 2 to absorb the red color or remove interfering substances so that the red color could be extracted and identified.

A PPO enzyme was isolated from mature green tobacco leaves using the method of Penn and Weybrew (1958). Powder containing the enzyme was stored in an ultracold freezer at −70 °C. The enzyme was combined with different substrates under various temperatures and time periods to produce red color.

Preparation of Enzyme for Assay. A half gram of the enzyme was suspended in 30 mL of 0.067 M phosphate buffer (1:1, KH_2PO_4 and Na_2HPO_4, pH 6.2) at 0 °C and centrifuged for 5 min at 5000 rpm. The supernatant was filtered and used as enzyme throughout this work (Wada and Ihida, 1957).

Concentration of Solutions. Chlorogenic acid [1,3,4,5-tetrahydrocyclohexanecarboxylic acid 3-(3,4-dihydrocinnamate)], caffeic acid (3,4-dihydroxycinnamic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid), nornicotine, nicotine, myosmine, proline, *p*-quinone, and pyrrolidine were prepared at a concentration of 0.02 M in deionized water.

Enzyme Test Reactions. Five milliliters of buffer, 2.5 mL of substrate (including chlorogenic acid, caffeic acid, nicotine, nornicotine, myosmine, proline, alanine, glutamic acid, asparagine, and pyrrolidine), and 1 mL of enzyme were used for individual assays. Nornicotine, chlorogenic acid, and buffer were treated at 85 °C to determine if red color was initiated from the reaction of nornicotine and chlorogenic acid by heat and could develop without enzyme. Caffeic acid, chlorogenic acid, proline, alanine, glutamic acid, asparagine, nicotine, and pyrrolidine were obtained from Aldrich Chemical Co., Milwaukee, WI. Nornicotine was obtained from Philip Morris Inc., Richmond, VA. Myosmine was synthesized according to the method used by Brandange and Lindblom (1976).

Measurements. Spectra were obtained with an IBM-9430 UV–visible spectrophotometer from 380 to 900 nm.

Chemical Reactions for Model System. Chemical reactions with nornicotine, *p*-quinone, and *o*-quinone were used to produce a model reaction to understand enzyme mechanism and formation of red color. The oxidation of caffeic acid to *o*-quinone was attempted using caffeic acid and silver carbonate on Celite in diethyl ether, acetone, and tetrahydrofuran (THF) (Balogh et al., 1971); sodium metaperiodate (Adler and Magnusson, 1971); silver oxide in THF (Fieser and Fieser, 1975); sodium dichromate in aqueous acetic acid (Gates and Newhall, 1948); and aqueous ferric chloride (Edwards and Lewis, 1957). Caffeic acid methyl ester was prepared by allowing a solution of caffeic acid in methanol plus three drops of 36 N H_2SO_4 to stand overnight at 21 °C. Caffeic acid methyl ester was subjected to the same oxidation procedures as caffeic acid to produce *o*-quinone.

Nornicotine was reacted with *p*-quinone in methylene chloride, water, and methanol to form red color. Products from reaction in methylene chloride were separated on a 2.5 × 30-cm, 250–400-mesh silica gel column using (92.5:7.5 v/v) chloroform/methanol solution. Twenty-milliliter fractions were collected; fractions containing red color were combined and concentrated to 1 mL for GC/MS. The components were separated on a Supelco DB-5, 0.28-mm i.d. (WCOT) 60-m fused silica capillary column using a HP 5880 with a 5970 GC/MS system. A temperature range from −40 to 240 °C with 5 °C/min temperature program was used to obtain a total ion chromatogram.

Nornicotine was reacted with *o*-quinone prepared from pyrocatechol according to the procedure of Adler and Magnusson (1971) to obtain red color. The color was separated from the reaction mixture using a reversed-phase C_{18} thin-layer plate (C_{18} TLC) with methanol/water (2:1 v/v); color was eluted from the TLC plate with methanol. Mass spectra were obtained using a Waters/VG 2250 (HPLC/MS) system equipped with a Bio-Rad–Bio-Sil Amino-5-S (4.6 × 250-mm) column (Bio-Rad, 1414 Harbor Wade S, Richmond, CA). Separation was obtained using CH_3CN/HOH (9:15 v/v) isocratically as an eluant 1 mL/min.

Table I. Percent Alkaloids in Flue-Cured Tobaccos

tobacco	total[a]	nicotine[b]	nornicotine[b]	% conv
F grade	3.18	3.04	0.15	4.72
FR grade	3.06	2.70	0.34	11.11
CR[c]	2.99	2.33	0.66	22.07

[a] Average of three determinations from the Autoanalyzer. [b] Average of three determinations from the Cundiff-Markunas and capillary GC. [c] Cherry red tobaccos.

Table II. Polyphenols of Flue-Cured Tobaccos[a]

	mg/g		
polyphenol	F grade	FR grade	CR[b]
chlorogenic acid	7.55	8.52	9.83
scopoletin	0.54	0.53	0.55
rutin	4.47	4.90	6.29

[a] Average of three determinations. [b] Cherry red tobaccos.

Table III. Amino Acid Data for F and FR Grade and Cherry Red Tobaccos[a]

	mg/g		
amino acid	F grade	FR grade	CR
aspartic acid	1.09	0.91	0.48
glutamic acid	0.70	0.62	0.38
asparagine	3.60	0.62	1.18
serine	0.12	0.13	0.17
glutamine	1.95	2.15	1.59
glycine	1.03	0.91	0.44
histidine	0.30	0.22	0.03
arginine	0.04	0.04	ND[b]
threonine	0.81	0.72	0.36
alanine	0.81	0.72	0.08
proline	7.12	6.80	6.51
tyrosine	0.17	0.51	ND[b]
valine	0.09	0.06	0.18
isoleucine	0.14	0.13	0.06
leucine	0.06	0.09	0.01
phenylalanine	0.36	0.27	0.62
total	18.39	14.90	11.78

[a] Average of three determinations. [b] ND, not determined.

RESULTS AND DISCUSSION

Leaf color from FR grade and CR tobaccos suggested the same chemistries producing red color; however, the F grade appeared to be different in color. Table I shows percent difference in nicotine and nornicotine among F and FR grades and CR tobaccos. These data showed that nornicotine was higher in FR than in F, but nornicotine in CR was twice the concentration of nornicotine in FR. Polyphenols (Table II) were 11 and 32% greater in FR and CR than in F. Chlorogenic acid and rutin accounted for the major differences in polyphenol concentration among the three tobaccos, and the increasing concentration of polyphenols exhibited the same pattern as nornicotine. Amino acid (Table III), nornicotine, and polyphenol concentrations from the three tobaccos were not definitive in explaining the difference in color.

Solutions obtained from aqueous 80% ethanol extracts suggested FR and CR were similar in red color, but the solution obtained from F grade tobacco was dark brown. Chloroform removed nicotine and nornicotine from the extracts at pH 10 without removing red color. Extractions at pH 2 with chloroform failed to remove the red color. No red color was removed with *n*-hexane, diethyl ether, and 1-butanol at any pH. Further attempts to remove red color from aqueous ethanolic extracts at pH 2 with poly(vinylpyrrolidone) failed. The chemistry responsible for red color in the aqueous solutions obtained from the FR and CR tobaccos could not be removed; therefore, the structure of the red pigment from the tobacco extracts could not be determined.

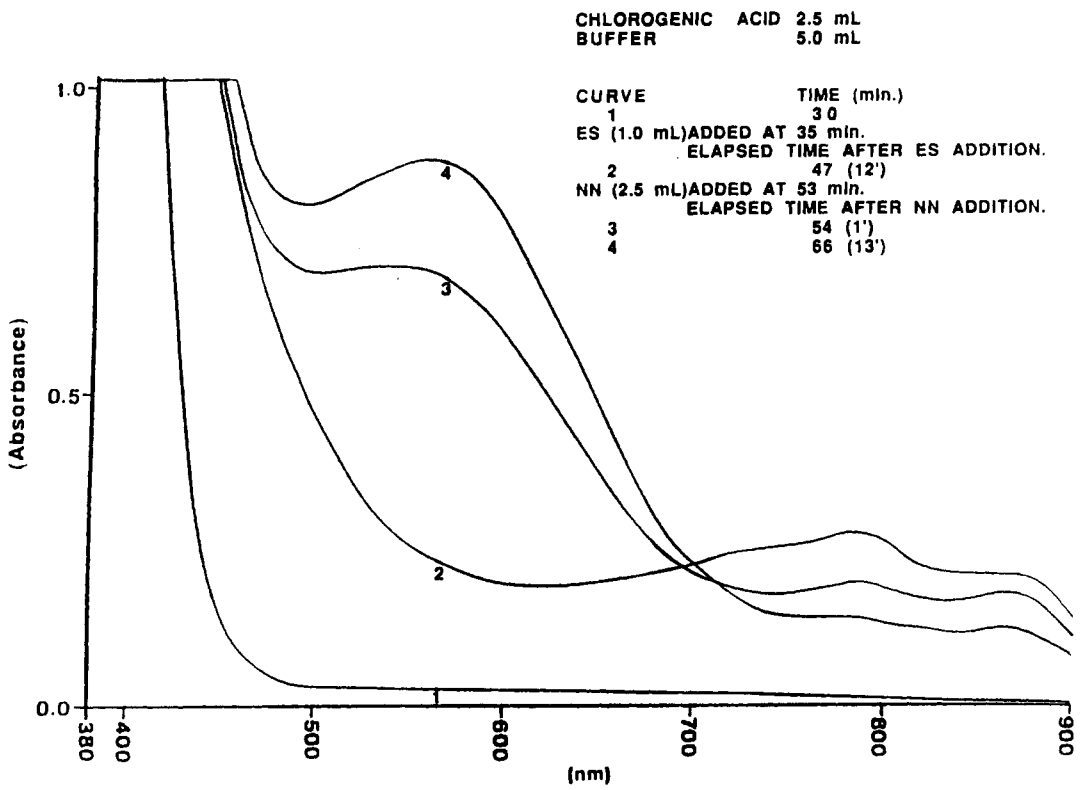

Figure 1. Enzyme assays at 21 °C.

The reaction of nornicotine and *o*-quinones, formed from oxidized *o*-diphenols, is well documented. Thus, the in vitro assays were initiated to determine enzyme activity as influenced by time and temperature on the formation of red color. The enzyme and buffer were tested at 21 °C in the visible region from 380 to 900 nm to determine if absorption occurred from the enzyme; no absorption at 570 nm was observed. Chlorogenic acid and buffer were combined and monitored for 30 min in the first assay, and no absorption was detected (Figure 1, curve 1). Absorption was observed at 785 or 865 nm 1 min after the enzyme was added and increased for 12 min (Figure 1, curve 2) until nornicotine was added to the solution. This indicated that the enzyme altered the structure of chlorogenic acid. When nornicotine was added, chlorogenic acid reacted to produce red color as seen from the increase in absorption for the red color at 570 nm and simultaneously the reduction in absorption at 785 and 865 nm as a function of time (Figure 1, curves 3 and 4).

To validate the effect of enzyme upon the reaction, chlorogenic acid, nornicotine, and buffer were monitored for 90 min at 0 °C with no change in absorption (Figure 2, curve 1). Red color, formed instantly after the addition of the enzyme, was monitored from 1 to 12 min (Figure 2, curves 2 and 3).

Flue curing requires 148–172 h. Yellowing requires 36–48 h at a temperature below 38 °C, and the drying-out phase reaches temperatures of 66 °C before color is fixed (Boyette and Watkins, 1984). Chlorogenic acid, nornicotine, buffer, and enzyme were assayed at 70, 75, 80, and 85 °C to test enzyme integrity and activity with *o*-diphenols as a function of temperature. Absorption of the solutions was measured 30 s later. Figure 3, curve 1, shows stability and activity of the enzyme measured over the temperature range from 21 to 70 °C. The enzyme showed loss of activity at 75 °C as indicated by loss in absorption at 570 nm (Figure 3, curve 2). Some activity was observed at 80 °C (curve 3) with no absorption at 85 °C, indicating that the enzyme was no longer active (curve 4). These results show that the enzyme is heat stable and could react during curing until water is lost from the leaf.

To verify that absorption at 570 nm was an enzymatic reaction and not chemical, an assay using chlorogenic acid, nornicotine, and buffer was subjected to temperatures from 50 to 85 °C. No absorption occurred from the mixture at 570 nm at 50 °C; however, absorption did occur at 85 °C, but it was not comparable to color obtained at 75 °C (Figure 3, curve 2). The reaction ran for 1 h without change. Therefore, it was concluded that the enzyme was necessary to produce the reaction responsible for absorption at 570 nm.

Wernsman and Matzinger (1968) established that nornicotine occurs predominantly during yellowing. An assay was run to determine if proline generated during yellowing would compete with nornicotine and inhibit the reaction of nornicotine and oxidized chlorogenic acid. Similar patterns were observed in the reaction mixture with proline to the assay obtained in Figure 1, curve 2, showing only the activity of the enzyme upon chlorogenic acid. Addition of nornicotine to the assay after 20 min gave instant color, indicating no competition between proline and nornicotine.

Previous assays that contained enzyme, nornicotine, and chlorogenic acid gave positive activity producing red color. Additional experiments were conducted to determine if both quinic acid and caffeic acid moieties of chlorogenic acid needed to be intact for enzyme activity. No absorption was obtained from caffeic acid, buffer, and nornicotine during the 10 min the reaction was monitored. When the enzyme was added, red color appeared instantly at 570 nm. Activity of the enzyme was observed at 480 nm after 5 min. When nornicotine was added to the assay, instant red color developed at 570 nm. This suggests PPO reacted with *o*-diphenol groups of either chlorogenic or caffeic acid to form an *o*-quinone. Further evidence was obtained to confirm PPO specificity for *o*-diphenols by testing ferulic acid. An assay was run using ferulic acid, buffer, nornicotine, and enzyme for 1 h; no absorption was detected at 570 nm.

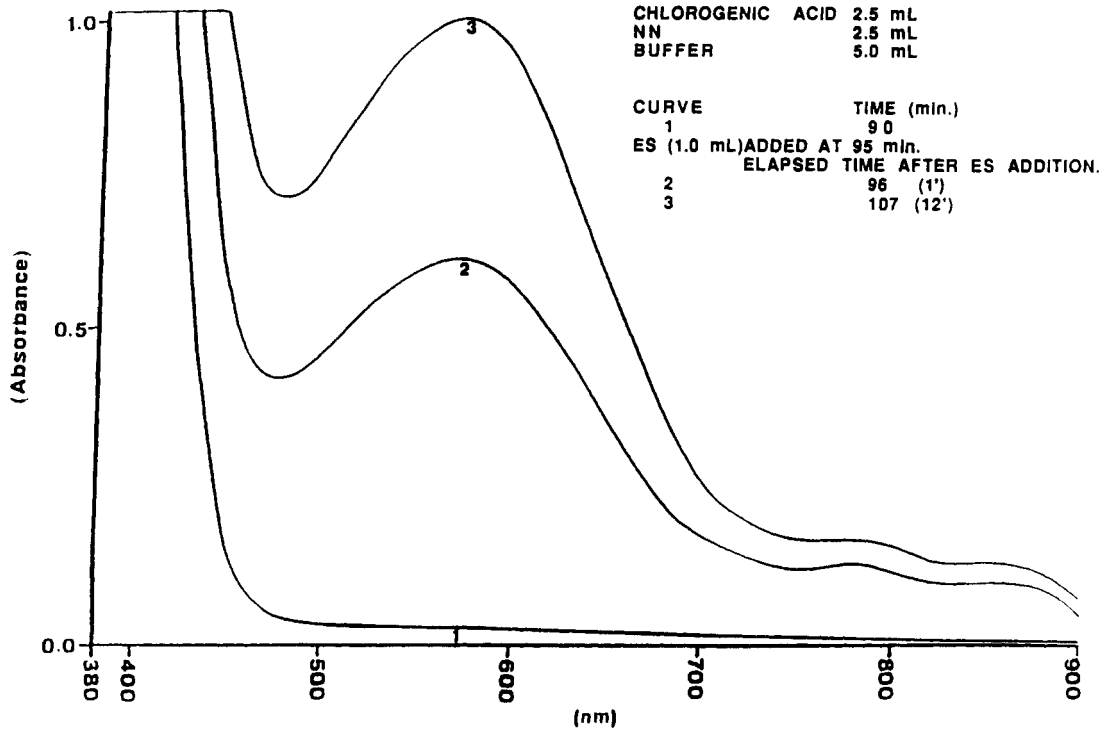

Figure 2. Enzyme assays at 0 °C.

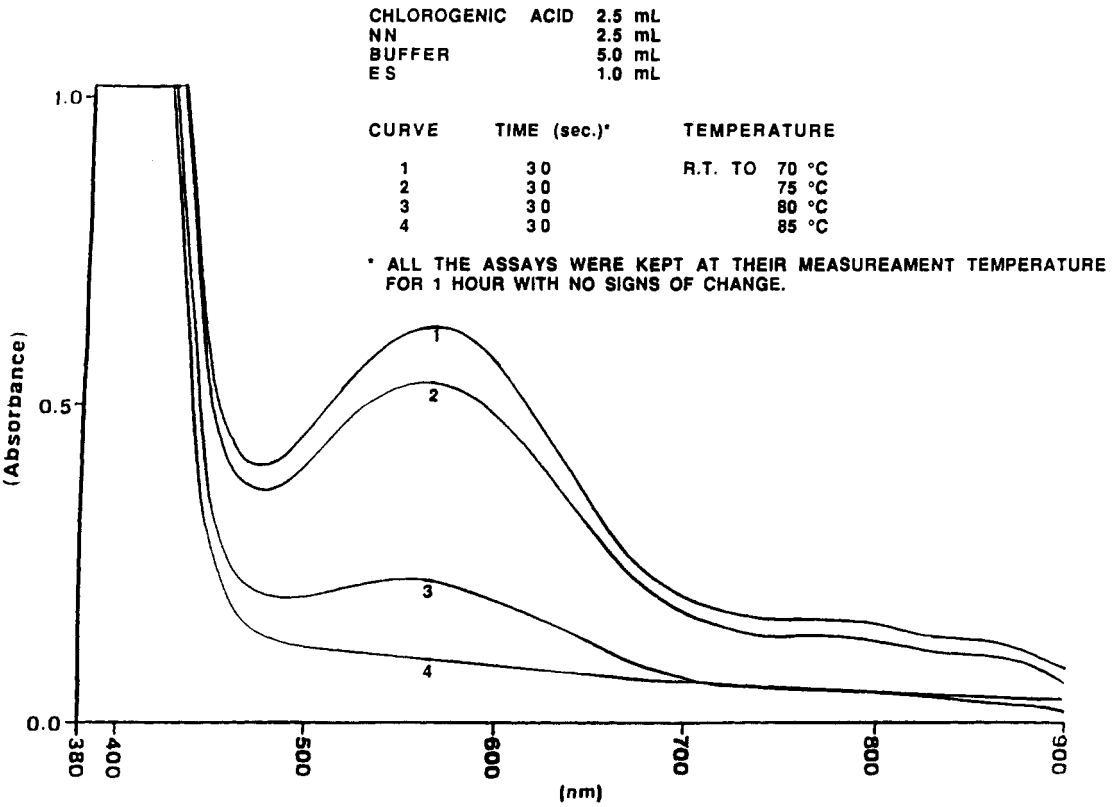

Figure 3. Enzyme assays at different temperatures.

Red color was formed in the assays with nornicotine and oxidized *o*-diphenols, but this did not exclude the possibility that tobacco alkaloids other than nornicotine also react with *o*-quinones producing red color. Therefore, reactions using the enzyme, nicotine, and myosmine were assayed with chlorogenic and caffeic acids. Only a pale yellow color was observed in the reactions (Table IV). Anabasine and anatabine, other alkaloids commonly found in flue-cured tobacco, were unavailable for assay. Some primary and secondary amines (Table IV), commonly found in tobacco, were assayed to determine if these compounds interfered with color development obtained from oxidized *o*-diphenols and nornicotine. The absence of red color indicates that primary and secondary amines tested did not react.

The observation that quinone formation from *o*-diphenols was mediated by enzyme led to studies with authentic *p*-quinone. Red color developed immediately with an absorption at 530 nm. However, the color changed, slowly with time, to a red-brown, and the solution turned

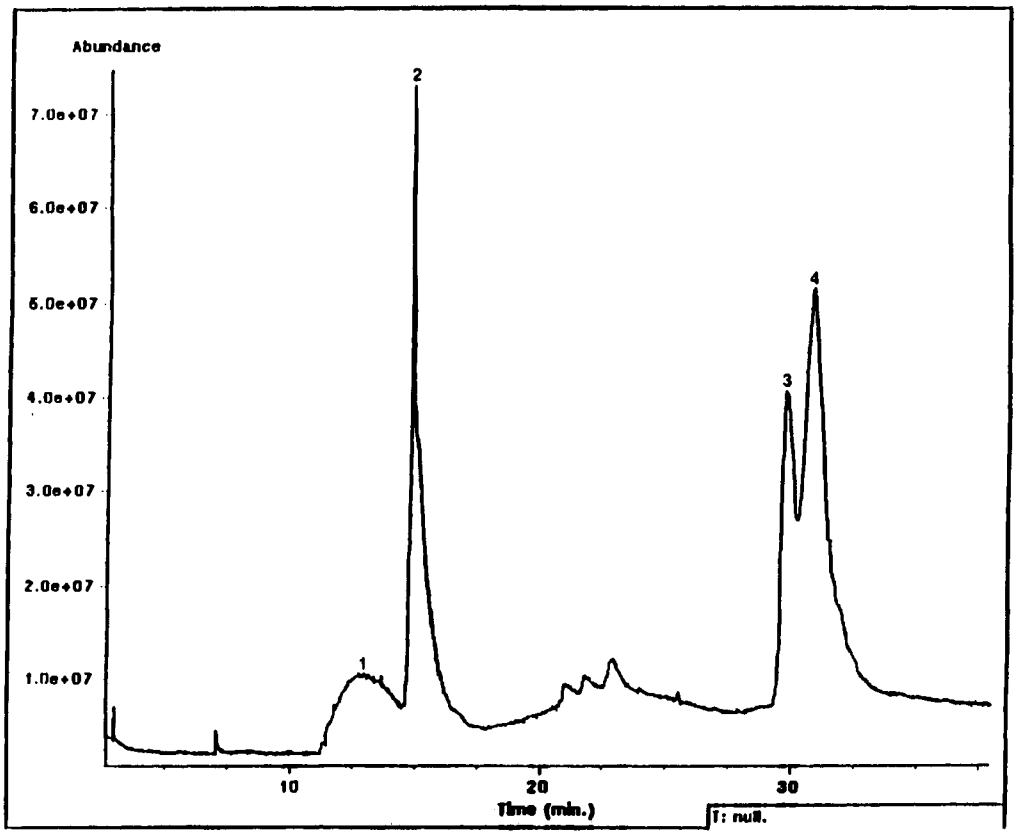

Figure 4. GC/MS of total ion chromatogram of nornicotine and *p*-quinone reaction.

Table IV. Color Reaction of Amino Acids and Amines with Oxidized Phenols

	oxidized phenol	
amine/amino acid	chlorogenic acid	caffeic acid
pyrrolidine	light brown	light pink
proline	pale yellow	pale yellow
alanine	yellow	pale yellow
asparagine	yellow	pale yellow
glutamic acid	yellow	pale yellow
myosmine	pale yellow	pale yellow
nicotine	pale yellow	pale yellow

completely brown after sitting overnight at 21 °C.

Attempts failed to obtain *o*-quinone from caffeic acid using different reagents and conditions similar to oxidation of pyrocatechol. The same methods used to attempt chemical oxidation of caffeic acid to its *o*-quinone also failed with caffeic acid methyl ester.

The chromatogram from nornicotine 1,4-dihydroquinone (Figure 4) shows 1,4-dihydroquinone (peak 1), unreacted nornicotine (peak 2), and two large peaks (3 and 4). Peak 3 gave mass spectra (Figure 5) *m/e* 240 with fragmentation 162 (100%) that accounts for loss of pyridine, 120 (21%) that accounts for loss of C_3H_6 from the 162 ion, 65 (17%) that comes from the rearrangement of the phenol ion, and 93 and 211 (12%) that comes from the phenoxy ion and loss of CHO (29), a typical fragmentation of phenols. On the basis of the spectra, the structure was proposed as 1-(*p*-hydroxyphenyl)nornicotine (Figure 5). To confirm the structure, a GC/IR spectrum was run (Figure 6A). This confirmed para substitution on the phenolic ring (Figure 6A) which shows the IR spectrum of the proposed structure as compared to the spectrum of authentic *p*-aminophenol (Figure 6B). Investigation of peak 4 (Figure 4) indicated a mixture of compounds from which mass spectra for two of the compounds was obtained. The two compounds gave *m/e* 254 and 256, respectively, and the following fragmentation pattern (Figure 7): *m/e* base peak 254 (100%); 225 (85%), loss of 29 (CHO); 176 (35%), loss of pyridine from the molecular ion peak; 136 (32%), possible loss of C_3H_4 from the pyrrolidine ring from the 176 fragment; and 118 (32%), for which we could not account. These were the major ions from the mass spectra of the structure proposed in Figure 7.

The molecular ion at 256 (Figure 8) was also the base peak (100%) with the following fragmentation: 138 (67%), loss of pyridine plus C_3H_4 from the molecular ion peak; 178 (62%), loss of pyridine from the base peak; and 106 (55%), probably accounts for pyridine conjugated with Schiff base (CH=NH). These are the major ions for the structure proposed in Figure 8. These molecular ions were obtained from peak 4 from the total ion chromatogram that was a mixture. An IR spectra could not be obtained for these compounds to verify the proposed structure.

The results obtained from the reaction of *p*-quinone and nornicotine gave at least two competing reactions. The first reaction that formed the molecular ion *m/e* 240 was a Schiff base reaction between nornicotine and *p*-quinone and followed by aromatization of the conjugated ring to a phenol. The second product from the reaction of *p*-quinone with nornicotine was a Michael addition reaction that gave two identifiable products (MS 254 and 256).

The first chemical reactions involved *p*-quinone, but the enzyme assays produced evidence that a chlorogenic or caffeic acid *o*-quinone was involved in formation of red color from oxidation of the *o*-diphenols. Chemical reaction with nornicotine and *o*-quinone, obtained from oxidation of pyrocatechol, was very labile. Reaction with nornicotine and *o*-quinone in four different solvents (water, methanol, methylene chloride, and *n*-hexane) resulted in a color change in which the red color of the reaction darkened instantly. The product had to be retained at −40 °C to maintain color stability. Methylene chloride was the only solvent used in which the color was stable for any period.

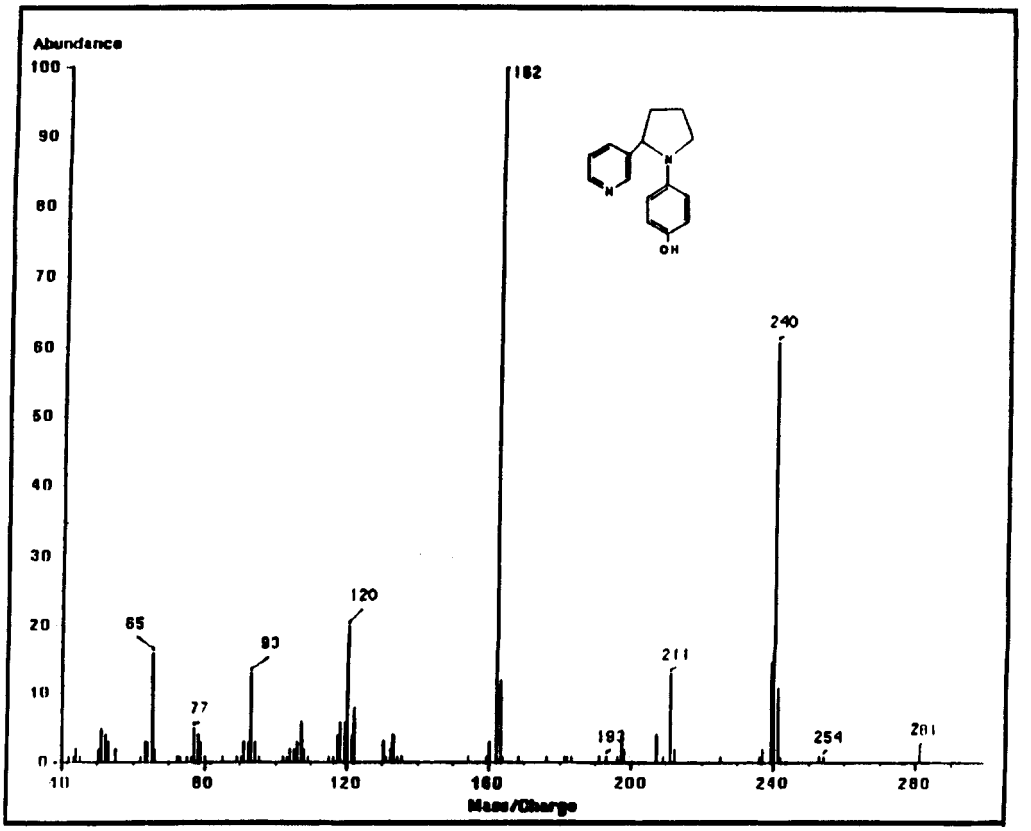

Figure 5. MS of peak 3 from total ion chromatogram.

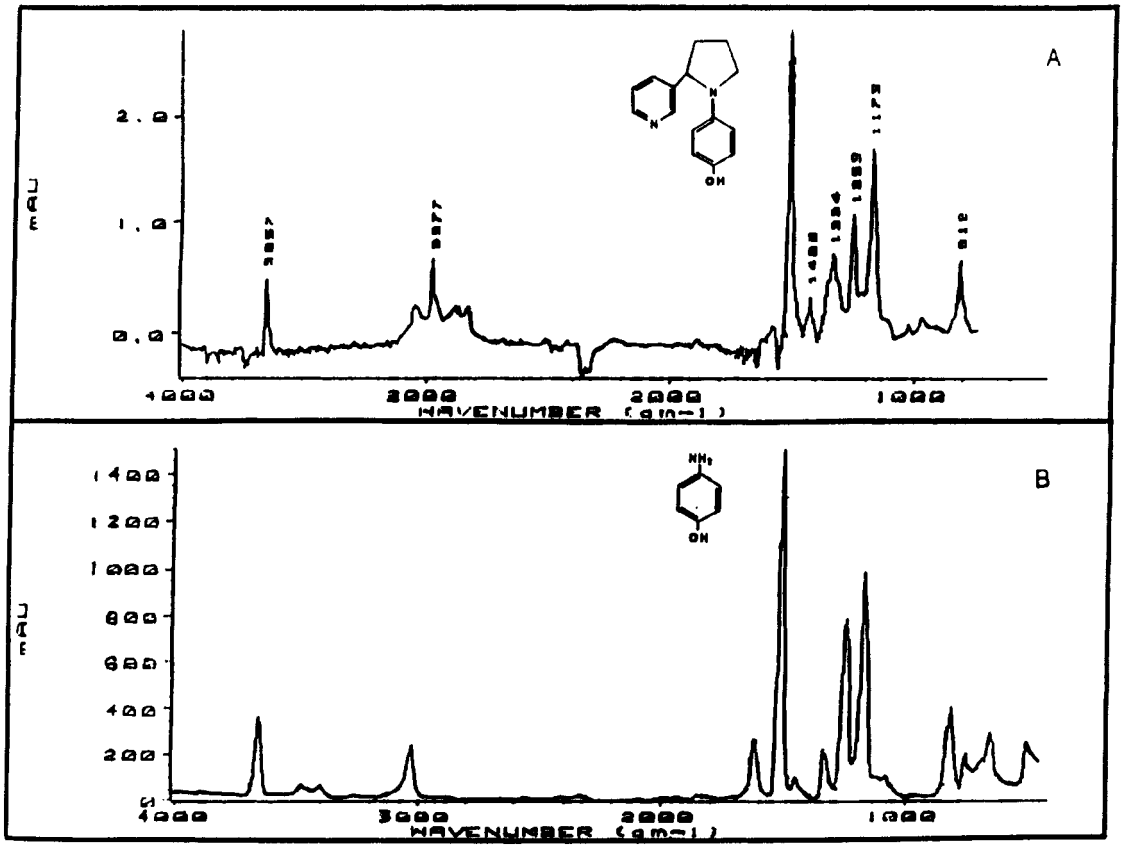

Figure 6. (A) GC/IR of peak 3 from total ion chromatogram. (B) IR of *p*-aminophenol.

A reversed-phase (RP) TLC (C_{18}) plate was used and developed in methanol and water (2:1 v/v). The red compound was removed from the TLC plate and extracted from the C_{18} phase with methanol. An attempt to identify the components of the red substance by HPLC/MS resulted in several compounds for which structures could not be proposed. An examination of the fragmentation pattern (Figure 9) of the mass spectra compares similarly with the compound from Michael addition product in Figure 8 with fragmentation at 256, 178, and 118.

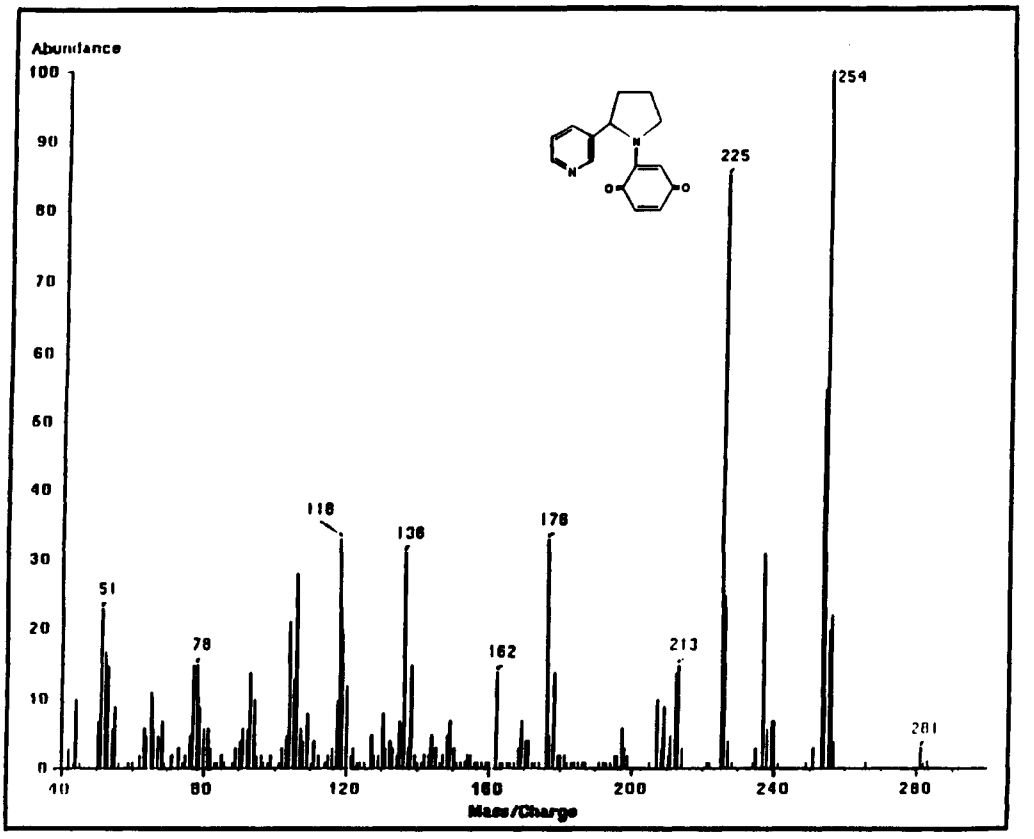

Figure 7. GC/MS of peak 4 mass of 254.

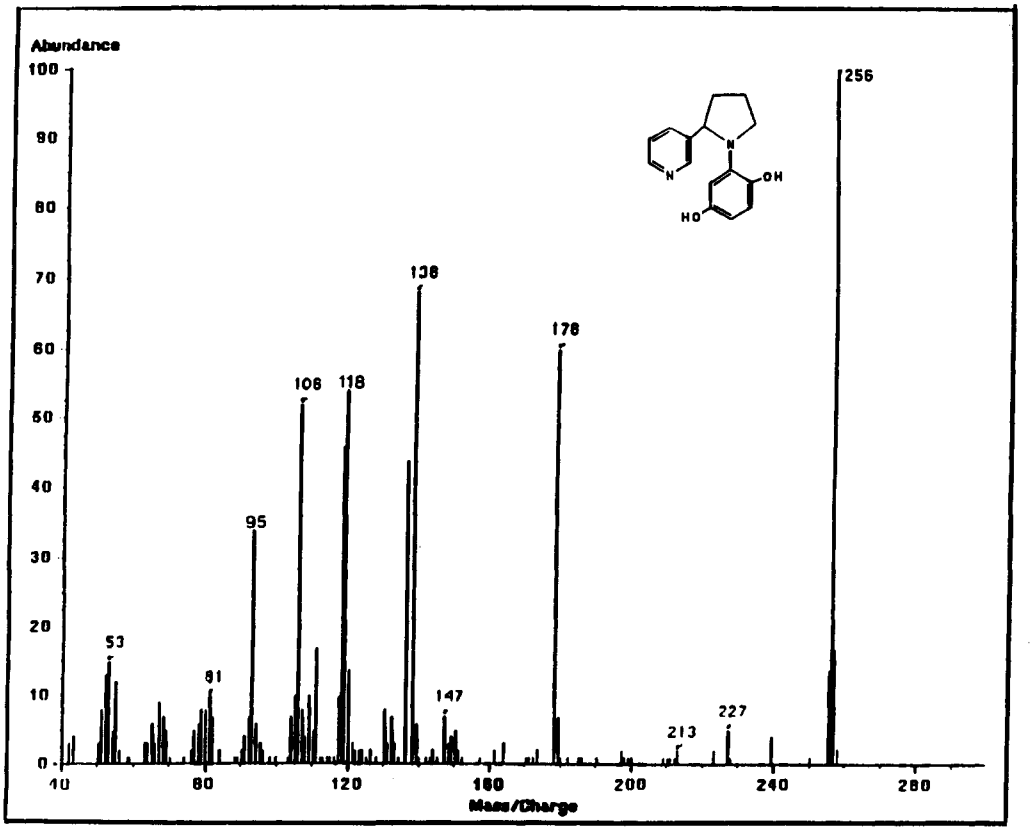

Figure 8. GC/MS of peak 4 total mass 256.

CONCLUSION

A PPO enzyme isolated from green tobacco leaves was active in oxidizing chlorogenic acid and caffeic acid to *o*-quinones that coupled with nornicotine to produce red color. Data from this study differed from the literature in which H_2O_2 was a necessary substrate to obtain the red pigment from the quinones produced following the enzyme action upon chlorogenic and caffeic acids. In the reaction mixtures used in this study, H_2O_2 was not added, but red color developed in all of the solutions in which enzyme, chlorogenic or caffeic acids, and nornicotine were combined.

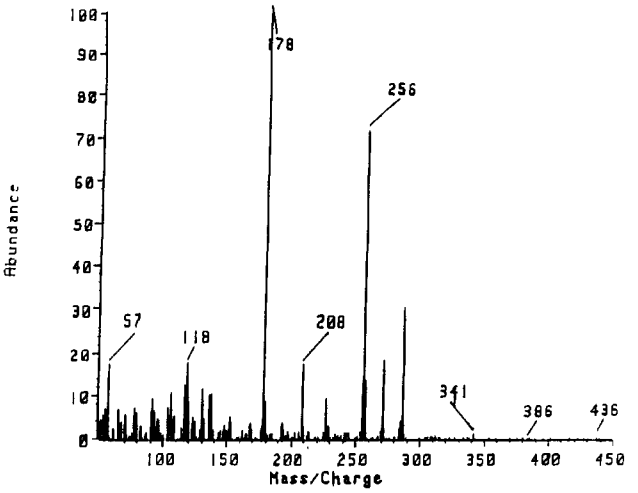

Figure 9. HPLC/MS of nornicotine and *o*-quinone reaction.

Variable temperature data showed that red color developed up to 75 °C for 1 h after the enzyme was added to the solutions without loss of activity. Temperature of this magnitude is greater than the temperature used to dry tobacco and could produce chemical reactions. Previous investigators treated only the enzyme without substrates at temperatures comparable to the temperatures used in this study; therefore, there was no evidence from previous work that enzyme plus substrate was affected by temperature because only the enzyme was heat treated.

The model systems used in the study gave reactions that produce red color similar to the color obtained from enzyme, nornicotine, and oxidized phenols. Chemical reactions used as model systems indicated two types of bonding, Michael addition and Schiff base, that could occur in the leaf to produce red color in cured tobacco. Further work is needed to chemically produce *o*-quinones from chlorogenic and caffeic acids and to react these compounds with nornicotine, since it was not possible to isolate the red color from tobacco extracts.

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