Incorporation of Leaf Chemical Constituents into Tobacco Brown Pigments*

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INTRODUCTION

Wright et al. (18) isolated a dark-brown pigment by acid precipitation of a soluble, non-dialyzable fraction of aged Burley tobacco and characterized it as an iron-protein-chlorogenic acid-rutin complex. The remaining acid-soluble brown pigment of the same non-dialyzable fraction showed a similar qualitative composition. Jacobson (7) reported that brown pigments from a Connecticut shade tobacco were also composed of proteins and polyphenols. He substantiated this conclusion by incorporating ^{14}C -chlorogenic acid and rutin into the pigments in excised leaves. The chemical composition of tobacco brown pigment is further complicated by the presence of alkaloids, pyridine derivatives, carbohydrates, a silicon and possibly other leaf constituents (5). Brown pigments determine the color of cured leaves which has been established as an important criterion of tobacco quality. Recently, they have been implicated as the possible precursors of tumorigenic agents in cigarette smoke (3). The quantitative and qualitative modification of brown pigments may provide a means to minimize smoking hazards without sacrificing leaf color quality. To achieve this goal, the relation of polyphenol and protein content with the amount and kind of brown pigments should be investigated.

Chlorogenic acid (3-O-caffeoylquinic acid) and rutin (quercetin-3-rhamnosidoglucoside) are the predominant polyphenols in tobacco leaves. These polyphenols in green leaves are quantitatively correlated with brown pigments in air-cured products (16). The formation of brown pigments is attributed to the oxidation of polyphenols by chlorogenic acid oxidase (CAO) and peroxidase (PRO) during leaf senescence and curing. Inhibition of these oxidases resulted in a decreased amount of brown pigments (14). It has been suggested that both oxidases may become the protein moiety of the pigments (13, 17). However, a recent study revealed that the amino acid composition in acid hydrolysates of the acid-precipitated, alcohol-insoluble brown pigments from both air-cured and flue-cured leaves resembles that of Fraction I protein in tobacco (15). The latter degrades rapidly during the early phase of leaf curing (8). Little information is available concerning the incorporation of Fraction I protein, oxidases and polyphenols into brown

pigments. Such information would give insights on the precursor-product relationship that may be used as a guide-line for quantitative and qualitative modifications of brown pigments in cured tobacco. *In vivo* studies of interactions among leaf constituents as they relate to the formation of brown pigments are not possible. The present study applied *in vitro* synthesis of brown pigments using varying quantities of ^{14}C -labeled polyphenols and proteins in order to determine the percent incorporation of these compounds into the pigments.

MATERIALS AND METHODS

Different plant parts of tobacco, *Nicotiana tabacum* cv. Burley 21, were the source of isotope-labeled leaf constituents used in the *in vitro* synthesis of brown pigments. ^{14}C -Chlorogenic acid was produced from green capsules which were longitudinally split and incubated in Petri dishes in darkness for 24 hours at ambient temperatures with 5 ml of 20 mM phosphate buffer, pH 6.5, containing L-phenylalanine- μ - ^{14}C (3.1×10^6 cpm), 50 mM quinic acid, 50 mM KCl and 25 μg neomycin. Purification of ^{14}C -chlorogenic acid employed Soxhlet extraction in 80% isopropanol, gel filtration with Sephadex LH-20 and two-dimensional paper chromatography as the procedures described in a previous paper (12). ^{14}C -Chlorogenic acid so isolated excluded the isomers 4- and 5-caffeoylquinic acids and had UV spectra superimposable with that of authentic compound. Its specific radioactivity was 2.7×10^6 cpm/mmol.

Burley 21 seedlings grown in hydroponic culture were used for production of ^{14}C -CAO and ^{14}C -PRO. The plants were kept in a growth chamber where temperatures were maintained at 80 °C during a 14-hour day and 65 °C at night. At the 5–6 leaf stage, the seedlings were transferred into aluminum-foil-covered beakers (250 ml) filled with Hoagland solution just enough to submerge the roots and were sprayed with 1000 ppm of Ethrel, an ethylene-releasing agent, in 0.1% Tween 80 solution. ^{14}C -Amino acids in 0.1 M sodium phosphate buffer, pH 7, were added into the beakers in an amount of 2 μCi per plant. Ethrel applications were repeated daily and, whenever needed, Hoagland solution was added to maintain the moist condition of the roots. On the fifth day, the leaves beginning to yellow were harvested and ground in cold acetone (–40 °C) con-

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taining 0.8 mM 2-mercapto-ethanol to make an acetone powder. The CAO and PRO were partially purified from acetone powder extract by saturation with 35% and 70% ammonium sulfate as previously described (10). The specific radioactivities of ^{14}C -CAO and ^{14}C -PRO were 74×10^{-3} and 35×10^{-3} cpm/mg protein, respectively.

For production of ^{14}C -Fraction I protein, a ^{14}C -amino acid feeding experiment identical to the one described above was carried out except that Ethrel was not used and the seedlings were harvested on the third day after treatment. Fraction I protein was purified into a crystalline form by the method of Chan et al. (1). The specific radioactivity of the Fraction I protein was 32×10^{-6} cpm/ μmol .

In vitro synthesis of brown pigments was performed in 40 ml of 0.1 M sodium phosphate buffer, pH 6, containing varying quantities of chlorogenic acid and CAO or PRO with or without radioactivity. Non-radioactive CAO and PRO were prepared from the acetone powder of mature field-grown Burley 21 leaves in the same manner as the afore-mentioned procedures for partial purification of ^{14}C -oxidases. Rutin (10 mg in ethanol) was added to all reaction mixtures and, in the case of PRO reaction, 100 μmol of H_2O_2 was also added. The reaction mixtures were gently shaken for one hour at ambient temperatures. To isolate the brown pigments, the reaction mixtures were adjusted to pH 10 with NH_4OH and subsequently acidified to pH 1 with concentrated H_2SO_4 . The brown pigments precipitated by this procedure are acid-insoluble and those remaining in the solution are acid-soluble. The acid-insoluble pigments were solubilized in dilute NH_4OH solution, pH 10. Both acid-soluble and acid-precipitated pigment solutions were dialyzed in dialyzing tubing with several changes of deionized water. The non-dialyzable fractions were freeze-dried and dissolved into 10 ml of dilute NH_4OH , pH 10, for spectrophotometric determination of browning intensity at 420 nm. Aliquots were pipetted into scintillation vials, mixed with 15 ml of Instagel, and radioactivities were counted in a Packard Automatic Tri-Carb liquid scintillation spectrometer (Model 4322) at 6 °C. A quenching curve based on a dilution series of brown pigments was used to correct quenching effect.

The same two brown pigment fractions were also isolated from air-cured Burley 21 leaves. Their gel permeation profiles were compared with those of the *in vitro* synthesized pigment fractions. Gel permeation studies employed a Sephadex G-100 column (1 $\times$ 75 cm) equilibrated with dilute NH_4OH solution, pH 10. Five milligrams of the pigments was dissolved in 1 ml of the elution solution and passed through the column at a flow rate of 40 ml per hour. Eluates were monitored for pigment distribution with an UV analyzer at 280 nm and collected in 5 ml fractions whose browning intensity was spectrophotometrically measured at 420 nm. A molecular weight series of proteins (aldolase, ovalbumin, chymotrypsinogen A and ribonuclease A) were chromatographed using the same procedure in order to

estimate the molecular weight distribution of the pigment fraction.

Under electrophoresis in a polyacrylamide gel with a discontinuous buffer system, the non-dialyzable brown pigments appeared to have at least one major band migrating at the same rate as the indicator dye, bromophenol blue. The *in vitro* synthesized ^{14}C -brown pigments were applied onto a 7.5% polyacrylamide gel containing 0.3% N,N-diallyltartardiamide. Disc electrophoresis with Tris-glycine buffer, pH 8, was the method of Davis (4). After the completion of electrophoresis, the gel was sliced into 1 mm sections and solubilized in 1 ml of 2% periodic acid. Radioactivity was counted by the foregoing procedure, and the percent incorporation of radioactivity into the major brown pigment band was calculated.

In certain experiments, the quantity of chlorogenic acid and rutin and the activity and isozymes of CAO and PRO in the reaction mixtures before and after the *in vitro* synthesis of brown pigments were determined. Polyphenol quantification was determined by two-dimensional paper chromatography and spectrophotometry, whereas oxidase assays employed spectrophotometric, electrophoretic and histochemical methods. Detailed procedures of these analyses have been described elsewhere (12).

RESULTS AND DISCUSSION

The CAO and PRO preparations from mature Burley 21 leaves exhibited 0.9 ($\Delta\text{A}/\text{mg}/\text{min}$) and 12.76 ($\Delta\text{A}/100\mu\text{g}/\text{min}$) activities, respectively. The CAO activity in the PRO preparation was negligible. Conversely, PRO activity in the CAO fraction was less than 15% of that in the PRO fraction. The addition of CAO into the reaction mixture containing chlorogenic acid and rutin changed the solution instantaneously from a light yellow into a dark brown color, whereas a similar change of coloration in the PRO catalyzed solution occurred only in the presence of H_2O_2 . In either case, there was no formation of brown substances in the absence of polyphenols or oxidases. This confirms a general belief that enzymatic oxidation of polyphenols is the process of brown pigment formation. Reaction mixtures containing more than 1 mg CAO or PRO per milliliter were assayed for oxidase activity after the completion of *in vitro* synthesis of the brown pigments. Oxidase activity was not measurable in either case. Neither the CAO nor the PRO isozymes were detected after disc gel electrophoresis. In contrast, the reaction mixtures devoid of chlorogenic acid and rutin showed a slightly yellow coloration and retained oxidase and isozyme activity after one hour of shaking. These results support the previous conclusions that polyphenoloxidase and PRO are the protein moiety of tobacco brown pigments (13, 17). This was further substantiated by the recovery of about 0.06% of total PRO activities in the acid-soluble brown pigments synthesized *in vitro*; however, there was no measurable activity in the acid-precipitated fraction.

Figure 1. Gel permeation chromatographic profiles of acid-precipitated and acid-soluble, non-dialyzable brown pigments from air-cured Burley 21 leaves. Chromatographic conditions are detailed in Materials and Methods.

The amount of residual activity reflects nearly total inactivation of PRO. Both brown pigment fractions exhibited no CAO activity.

Figure 1 shows the differences in molecular weight distribution between acid-precipitated and acid-soluble brown pigments as resolved by gel permeation chromatography. In comparison of the same pigment fractions from air-cured leaf and *in vitro* synthesis with CAO or PRO, a difference was not apparent. In general, the acid-precipitated pigments contained an absorption peak at the solvent front which was accompanied by an intense brown coloration. This was followed by a broad distribution of absorbents with a yellow color. These absorbents must represent the aggregates of proteins and polyphenols. According to Chortyk (2), the major portion of the acid-precipitated brown pigments was in the 10,000–60,000 range of molecular weight. This may correspond to the protein distribution but does not coincide with the browning intensity. Whether the latter is determined by the nature of protein-polyphenol complex or the amount of polyphenols in the aggregates cannot be elucidated without further fractionation and analysis of the pigments.

The solvent front of the acid-soluble pigments in the gel permeation profile was less conspicuous and exhibited a light yellow color. A large proportion of protein-polyphenol complex appeared in the 8,000–25,000 range of molecular weight along with an intense brown color. When both fractions of brown pigments, isolated either from air-cured leaves or synthesized *in vitro*, were subjected to disc gel electrophoresis, they showed an identical mobility toward the anode as the indicator, bromophenol blue. This confirms Wright's finding that the pigments are negatively charged (18). The foregoing physicochemical properties assure that the acid-precipitated and acid-soluble brown pigments synthesized *in vitro* by the present method are similar to those isolated from air-cured Burley leaves. Brown pigments are also known to be present in the dialyzable fraction of leaf extract (19).

Table 1. In vitro Incorporation of ^{14}C -chlorogenic acid into tobacco brown pigment fractions.

Oxidase (10 mg)	^{14}C -Chlorogenic acid (mg)	Acid-precipitated, non-dialyzable fraction				Acid-soluble, non-dialyzable fraction			
		Absorbance (420 nm)	^{14}C Activity (dpm)	^{14}C Recovery (%)	^{14}C in the pigment band (%)	Absorbance (420 nm)	^{14}C Activity (dpm $\times 10^{-3}$)	^{14}C Recovery (%)	^{14}C in the pigment band (%)
CAO	50	1.50	5033	0.42	64	8.00	280	23.22	97
	25	1.40	3161	0.52	60	6.25	172	28.56	91
	12.5	1.37	2446	0.81	48	4.95	84	27.75	91
	6.3	1.24	1418	0.94	37	4.13	46	30.28	88
	3.1	1.10	550	0.73	24	3.38	23	30.21	96
	0	0.82	—	—	—	0.35	—	—	—
PRO	50	1.30	950	0.08	57	4.50	138	11.46	98
	25	1.20	456	0.08	46	2.45	55	9.15	95
	12.5	0.88	461	0.15	25	2.37	33	10.96	93
	6.3	0.92	198	0.13	35	1.78	20	13.04	94
	3.1	0.72	338	0.45	22	1.60	10	13.77	96
	0	0.24	—	—	—	0.28	—	—	—

CAO = chlorogenic acid oxidase
PRO = peroxidase

Table 2. In vitro incorporation of ^{14}C -chlorogenic acid oxidase into tobacco brown pigment fractions.

^{14}C -CAO (mg)	Chloro- genic acid (mg)	Acid-precipitated, non-dialyzable fraction				Acid-soluble, non-dialyzable fraction			
		Absorbance (420 nm)	^{14}C Activity (dpm $\times 10^{-3}$)	^{14}C Recovery (%)	^{14}C in the pigment band (%)	Absorbance (420 nm)	^{14}C Activity (dpm $\times 10^{-3}$)	^{14}C Recovery (%)	^{14}C in the pigment band (%)

Experiment 1

50	50	4.65	1896	51.35	73	10.75	740	20.45	60
10	50	1.30	418	56.53	84	8.10	142	19.16	58
5	50	0.80	160	43.43	44	7.80	93	25.25	51
1	50	0.45	28	38.09	35	3.75	10	13.88	45

Experiment 2

10	100	1.40	388	52.62	83	14.10	143	19.33	62
10	50	1.30	418	56.53	84	8.10	142	19.16	58
10	10	1.10	387	52.40	80	2.35	100	13.47	65
10	5	1.05	427	57.77	79	1.95	103	13.91	65
10	0	0.95	400	54.21	86	0.15	78	10.53	57

CAO = chlorogenic acid oxidase

Table 3. In vitro incorporation of ^{14}C -peroxidase into tobacco brown pigment fractions.

^{14}C -PRO (mg)	Chloro- genic acid (mg)	Acid-precipitated, non-dialyzable fraction				Acid-soluble, non-dialyzable fraction			
		Absorbance (420 nm)	^{14}C Activity (dpm $\times 10^{-3}$)	^{14}C Recovery (%)	^{14}C in the pigment band (%)	Absorbance (420 nm)	^{14}C Activity (dpm $\times 10^{-3}$)	^{14}C Recovery (%)	^{14}C in the pigment band (%)

Experiment 1

50	50	1.60	658	36.97	85	7.90	668	37.50	50
10	50	1.35	102	28.51	75	4.80	112	31.11	42
5	50	0.80	56	31.39	77	4.40	53	29.78	45
1	50	0.75	13	36.88	72	2.20	10	27.39	55

Experiment 2

10	100	3.55	117	32.92	80	12.75	132	37.01	50
10	50	1.35	102	28.51	75	4.80	112	31.11	42
10	10	1.30	126	35.46	56	1.05	105	29.49	55
10	5	1.30	132	37.25	86	0.70	90	25.14	50
10	0	0.55	111	31.29	72	0.55	116	32.65	55

PRO = peroxidase

An intense brown coloration of the dialysate from the reaction mixture is in agreement with the fact that there are low molecular weight polymers containing proteins and/or oxidized polyphenols. Complex polyphenols with a brown appearance have been isolated from cured tobacco (9).

The absorbance of brown color at 420 nm in both acid-precipitated and acid-soluble brown pigments was positively correlated with the concentration of chlorogenic acid in the reaction mixture (Table 1). This is in keeping with the positive correlation between chlorogenic acid content in green leaves and brown pigment quantity in air-cured tissues (16). Chlorogenic acid concentration in the reaction mixture also determined its amount of incorporation into brown pigments. For the acid-precipi-

tated pigments, incorporation appeared more in the CAO-mediated reaction than that treated with PRO. However, there was less than one percent recovery of radioactivity in both cases. When the concentration of chlorogenic acid decreased in the reaction mixture, the percent recovery became higher, but its incorporation into the brown pigment band in the polyacrylamide gel was lower. This indicates that quinones formed from polyphenol oxidation are not randomly complexed with proteins, and its aggregation tends to be in favor of large molecular weight proteins. The incorporation of ^{14}C -chlorogenic acid into the acid-soluble brown pigments appeared to be independent of chlorogenic acid concentration, and more than 90% of the radioactivity was associated with the brown pigment band. On the

average, about 30% of ^{14}C -chlorogenic acid was recovered in both of the pigment fractions formed by CAO catalysis. In contrast, there was only a 12% recovery in the PRO-mediated reaction.

A positive correlation between the quantity of chlorogenic acid and the absorbance (420 nm) of brown pigments also existed in the studies on the *in vitro* incorporation of ^{14}C -CAO and ^{14}C -PRO into brown pigments (Tables 2 and 3). In addition, varying CAO quantity affected the absorbance and the percent incorporation of radioactivity in both acid-precipitated and acid-soluble pigments. The more the CAO in the reaction mixture, the higher the percentage of radioactivity was in the pigment band. On the other hand, the quantity of chlorogenic acid in the reaction mixture did not alter the percent of CAO incorporation into the precipitated pigments. It did, however, enhance CAO incorporation into the acid-soluble fraction. In either case, the radioactivity in the pigment band remained relatively constant. Similar patterns of isotope incorporation were evident in the ^{14}C -PRO experiments. The exception was that there was less isotope recovery in the acid-precipitated pigments but more in the acid-soluble ones. Overall, 60–70% of oxidase preparations were recovered in the two pigments, and 50–80% of this radioactivity can be accounted for in the pigment band. The absence of chlorogenic acid in the reaction mixtures did not lower the percent recovery of radioactivity in the pigments and pigment bands in most cases. From this, one may conclude that the major proportion of the pigments is the degradation of proteins which may or may not be reacted with quinones, the oxidation products of polyphenols.

It is known that 50% of soluble proteins in tobacco leaves is Fraction I protein and it is totally degraded during leaf senescence and curing (6, 8). The ^{14}C -amino acid feeding experiment employing senescent seedling leaves in the present study might result in an incorpor-

ation of radioactivity into Fraction I protein in addition to CAO and PRO. To establish the relationship of the oxidase purification procedure with the recovery of Fraction I protein, the crystalline Fraction I protein of Burley 21 was subjected to the same procedures employed for the isolation of CAO and PRO. About 90% of the Fraction I protein was salted out with 35% ammonium sulfate saturation. Of this, 60% was precipitated out as an acid-precipitable fraction. This implies that Fraction I protein and its degradation products are present in the CAO and PRO preparations and, thus, may contribute the protein moiety of brown pigments.

Data on the incorporation of ^{14}C -Fraction I protein into brown pigments are given in Table 4. The increased quantity of Fraction I protein slightly enhanced the browning intensity. However, only about 10% of Fraction I protein was recovered as the acid-precipitated pigment and 50% of which appeared in the pigment band. The maximal level of incorporation reached only 1–3% in the acid-soluble pigments. These results may be explained by the fact that proteases in the CAO preparation catalyzed the degradation of Fraction I protein. Particularly, the pH of the reaction mixture is very close to the pH optimum (pH 5.5) of one protease that reportedly may be related to the catabolism of Fraction I protein (8). Enzymatic degradation of Fraction I protein was less extensive in the PRO mixture where approximately 30% of the protein was recovered in the acid-precipitated pigments. Similarly, the percent incorporation into the acid-soluble pigments was three times greater in the PRO mixture than in the CAO solution. Most of the radioactivity appeared in the pigment band except for the acid-precipitated pigment from the CAO reaction where the pigment band contained 50–60% of the total radioactivity. Nevertheless, the appearance of radioactivity in pigment bands provides additional evidence on the degradation of Fraction I protein in the

Table 4. *In vitro* incorporation of ^{14}C -Fraction I protein into tobacco brown pigment fractions.

Oxidase (10 mg)	^{14}C -Fraction I protein (μg)	Acid-precipitated, non-dialyzable fraction				Acid-soluble, non-dialyzable fraction			
		Absorbance (420 nm)	^{14}C Activity (dpm)	^{14}C Recovery (%)	^{14}C in the pigment band (%)	Absorbance (420 nm)	^{14}C Activity (dpm)	^{14}C Recovery (%)	^{14}C in the pigment band (%)
CAO	900	4.20	6250	10.85	55	11.20	1388	2.41	100
	450	3.50	3150	10.95	51	10.80	800	2.78	100
	150	3.05	1375	14.32	56	9.91	150	1.56	99
	50	2.90	350	10.93	68	8.60	50	1.56	100
	0	1.45	—	—	—	8.70	—	—	—
PRO	900	3.35	11863	20.60	96	6.55	1800	3.13	87
	450	2.15	9575	33.25	71	6.15	975	3.39	69
	150	1.83	2975	30.99	90	5.15	838	8.72	100
	50	1.29	1000	31.25	80	4.95	100	3.63	100
	0	1.28	—	—	—	4.95	—	—	—

CAO = chlorogenic acid oxidase
PRO = peroxidase

reaction mixture because this protein is too large to migrate toward the anode through the gel matrix of the present electrophoretic system.

The degradation rate of Fraction I protein may affect the quantity of acid-precipitated and acid-soluble brown pigments. This is evidenced by the previous finding that flue-cured leaves contain more acid-precipitated brown pigment than air-cured tobacco (15). In other words, the curing process plays a major role in determining the quantity and quality of the brown pigments in cured tobacco. The present results also substantiate the previous findings that the concentration of polyphenols, especially chlorogenic acid, determines the extent of leaf browning, and CAO and PRO incorporate into the pigment as a result of product inhibition (13, 17). Genetic variability in the content of polyphenols and oxidases exists among *Nicotiana* species and within *N. tabacum* (11.) Therefore, breeding and cultural practices would provide an effective means to modify tobacco leaf qualities that are associated with brown pigments.

The complexity of brown pigments can be further manifested by incorporation of other leaf constituents since alkaloids, pyridine derivatives and carbohydrates have been identified in brown pigments (5). Wright et al. (18) found a large quantity of a polysaccharide in the non-dialyzable fraction of the pigments. Brown pigments synthesized *in vitro* by the present method may contain glycoproteins but not a polysaccharide *per se*. This delineates any possible effect of this polysaccharide on the browning reaction on the one hand and on the pyrolytic products of brown pigments on the other. The *in vitro* synthesis of brown pigments in the present study demonstrated the feasibility of selective labeling of pigment components. This facilitates future research in elucidation of the precursor-product relationship in the tobacco and health related problems, since the pyrolysate of brown pigments contains polynuclear aromatic hydrocarbons and phenols, which are respectively identified as tumor initiators and promoters (20).

SUMMARY

The percent incorporation of chlorogenic acid, chlorogenic acid oxidase (CAO), peroxidase (PRO) and Fraction I protein into the non-dialyzable fraction of acid-precipitated and acid-soluble brown pigments of tobacco was studied by use of ^{14}C -precursors and appropriate substrates in the *in vitro* synthesis of brown pigments. The *in vitro* synthesized pigments showed the same gel permeation profiles as the brown pigments isolated from air-cured leaves. The incorporation of chlorogenic acid was correlated with its quantity in the reaction mixture, and a significant amount of incorporation appeared in the acid-soluble pigments which also correlated with browning intensity. The amount of protein fractions containing CAO and PRO affected the percent incorporation of radioactivity, but this was independent of chlorogenic acid concentration. In addition to the loss of CAO and PRO during the formation of brown pigments, the de-

gradation of proteins, primarily Fraction I protein, occurred in the reaction mixture. The degradation products of Fraction I protein were recovered in both brown pigments. Results indicate that chlorogenic acid oxidation is the major factor for browning, and the degradation products of Fraction I protein and CAO and PRO contribute to the protein moiety of the pigments.

ZUSAMMENFASSUNG

Bei der *in-vitro*-Synthese von braunen Pigmenten wurde unter Verwendung von ^{14}C -markierten Vorläufern und geeigneten Substraten untersucht, in welchem Verhältnis Chlorogensäure, Chlorogensäureoxidase (CAO), Peroxidase (PRO) und Fraktion-I-Protein in die nicht dialysierbare Fraktion der säuregefällten und säurelöslichen braunen Pigmente des Tabaks aufgenommen werden. Die *in vitro* synthetisierten Pigmente zeigten die gleichen Gel-Permeationsprofile wie die aus „air-cured“-Blattabaken gewonnenen braunen Pigmente. Die Inkorporation von Chlorogensäure korrelierte mit deren Gehalt in der Reaktionsmischung, und ein beträchtlicher Teil der aufgenommenen Menge fand sich in den säurelöslichen Pigmenten wieder, die ebenfalls mit der Bräunungsintensität korrelierten. Die Menge der Proteinfractionen, die Chlorogensäureoxidase und Peroxidase enthielten, beeinflusste die prozentuale Aufnahme von Radioaktivität; dies war jedoch unabhängig von der Chlorogensäurekonzentration. Außer dem Verlust an Chlorogensäureoxidase und Peroxidase während der Bildung von braunen Pigmenten fand in dem Reaktionsgemisch der Abbau von Proteinen, vor allem von Fraktion-I-Protein, statt. Die Abbauprodukte von Fraktion-I-Protein fanden sich in beiden braunen Pigmenten. Aus den Ergebnissen läßt sich schließen, daß die Bräunung hauptsächlich auf der Oxidation der Chlorogensäure beruht und daß die Abbauprodukte des Fraktion-I-Proteins mit Chlorogensäureoxidase und Peroxidase zu dem Proteinanteil in den Pigmenten beitragen.

RÉSUMÉ

Le pourcentage d'incorporation d'acide chlorogénique, d'oxidase d'acide chlorogénique (CAO), de peroxidase (PRO) et de protéines de la fraction I dans la fraction non-dialysable de pigments bruns précipités dans l'acide et de pigments bruns solubles dans l'acide, a été étudié au moyen de précurseurs ^{14}C et de substrats appropriés dans la synthèse *in vitro* des pigments bruns. Les pigments synthétisés *in vitro* ont présenté les mêmes profils d'imprégnation du gel que les pigments bruns extraits de feuilles «air-cured».

On a trouvé une corrélation entre l'incorporation de l'acide chlorogénique et sa concentration dans le mélange de réactifs, et un taux significatif d'incorporation est apparu dans la fraction des pigments solubles dans l'acide qui étaient également en corrélation avec l'inten-

sité du brunissement. La quantité de fractions protéiques contenant du CAO et du PRO a influencé le taux d'incorporation de radioactivité, ceci indépendamment de la concentration d'acide chlorogénique. En plus de la perte en CAO et en PRO durant la formation des pigments bruns, les protéines, principalement celles de la fraction I, se sont dégradées dans le mélange de réactifs. Les produits de dégradation des protéines de la fraction I furent retrouvés dans les deux sortes de pigments bruns. Les résultats indiquent que l'oxydation de l'acide chlorogénique est le facteur principal de brunissement et que les produits de dégradation des protéines de la fraction I et le CAO et le PRO contribuent à la formation de la partie protéique des pigments.

REFERENCES

1. Chan, P. H., K. Sakano, S. Singh and S. G. Wildman: Crystalline Fraction I protein: Preparation in large yield; *Science* 176 (1972) 1145-1146.
2. Chortyk, O. T.: Comparative studies on the brown pigments of tobacco; *Tob. Sci.* 11 (1967) 137-139.
3. Chortyk, O. T., W. S. Schlotzhauer and R. L. Stedman: Composition studies on tobacco, XXIII. Pyrolytic and structural investigations on the polyphenol-amino acid pigments of leaf; *Beitr. Tabakforsch.* 3 (1966) 422-429.
4. Davis, B. J.: Disc electrophoresis, II. Method and application to serum human protein; *N.Y. Acad. Sci. Ann.* 121 (1964) 404-427.
5. Dymicky, M., O. T. Chortyk and R. L. Stedman: Composition studies on tobacco, XXVII. Polyphenol-amino acid leaf pigment: further structural investigations; *Tob. Sci.* 11 (1967) 42-44.
6. Gains, T. P., and J. D. Miles: Protein composition and classification of tobacco; *J. Agric. Food Chem.* 23 (1975) 690-694.
7. Jacobson, J. S.: The brown pigments of autolyzed tobacco leaves, I. Isolation and characterization; *Arch. Biochem. Biophys.* 93 (1961) 580-590.
8. Kawashima, N., and E. Tamaki: Studies on protein metabolism in higher plant leaves, I. Amino acid compositions of tobacco leaf proteins at various stages; *Phytochem.* 6 (1967) 329-338.
9. Runeckles, V. C.: Tobacco polyphenols, IV. A complex polyphenol in flue-cured tobacco; *Arch. Biochem. Biophys.* 102 (1963) 354-358.
10. Sheen, S. J.: The distribution of polyphenols, chlorogenic acid oxidase and peroxidase in different plant parts of tobacco, *Nicotiana tabacum* L.; *Phytochem.* 8 (1969) 1839-1847.
11. Sheen, S. J.: Comparative quantities of polyphenols and oxidases in *Nicotiana* species; *Tob. Sci.* 14 (1970) 16-19.
12. Sheen, S. J.: Changes in amount of polyphenols and activity of related enzymes during growth of tobacco flower and capsule; *Plant Physiol.* 51 (1973) 839-844.
13. Sheen, S. J.: Polyphenol oxidation by leaf peroxidases in *Nicotiana*; *Bot. Gazette* 135 (1974) 155 to 161.
14. Sheen, S. J.: Quantitative modification of polyphenols and brown pigments by foliar application of certain chemicals in Burley tobacco; *Tob. Res.* 1 (1975) 59-68.
15. Sheen, S. J., and H. R. Burton: Amino acid composition in tobacco fractions containing brown pigments; *J. Ag. Food Chem.* 26 (1978) 380-385.
16. Sheen, S. J., C. Grunwald and J. Sims: Effects of nitrogen fertilization and stalk position on polyphenols and carbohydrates in air-cured leaves of three tobacco genotypes; *Can. J. Plant Sci.* 53 (1973) 897-905.
17. Weybrew, J. A., and R. C. Long: More on tobacco browning: The first pigment; *Tob. Sci.* 14 (1970) 167-169.
18. Wright, H. E., Jr., W. W. Burton and R. C. Berry, Jr.: Soluble browning reaction pigments of aged Burley tobacco, I. The non-dialyzable fraction; *Arch. Biochem. Biophys.* 86 (1960) 94-101.
19. Wright, H. E., Jr., W. W. Burton and R. C. Berry, Jr.: Soluble browning reaction pigments of aged Burley tobacco, II. The dialyzable fraction; *Phytochem.* 3 (1964) 525-533.
20. Wynder, E. L., and D. Hoffmann: Tobacco and tobacco smoke, Studies in experimental carcinogenesis; Academic Press, Inc., New York, 1967, p. 730.

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