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THE CARBOHYDRATE CONSTITUENTS OF THE CELL WALL OF SUSPENSION CULTURES OF *ROSA GLAUCA*

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Abstract—Sequential extractions of 14-day-old *Rosa glauca* cell walls cultured *in vitro* showed that two different types of acidic polysaccharide were present. One was extracted with EDTA or ammonium oxalate solutions, and the other remained in close association with cellulose even after 4.3 N NaOH extractions or 2 N H_2SO_4 hydrolysis. The cell wall has a low content in structural protein. The behaviour of each constituent sugar was followed during the course of the various extraction steps, and a complete quantitative account of the protein, uronic acid and neutral sugar components is given at each stage.

INTRODUCTION

Primary walls play an active role in elongation or extension processes during growth [1–3]. They are found in organs like coleoptiles, hypocotyls, internodes of very young stems, young roots, cambial tissue in trees and also in cells cultured *in vitro*. It has been recognized during the last five years that they are essentially built of a fibrillar cellulosic framework, an amorphous matrix of pectic and hemicellulosic polysaccharides and a structural protein, frequently called extensin and characterized by a high hydroxyproline content and a high degree of glycosidation [4–10].

Several chemical and biochemical methods have been applied to this multimolecular complex for extracting and studying the separated constituents. Methods differ depending on the objective, the non-cellulosic polysaccharide or the protein moeity [5, 7, 9, 11–14]. Some polysaccharides have been isolated from primary walls: a pectic acid [5, 10, 15], an arabinogalactan [5, 9], a galactan [16], a xyloglucan [8, 17, 18] and a xylan [10]. Significant differences have been established within the same type of polysaccharide according to the cells or tissues studied.

In a series of investigations on primary cell walls from lupin and mung bean hypocotyls. Monro *et al.* have established that much of the pectin, hemicelluloses and glycoprotein is not covalently linked but that the glycoprotein and microfibrils have direct interaction [12–14]. The wall glycoprotein has been the subject of several papers [6, 11, 19]. In spite of apparent similarities, the model proposed in 1973 by Albersheim and co-workers [20] is not representative of all types of primary walls. Several results on hypocotyls [21], rose cells cultured *in vitro* [22] or cambial cells of *Populus* and *Tilia* [8–10] do not fit an initial model in which all the polymers appear cross-linked. These discrepancies suggest that cell walls of different plants or organs or cellular types of a same plant have their own molecular characteristics, probably in relation to their different functions; also primary walls are changing *per se* as a function of cell maturation.

In our view, the pectic polysaccharides must be further studied because of their quantitative importance in the wall. Lamport has estimated the pectic components of *Acer pseudoplatanus* to be as much as 36% of the wall [4]. Talmadge *et al.* propose the figure of 34% in the wall of the same strain [5]. Simson and Timell found in the cambial tissues of *Populus tremuloides* and *Tilia americana* that 40–50% and more than 50% of the total polysaccharides of the wall, respectively, are pectic material (acidic polysaccharides plus arabinan and galactans) [7]. Their nature as large charged molecules [23, 24] suggests that they probably have a physiological role of the utmost importance in the primary wall.

We have studied these wall polymers in the cells of a *Rosa glauca* strain cultured in our laboratory, as a suspension culture, since 1974 [25]. In this paper, the first of a series, results on our sequential fractional extractions are presented with special attention to the acidic polymers of the walls. At each step of the extraction procedure to which the cell walls were submitted (Fig. 1), a complete and accurate balance of the constituents has been established both for the solubilized fractions and for the residues. This allowed us to determine the yield and nature of the polysaccharides extracted and also to follow the movements of each constituent sugar during the fractionation process.

RESULTS

Characteristics of the walls

The cells used in these experiments were selected at the stage corresponding to the end of the exponential phase. At this stage the cells are small in size (half the final size observed in the stationary phase) and are strongly aggregated in clumps of 20 or 40 cells. The washed walls represent about 1% in weight of the fresh cells. The overall analytical data obtained on the wall composition reveal that they are made up of 70% neutral sugars, 24% acidic sugars and only 2.5% protein. Amongst the neutral sugars, glucose was the major component with galactose

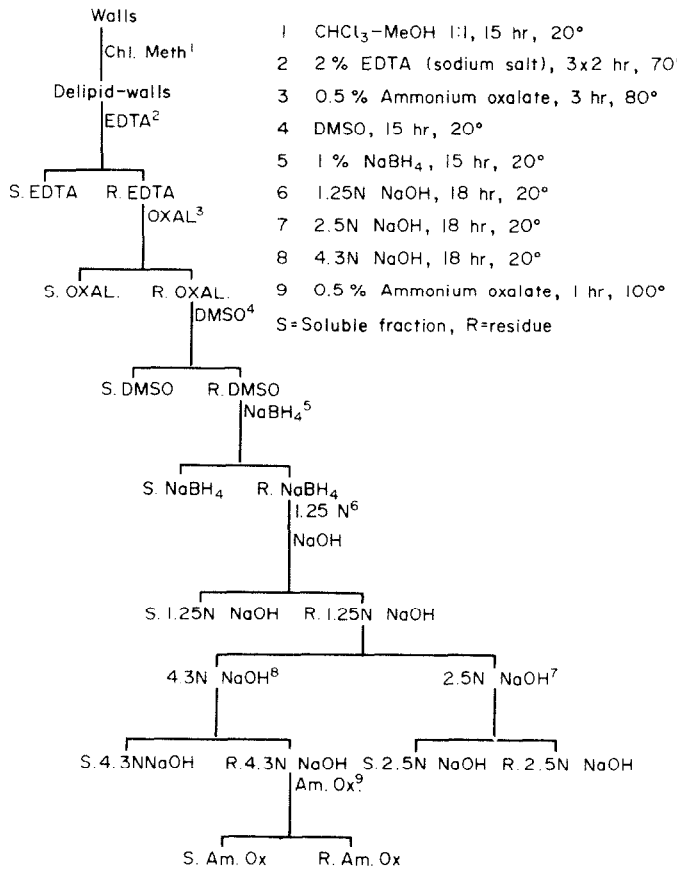

Fig. 1. Scheme of sequential extractions of *R. glauca* cell walls.

and arabinose being the next two most abundant sugars. The protein content is particularly low (2.5%) as compared to the 8–10% found in other types of primary cell walls. Hydroxyproline, which is the most characteristic amino acid of the wall protein, represents 13.2% of the protein.

Interesting information on the walls was obtained by applying the selective hydrolysis conditions of Bartley (2 N H_2SO_4, 120°, 1 hr) [26] which were shown to solubilize the pectic and hemicellulosic constituents of young plant tissues, leaving an insoluble part consisting mainly of cellulose. Using this hydrolytic procedure, a comparison was made between the walls from 10-, 14- and 28-day-old cells of *Rosa glauca* (Table 3). The 14-day-old cells had the highest content of material in the non-soluble fraction. This fraction had also the maximum content of uronic acids. The youngest cells were richer in hydrolysable polymers. It must be noted that an important part of the sugars extracted during the acid hydrolysis are uronic acids. In the three series studied, it is remarkable that a part of the polyuronides was solubilized and the other part, which was approximately 50% of the bulk, remained polymeric, in close association with the non-hydrolysable cellulosic microfibrils or the resistant wall glycoprotein still present in the residues of hydrolysis.

Scheme of extraction

In order to determine the distribution of the pectic polysaccharides in the different fractions extracted from the walls, the conventional sequential treatments, with solvents of increasing ability to dissolve the different constituents, have been applied to the walls of *Rosa glauca* cells. The general scheme followed in this investigation is presented in Fig. 1. Data in Tables 1 and 2 show the percentage of the wall components (neutral sugars, uronic acids, protein) that have been extracted in sequence.

EDTA *and ammonium oxalate extracted polymers*

These polymers are mainly composed of uronic acids (around 50%). The neutral sugar components are essentially arabinose with a smaller proportion of galactose. The content of rhamnose is high, as expected from the pectic nature of the fraction. It must be observed that 21% of the total polyuronides are extracted in this step although only 10% of the total wall is solubilized (Tables 1, 2 and 5).

DMSO *extracted polymers*

Although DMSO is a powerful solvent for many polysaccharides, it only removed 0.5% of the wall polymers of the cells studied. The fraction obtained is made mainly of xylose with a much smaller quantity of glucose (Tables 1 and 2). There is apparently no extraction of a xyloglucan.

$NaBH_4$ *extracted fraction*

Sodium borohydride is often used as a reducing agent prior to, or accompanying, alkaline extraction [7] in order to prevent the polysaccharides from alkaline

Table 1. Percentage of different components extracted in the sequential treatment of the walls and proportions of the different components

Fraction	Percentage of total wall	Neutral sugar content (% by wt)	Uronic acid content (% by wt)	Protein content (% by wt)
EDTA	6.7	40.5	51	1.4
Oxal.	3.5	35.5	48	1.5
DMSO	0.5	46	17	2.2
$NaBH_4$	1.2	tr	49	7.5
1.25 N NaOH	4.7	48.5	20	24.3
2.5 N NaOH	10.8	56	4.5	7.3
4.3 N NaOH	13.3	66	9	6.0
2.5 N NaOH residue	55.5	71	29	0.33
4.3 N NaOH residue	61.7	68	25	0.35

Table 2. Neutral sugar composition of the different fractions

Fraction	% in the fraction (by wt)	Neutral sugars in % of total (by wt)	Molar ratio						
			Rha	Fu	Ara	Xyl	Ma	Gal	Glc
A_0*	70	70	3.5	1	13	8	3	16.5	55
EDTA	40.5	2.7	10.5	—	53	3.5	—	19	14
Oxal.	35.5	1.2	11.5	—	49.5	4	—	22	13
DMSO	46	0.2	9	—	25	38	—	14.5	13.5
1.25 N NaOH	48.5	2.3	4	4	19	14.5	5.5	16	37
2.5 N NaOH	56	6	2.5	3	10	18.5	13	14.5	38.5
4.3 N NaOH	66	8.7	3.5	3.5	14	15.5	11	16	36.5
2.5 N NaOH residue	71	39.4	4.5	1.5	20.5	9.5	—	16	48
4.3 N NaOH residue	68	42	2	1.5	14.5	6.5	—	14	61.5

* A_0 represents the starting cell wall material (see Experimental).

Table 3. Carbohydrate composition of soluble and insoluble parts of the walls after 2 N H_2SO_4 hydrolysis

Fraction	Percentage total of wall	Uronic acid content (% by wt)	Neutral sugar content (% by wt)	Molar ratio						
				Rha	Fu	Ara	Xyl	Ma	Gal	Glc
Residue (10-day-old cells)	26.5	18	73	—	—	tr	1	3.5	—	95.5
Residue (14-day-old cells)	42	29	62	—	—	—	2.5	5	—	92.5
Residue (28-day-old cells)	35.5	13	72	—	—	—	tr	1.5	—	98.5
Soluble (10 days)	73.5	15	61	12	3	31	13.5	2	19	19.5
Soluble (14 days)	58	18	56	5	3	33	17	1	29	12
Soluble (28 days)	64.5	14	42	10	3	21.5	13.5	2.5	25.5	24

degradations. Apart from this role, sodium borohydride, when used in dilute solutions, has been shown [27, 28] to be an efficient solvent for acidic polysaccharides not solubilized by aqueous solutions. The 1% solution of borohydride solubilized only a small percentage of the wall but the fraction was strongly acidic (49% uronic acids). It appears that this treatment effectively acts as a complement to the usual solvents of the pectic material. This fraction also had 7.5% protein (Tables 1, 4 and 5).

1.25 N NaOH *extracted polymers*

In this mild alkaline treatment, one can observe that only a small part of the wall is solubilized. The main feature of this treatment is the solubilization of 46% of the wall protein (Table 4). It is to be noted that this protein fraction contains only 4% hydroxyproline. Polymers containing glucose are the major constituents of the fraction. Those containing arabinose or galactose are also extracted (Tables 1, 2 and 4).

Table 4. Data on protein extraction during the sequential treatment of the wall

Fraction	% of the total wall extracted	% of protein in the fraction (by wt)	Protein as % of the total wall	% of total protein extracted	Hydroxyproline content*
A_0	100	2.5	2.5	100	13
EDTA	6.7	1.4	0.09	3.6	—
Oxal.	3.5	1.5	0.05	2	—
DMSO	0.5	2.2	0.01	0.4	—
$NaBH_4$	1.2	7.5	0.09	3.6	16.5
1.25 N NaOH	4.7	24.5	1.15	46	4
2.5 N NaOH	10.8	7.4	0.8	32	9
4.3 N NaOH	13.3	6	0.8	32	10.5
2.5 N NaOH residue	55.5	0.33	0.18	7.2	44
4.3 N NaOH residue	61.7	0.35	0.21	8.4	21.5

* As a percentage of the protein of the fraction.

Table 5. Data on uronic acid extraction during the sequential treatment

Fraction	% of total wall extracted	Uronic acid content of the fraction (% by wt)	Uronic acids in percent of total wall (by wt)	% of total uronic acids extracted
A_0	100	24	24	100
EDTA	6.7	51	3.4	14.2
Oxal.	3.5	48	1.7	7.1
DMSO	0.5	17	0.08	0.3
$NaBH_4$	1.2	49	0.6	2.5
1.25 N NaOH	4.7	20	0.9	3.7
2.5 N NaOH	10.8	4.5	0.5	2
4.3 N NaOH	13.3	9	1.2	5.0
2.5 N NaOH residue	55.5	29	16	66.6
4.3 N NaOH residue	61.7	25	15.4	64

2.5 N NaOH *extracted polymers*

From Table 1 it can be observed that around 11% of the initial walls are extracted. This fraction is rich in protein. Polymers of glucose and xylose are solubilized and to a lesser proportion all the neutral sugars are found in the hydrolysates. There is no selective extraction of one type of polymer, suggesting that all these sugars belong to linked macromolecules (Tables 2 and 4).

4.3 N NaOH *extracted polymers*

Applied to the 1.25 N NaOH treated walls, this alkaline treatment extracts 13% of the wall components. These are mainly neutral polymers (glucose and xylose are the main sugars found). There is a low percentage of uronic acids as previously noted in the 2.5 N NaOH extracted fraction. Again the wall protein is dissolved to a high degree, comparable to that of the previous fraction. All these data show that these strong alkaline conditions do not have the ability to extract from the walls more constituents than the 2.5 N NaOH solutions; all these alklaine extractions resulted in the solubilization of up to 82% of the total wall protein (Table 4).

Table 6. Percentage of each monosaccharide extracted at different stages of the sequential treatment

	Monosaccharides (% by wt)						
Fraction	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
A_0*	100	100	100	100	100	100	100
EDTA	11.5	—	13	1.5	—	5	1
Oxal.	6	—	5.5	0.8	—	2.5	0.5
DMSO	1	—	0.5	1.6	—	0.5	0.1
1.25 N NaOH	4	8	3.5	6	7.5	3.5	2
2.5 N NaOH	5	17	5.5	20.5	48	8	6
4.3 N NaOH	11	32	10	25	58.5	13.5	8
2.5 N NaOH residue	69	55.5	70.5	68	—	59	48
4.3 N NaOH residue	30.5	50	51.5	46	—	55	64.5

* For composition see Table 2.

Characteristics of the residual walls

From the residue after 2.5 N NaOH extraction, it can be seen that little protein was present with a high content of hydroxyproline (44%). This hydroxyproline represents 25% of the total hydroxyproline of the wall (Table 4). This result, added to the fact that in this residue there is still 70% of the arabinose initially present in the wall, suggests the possible role of the hydroxyproline–arabinoside linkage in the connection of the cellulosic and non-cellulosic polymers (Table 6). From the data in Table 1, it can be seen that after the NaOH treatments, as much as 40% of the wall components have been solubilized. The residue (*ca* 60%) which has a very low protein content is still composed of approximately 70% neutral sugars and 25–29% uronic acids (Tables 2, 4 and 5). When the above residue was subjected to the 2 N H_2SO_4 hydrolysis, all the arabinose and galactose were found in the soluble part and nearly all the glucose and a part of uronic acids remained insoluble. This result suggests that cellulose and a polyuronide must be associated through strong linkages.

Extraction with ammonium oxalate

In order to obtain a more complete extraction of the polyuronides, the residual walls after NaOH extraction were treated with ammonium oxalate at pH 7 (Fig. 1). The solubilized material (about 20%) was acidic in nature since it contained 33% uronic acids. Among the neutral sugars, arabinose (50%) and galactose (30%) were the most important. Here again the insoluble part essentially constituted of cellulose but 26% of the initial polyuronides of the wall remained tightly associated to it.

DISCUSSION

In comparison to the results obtained with other types of primary cell walls, it appears that the 14-day-old cell walls of *Rosa glauca* have their own molecular characteristics, related to the cellular morphology and physiology. At the stage of sampling, the young and small cells are limited by a thin wall which undergoes important changes in dimensions. These walls must be able to undergo a fast extension since the cell size doubles in approximately 10 days. The low level of the hydroxyproline-rich wall protein must be also related to the stage of sampling, at a time where the cells are continuously dividing. In the *R. glauca* cell walls studied the main characteristic is the high percentage of pectic components, a type of hydrophilic matrix which allow the processes of isodiametric extension of the ovoïd rose cells. The results obtained with the sequential treatments followed in this study suggest that most of the neutral polymers are connected to the pectic acids of these walls.

If we assume that a pectic acid similar to that described by Simson and Timell [10] in the cambial walls of *Populus* and *Tilia* is also present in the walls of *R. glauca*, then it can be understood why at the end of the extraction sequence about 50% of the constituents are still in the form of undissociated polymers. It must be noted that at every stage, all the constitutive monomers (neutral sugars and uronic acids) are present in the polymers extracted. There is no selective extraction but a simultaneous solubilization of a mixture of polymers or a solubilization of fragments of a large pectic acid molecule.

From our results it can be seen that pectic acid molecules have to be studied in more detail at two stages of the sequence. The first corresponds to the acidic polyuronides extracted by EDTA–ammonium oxalate solutions. The second remains in the insoluble material after the 2.5 N NaOH treatment, there being no indication that more drastic alkaline conditions remove any more material. At this stage of the sequential treatments, we have shown that ammonium salts can extract a part of the insoluble pectic polymer.

The presence in the final residue from the extraction process of hemicellulosic material and of pectin still associated with cellulose has been observed in several instances [7, 29, 30]. It is well known that it is very difficult to obtain a cellulose totally free of contaminating sugar residues. It seems, however, that the large percentage of uronic acids remaining in close association with our cellulosic residue after 2.5 N NaOH, as well as 2 N H_2SO_4, treatment is a new facet of the characteristics of these primary walls.

EXPERIMENTAL

Origin of cell walls. The walls used throughout were prepared from a cell suspension culture of *R. glauca* isolated in our laboratory in 1973. This strain (A_0) growing on sucrose and mineral salts has no hormone dependence (habituated strain) [25]. After 14 days of growth at 22°, the cells were washed, ground in a phosphate buffer (pH 7.2 at 2–4°), washed several times [31, 32] on a scintered glass funnel, and lyophilized. Before use, the walls were delipidated [33] with $CHCl_3$–MeOH (1:1) overnight at 20°.

Analytical procedures. The protein content was determined using the method of Lowry [34]. Uronic acids were estimated by the carbazole method [35, 36] and controlled by decarboxylation according to Bylund and Donetzhuber [37]. Hydroxyproline was assayed following the method of Kivirikko [38, 39] after hydrolysis at 100 with 6 N HCl in sealed tubes for 20 hr. Neutral sugars were obtained from the lyophilized fractions by the hydrolytic procedure previously described [40]. After neutralization with $BaCO_3$ the monosaccharides were qualitatively and quantitatively determined by GLC of their alditol acetate derivatives [41]. For GLC, a Packard–Becker 417 instrument fitted with a flame-ionization detector was used. Separations were performed on glass columns (2 m × 0.15 cm) containing 3% of ECNSS-M on Gas Chrom. Q (100–120 mesh) at 185°. Peak areas were calculated with a 3880 A Hewlett–Packard integrator. *Meso*-inositol was used as the internal standard.

Sequential extraction of the walls. The different steps of the extraction procedure are shown in Fig. 1. Water-soluble polysaccharides were extracted from the walls by hot solutions (80°) of 2% EDTA (3 × 2). Ammonium oxalate (0.5%) was used at 80° for 2 hr. Alkaline extractions were performed successively with 1.25, 2.5 and 4.3 M NaOH soln (20°, 18 hr under N_2).

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