Carbohydrate Research, 138 (1985) 247–255
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURAL INVESTIGATION OF THE SODIUM HYDROXIDE-SOLUBLE POLYSACCHARIDES OF TOBACCO (*Nicotiana tabacum*): AN ARABINOXYLOGLUCAN*

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(Received June 15th, 1984; accepted for publication, October 3rd, 1984)

ABSTRACT

An arabinoxyloglucan (amyloid) isolated from bright tobacco (*Nicotiana tabacum*, L. cv., Delhi 76) consists of L-arabinose, D-xylose, and D-glucose residues in the molar ratios 1:2.2:6.8. Sedimentation data indicate that the polysaccharide is homogeneous. The methylation analysis data show a statistical unit of 20 sugar residues with 5 terminal, non-reducing end-groups (3 D-xylosyl and 2 L-arabinosyl). There are 5 residues of D-glucose involved in branching through positions 4 and 6. The remaining 10 non-terminal residues consist of two (1→2)-linked D-xylosyl residues and eight (1→4)-linked D-glucosyl residues. The proposed statistical unit accords with the periodate-oxidation results.

The formation of ~0.1 mol each of 2,3,4,6-tetra-*O*-methyl- and 2,3,4-tri-*O*-methyl-D-glucose suggests that an average unit may contain ~200 sugar residues.

INTRODUCTION

Several xyloglucans (amyloids) from a variety of sources have been characterised[1,2]. A xylan[3] from the stalk and a 4-*O*-methylglucuronoxylan[4] and an arabinoxyloglucan[1] from the midrib of the leaves of uncured, greenhouse, bright tobacco have been isolated and characterised, and the extracellular polysaccharides[5] from suspension-cultured cells of tobacco have also been studied.

We have reported the isolation and characterisation of an Amadori compound, the low-molecular-weight carbohydrates[7], and the water-soluble pectic polysaccharides[8] from the cured-leaf laminae of tobacco, and we now describe further work on the sodium hydroxide-soluble polysaccharide fraction.

*Contribution No. 598 from the Food Research Institute. Presented at the XIIth International Carbohydrate Symposium, Utrecht, The Netherlands, 1–7 July, 1984.

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RESULTS AND DISCUSSION

Fractionation of the sodium hydroxide-soluble polysaccharides from the oxalate-insoluble residue of cured-leaf laminae of tobacco yielded a major hemicellulose-B fraction. Fractionation of B by chromatography on DEAE-cellulose (CO_3^{2-}) yielded, *inter alia*, a major neutral fraction (W_1) and a series of acidic fractions. Further fractionation of W_1 on DEAE-cellulose (borate form) yielded, *inter alia*, an arabinoxyloglucan.

The arabinoxyloglucan, gave a single, symmetrical peak on sedimentation analysis[9], had $[\alpha]_D$ +29°, gave a greenish-blue colour with iodine–potassium iodide[10], and had an i.r. band at 897 cm^{-1} characteristic of amyloids. Partial acetolysis of the polysaccharide yielded α-cellobiose octa-acetate, and deacetylation of the components moving more slowly in t.l.c. gave components corresponding (h.p.l.c.) to cellotriose and cellotetraose. These results demonstrated the polysaccharide to be an amyloid.

The amyloid yielded, on hydrolysis, L-arabinose, D-xylose, and D-glucose in the molar ratios 1:2.2:6.8. Preparative p.c. of the mixture gave crystalline L-arabinose and D-xylose; D-glucose was identified as the crystalline *p*-nitroaniline derivative. Methylation of the amyloid gave a product, $[\alpha]_D^{25}$ +2.4°, which was methanolysed and then hydrolysed. G.l.c.–m.s.[12] and g.l.c.–c.i.-m.s.[13] of the derived alditol acetates (see Table I) indicated the molar proportions of the parent sugars to be as follows: 2,3,5-tri-*O*-methyl-L-arabinose, 1; 2,3,4-tri-*O*-methyl-D-xylose, 1; 3,4-di-*O*-methyl-D-xylose, 1; 2,4-di-*O*-methyl-D-xylose, 0.55; 2,3,6-tri-*O*-methyl-D-glucose, 3.5; and 2,3-di-*O*-methyl-D-glucose, 3. Among the minor components, ~0.05 mol each of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-glucose were also detected.

The amyloid reduced 1.2 mol of periodate, with the liberation of 0.17 mol of

TABLE I

G.L.C. AND C.I.-M.S. DATA FOR SUGARS FROM THE METHYLATED ARABINOXYLOGLUCAN

Acetate of	*Retention time (min)*	*C.i.-m.s. data:* m/z *(relative abundance, %)*
2,3,5-Tri-*O*-methylarabinitol	0.67	$[MH]^+$ 279 (100), 280 (15), 247 (20), 219 (20), 177 (8), 117 (10)
2,3,4-Tri-*O*-methylxylitol	0.76	$[MH]^+$ 279 (45), 220 (10), 219 (100)
2,3,4,6-Tetra-*O*-methylglucitol	1.00	—
3,4-Di-*O*-methylxylitol	1.04	$[MH]^+$ 307 (90), 308 (15), 248 (12), 247 (100)
2,4-Di-*O*-methylxylitol	1.15	$[MC_4H_9]^+$ (0), 309 (100), 310 (12), 291 (20), 279 (20), 249 (70)
2,3,4-Tri-*O*-methylglucitol	1.25	—
2,3,6-Tri-*O*-methylglucitol	1.29	$[MH]^+$ 351 (50), 292 (15), 291 (100)
2,3-Di-*O*-methylglucitol	1.51	$[MH]^+$ 379 (98), 380 (20), 337 (20), 320 (15), 319 (100)

TABLE II

SUGARS (MOL) OBTAINED ON HYDROLYSIS OF METHYLATED ARABINOXYLOGLUCAN

Component	*Molar ratio of glycitol acetates by g.l.c.*	
	Cured-leaf laminae	*Uncured-leaf midrib*[1]
2,3,5-Tri-*O*-methyl-L-arabinose	2	2
2,3,4-Tri-*O*-methyl-D-xylose	3	3
2,3,4,6-Tetra-*O*-methyl-D-glucose	0.1	—
3,4-Di-*O*-methyl-D-xylose	2	2
2,3,4-Tri-*O*-methyl-D-glucose	0.1	—
2,3,6-Tri-*O*-methyl-D-glucose	8	6
2,3-Di-*O*-methyl-D-glucose	5	5

formic acid per "anhydro sugar" residue. Smith degradation[14] of the resulting oxopolysaccharide yielded erythritol, glycerol, ethylene glycol, glycolaldehyde, and glyceraldehyde (arising from 2-*O*-linked D-xylose residues); no reducing sugars were detected. These results demonstrated that the 2,4-di-*O*-methyl-D-xylose originated from incomplete methylation of the D-xylose end-groups. This conclusion is consistent with the difficulties experienced in the methylation of amyloids in general[15]. The imbalance between the end groups and branch points is attributed to demethylation of the 2,3,6-tri-*O*-methyl-D-glucose residues at position 6, resulting from prolonged hydrolysis. The revised data with respect to 3,4-di-*O*-methylxylose multiplied by 2 are summarised in Table II.

The methylation analysis data show a statistical unit of 20 sugar residues consisting of 5 terminal, non-reducing end-groups (3 D-xylosyl and 2 L-arabinosyl). There are 5 residues of D-glucose involved in branching, through positions 4 and 6. The remaining 10 non-terminal residues consist of two (1→2)-linked D-xylosyl residues and eight (1→4)-linked D-glucosyl residues. The proposed statistical unit accords well with the results of periodate oxidation, in requiring a reduction of 1.2 mol with the liberation 0.16 mol of formic acid per "anhydro sugar" residue. The molar ratios of arabinose–xylose–glucose (1:2.5:6.5), calculated from the proportion of methylated sugars, agree well with those (1:2.2:6.8) found for the original polysaccharide. The formation of ~0.1 mol each of 2,3,4,6-tetra-*O*-methyl- and 2,3,4-tri-*O*-methyl-D-glucose suggests that an average unit may contain ~200 sugar residues. Such units, although not found by Eda *et al.*[1] in their original amyloid, were found in substantial amounts in the oligosaccharide preparations[1] obtained by enzymic degradation. The amyloid isolated by these workers from uncured tobacco-leaf midrib (variety BY) shows (Table I) a similar repeating-unit consisting of 18 sugar residues and differs in having only 6 non-terminal (1→4)-linked D-glucosyl residues instead of the eight found in the present study.

The $[\alpha]_D$ values (+29° for the unmethylated and +2.4° for the methylated amyloid) support the evidence that the glucosidic bonds are preponderantly β; by

analogy with the tobacco midrib amyloid[1], it is assumed that the L-arabinose and D-xylose residues are α.

Amyloids from various sources[1,2] fall into three classes: (*1*) xyloglucans containing D-galactose residues, (*2*) fucoamyloids[15] containing L-fucose residues in addition to the above sugars, and (*3*) arabinoxyloglucans containing α-L-arabinofuranosyl-(1→2)-D-xylopyranosyl side-chains. Terminal non-reducing residues of L-arabinose have been found[16–18] in amyloid preparations. The isolation and characterisation of arabinoxyloglucans from tobacco[1] suggests that the previously reported amyloid preparations contained small proportions of this amyloid. The arabinoxyloglucan from tobacco midrib, and the one presented in this study, therefore, constitute the third group, and they appear to modify the burning rate of cellulose and ameliorate the associated harshness. A definition of their role in shatterability, retention of moisture, texture, and the formation of pyrolysis products could provide a basis for the improvement of tobacco quality, safety, and processability. Since the tobacco amyloid is soluble due to the presence of covalently linked sugar moieties other than glucose, it could, as a by-product isolated during the production of tobaco-leaf proteins, find applications in the food industry.

EXPERIMENTAL

General. — I.r. spectra were recorded with a Beckman IR 4250 spectrophotometer. G.l.c.–m.s. was performed with a Finnigan Incos Model 2100 spectrometer operating at 70 eV (separator temperature, 250°; transfer line temperature, 230°; analyser temperature, 80°), with a column (5 ft × 0.25 in.) of 3% of OV-225 on Chromosorb WHP (80–100 mesh), a temperature programme 100→200° at 4°/min, and a helium flow-rate of 30 mL/min. G.l.c.–c.i.-m.s.[13] was performed with a Finnigan Incos MAT-312 system with butane as the reagent gas, operating with a c.i. ion-source temperature of 160° (250 eV; filament emission, 0–5 mA; accelerating voltage, 3 kV; electron multiplier, 2 kV; scanning range, 60–500 at 2 s/scan) and a column (6 ft × 0.25 in.) of 3% of OV-225 on Chromosorb WHP (80–100 mesh), a temperature programme 120→230° at 5°/min, and a helium flow-rate of 30 mL/min. Routine g.l.c. was performed with a Pye 104 Gas Chromatograph, with flame-ionisation detectors, dual glass columns (5 ft × 0.125 in.) packed with (*a*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) and (*b*) 3% of OV-225 on Chromosorb WHP (80–100 mesh), a temperature programme 100→210° at 2°/min, and a nitrogen flow-rate of 30 mL/min. Peak areas were evaluated with a Pye–Unicam CPDI Computing integrator. Optical rotations were measured with a Perkin–Elmer 141 polarimeter and are equilibrium values.

Descending p.c. was performed on Whatman No. 1 paper with the organic phases of *A*, ethyl acetate–pyridine–water (8:2:1); *B*, 1-butanol–pyridine–water (10:3:3); *C*, 1-butanol–acetic acid–water (4:1:5); and *D*, butanone saturated with water containing 2% of ammonia. Paper electrophoresis[19] was performed on What-

man No. 3MM paper in *A*, 0.2M borate buffer (pH 10); *B*, 0.2M acetate buffer (pH 5); and *C*, borate–calcium chloride buffer[20] (pH 9.2) at a potential gradient of 14.0 V/cm. Sugars were detected with *A*, aniline hydrogenphthalate; and *B*, alkaline silver nitrate. T.l.c.[21] was performed on Silica gel G, with detection by charring with 5% sulphuric acid. H.p.l.c. was carried out at 85° using a Waters Associates High Pressure Liquid Chromatograph, with differential refractometer detection, using a column (30 × 0.78 cm) of Aminex HPx-85 eluted with water (0.6 mL/min). Peak areas were evaluated with a Columbia Scientific integrator coupled to a Fisher Recorder Series 5000. Concentrations were carried out at 35° on a rotary evaporator. Melting points were determined on an Electrothermal apparatus and are uncorrected.

Routine hydrolyses of the polysaccharide samples (2–3 mg) were performed with M sulphuric acid (0.3 mL) for 3 h at 100°. Amyloid fractions (5 mg) were hydrolysed with aqueous 72% sulphuric acid (0.12 mL) at 5° for 2–3 h and, following dilution to M acid, for 3 h at 100°. The hydrolysates were neutralised with barium carbonate. The sugar samples (5 mg or less) were reduced with borohydride[14], and the alditols were acetylated essentially as described by Bjorndal *et al.*[12] but for 1 h at 100° followed by storage overnight at room temperature. Deacetylations were performed at 0° with methanolic 1% sodium methoxide. Demethylations[22] were carried out at −40° with boron trichloride.

Extractions. — *(a) With ammonium oxalate.* The water-insoluble residue (~284 g), obtained by extraction of tobacco powder (Delhi 76, flue-cured lamina, 966 g) with boiling, aqueous 80% ethanol and hot water[6,8], was stirred with aqueous 0.5% ammonium oxalate at 92 ±3°. The combined filtrate and washings (7.5 L) were adjusted to pH 4.5 with glacial acetic acid and mixed with ethanol (1.5 vol.), yielding the oxalate-soluble fraction I (30.8 g; moisture, 7.37; N, 3.36; ash, 2.51%). Two further extractions of the residue gave fractions II (15.9 g; moisture, 8.7; N, 4.12; ash, 2.38%) and III (3.6 g; moisture, 7.72; N, 3.11%).

P.c. of the hydrolysates of I–III revealed galactose, arabinose, rhamnose, and uronic acids, with traces of glucose and xylose (in I and II); III gave a higher proportion of glucose.

(b) With sodium hydroxide. The oxalate-insoluble residue (234 g) was stirred with aqueous 10% sodium hydroxide (6.6 L) at 25° for 24 h under nitrogen. The insoluble residue was collected by filtration and centrifugation, and washed twice with aqueous 10% sodium hydroxide (2.5 L). The combined extract (9 L) was adjusted to pH 4.5 with glacial acetic acid, and the precipitate was collected by centrifugation, washed with aqueous 80% ethanol, ethanol, and acetone, and then dried to yield the hemicellulose-A fraction (8.78 g; moisture, 6.66; N, 8.65; ash, 9.87%).

Ethanol (3 vol.) was added to the supernatant solution to yield the hemicellulose-B fraction (29.9 g; moisture, 7.46; N, 1.31; ash, 15.26%).

The sodium hydroxide-insoluble material was neutralised with glacial acetic acid, collected by centrifugation, washed with aqueous 80% ethanol, ethanol, and

acetone, and then dried (residue, 82.1 g; moisture, 5.2; N, 0.5; ash, 15.38%).

The aqueous 75% ethanolic supernatant was concentrated to 3.6 L, and a portion (500 mL) was dialysed, concentrated, and freeze-dried to yield the hemicellulose-C fraction (0.72 g).

P.c. of the hydrolysates of A–C and the residue revealed mainly glucose with traces of galactose, arabinose, xylose, and uronic acid in A; galactose, glucose, arabinose, and small proportions of mannose and rhamnose in B; traces of arabinose in C; and glucose with traces of galactose, mannose, arabinose, xylose, and rhamnose in the residue.

Fractionation of hemicellulose-B. — A solution of the hemicellulose-B (20 g) in water (1400 mL) was mixed with Rexyn-101 (H^+) resin, giving a clear filtrate and a water-insoluble and sodium hydroxide-soluble material (4.55 g) recovered from the resin by washing with M sodium hydroxide. The filtrate and washings (1.9 L) were mixed with ethanol (4 vol.), and the precipitate was recovered by centrifugation. The supernatant solution was concentrated to dryness, to give a tarry product (3.19 g). A solution of the precipitate in water was concentrated to remove traces of ethanol, and the resulting syrupy solution (450 mL) was added to a column (4 × 15 in.) of DEAE-cellulose (CO_3^{2-} form)[23]. Elution with water (1.9 L) removed the bulk of the neutral fraction W_1 (2.34 g). Further elution with water (1.7, 4, and 0.5 L) yielded fractions W_2–W_4 (0.15 g, 0.13 g, and 0.01 g, respectively). Elution with 0.25M ammonium carbonate (1.2 L) yielded a fraction which was concentrated to 400 mL, acidified with acetic acid, and mixed with ethanol (4 vol.). The precipitate was collected by centrifugation, washed with aqueous 80% ethanol and then ethanol, and dissolved in water (250 mL). This solution was deionised with Rexyn-101 (H^+) resin, filtered, concentrated, and freeze-dried to yield an acidic fraction A_1 (0.77 g). Further elution with 0.25M ammonium carbonate (1.7 L) gave the major acidic fraction A_2 (3.07 g). Elution with more 0.25M ammonium carbonate (4.8 L) yielded fraction A_3 (0.29 g). The sodium hydroxide fractions 1–4, eluted with 0.25M sodium hydroxide (1.1, 1.2, 2, and 3.5 L), were similarly recovered (yields 0.04, 0.54, 0.69, and 0.19 g, respectively).

P.c. of the hydrolysates (M sulphuric acid, 100°, 3 h) of W_1–W_4, A_1–A_3, sodium hydroxide fractions 1–4, the tarry product, and the water-insoluble sodium hydroxide-soluble fraction revealed mainly glucose, arabinose, and xylose, with traces of galactose and mannose, in W_1. Fractions W_2 and A_1 gave the same components, but with somewhat higher proportions of galactose and mannose. Fraction A_2 gave mainly galactose, arabinose, rhamnose, and uronic acids, with small proportions of glucose and xylose. Fractions A_3 and 1 gave galactose, glucose, arabinose, xylose, and uronic acids, with traces of mannose and rhamnose. Fractions 2–4 gave mainly glucose and xylose, with relatively lesser amounts of galactose, arabinose, and uronic acids, and minor amounts of mannose and rhamnose.

Fractionation of the polysaccharide W_1. — A solution of W_1 (2 g) in water (100 mL) was filtered to yield an insoluble residue (0.23 g) and a soluble portion

(1.77 g). A solution of the soluble fraction in water (25 mL) was added to a column (1 × 25 in.) of DEAE-cellulose (borate form)[24] and eluted with water (1.5 L, 10-mL fractions). Fractions were dialysed and then freeze-dried (residue, 1.26 g). Gradient elution with 0→0.5M sodium metaborate (3 L), with dialysis and freeze-drying of fractions 175–201, yielded a further product (0.2 g).

Acid hydrolysis (M sulphuric acid, 100°, 3 h) of the water fraction gave only glucose, xylose, and arabinose.

Analysis of tobacco arabinoxyloglucan (amyloid). — The tobacco amyloid had $[\alpha]_D^{25}$ +29° (*c* 1, water); ν_{max}^{KBr} 757, 897, and 945 cm^{-1} (Found N, 1.33; moisture, 7.32%). Sedimentation analysis[9] of a 0.5% solution in 0.02M sodium acetate buffer (pH 5.03) at 52,000 r.p.m. gave a single, symmetrical peak. The amyloid (0.2 g) was treated with aqueous 72% sulphuric acid (10 mL) at 5° for 3 h, and the hydrolysate was then made up to 125 mL (*i.e.*, to M acid) and kept at 100°; $[\alpha]_D^{25}$ +46° (initial) → +57° (constant) in 3 h. The hydrolysate was neutralised ($BaCO_3$), filtered, and concentrated to dryness. A portion (3 mg) of the residue was reduced with borohydride and then acetylated. G.l.c. of the alditol acetates revealed derivatives of arabinose–xylose–glucose in the ratios of 1:2.2:6.8. Preparative p.c. of the remainder yielded L-arabinose, m.p. 152–153°, $[\alpha]_D^{25}$ +103° (*c* 1, water), and D-xylose, m.p. 145–146°, $[\alpha]_D^{25}$ +18° (*c* 1, water). D-Glucose was identified as the dihydrate of the *p*-nitroaniline derivative, m.p. 183–184°, $[\alpha]_D^{25}$ −206° (*c* 1, pyridine).

Acetolysis of the amyloid. — The amyloid (50 mg) was acetolysed essentially as described by Eda and Kato[1]. T.l.c. (3 developments with 5% methanol in benzene) of the product mixture yielded 4 fractions having R_{GlcAc_5} 1.0, 0.77–0.72, 0.62, and 0.54–0.16, respectively. Deacetylation of fraction *1* (31.5 mg) gave (p.c.) arabinose, xylose, and glucose. Fraction *2* (14.4 mg) gave α-cellobiose octa-acetate, m.p. 229° (from ethanol), $[\alpha]_D^{25}$ +41° (*c* 1, chloroform). Fractions *3* and *4* were combined with the mother liquor from fraction *2* and deacetylated. H.p.l.c. then revealed components with retention times (8.63, 9.52, and 11.00 min) identical to those of cellotetraose, cellotriose, and cellobiose, respectively.

Methylation analysis of the amyloid. — The dried amyloid (108 mg) in dry methyl sulphoxide (5 mL) was methylated by the Hakomori method[11], using methylsulphinyl carbanion (5 mL; reaction time, 5 h) and methyl iodide (5 mL; reaction time, 45 min). The product (80 mg), recovered by dialysis, was methylated again by the above procedure and the product (70 mg), recovered following dialysis, showed OMe 10%. A portion (38 mg) was methylated again using freshly distilled methyl sulphoxide (2 mL) and freshly prepared methylsulphinyl carbanion (2 mL, reaction time, 8 h) using a Vortex high-speed shaker. Following addition of methyl iodide (2 mL) under constant agitation, the sample was recovered by continuous extraction with chloroform and, following dialysis, yielded a product (29 mg), $[\alpha]_D^{24}$ +2.4° (*c* 1, chloroform), which showed no i.r. absorption for hydroxyl.

The methylated polysaccharide (29 mg) was methanolysed (methanolic 3% hydrogen chloride, 7 mL; 16 h, reflux; neutralisation with Ag_2CO_3) and hydrolysed

(M H_2SO_4, 100°, 30 h; neutralisation with $BaCO_3$). P.c. (solvent *D*) and p.e. (buffer *A*) of the syrupy product (27 mg) showed 4 major components (R_F 0.84, 0.79, 0.49, and 0.24) and 3 major components (M_{Glc} 0.00, 0.19, and 0.35), respectively, and traces of 3 other components.

A portion (5 mg) of the mixture was reduced[14] ($NaBH_4$) and then acetylated, and the products were examined by g.l.c. (column *b*), g.l.c.–m.s.[12], and g.l.c.–c.i.-m.s.[13]. The corrected g.l.c. data for the components (see Results and Discussion) are presented in Table II. The respective $[MH]^+$ ions for the major components were confirmed from c.i.-m.s. Although no standard for 2,4-di-*O*-methylxylitol triacetate was available, the presence of a primary fragment[12] ion with *m/z* 117 indicated the identity of this component. This conclusion was supported by the results of c.i.-m.s.; the ion with *m/z* 309 (100%) could have arisen from an unstable $[M + C_4H_9]^+$ ion by loss of 3 H_2O.

Periodate oxidation. — Tobacco amyloid (12.1 mg) was treated with sodium metaperiodate and the product was subjected to Smith degradation as described previously[2]. The results per "anhydro sugar" residue were: periodate reduced, 1.2 mol; formic acid produced, 0.17 mol. The oxopolysaccharide gave ethylene glycol, glycerol, erythritol, glycolaldehyde, and glyceraldehyde on hydrolysis: no reducing sugars were detected.

ACKNOWLEDGMENTS

The authors thank Mr. G. Morris (microanalysis), Mr. P. Lafontaine (g.l.c.–m.s. and g.l.c.–c.i.-m.s.), and Mr. J. Emery for technical assistance.

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