Phytochemistry, Vol. 20, No. 11, pp. 2507–2510, 1981.
Printed in Great Britain.

0031-9422/81/112507-04 $02.00/0

AN EXTRACELLULAR ARABINOGALACTAN-PROTEIN FROM *NICOTIANA TABACUM*

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(*Revised received* 3 *April* 1981)

Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; cell cultures; arabinogalactan-protein; hydroxyproline; glucuronic acid.

Abstract—An extracellular arabinogalactan-protein was obtained from suspension-cultured tobacco cells. It seemed to be a homogeneous preparation from the results of gel-filtration, ultracentrifugation and disc gel electrophoresis. Its MW was estimated to be 2.24×10^5 and its sedimentation coefficient ($s_{20,w}$) was calculated to be 5.07 S. It consisted of arabinose (40.0%), galactose (36.2%), rhamnose (0.8%), glucuronic acid (10.0%), glucosamine (0.2%), galactosamine (0.1%) and protein (5.5%). The sugar moiety appeared to be a typical arabino-3,6-galactan. A D-glucuronic acid residue was present as the non-reducing terminal group and was attached to C(O)-6 of a D-galactosyl residue by β-linkage.

INTRODUCTION

Arabinogalactan-proteins (AGPs) have been found in various plants [1]. The characteristics of AGPs are (1) the polymer is a glycoprotein with a very high carbohydrate content (typically 80–95%), (2) the carbohydrate portion is an arabino-3,6-galactan, (3) the protein portion is rich in hydroxyproline, alanine and serine and (4) the polymer precipitates with β-glycosyl Yariv antigen. Although AGPs are widely distributed in the plant kingdom, their biological roles are far from clear [1]. Structural information about these glycoproteins is therefore a prerequisite for any understanding of their significance.

Growth medium of suspension-cultured cells is one of the sources for AGPs and two groups have reported on these extracellular AGPs [2, 3]. This paper describes the purification and some structural investigations of an AGP obtained from the extracellular polysaccharide (ECP) of suspension-cultured tobacco cells.

RESULTS

Extracellular AGP was purified from the buffer extract of ECP by ion-exchange chromatography, deproteinization and gel-filtration. Table 1 shows the general data of each fraction.

The AGP obtained seemed to be a homogeneous preparation from the results of three different experiments. First, the material showed a single symmetrical peak at $1.7\,V_0$ on gel-filtration with Sepharose 4B using 10 mM Tris–HCl buffer (pH 8.0) as the eluant. If water was used as the eluant, the AGP appeared at the void volume. From the elution position, the MW was estimated to be 2.24×10^5. Second, the AGP showed a single symmetrical peak in an ultracentrifugational study. The logarithms of the boundary positions were linear with time and the sedimentation coefficient ($s_{20,w}$) was calculated to be 5.07 S. Taking into account the MW from gel-filtration and assuming the partial specific volume as 0.62 ml/g [4], the frictional ratio of the AGP was calculated to be 3.89. Third, in polyacrylamide disc gel electrophoresis (PAGE), the material migrated as a single band which was stained both by thymol–sulphuric acid for carbohydrate and by Coomassie blue for protein. The relative mobility to tracking dye (bromophenol blue) was 0.25. When the

Table 1. General data of each fraction*

Fractions	Wt (mg)	Hydroxyproline (μg)	Protein (wt %)	Uronic acid (wt %)	Neutral sugar (wt %)	Neutral sugar composition (mol %)						Reaction with Yariv antigen
						Rha	Ara	Xyl	Man	Gal	Glc	
ECP	3000	6600	22.0	n.d.†	44	1	19	17	21	14	29	n.d.
Buffer extract	1200	2560	0.4	7	83	3	39	2	10	35	11	+
DEAE-cellulose 0.1 M NaCl	130	1560	2.4	9	72	1	47	14	3	33	8	+
Borate–phenol buffer layer	107	850	5.5	11	77	1	52	0	0	47	0	+
Sepharose 4B (AGP)	90	820	5.5	10	77	1	52	0	0	47	0	+
GP	36	660	15.0	26	58	0	8	0	0	92	0	+

* From 2 l. of growth medium.

† n.d., not determined.

electrophoresis was performed with the usual gels [5], the diffused zone at the top of the gel was observed with thymol–sulphuric acid stain. No band was seen with Coomassie blue stain.

No reducing end sugar was detected, indicating that the AGP was a glycoprotein.

Table 2 shows the amino acid and amino sugar analyses of the AGP. The major amino acids were hydroxyproline (16.5%), alanine (13.0%) and serine (10.7%). Total protein was calculated to be 5.5% from the amino acid analysis. The neutral sugars were made up of L-arabinose and D-galactose, and small amounts of L-rhamnose (Table 1). The mole ratio of arabinose to galactose was 1.1. In addition to the neutral sugars, a fair amount of uronic acid was detected (Table 1). GLC analysis (dithioacetal method) showed that the uronic acid was D-glucuronic acid.

In the ^{13}C NMR spectrum of the AGP, the anomeric carbon region contained two signals at δ 110.74 and 104.84 which were assigned to C-1 of α-L-arabinofuranose and β-D-galactopyranose, respectively, by reference to published data [6, 7]. This indicated that the anomeric configurations of arabinosyl and galactosyl residues in the AGP were α and β, respectively.

Methylation analysis of the AGP (Table 3) indicated that the sugar moiety was a typical arabino-3,6-galactan.

Under mild conditions, partial acid hydrolysis of the AGP resulted in selective cleavage of arabinosyl residues, and a galactan-protein (GP) was obtained. It consisted of neutral sugars (58%, mainly galactose), glucuronic acid (26%) and protein (15%), and it reacted with β-glucosyl Yariv antigen. Methylation analysis of the GP (Table 3) showed a remarkable increase of 2,3,4-tri-*O*-methyl galactose (6-linked) and a decrease of 2,4-di-*O*-methyl galactose (3,6-linked) compared with the data of AGP, indicating that the arabinosyl residues were attached to galactosyl residues at C-3. 2,3,4,6-Tetra-*O*-methyl galactose (terminal) was also present in the GP, however, the amounts of the terminal residue were rather low considering the number of the branching point (i.e. the amounts of 3,6-linked galactose), suggesting that uronic acids might be present as non-reducing terminal groups.

Table 2. Amino acid and amino sugar composition of AGP

Amino acid	Mol %
Hyp	16.5
Asp	9.9
Thr	6.0
Ser	10.7
Glu	9.2
Pro	4.9
Gly	7.1
Ala	13.0
Cys	0
Val	4.0
Met	1.2
Ile	1.8
Leu	3.5
Tyr	4.0
Phe	1.9
His	1.1
Lys	4.0
Arg	1.2
Amino sugar	**(μg/mg AGP)**
Glucosamine	1.69
Galactosamine	0.73

Acid hydrolysis of AGP with 1 N TFA at 100° for 1 hr [8] yielded acidic oligosaccharides and the main product was purified. On complete acid hydrolysis, it gave galactose and glucuronic acid in almost equimolar amount (dithioacetal method). After reduction of the saccharide with sodium borohydride, GLC analysis (dithioacetal method) of the acid hydrolysate showed that galactose had been converted to galactitol, indicating that the oligosaccharide was glucuronosyl galactose. Methylation analysis of the saccharide (Table 3) gave 2,3,4-tri-*O*-methyl galactose (6-linked), indicating a 1 → 6 linkage between glucuronic acid and galactose. The anomeric configuration was determined by NMR spectroscopy. In the ^{1}H NMR spectra, the anomeric proton signals of β-D-galactose and D-glucuronic acid were overlapped and it was difficult to assign the configuration. However, in the ^{13}C NMR spectra, three signals in the anomeric carbon region (δ 92.86, 97.30 and 103.33) were readily assigned to C-1 of α-D-galactose, β-D-galactose and β-D-glucuronic acid, respectively, by reference to published data [7, 8]. The signal of C-6 of D-galactose (δ 62) was not seen in the spectrum, which was thought to be due to the substituting effect, confirming the 1 → 6 linkage between galactose and glucuronic acid. From the data above, the aldobiouronic acid was assigned the structure: *O*-β-D-glucuronosyl-(1 → 6)-D-galactose.

DISCUSSION

The growth medium of suspension-cultured cells is one of the sources for AGPs. Katō *et al.* [9] isolated an arabinogalactan (AG) from ECP of tobacco and investigated its structure. Our AGP was thought to be the same kind of material as the AG, since the AG contained protein (1%) and uronic acid (3%) in addition to arabinogalactan. In fact, the results of methylation analysis of both materials were similar. Hori and Sato [3] also purified an extracellular AGP from tobacco and provided the chemical analysis data. Both AGPs consisted of the same components: arabinose, galactose, uronic acid, hexosamines [10] and protein. However, their AGP contained more uronic acid, galactose and hydroxyproline than ours. Furthermore, on Sepharose 4B chromatography their AGP appeared in the void volume whereas ours entered the gel. These discrepancies are probably due to the strain differences in the tobacco cells (Xanthi vs Bright Yellow) used in two studies. Anderson *et al.* [2] isolated extracellular and intracellular AGP from *Lolium multiflorum* using Yariv antigen precipitation. Their AGPs were similar to ours with regard to MW, protein and carbohydrate content, and amino acid compositions, however, there were some differences between the two AGPs. For example, their AGPs did not contain rhamnose and uronic acid, and on methylation analysis, there were no (or few) 2,3-di-*O*-methyl arabinofuranosyl residues (5-linked). Our data and those of Katō *et al.* [9] revealed that tobacco AGP contained an appreciable amount of 5-linked arabinofuranosyl residues. Thus D-glucuronic acid and 5-linked arabinofuranose might be characteristic of tobacco AGP. Intracellular AGP obtained from 0.1 M potassium chloride fraction of ref. [8] also contained D-glucuronic

Table 3. Methylation analyses of AGP, GP and aldobiouronic acid

Methylated sugars	RR_t*		Composition (mol %)		
	Column 1	Column 2	AGP	GP	Aldobiouronic acid
2,3,5-Ara†	0.66	0.68	31	+	—
2,3,4-Ara	0.71	0.74	7	+	—
2,3-Ara	0.97	0.89	16	+	—
2,3,4,6-Gal	1.00	1.00	+	11	—
2,4,6-Gal	1.16	1.14	10	13	—
2,3,4-Gal	1.34	1.22	3	47	100
2,4-Gal	1.73	1.37	32	29	—

* Relative to that of 2,3,4,6-tetra-*O*-methyl-D-galactitol diacetate.
† 2,3,5-Ara = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl arabinose, etc.

acid and a 5-linked arabinofuranosyl residue (unpublished data).

AGP reacts with β-glycosyl Yariv antigen. Although the exact reaction mechanism between AGP and the antigen has not yet been established [1], our observations provided some hints. Thus the GP reacted with the antigen, suggesting that arabinosyl residue does not participate in the recogniton. As reported by Pope [11], alkaline treatment of AGP followed by gel-filtration yielded the hydroxyproline-arabinogalactan (HAG). The HAG obtained from tobacco AGP did not react with the antigen, suggesting that the protein portion might be essential for the reaction. In order to get more information concerning the reaction mechanism, it will be necessary to obtain the protein portion intact. Deglycosylation of the AGP with anhydrous hydrogen fluoride [12] or with hydrogen fluoride in pyridine [13] might help to solve the problem.

Hexosamines which were reported to be present in many AGPs [2, 10, 14] were also present in tobacco AGP, although their roles are not known.

In addition to neutral sugars, hexosamines and protein, tobacco AGP contained D-glucuronic acid, which appeared to be the non-reducing terminal group and is attached to C(O)-6 of D-galactosyl residue by β-linkage. A disaccharide, *O*-β-D-glucuronosyl-(1 → 6)-D-galactose, which was isolated from tobacco AGP by partial acid hydrolysis, has been obtained from *Acacia senegal* gum (gum arabic) [15]. The structure and the sugar compositions of the carbohydrate portion of the tobacco AGP are quite similar to the *A. senegal* gum, suggesting that they are closely related to each other.

EXPERIMENTAL

Isolation of AGP. Cell-suspension cultures of tobacco (*Nicotiana tabacum*, cv. BY-2) were grown as previously described [16]. Six-day-old cells were filtered and to the filtrate (2 l.) EtOH (4 l.) was added and kept in the cold room overnight. The ppt obtained by centrifugation was dialysed and freeze-dried to give an extracellular polysaccharide (ECP, 3 g). The hydroxyproline content of the ECP was usually 2–3 μg/mg. The ECP was extracted (2 ×) with 10 mM Tris–HCl (pH 8.0) containing 0.5 M NaCl in a Waring blender and the extract was dialysed and freeze-dried (1.2 g). The residue was dissolved in 10 mM Tris–HCl (pH 8.0) and applied to a DEAE-cellulose column (2 × 15 cm) equilibrated with the same buffer. The column was eluted stepwise with buffer containing no NaCl, 0.1 M and 0.5 M NaCl. As the second fraction contained most of the hydroxyproline, it was collected, dialysed and freeze-dried (130 mg). This material was dissolved in 40 vol. of 0.05 M sodium tetraborate buffer (pH 9.8) and 20 vol of PhOH were added. The mixtures were stirred vigorously for 1 hr and then centrifuged. The buffer layer was dialysed and freeze-dried (107 mg). The deproteinized fractions were redissolved in 10 mM Tris–HCl (pH 8.0) and applied to a column (1.5 × 80 cm) of Sepharose 4B, conditioned with the same buffer and previously calibrated using Dextran T-series. When monitored for carbohydrate [17] and protein [18], a single symmetrical peak was obtained at $1.7V_0$. This fraction was dialysed and freeze-dried to give AGP (90 mg). Its specific rotations at 365, 435, 546, 577 and 589 nm were −92°, −58°, −34°, −30° and −28°, respectively (H_2O, *c* 0.5). Its absorbance at 280 nm was too low to be recorded.

Amino acid analysis. Amino acids were analysed in a Nihon Denshi JLC-6AH amino acid analyser after hydrolysis of the sample in 6 M HCl at 110° for 18 hr in a sealed tube under N_2. Non-peptide bound hydroxyproline was measured colorimetrically [19]. Protein was assayed by Lowry's method [18] with BSA as the standard.

Carbohydrate analysis. The total sugar content was estimated by the PhOH–H_2SO_4 method [17] using a 1:1 mixture of L-arabinose and D-galactose as the standard. To analyse the neutral sugar composition, the sample was hydrolysed with 2 N TFA for 1 hr at 120°. The sugars liberated were converted into alditol acetates [20] and estimated by GLC on a glass column (200 × 0.3 cm) containing Gas Chrom P (100/200 mesh) coated with a mixture of 0.2% ethylene glycol adipate polyester, 0.2% ethylene glycol succinate polyester and 0.4% silicone XF-1150 (column 1) [21]. Uronic acid contents were determined by the modified method of ref. [22] (*m*-hydroxydiphenyl method). For simultaneous determination of neutral sugars and uronic acids by GLC, the dithioacetal method of ref. [23] was employed. The sugars liberated by hydrolysis with 2 N TFA for 1 hr at 120° were converted to their diethyl dithioacetal trimethylsilyl derivatives (BSTFA was used as Me_3Si-reagent) and estimated by GLC on a glass capillary column (40 m × 0.28 mm) coated with 3% OV-101 (column 2). Separations were carried out at 225° with He as the carrier gas (1 ml/min). Hexosamines were determined after the hydrolysis of the sample in 4 M HCl at 110° for 4 hr [10]. After removal of HCl, the hydrolysate was passed through a column of Dowex 50 (H^+) and the hexosamines eluted with 2 M HCl. The hexosamine content was estimated by GLC on column 2 [2] using *myo*-inositol as the int. standard. All GLC analyses were done with a FI-detector.

Ultracentrifugation. Sedimentation studies were carried out in a Hitachi 282 analytical ultracentrifuge. Samples (5 mg/ml) were dialysed to equilibrium against 10 mM Pi buffer (pH 7.0) containing 0.1 M NaCl. Runs were performed at 56000 rpm at 20°.

PAGE. The system of Laemmli [5] was modified by the use of borate buffer [25] instead of Tris–glycine. Electrophoresis was performed at 3 mA/tube for 2 hr and the gel (10%) was stained with thymol–H_2SO_4 [26] for carbohydrate and Coomassie blue [5] for protein.

Methylation analysis. Methylation of the sample was performed as previously described [21]. The partially methylated alditol acetates were separated by GLC on column 1 [21] or on column 2 (150°–220° at 2°/min; carrier gas, He 1 ml/min).

Preparation of GP. AGP (30 mg) was hydrolysed in 0.05 N TFA (3 ml) at 100° for 1 hr [9]. After cooling, EtOH (9 ml) was added and the ppt was collected by centrifugation, washed with 80% EtOH and freeze-dried to give GP (11 mg), $[\alpha]_D$ +4° (H_2O, *c* 0.1). Sugar analysis of GP indicated that more than 90% of the arabinose was removed. The recoveries of uronic acid, protein and hydroxypyroline were 94, 98 and 80%, respectively.

Preparation and characterization of aldobiouronic acid. AGP (100 mg) was hydrolysed in 2 N TFA (10 ml) at 100° for 1 hr. After cooling, the hydrolysate was evap. to dryness, dissolved in H_2O, applied onto a column of Dowex 1 (acetate) and separated into a neutral fraction (eluted with H_2O, 77 mg) and an acidic fraction (eluted with 4 M HOAc, 10 mg). On TLC with EtOAc–HOAc–H_2O (10:5:6), the acidic fraction gave three spots; R_{gal} 0.81, 0.57 (main) and 0.31. The main product was purified on a cellulose column (2 × 15 cm) using the above solvent system, to yield 4 mg of pure material. The material was homogeneous on TLC using the above solvent system (R_{gal} 0.57) and EtOAc–HOAc–HCO_2H–H_2O (18:8:3:9) (R_{gal} 0.78).

Other methods. β-Glucosyl Yariv antigen was synthesized according to ref. [27]. Authentic antigen was a generous gift from Dr. A. E. Clarke. The gel-diffusion plate was made from 1% agarose in 10 mM Tris–HCl (pH 8.0) containing 0.1 M NaCl. Reducing end group analysis was carried out by the method of ref. [28]. ^{13}C NMR (25.1 MHz) [29]: AGP (~50 mg), 70°, 10 mm tube, TMS as int. standard; aldobiouronic acid, RT, 1 mm tube, MeOH as int. standard (49.80 ppm from TMS).

Acknowledgements—The authors thank Drs. M. Iijima and A. Kato of this institute for providing the growth medium of suspension-cultured tobacco cells, Dr. A. E. Clarke of University of Melbourne for the gift of β-glucosyl Yariv antigen, and Dr. H. Kodama of this institute for the synthesis of *p*-nitrophenyl-β-D-glucopyranoside.

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