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DIFFERENTIAL ALKALI-EXTRACTION OF HEMICELLULOSE AND HYDROXYPROLINE FROM NON-DELIGNIFIED CELL WALLS OF LUPIN HYPOCOTYLS

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ABSTRACT

The extraction by alkali of hemicellulose polysaccharides and polymer hydroxyproline from non-delignified, primary cell-walls of lupin hypocotyls has been studied, using sequential extractions at 0° and 18–22° 10% Aqueous potassium hydroxide at 0° rapidly removed about two-thirds of the hemicellulose normally extracted in 10% KOH at 18–22° and including nearly all of the hemicellulose-A Little hydroxyproline was released at 0° When the temperature was subsequently raised to 18–22°, the remaining 10% KOH-soluble hemicellulose, along with most of the hydroxyproline, was released The monosaccharide composition of these fractions changed markedly with the time of extraction Arabinose increased from 35% of the polysaccharide extracted during the first hour at 18–22° to ~65% of the polysaccharide extracted between 16 and 20 h at 18–22°. Hydroxyproline changed similarly as a proportion of polymer The implications of these and other results are discussed in relation to polysaccharide and polymer extraction without prior delignification and to models of the primary cell-wall

INTRODUCTION

Alkaline extraction of hemicellulose from plant cell-walls has usually involved delignification followed by successive treatments with dilute and concentrated alkali at room temperature These methods have been developed largely for plant tissue having a high degree of secondary cell-wall They are not so suitable for primary cell-walls where lignin levels are very low, and the walls contain glycoprotein rich in hydroxyproline Chemical delignification not only destroys this glycoprotein but may also result in a substantial loss of polysaccharide sugar[1] The hydroxyproline-rich glycoprotein has been postulated to play a role in controlling cell-wall elongation and has been named extensin[2] We have found[3] that extraction of non-delignified cell-walls of lupin hypocotyls with 10% KOH at 0° for 4 h dissolves most of the wall

hemicellulose-type polysaccharide but only a small amount of the hydroxyproline Subsequent overnight-extraction of the walls with 10% KOH at room temperature does, however, extract nearly all of the extensin glycoprotein (using hydroxyproline as an index of extensin) and a further fraction of hemicellulose differing in composition from that extracted at 0°

Because the marked difference in composition of the polymers extracted at 0° and 18–22° may be related to the involvement or non-involvement of conventional hemicellulose in the extensin–polysaccharide complex, a more-detailed study of the time course of alkali extraction of polymers from cell walls of lupin hypocotyls at 0° and 18–22° has been undertaken and is now reported

RESULTS AND DISCUSSION

The time course of polymer extraction with 10% KOH at 0° for 12 h and then at 18–22° for a further 48 h was established by extracting a batch of depectinated cell-walls successively with portions of alkali The numbers and durations of the stages in the extraction sequence and the corresponding total extraction times are shown in Table I Results for the extraction of total non-dialysable polymer and its associated hydroxyproline are given on a cumulative basis in Fig 1 A plot of non-dialysable polymer and hydroxyproline removed per hour in each successive extraction is given in Fig 2 Hydroxyproline appears to be a satisfactory index of wall protein in lupin hypocotyl-walls (unpublished data), so that the hydroxyproline results for polymer extracted and for the cell walls after extraction also pertain to the wall protein

At each temperature, there is a rapid, initial extraction of polymer which soon decreases Polymer soluble at 0° represents about two-thirds of the total polymer conventionally extracted over 24 h with 10% KOH at 18–22°. As Table II shows, the sum of the polymer soluble at 0° and that subsequently extracted at 18–22° includes most of the cell-wall hemicellulose, although there is some further extraction with 24% KOH and a small amount remains in the wall residue The initial rate of extraction of polymer extracted at 0° is much greater (4 7 times in the first hour) than that of the material soluble at 18–22°. Fig 2 shows that, although the initial rates of extraction of hydroxyproline at 0° and 18–22° are similar, there is a sharp decline at 0° so that the average rate of extraction is much higher at 18–22°

Table II shows that the recoveries of total polymer and of hydroxyproline were high, except for the 24% KOH-treatment where ~43% of the weight loss and 48% of the hydroxyproline loss from the 10% KOH-extracted cell-walls upon 24%-KOH extraction are not accounted for in the dialysed 24%-KOH extract Polymer extracted at 0° and at 18–22° contains ~9 5% and ~77% of the depectinated-wall hydroxyproline, respectively As each extract was neutralized immediately after filtering off the cell walls, alkaline degradation of the extracted polymers was minimized, leading to better recoveries than previously reported[3] when such degradation would have continued during the entire duration of the alkali-treatment.

TABLE I

SEQUENCE OF ALKALINE EXTRACTIONS OF DEPECTINATED CELL-WALLS OF LUPIN HYPOCOTYLS

Stage in sequence	1	2	3	4	5	6	7	8	9	10	11	12	13
Filtering time (from sequence start) (h)	1	2	4	8	12	13	14	16	20	24	36	60	84
Duration of extraction (h)	1	1	2	4	4	1	1	2	4	4	12	24	24
Temperature (degrees)	←		0		→	←			18–22			→	18–22
Extractant	←				10% KOH (w/v)							→	24% KOH (w/v)

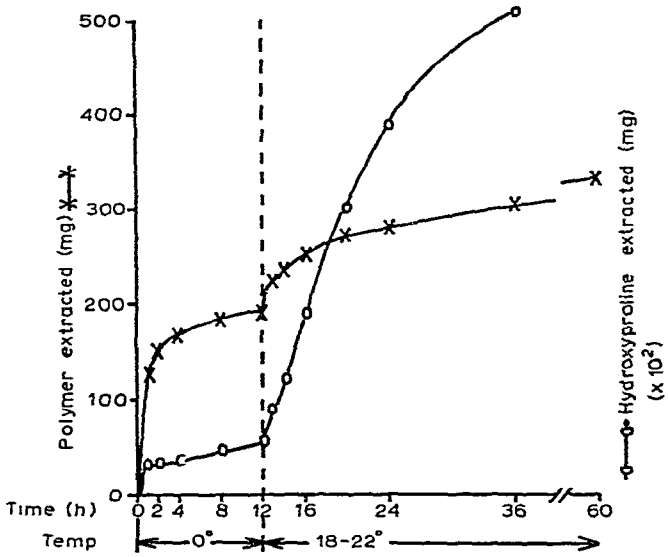

Fig 1 Extraction of total polymer and hydroxyproline from cell walls of lupin hypocotyls by 10% KOH at 0° and at 18–22° as a function of time

TABLE II

ALKALINE EXTRACTION OF HYDROXYPROLINE AND POLYMER FROM CELL WALLS[a] OF LUPIN HYPOCOTYLS

Extraction conditions[b]	*Wall or wall residue*	*Total extracted polymer*	*Hydroxyproline content*	
			Extracted wall	*Dialysed extract*
Neutral detergent, 4 h, 100°	1000	—	5 94	—
10% KOH, 0°, 12 h	784 1	193 9	5 36	0 57
10% KOH, 18–22°, 48 h	635	139 1	0 96	4 56
24% KOH, 18–22°, 24 h	527	61 3	0 42	0 28

[a]All results are expressed as mg/g of neutral detergent-extracted cell-wall [b]Extractions in sequence

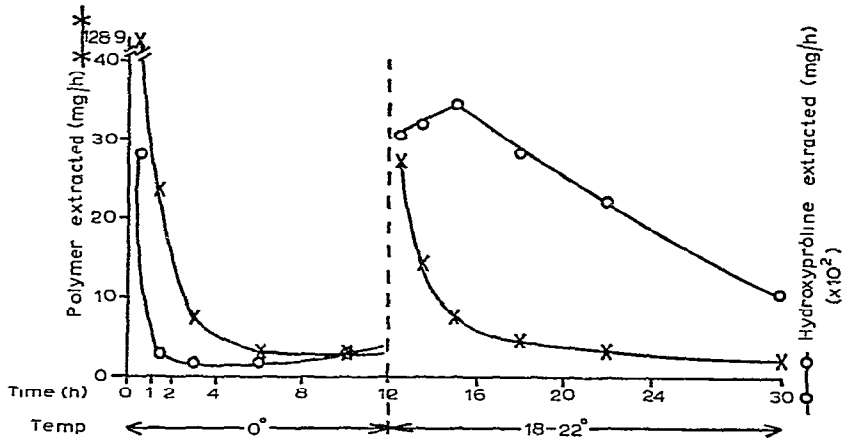

Fig 2 Rates of extraction of polymer and hydroxyproline from cell walls of lupin hypocotyls by 10% KOH at 0° and 18–22°

In Fig 3, hydroxyproline in the dialysed extracts is plotted as a percentage of polymer present The curves for hydroxyproline and total polymer at 0° in Fig 2 and the curve in Fig 3 at 0° taken over the same initial times suggest that, at this stage, the hydroxyproline and polymer are in constant proportion A different situation operates at room temperature (Fig 2) In this case, the two curves diverge markedly, so that, in effect, there is a marked rise in the hydroxyproline content of the extracted polymer In the extraction between 8 and 12 h at 18–22°, hydroxyproline reaches its highest level as a proportion (7%) of extracted polymer (Fig 3) Hydroxyproline accounts for ~30% of the wall-protein amino acids (unpublished data), so that polymer extracted at this stage would be 20% protein or peptide

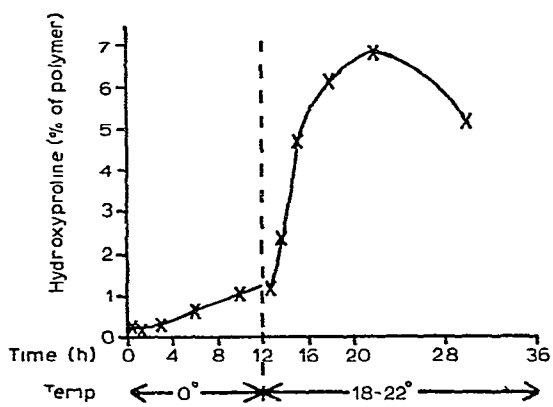

Fig 3 Hydroxyproline as a percentage of the polymer from each stage in the extraction sequence of Table I

Fractionation of polymers. — The polymer soluble at 0° from steps 1 and 4 and the polymer soluble at room temperature from steps 6 and 10 of the extraction sequence (see Table I) were fractionated by dissolution in aqueous $CaCl_2$, precipitating predominantly (1→4)-linked polysaccharide with I_2–KI, and recovering the more-branched heteroglycan from the supernatant. Previous results[3] have shown

TABLE III

FRACTIONATION OF HEMICELLULOSE EXTRACTED[a] FROM CELL WALLS OF LUPIN HYPOCOTYLS

Parent hemicellulose			*Derived fractions*		
Stage in extraction sequence[b]	*Extraction temperature (degrees)*	*Weight taken (mg)*	*Hemicellulose-B, predominantly (1→4)-linked polysaccharide*	*Heteroglycan-B*	*Recovery (%)*
1	0	100	94 6	7 5	102
4	0	100	65	18 2	83
6	18–22	100	71 0	13 0	84
10	18–22	100	74 6	19 2	94

[a]With 10% KOH at 0° and 18–22°. [b]See Table I.

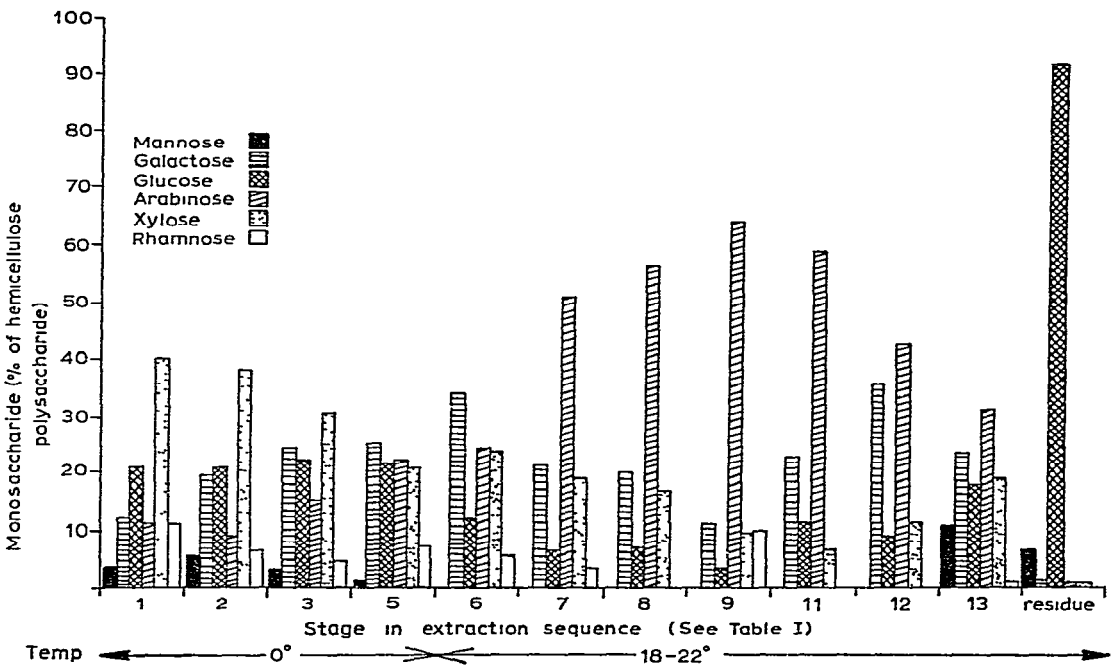

Fig 4. Monosaccharide composition of hemicellulose from sequential extractions of cell walls of lupin hypocotyls with 10% KOH at 0° and 18–22°.

that most of the hydroxyproline in the polymer extracted at 18–22° is found in the iodine-precipitable fraction Table III shows that the heteroglycan fraction is always a minor one Although I_2–KI in $CaCl_2$ solution is considered to precipitate (1→4)-linked xylans, glucans, and mannans, the polysaccharide from extraction step 10, although containing only 10% of xylose (see Fig 4 below), is still 72% precipitable by iodine.

In a second extraction experiment, which to some extent served as a replicate for that shown in Table I, the extraction of the various polysaccharide fractions [hemicellulose-A, hemicellulose-B (1→4)-linked polysaccharide, and hemicellulose-B heteroglycan] which comprised the total polymer was studied The course of extraction of the hemicellulose-B fractions was similar to that for extraction of total polymer shown in Fig 1, although the ratio of iodine-precipitable to non-precipitable heteroglycan polysaccharide decreased in the latter stages of extraction at each temperature However, a striking feature was that the hemicellulose-A (xylose, 74 2, arabinose, 8 7, rhamnose, 1 6, galactose, 9 4, glucose, 5 8%) was almost totally extracted (95 5%) within the first 4 h at 0°, in contrast to the slower release of total polymer or the hemicellulose-B fractions As reported previously[3], the hemicellulose-A was, however, a minor component of the total hemicellulose, comprising only 12% of the polymer extracted over the first 4 h at 0° or only 4% of total polymer extracted by 10% KOH at 0° and 18–22°

Monosaccharide composition — The compositions of the total polysaccharide obtained at each stage of the extraction sequence (except for 4 and 10) are shown in Fig 4 They confirm the earlier results[3], which indicated a higher proportion of galactose and arabinose in the polysaccharide extracted at 18–22° compared with the 0°-polysaccharide The monosaccharide content of the polysaccharide extracted at 0° changes quite markedly from the earlier to the later extractions in the sequence During the first hour, when the bulk of 0°-polysaccharide is dissolved, xylose and glucose, in the ratio of 2 1, comprise nearly 60% of the polymer The proportion of xylose then falls until, in the last of the 0°-extracts, the polysaccharide consists largely of equal amounts of xylose, arabinose, glucose, and galactose The initial, high level of xylose reflects the rapid extraction of hemicellulose-A (74 2% of xylose) The increasing galactose and arabinose may be partly due to an increasing proportion of heteroglycan-B, which is typically rich in arabinose and galactose It could also be due to the presence of extensin fragments, especially during the latter part of the 0°-sequence where the increase in arabinose and galactose is paralleled by an increase in hydroxyproline Extensin has been shown to be covalently linked to arabinose[5] and galactose[6]

A small and decreasing amount of mannose and rhamnose is extracted at 0°, although little rhamnose and no mannose are found in the extracts at 18–22° The distribution of mannose is interesting in view of the usual insolubility of cell-wall mannose polymers in 10% alkali and their solubility in 24% alkali or alkali–borate[7].

The monosaccharide composition of the polysaccharide soluble at room temperature shows several changes with time There is a drop in the xylose level from

24 to 7%, a drop in galactose to about a third of its initial level by 20–24 h followed by a rise, and a threefold increase in arabinose content from the 12–13-h to the 20–24-h extraction followed by a decrease The increase in arabinose parallels the increase in hydroxyproline in the polymer soluble at room temperature This is in agreement with much of the arabinose extracted during the sequence at 18–22° being associated with hydroxyproline in extensin fragments In higher plants, most of the hydroxyproline appears to be glycosylated with (1→4)-linked-arabinose oligosaccharides[5,8] The present data indicate that at stage 9 of the extraction sequence (4–8 h at 18–22°), where the polymer is 6 2% of hydroxyproline and 64% of arabinose, the molar ratio of hydroxyproline to arabinose is 1 8 7 This suggests that about half of the arabinose extracted at this stage could be associated with extensin hydroxyproline

It is evident from the present work that considerable extraction of polymer from plant cell-walls low in lignin can be achieved without delignification, which in any case seriously degrades the wall[1] The extent to which the polymers extracted are free polysaccharide or protein–polysaccharide (extensin fragments) remains to be investigated, although the two-temperature separation used here could be achieving a separation of polysaccharide not bound to the wall network (extracted at 0°) and of polysaccharide–glycoprotein (extracted at 18–22°) that is linked to other insoluble constituents of the wall

Increasing evidence[9] points to linkage of the various amorphous and fibrous or crystalline components of the primary wall, as opposed to the older concepts of a mixture of the amorphous components (hemicellulose, lignin, protein, etc) merely encrusting the cellulose fibres In the present study, nearly all of the pectin has been removed without dissolving the hemicellulose or rendering the extensin extractable in 10% KOH at 0° This suggests that these polymers are linked to the alkali-insoluble portions of the wall other than through pectin An independent linkage of pectin and hemicellulose to the microfibrils is further suggested by the report[10] that hydroxyproline and hemicellulose can be removed from primary cell-walls with 10% KOH, while leaving the pectic polyuronide unextracted The present results suggest that the hemicellulose extracted at 0° is either not attached to other wall components and is insoluble in hot detergent, or is only attached by very alkali-labile links such as ester links The glycoprotein part of the wall complex requires alkali treatment at a higher temperature to release it, possibly by β-elimination of galactosylserine links[6] Presumably, this means that this polymer is more firmly bound to the alkali-insoluble wall component other than through the pectic material In one wall-model recently proposed[9] for sycamore-cell suspension cultures, a sequence of linkages was envisaged involving extensin covalently linked to pectin, which was itself covalently linked to a xyloglucan which, in turn, was hydrogen-bonded to the cellulose microfibrils The present results suggest that much of the hemicellulose and glycoprotein is not bound to the other wall constituents in this manner, at least in differentiated hypocotyl tissue

EXPERIMENTAL

Plant tissue — Lupin hypocotyls were grown, as previously described[11], until 5–6 cm in length

Cell-wall preparation. — Hypocotyls (300 g) were ground for 30 sec in distilled water (750 ml) in a Waring blender, filtered over sintered glass, and immediately extracted under reflux for a total of 4 h in two changes of 1 5 litres of a neutral detergent[12]. The neutral detergent removes cytoplasmic material, and non-covalently bound protein and pectins from the wall The walls were filtered off, washed twice by resuspending in hot distilled water and filtering, finally washed with ethanol and then acetone, and dried at 40° overnight This depectinated plant material was not examined microscopically but was concluded to be composed mainly of cell-wall material and is referred to as cell wall throughout the paper

Extractions. — Depectinated cell-wall (5 52 g) was subjected to a series of extractions by stirring with 200-ml volumes of aqueous KOH in a flask which had been thoroughly flushed with nitrogen The number of each extraction in the sequence, the time of filtering of each extraction (taken from the start of stage 1 in sequence), and the duration of each stage at the different temperatures and KOH concentrations are shown in Table I

For stages 1 to 5, the temperature was maintained at 0° The remaining extractions were carried out at laboratory temperature (18–22°) At the end of each extraction, the walls were filtered off quickly on a sintered-glass funnel (porosity 1) and then immediately submitted to the next extraction in the sequence All extracts were adjusted to pH 4.5 with acetic acid immediately after filtering, passed through a sintered-glass funnel (porosity 4), and dialysed for 24 h before freeze-drying

Residue weights after stages 5, 12, and 13 were obtained by neutralizing the walls with dilute acetic acid, washing with distilled water, ethanol, and acetone, and drying overnight at 40° before weighing

Polysaccharide fractionation — Separation of hemicellulose-A and hemicellulose-B was achieved by centrifugation of the acidified alkali-extracts at 70,000*g* for 1 h after standing at 0° for 16 h The precipitate (hemicellulose-A) was washed, resuspended in water, and freeze-dried The hemicellulose-B supernatant (or total polymer where hemicellulose-A was not first isolated) was dialysed and freeze-dried, prior to fractionation into linear (1→4)-linked polysaccharide and heteroglycan-B by precipitation of the (1→4)-linked species from solution in $CaCl_2$ (sp gr. 1 3) as described by Gaillard and Bailey[13].

Carbohydrate analyses — Freeze-dried extracts and derived polysaccharide fractions (20 mg) were placed in 50-ml boiling tubes, and 72% H_2SO_4 (0 7 ml) was added After 3 h, distilled water (20 ml) was added, and each tube was covered with a watch-glass and placed in a water bath at 100° for 3 h The hydrolysates were neutralized with $BaCO_3$, filtered, and freeze dried The monosaccharides in the hydrolysate were analysed by g l c of their alditol acetates

The alditol acetates were prepared by the method of Sloneker[14], and were

separated with a Varian Aerograph series 1400 gas chromatograph using a 2-m stainless-steel column (outside diameter, 0 125 inch) packed with 3% of ECNSS-M by weight on Gas-Chrom Q (100/120 mesh, Applied Science Laboratories Inc)

Hydroxyproline analyses — Wall residues and freeze-dried cell-wall extracts (20 mg) were hydrolysed under gentle reflux with 6M HCl (8 ml) for 18 h The hydrolysates were analysed for hydroxyproline by the method of Switzer and Summer[15]

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