Phytochemistry, Vol. 21, No. 6, pp. 1325–1329, 1982.
Printed in Great Britain.

0031-9422/82/061325-05$03.00/0

METHYLATION ANALYSIS OF EXTRACELLULAR POLYSACCHARIDES FROM SUSPENSION-CULTURED CELLS OF *NICOTIANA TABACUM*

YUKIO AKIYAMA and KUNIO KATŌ

Central Research Institute, The Japan Tobacco and Salt Public Corporation, 6-2 Umegaoka, Midori-ku, Yokohama 227, Japan

(*Received* 9 *September* 1981)

Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; cell cultures; extracellular polysaccharide; methylation analysis.

Abstract—The soluble extracellular polysaccharides (ECP) of suspension-cultured tobacco cells were fractionated by DEAE-Sephadex CC into seven sub-fractions. Sugar composition and methylation analysis of each fraction were performed and the following polysaccharides were suggested to be present in the ECP: arabinoxyloglucan, galactoglucomannan, (arabino)xylan, arabinogalactan-protein, and arabinoglucuronomannan. The glycosidic linkage analysis of the ECP was also performed with the advance of the culture age.

INTRODUCTION

Suspension-cultured plant cells have been shown to produce polysaccharides similar both in composition and structure to those of primary cell walls [1, 2]. These polysaccharides may be isolated as extracellular polysaccharides (ECP) from the growth medium without using drastic extraction procedures [3]. From ECP of sycamore (*Acer pseudoplatanus*), rhamnogalacturonan, arabinogalactan and xylogucan have been separated by Aspinall *et al.* [4] and by Albersheim *et al.* [5–7]. Xyloglucan, 3,6-linked arabinogalactan, and polyuronide have been suggested to be present in the ECP of *Vinca rosea* from the results of methylation analysis by Takeuchi and Komamine [8]. Arabinogalactan, arabinogalactan-protein, and interior chain of glucuronomannan have been isolated from the ECP of tobacco (*Nicotiana tabacum*) by Katō *et al.* [9–11]. The purpose of the present study was to survey what kind of polysaccharides are present in tobacco ECP. The soluble polysaccharides were divided into seven sub-fractions by ion-exchange chromatography and the linkage analysis of each fraction was performed.

RESULTS AND DISCUSSION

The ECP has been usually obtained by EtOH precipitation followed by freeze-drying. However, it has been reported that the part of the ECP (*ca* 1/3) thus obtained became water insoluble [4, 9]. In this study, the ECP was dialysed against phosphate buffer immediately after harvest so that the insoluble material was not produced. The soluble polysaccharides were then fractionated by DEAE-Sephadex A-25 CC into seven fractions (F-1–F-7). The neutral sugar composition and the results of the methylation analysis of each fraction are shown in Tables 1 and 2, respectively. The neutral sugar composition from the TFA hydrolysis and from the methylation analysis are in broad agreement.

The ECP contained xylose, arabinose, rhamnose, glucose, mannose and galactose residues in the molar ratios 1:1.47:0.07:2.07:1.00:1.07 and uronic acids, which were identified as glucuronic acid (main) and galacturonic acid (trace) by GLC. The methylated polysaccharides from the ECP were hydrolysed, reduced, acetylated, and the partially methylated alditol acetates (PMAA) were subjected to GLC and GC/MS analysis. GC/MS detected 16 peaks which contain carbohydrate derivatives.

The ECP solution was applied to a DEAE-Sephadex column to separate it into sub-fractions. About 55% of the sugars was eluted from the column by phosphate buffer washing (F-1) and the remainder was roughly equally distributed in the following five fractions (F-2–F-6) whereas the final NaOH fraction (F-7) contained *ca* 2% of the original sugars.

F-1 contained xylose, arabinose, glucose, mannose and galactose residues in the molar ratios 1:0.36:1.96:0.59:0.59. Electrophoresis of F-1 gave two main spots, indicating that it consists of two kinds of polysaccharides, which were thought to be arabinoxyloglucan and galactoglucomannan on the basis of methylation analysis. Arabinoxyloglucan has been isolated from the cell-wall preparation of the midrib of tobacco leaves and its structure characterized [12–14]. It consisted of xylose, arabinose and glucose residues in the molar ratios 1:0.39:1.64 and gave in methylation analysis terminal arabinose, terminal xylose, 2-linked xylose, 4-linked glucose and 4, 6-linked glucose residues, which were all detected in F-1, although the amount of the 4-linked glucose residue was rather more than expected, provided that the structures of arabinoxyloglucan in the ECP and the leaf midrib were same. The ECP of sycamore

Table 1. General data of ECP and sub-fractions

Fraction	Eluted by	Total sugar (mg)	Uronic acid* (wt %)	Protein (mg)	Sugar composition (mol %)					
					Xyl	Ara	Rha	Glc	Man	Gal
Original ECP soln (200 ml)		349.5	20.5	37.4	15	22	1	31	15	16
F-1	Buffer alone	190.4	1.6	7.5	22	8	0	43	13	13
F-2	Buffer + 0.05 M NaCl	21.3	12.6	3.4	50	15	0	13	7	16
F-3	Buffer + 0.1 M NaCl	16.6	26.3	3.3	52	28	tr	tr	tr	20
F-4	Buffer + 0.2 M NaCl	22.7	26.1	5.3	25	43	2	tr	3	28
F-5	Buffer + 0.3 M NaCl	13.8	64.2	6.0	tr	59	2	tr	9	29
F-6	Buffer + 0.5 M NaCl	13.1	69.5	1.7	tr	67	tr	9	9	15
F-7	0.5 M NaOH	5.6	32.4	9.6	29	6	0	49	11	5

*Expressed in wt % against total sugar.

[4–7] and *Vinca rosea* [8] have been reported to contain xyloglucan but no arabinoxyloglucan. Assuming that all arabinose and xylose, and most of the glucose residues in F-1 were derived from arabinoxyloglucan, the remaining sugars were galactose, mannose, and glucose. Methylation analysis of F-1 gave terminal galactose, 4-linked mannose, and 4,6-linked mannose residues other than PMAAs derived from arabinoxyloglucan. These data suggested that the second polysaccharide in F-1 may be galactoglucomannan, which would have the backbone of (1→4)-linked glucose and mannose residues to which the galactose residue attaches at C-6. Galactoglucomannan with a similar structure has been reported in wood hemicellulose [15], but it has not been previously detected in the cell wall of tobacco.

Sugar composition analysis showed that F-2 and F-3 are rich in xylose, which was found to be mainly 2- or 4-linked with some terminal and 2, 4-linked by methylation analysis. β-(1-4)-Linked xylan [16] and 4-*O*-methyl glucuronoxylan [17] have been isolated from the midrib of tobacco leaves. The former was found to be a straight (1→4)-linked xylan and the latter a (1→4)-linked xylan backbone carrying a side-chain of 4-*O*-methyl glucuronic acid at C-2. Thus, F-2 and F-3 were thought to contain some xylan-type polysaccharides which would have a (1→4)-linked xylan backbone carrying some side-chains. Arabinose seemed to be one possible side-chain since appreciable amounts of terminal arabinose residue were detected in F-2 and F-3 by methylation analysis. Aspinall *et al.* [4] suggested the presence of (1→4)-linked xylan-type chains in the ECP of sycamore.

Terminal arabinose, 5-linked arabinose, 3-linked galactose, 6-linked galactose, and 3, 6-linked galactose residues, which were thought to be derived from arabinogalactan-protein [9, 10], were detected in the fractions from F-2 to F-5. Gel-diffusion experiment showed that F-2, 3, 4 and 5 reacted with Yariv antigen, which confirmed the presence of arabinogalactan-protein in these fractions, but did not rule out the possibility of the occurrence of arabinogalactan. The distribution of arabinogalactan-protein in four fractions was probably due to the difference in amounts of glucuronic acid residue. The arabino-3, 6-galactan has also been reported to be present in the ECP of sycamore [4–7] and *Vinca rosea* [8]. Pope [18] has isolated hydroxyproline-arabinogalactan from the ECP of sycamore by alkaline hydrolysis followed by gel-filtration. The hydroxyproline-arabinogalactan is thought to be derived from arabinogalactan-protein.

The 2-linked and 2, 3-linked mannose residues were detected in F-4, 5 and 6. It was reported that glucuronomannan interior chain from tobacco ECP [11] and arabinoglucuronomannan from suspension-cultured tobacco cells [19] gave 2-linked and 2, 3-linked mannose residues in methylation analysis. Thus, these two PMAAs of mannose residues in F-4, 5 and 6 were thought to be derived from arabinoglucuronomannan which has not yet been found in tobacco cell wall. The sugar compositions and the methylation analysis of F-7 were similar to those of F-1.

By comparing the results of sugar composition and methylation analysis of sub-fractions (F-1–F-7) with those of the polysaccharides which were isolated from tobacco, it was suggested that tobacco ECP contains at least five kinds of polysaccharides: arabinoxyloglucan, galactoglucomannan, (arabino) xylan, arabinogalactan-protein, and arabinoglucuronomannan. It is of course necessary to isolate and purify each polysaccharide from the ECP to confirm the above conclusion, which is currently under way.

ECPs were obtained from the growth medium after 1, 3 and 5 days from subculture and the sugar composition and the methylation analyses were performed (Tables 3 and 4). ECP contained 70–80% neutral sugar, 13–17% uronic acid, and 6–9% protein. Little change was observed in the neutral sugar composition of the ECP during culture. Similar results were reported on ECP of *Vinca rosea* [8] and *Nicotiana tabacum* [20]. Methylation analyses of the three ECPs gave the same 16 PMAAs, but those relative ratios were different, suggesting that the amounts of

Table 2. Methylation analyses of ECP and sub-fractions

Methylated sugars*	RR_t†		Composition (mol %)							
	Column A	Column B	ECP	F-1	F-2	F-3	F-4	F-5	F-6	F-7
2, 3, 5-Me_3-L-arabinose	0.77	0.56	17.1	3.6	6.5	12.9	24.2	28.9	54.3	4.9
2, 3, 4-Me_3-D-xylose	0.82	0.72	6.4	7.4	9.0	10.4	5.8	3.2	—	4.4
2, 3-Me_2-L-arabinose	0.94	1.11	3.5	1.1	3.0	5.4	9.1	10.4	—	—
2, 3- or 3, 4-Me_2-D-xylose	0.95	1.18	6.5	7.6	28.8	44.6	14.9	3.5	—	16.4
2, 3, 4, 6-Me_4-D-glucose	1.00	1.00	0.9	1.6	2.1	—	—	—	—	1.7
2, 3, 4, 6-Me_4-D-galactose	1.03	1.14	7.8	11.4	9.0	—	4.0	5.5	8.0	2.8
3-Me-D-xylose	1.08	1.58	0.7	6.7	8.7	11.5	3.3	—	—	3.6
3, 4, 6-Me_3-D-mannose	1.14	1.36	2.3	—	—	—	4.4	12.4	15.3	—
2, 3, 6-Me_3-D-mannose	1.15	1.46	2.2	4.5	2.2	—	—	—	—	2.7
2, 3, 6-Me_3-D-glucose	1.17	1.55	22.0	27.9	11.3	—	—	—	11.8	56.7
2, 4, 6-Me_3-D-galactose	1.18	1.46	4.8	5.8	5.7	3.2	7.4	6.6	—	—
2, 3, 4-Me_3-D-galactose	1.26	1.72	0.8	1.5	2.1	2.8	7.6	5.8	—	—
4, 6-Me_2-D-mannose	1.29	1.64	8.2	—	—	—	2.3	9.2	10.6	—
2, 3-Me_2-D-mannose	1.38	1.88	3.5	8.0	4.3	—	—	—	—	2.0
2, 3-Me_2-D-glucose	1.40	1.96	9.0	13.1	2.1	—	—	—	—	4.9
2, 4-Me_2-D-galactose	1.48	2.06	4.2	—	5.2	9.3	16.9	14.5	—	—

*2, 3, 5-Me_3-L-arabinose: 2, 3, 5-tri-*O*-methyl-L-arabinose, etc.

†RR_t retention times of the corresponding alditol acetates relative to that of 1, 5-di-*O*-acetyl-2, 3, 4, 6-tetra-*O*-methyl-D-glucitol.

Table 3. Chemical and neutral sugar compositions of three ECPs

Sample	Neutral sugar (wt %)	Uronic acid (wt %)	Protein (wt %)	Neutral sugar composition (mol %)					
				Xyl	Ara	Rha	Glc	Man	Gal
1 day ECP	79.5	13.0	5.6	19	26	1	26	12	16
3 day ECP	81.8	17.3	7.8	17	22	1	31	15	15
5 day ECP	70.6	16.0	9.5	15	22	1	31	15	16

Table 4. Methylation analyses of three ECPs

Methylated sugars	Composition (mol %)		
	1 day ECP	3 days ECP	5 days ECP
2, 3, 5-Me_3-L-arabinose	19.9	19.7	17.1
2, 3, 4-Me_3-D-xylose	8.6	9.0	6.4
2, 3-Me_2-L-arabinose	8.1	4.4	3.5
2, 3- or 3, 4-Me_2-D-xylose	10.2	9.4	6.5
2, 3, 4, 6-Me_4-D-glucose	8.7	9.9	0.9
2, 3, 4, 6-Me_4-D-galactose	3.1	8.1	7.8
3-Me-D-xylose	1.0	0.6	0.7
3, 4, 6-Me_3-D-mannose	5.1	1.5	2.3
2, 3, 6-Me_3-D-mannose	5.9	2.6	2.2
2, 3, 6-Me_3-D-glucose	13.0	15.3	22.0
2, 4, 6-Me_3-D-galactose	2.0	3.6	4.8
2, 3, 4-Me_3-D-galactose	0.9	0.5	0.8
4, 6-Me_2-D-mannose	2.6	3.3	8.2
2, 3-Me_2-D-mannose	1.7	4.2	3.5
2, 3-Me_2-D-glucose	6.0	5.4	9.0
2, 4-Me_2-D-galactose	3.3	2.8	4.2

each polysaccharide may not be constant during culture. Especially terminal and 4-linked glucose residues were significantly changed. In the early stages of growth, appreciable amounts of terminal glucose residues were present which decreased with the advance of the culture age. On the contrary, the amount of 4-linked glucose residue was increased with the advance of the culture age. The elucidation of the polysaccharide(s) responsible for the terminal glucose residue is now in progress.

EXPERIMENTAL

General method. Concn was carried out under red. pres. at <40°. Electrophoresis was performed on a Whatman GF/A glass microfibre paper at 1500 V for 30 min with 0.1 M Na tetraborate (pH 9.2). Carbohydrates were detected by heating with H_2SO_4. Gel-diffusion experiment was done as described in ref. [10]. GC was performed with FID on a glass capillary column (50 m × 0.27 mm) coated with silicone OV-101 (column A) [21] and a column (2 m × 0.3 cm) containing Gas Chrom P coated with a mixture of 0.2% PEGA, 0.2% PEGS and 0.4% silicone XF-1150 (column B) [16]. Peak areas were measured with a Hewlett–Packard 3380 A digital integrator. For GC/MS (70 eV), a silicone OV-101 glass capillary column (30 m) was used. Total sugar content was determined by the phenol–H_2SO_4 method [22] (glucose standard), uronic acid by the *m*-hydroxydiphenyl method [23] (glucuronic acid standard), and protein by the method of ref. [24] (bovine serum albumin standard).

Fractionation of ECP. Suspension culture of tobacco cells was carried out as described [10]. After filtration and centrifugation to remove cells, the ECP soln was dialysed against 10 mM NaPi buffer (pH 6) for 24 hr. The clear soln was then applied to a column (15 × 2.5 cm) of DEAE-Sephadex A-25 equilibrated in the same buffer. The column was eluted with the buffer alone at first, followed by the same buffer containing 0.05, 0.1, 0.2, 0.3, 0.5 M NaCl and finally with 0.5 M NaOH to give F-1, 2, 3, 4, 5, 6, and 7, respectively.

Sugar composition analysis. Polysaccharide was hydrolysed with 2 M TFA at 120° for 1 hr. After removal of TFA under a stream of N_2, the hydrolysate was converted to diethyl dithioacetal TMSi derivatives [25]. *myo*-Inositol was used as the int. standard. Separations were performed by comparison of GC retention times (columns A and B) flow rate 1 ml/min.

Methylation analysis. Methylation was carried out by the method of ref. [26] using 5–10 mg of sample. The resulting soln was dialysed against H_2O, concd, and the product was hydrolysed, reduced and converted into partially methylated alditol acetates (PMAA). Separations were performed by GC using column A, from 150 to 220° at 2°/min, He 1 ml/min, and column B, from 120° (initial hold 10 min) to 190° at 1°/min, He 60 ml/min. The identification of each peak was made by the combined GC/MS analysis of PMAA [27] and by comparison of GC retention times (columns A and B) when standards were available [9–14, 16, 17, 19, 21].

REFERENCES

1. Roberts, R. M. and Loewus, F. (1966) *Plant Physiol.* **41**, 1489.
2. Burke, D., Kaufman, P., McNeil, M. and Albersheim, P. (1974) *Plant Physiol.* **54**, 109.
3. Becker, G. E., Hui, P. A. and Albersheim, P. (1964) *Plant Physiol.* **39**, 913.
4. Aspinall, G. O., Molloy, J. A. and Craig, J. W. T. (1969) *Can. J. Biochem.* **47**, 1063.
5. Talmadge, K. W., Keegstra, K., Bauer, W. D. and Albersheim, P. (1973) *Plant Physiol.* **51**, 158.
6. Bauer, W. D., Talmadge, K. W., Keegstra, K. and Albersheim, P. (1973) *Plant Physiol.* **51**, 174.
7. Keegstra, K., Talmadge, K. W., Bauer, W. D. and Albersheim, P. (1973) *Plant Physiol.* **51**, 188.
8. Takeuchi, Y. and Komamine, A. (1978) *Physiol. Plant.* **42**, 21.
9. Katō, K., Watanabe, F. and Eda, S. (1977) *Agric. Biol. Chem.* **41**, 533.
10. Akiyama, Y. and Katō, K. (1981) *Phytochemistry* **20**, 2507.
11. Katō, K., Watanabe, F. and Eda, S. (1977) *Agric. Biol. Chem.* **41**, 539.
12. Eda, S. and Katō, K. (1978) *Agric. Biol. Chem.* **42**, 351.
13. Mori, M., Eda, S. and Katō, K. (1979) *Agric. Biol. Chem.* **43**, 145.
14. Mori, M., Eda, S. and Katō, K. (1980) *Carbohydr. Res.* **84**, 125.
15. Timell, T. E. (1965) *Adv. Carbohydr. Chem.* **20**, 448.
16. Eda, S. and Katō, K. (1976) *Agric. Biol. Chem.* **40**, 359.
17. Eda, S., Watanabe, F. and Katō, K. (1977) *Agric. Biol. Chem.* **41**, 429.
18. Pope, D. G. (1977) *Plant Physiol.* **59**, 894.
19. Mori, M. and Katō, K. (1981) *Carbohydr. Res.* **91**, 49.
20. Katō, K. and Noguchi, M. (1976) *Agric. Biol. Chem.* **40**, 1923.
21. Eda, S. and Katō, K. (1980) *Agric. Biol. Chem.* **44**, 2793.
22. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Analyt. Chem.* **28**, 350.
23. Blumenkrantz, N. and Asboe-Hansen, G. (1973) *Analyt. Biochem.* **54**, 484.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
25. Honda, S., Yamaguchi, N. and Kakehi, K. (1979) *J. Chromatogr.* **169**, 287.
26. Hakomori, S. (1964) *J. Biochem.* **55**, 205.
27. Jansson, P. E., Kenne, L., Liedgren, H., Lindberg, B. and Lonngren, J. (1976) *Chem. Commun. Univ. Stockholm* **8**, 1.