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Water-Soluble Glycoproteins of Tobacco Leaves

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A dialyzed water-soluble extract of defatted tobacco leaves was chromatographed on a DEAE-cellulose column to yield two fractions, A and B, that were rich in carbohydrate and protein. The ratio of carbohydrate to protein in fraction A was about twice that in fraction B. When rechromatographed by gel filtration chromatography with Sepharose 4B, these fractions lost neither of these components. When further rechromatographed on a Sepharose CL-6B column, each of these fractions separated into three subfractions (a_1, a_2, and a_3 and b_1, b_2, and b_3), containing both carbohydrate and protein. The subfractions differed in molecular weight but contained the same sugar moieties (arabinose, galactose, glucose, and rhamnose), although in different mole percentages.

Glycoproteins have only recently come to be generally regarded as components of cell walls in all higher plants (Northcote, 1972) and necessary for their growth (Ridge and Osborne, 1970). Although information concerning the chemical composition of tobacco has increased markedly in recent years, little is known about its glycoproteins. Those that have been examined were shown to be hydroxyproline-rich glycoproteins and were isolated from tobacco cells grown in suspension cultures (intracellular glycoproteins) and from their culture media (extracellular glycoproteins) (Hori and Sato, 1977; Hori and Fujii, 1978, 1980). Also isolated from culture media of tobacco cells were the arabinogalactans of hydroxyproline-rich glycoprotein (Hori et al., 1980; Akiyama and Kato, 1981). Tobacco glycoproteins are important from the standpoint of smoking quality because they contain both amino acid and sugar moieties (Tso, 1972): When tobacco is pyrolyzed, as in cigarettes or other smoking products, its amino acids and sugars form products that may be carcinogenic (Wynder and Hoffmann, 1967; Patterson et al., 1969; Higman et al., 1970). Thus, because plant glycoproteins are important both for their physiological role in plants and for the possible effect of their pyrolysis products, we undertook to examine the water-soluble glycoproteins of tobacco.

MATERIALS AND METHODS

Tobacco Samples. *Nicotiana tabacum* L., cv. Maryland 609, plants were field grown at Beltsville, MD, by conventional cultural practices (McKee, 1978). Maleic hydrazide (MH), the sucker control agent recommended for this area, was applied on plants which had been decapitated after they had reached midbloom. Green leaves were removed at harvest (14 days after MH application) and freeze-dried until just before extraction. The freeze-dried material was ground to pass a 40-mesh screen. Fractions rich in both carbohydrate and protein were isolated and chromatographed by procedures similar to those developed by Hillestad et al. (1977). About 100 g of the dried, ground leaves was extracted sequentially with two chloroform–methanol–water mixtures (10:10:1 and 30:20:1 v/v) and a chloroform–methanol mixture (40:1 v/v), a procedure that removed all the chlorophyll. The leaf residue was then extracted with 2–3 L of water (50 °C), and the extract was evaporated to a small volume at reduced pressure, dialyzed overnight at room temperature against distilled water, and freeze-dried.

Chromatographic Separations. The freeze-dried water extract residue (1.0–1.5 g) was dissolved in buffer A—0.05 M Tris–HCl buffer (pH 6.7)—and applied on a DEAE-cellulose (DE-52) column (5 × 50 cm) that had been preequilibrated with the same buffer. The column was eluted with about 800 mL of buffer A and then with 800 mL of buffer B—0.5 M Tris-HCl buffer (pH 6.7) containing 0.25 M NaCl. Elution was maintained at a rate of 1 drop/8 s, and the eluate was collected at the rate of 17 mL/tube. The tube contents were analyzed spectrophotometrically for total carbohydrate and protein, and those containing high amounts of both these constituents were pooled according to the eluting buffers and called fractions A and B. These fractions were evaporated under reduced pressure, dialyzed overnight against distilled water at room temperature without preservative, and freeze-dried.

Freeze-dried fractions A and B (about 1.0 g each) were then subjected to gel filtration chromatography with a Sepharose 4B column (2 × 25 cm) that had been preequilibrated with 0.025 M Tris-HCl buffer (pH 7.2). This buffer was also used to elute fraction A or B from the column, and the flow rate was 3 drops/min. The eluate was collected at the rate of 6 mL/tube, and the tube contents that were high in both carbohydrate and protein were pooled, dialyzed overnight against distilled water at room temperature with no preservative, and freeze-dried.

Analytical Methods. Total carbohydrate was determined by a phenol–H_2SO_4 procedure described by Dubois et al. (1956), with a mixture of arabinose, galactose, and glucose (5.4:3.6:1) as the standard. Uronic acid content

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was estimated by the carbazole method of Bitter and Muir (1962), with D-glucuronic acid as the standard. Sugars in samples that had been hydrolyzed with 0.1 N H_2SO_4 at 100 °C for 12 h were reduced with $NaBH_4$ to form alditols; the alditols were converted to their acetates with acetic anhydride and H_2SO_4 as described by Borchardt and Piper (1970), and the alditol–acetates were analyzed quantitatively by gas–liquid chromatography (GLC) by using a Varian 3700 equipped with a flame ionization detector. A glass column (4 mm i.d. × 1 m) packed with 3% OV-225 on 80–100-mesh Supelcoport was used, and the instrument was operated at attentuation 8×10^9. The helium flow rate was 30 mL/min. Injection port and detector temperatures were 200 and 210 °C, respectively, and the oven temperature programming was started at 130 °C and went to 260 °C at the rate of 3 °C/min. Chromatographic peaks were identified by comparison of their retention times with those of known compounds and by coinjection experiments. Data were recorded by computer (Chromatopec-Ela).

Protein was assayed with Coomassie Brilliant Blue according to Bradford (1976), and bovine serum albumin was used as the standard. Amino acids were analyzed with an amino acid analyzer (Beckman 118-BL) after samples had been hydrolyzed with 6 N HCl for 20 h at 105 °C in sealed glass tubes, freed of HCl, and dissolved in buffer.

Molecular weights of fractions A and B (chromatographed with Sepharose 4B, dialyzed, and freeze-dried) were estimated with two serially connected Sepharose CL-6B columns (1.4 × 23 cm and 1.5 × 39 cm) that had been preequilibrated with 0.25 M Tris-HCl buffer (pH 7.2) containing 0.1 M NaCl. The same buffer was used to elute the samples (50 mg each) from the columns. The eluate was passed through a quartz flow cell, and its absorbance at 280 nm was automatically recorded on a chart. Bromophenol blue tracking dye was routinely run through the columns to check the void volume. The flow rate of the eluate was maintained at 1 drop/s, and the eluate was collected at the rate of 1.5 mL/tube. Tube contents were pooled according to absorbance peaks and indicated the separation of fractions A and B into subfractions. The molecular weights of the subfractions were estimated by use of a semilogarithmic plot relating the elution volumes and molecular weights of known proteins (Ansari and Mage, 1977). The known proteins and their molecular weights were lysozyme (14 300), trypsinogen (24 000), pepsin (34 700), egg albumin (45 000), bovine serum albumin (6600), and β-galactosidase (116 000). Molecular weight experiments were carried out at room temperature.

RESULTS AND DISCUSSION

The freeze-dried water extract of the defatted ground leaves was obtained in about 1% yield, and it was a lightweight product that resembled vermiculite. This product was chromatographed on DEAE-cellulose, and a plot of the elution tube numbers vs. total carbohydrate and protein contents showed two major fractions high in both components (Figure 1). Only these fractions (A and B) were retained for further analyses. Fraction A was colorless and was eluted with buffer A in the void volume, and fraction B was colored and was eluted with buffer B. Analyses of these fractions (before rechromatography on Sepharose 4B column) showed that the ratio of carbohydrate to protein was higher for fraction A than for fraction B (Table I). The elution time of the uronic acids on the DEAE-cellulose column was the same as that of the carbohydrates. The quantity of uronic acids present in fraction A was about half that in fraction B, but the individual uronic acids were not characterized in this study.

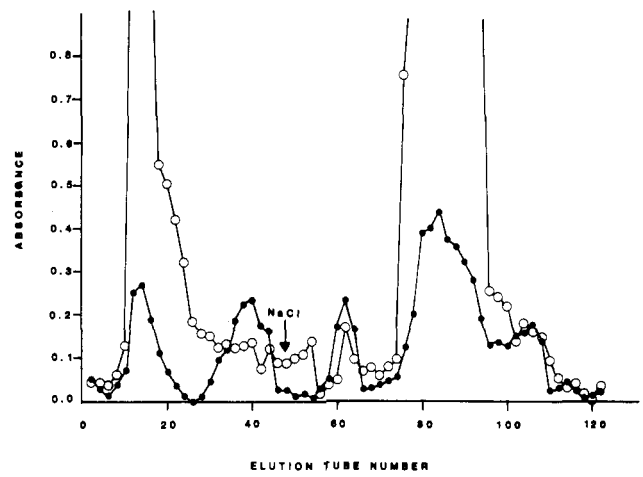

Figure 1. Chromatography of tobacco water-soluble extract on DEAE-cellulose (chloride form). NaCl was present in the eluate beginning from the point indicated by the arrow: (○) carbohydrate; (●) protein. Fraction A, tubes no. 1–27; fraction B, tubes no. 63–110; Experimental details are given in the text.

Table I. Carbohydrate and Protein Analyses of Fractions A and B Obtained from DEAE-cellulose Column

	fraction A	fraction B
ratio of carbohydrate to protein	6.04:1 (5.72:1)[a]	2.55:1 (3.63:1)[a]
uronic acid, μg/mg	5.12	11.68
sugars, mol %		
arabinose	51.2	50.5
galactose	27.3	34.0
glucose	12.0	6.0
rhamnose	6.7	8.7
xylose	3.4	0.8
amino acids,[b] %		
aspartic acid	10.40	9.16
hydroxyproline	trace	trace
threonine	6.72	6.23
serine	7.81	9.79
glutamic acid	7.16	6.41
proline	4.18	4.09
glycine	9.76	9.79
alanine	11.50	14.23
cysteine	ND[c]	ND[c]
valine	8.89	7.83
methionine	1.74	3.02
isoleucine	4.34	4.53
leucine	6.94	6.58
tyrosine	4.99	4.87
phenylalanine	3.04	3.38
lysine	4.99	4.98
histidine	2.00	2.14
arginine	3.90	2.85

[a] Ratio for fractions collected from the Sepharose 4B column. [b] In the order of elution from the amino acid analyzer. [c] ND = not detected.

Hydrolysates of fractions A and B had the same sugar moieties and had similar mole percentages of arabinose and galactose but different mole percentages of glucose, rhamnose, and xylose. Hydrolysates of fractions A and B also contained the same amino acids. The major amino acid was alanine in both fractions A and B. A high alanine content was one of the characteristics of the hydroxyproline-rich glycoproteins (Hori and Sato, 1977) isolated from cultures of tobacco cells. Fractions A and B, although high in alanine, contained only traces of hydroxyproline. The low peaks associated with the tubes separating fractions A and B (Figure 1) may have represented hydroxyproline-rich glycoproteins, which can be eluted with buffers containing a low concentration of NaCl (Hori and Fujii, 1978); glycoproteins of this type have been isolated from

Table II. Molecular Weights of and Sugar Moieties[a] in Subfractions Obtained by Chromatography of Fractions A and B on Sepharose CL-6B Column

	a_1	a_2	a_3	b_1	b_2	b_3
M_r	1.0×10^5	1.7×10^4	1.3×10^4	1.2×10^5	3.8×10^4	1.5×10^4
sugars, mol %						
arabinose	34.2	45.4		54.5	12.0	
galactose	56.9	29.3		35.3	49.0	
glucose	6.1	20.8		7.3	28.4	
rhamnose	2.8	4.5		12.7	10.6	
xylose	ND[b]	ND[b]		ND[b]	ND[b]	

[a] Subfractions a_3 and b_3 were not analyzed for sugars. [b] ND = not detected.

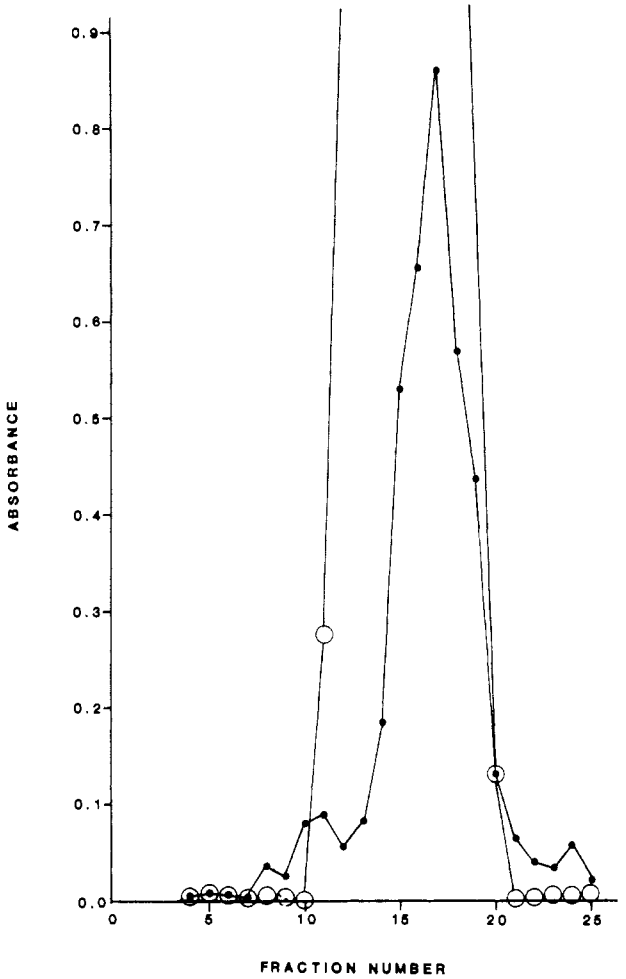

Figure 2. Gel filtration (Sepharose 4B) chromatography of fraction A. (○) Carbohydrates; (●) protein. Contents of tubes no. 8–21 were pooled. Experimental details are given in the text.

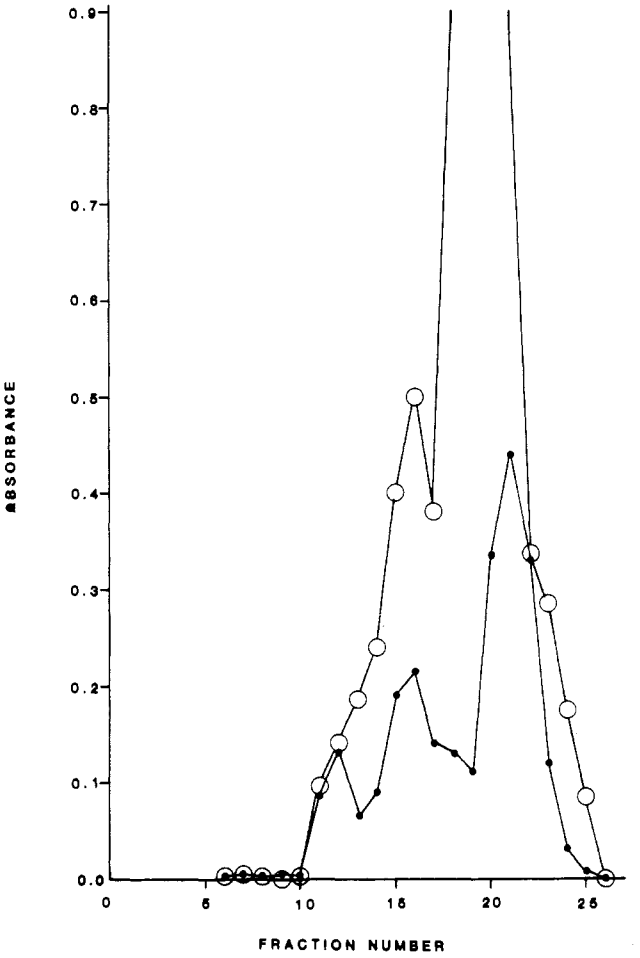

Figure 3. Gel filtration (Sepharose 4B) chromatography of fraction B. (○) Carbohydrate; (●) protein. Contents of tubes no. 10–26 were pooled. Experimental details are given in the text.

the leaves of *Vicia faba* L. (Pusztai and Watt, 1969). It is unlikely that the difference in hydroxyproline content between our tobacco glycoproteins and those previously isolated was due to the difference in plant materials used (tobacco green leaves vs. tobacco cells).

Fractions A and B were eluted in the void volume from the Sepharose 4B column and contained both protein and carbohydrate moieties (Figures 2 and 3) The ratios of carbohydrate to protein in the rechromatographed fractions A and B were slightly different from those determined after separation of the fractions with DEAE-cellulose (Table I). It is assumed that free proteins or monosaccharides that coeluted with the two fractions during and after separation by DEAE-cellulose were removed by Sepharose 4B.

During the molecular weight analysis with Sepharose Cl-6B, both fractions A and B separated into three subfractions—a_1, a_2, and a_3 and b_1, b_2, and b_3. Their estimated molecular weights ranged from 1.2×10^5 to 1.3×10^4 (Table II). The same sugars were present in subfractions a_1, a_2, b_1, and b_2 but in different mole percentages. Unlike fractions A and B, these subfractions contained no detectable xylose. The sugars in subfractions a_3 and b_3 as well as the amino acids in all subfractions were not determined for lack of sufficient sample. The chromatographic characteristics and compositions of these subfractions indicate that fractions A and B were mixtures of glycoproteins.

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Volatile Constituents of Dry Elder (*Sambucus nigra* L.) Flowers

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Three extracts of dry elder flowers, *Sambucus nigra* L., were analyzed: a steam-distilled essential oil, an ethanol concentrate of a petroleum ether extraction, and an isopentane extract of an ethanol concentrate. By use of gas chromatography, infrared spectroscopy and mass spectrometry, 79 compounds were identified: 16 hydrocarbons, 11 ethers and oxides, 7 ketones, 7 aldehydes, 16 alcohols, 6 esters, and 16 acids. The major constituents of the essential oil were *trans*-3,7-dimethyl-1,3,7-octatrien-3-ol (13%), palmitic acid (11.3%), linalool (3.7%), *cis*-hexenol (2.5%), and *cis*- and *trans*-rose oxides (3.4 and 1.7%, respectively). They were also principal components of the isopentane extract and of the ethanol concentrate. The three extracts had a good muscat odor.

Elder, *Sambucus nigra* L., is a wild shrub, which may grow to 10 m. Its white flowers possess a pleasant strong smell. A high-priced wine is produced in England from the berries. To our knowledge, the plant is not cultivated. Flowers are harvested around May–June and dried away from sunlight at a temperature lower than 40 °C, to minimize aroma loss.

Steiner and Von Kamiensky (1953) reported ethylamine, isobutylamine, and isoamylamine in elder flowers. Leifertova and Kudrnacova (1971) reported high levels of phenolic compounds in the buds and found choline in the flowers but not in the berries. Willuhn (1974) identified several alkanes in the elder leaves: heptacosane, nonacosane, and hentriacontane being quantitatively the most important ones. Richter and Willuhn (1974) reported that the essential oil of elder flowers was high in fatty acids (66%) and *n*-alkanes (7.2%). According to Bonnier and de Layens (1970), apples stored on a dry elder flowers bed have a pleasant muscat like aroma. This was in agreement with Bayonove (1973), who imitated muscat wine by adding dry elder flowers to a Grenache wine and reported that a sensory panel did not identify the true muscat wine. These facts led to our interest in studying further the composition of elder flowers aroma.

EXPERIMENTAL SECTION

Materials. Three extracts were studied: a steam distilled oil, an ethanol concentrate, and an isopentane extract. From 400 g of dry elder flowers with a water content of 96 ± 0.5 g/kg of dry matter and provided by Ducros Co. (Buis-les-Baronnies, France), an essential oil was steam distilled for 24 h on a modified Clevenger apparatus (Miquel et al., 1976). To avoid any loss of oil, 2 mL of ethyl ether–pentane (1:1) were introduced in the separator. Solvents were evaporated under a nitrogen stream and essential oil was kept at −18 °C. Its acid index was 70 ± 3.5 mg of KOH/g of essential oil. The yield was 0.53 g/kg of dry elder flowers. From a distilled ethyl alcohol extract obtained after soaking 150 g of the same sample of elder flowers in 1 L of alcohol (45%) at room temperature, a volatile essence was recovered by liquid–liquid extraction using purified isopentane. The yield of this isopentane extract was 0.15 g/kg of dry elder flowers. From an oleoresin prepared by soaking under reflux dry elder flowers in petroleum ether, an ethanol concentrate was provided by Payan-Bertrand (Grasse, France).

Purification of the Essential Oil and Identification of Its Constituents. *Separation by Column Chromatography.* A glass column, 30 cm × 2 cm i.d., filled with Florisil 60–100-mesh ASTM to the 20-cm level, was prewashed with 100 mL of distilled anhydrous petroleum ether. Elder flowers essential oil (1 g) was poured at the top of the column and eluted successively with petroleum ether, petroleum ether–ethyl ether (9:1), petroleum ether–ethyl ether (1:1), ethyl ether, and finally methanol. Solvents were removed under vacuum in a rotary evaporator and five final fractions were obtained.

Separation by Gas–Liquid Chromatography. A thermal conductivity detector was used with a helium flow rate of 70 mL/min. The column was programmed from 60 to 220 °C at 6 °C/min and held.

Further purification was achieved either on the same instrument with another 4 m × 4 mm i.d. glass column

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