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CELL WALL HYDROXYPROLINE-POLYSACCHARIDE ASSOCIATIONS IN *LUPINUS* HYPOCOTYLS

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Abstract—Extraction of lupin hypocotyl cell walls with guanidine thiocynate, both before and after dilute acid treatment, does not dissolve the hydroxyproline indicating that compounds containing this amino acid are probably covalently linked to insoluble wall constituents other than through acid labile arabinofuranose-hydroxyproline links. Dilute alkali does extract all of the wall hydroxyproline largely as non-dialysable material. Sequential extraction of cell walls with alkali at two temperatures (2° and 22–25°) removes most of the hemicellulose at the lower temperature but only dissolves the hydroxyproline at the higher temperature. Other studies show that the hydroxyproline containing polymer is co-precipitated with hemicellulose-B arabino-xylan. When cell walls from elongating and non-elongating hypocotyl sections are compared using this sequential extraction, the hemicellulose-B arabino-xylan containing hydroxyproline from the non-elongating wall has a much higher proportion of arabinose and galactose than the same polymer from the elongating wall. Much more of the hydroxyproline from the elongating wall is dialysable. These results indicate more bonding of the hydroxyproline-containing glycoprotein within the wall of non-elongating tissue consistent with its suggested role in stopping cell elongation. It is suggested that the glycoprotein is linked to insoluble wall constituents such as cellulose through galactose or by direct protein to cellulose links.

INTRODUCTION

STUDIES on the mechanical properties of elongating plant cell walls suggest that control of cell elongation depends on the cleavage and reformation of covalent links between the various wall polymers.[1–3] While several such links have been proposed none has ever been conclusively identified as playing a role in controlling extensibility. The ubiquitous occurrence of hydroxyproline in plant cell walls compared with its virtual absence in the cell cytoplasm,[4] coupled with its extensive *O*-glycosidic linkage to arabinose,[5] has led to the postulation of a hydroxyproline-rich glycoprotein called extensin as a controller of cell wall extensibility.[4] Some such activity by hydroxyproline is supported by the findings that a decrease in growth rate is often associated with an increase in the level of cell wall hydroxyproline.[6–8] Moreover hydroxyproline appears to be present in polymer which is covalently linked to the wall as it is not extracted by simple protein extractants and has generally only been released in small fragments after drastic enzymic[6] or chemical degradation.[5,9,10]

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In addition to hydroxyproline-arabinosides and other amino acids these fragments also contain much of the cell wall galactose.[11,12] Lamport,[4] has, therefore, proposed a cell wall model, relevant to the control of extensibility, in which protein is linked via hydroxyproline through arabinose to galactan and ultimately to the cellulose microfibrils. Acid labile glycosidic links are suggested between the arabinose units and some alkali labile link between the arabinose and galactose. However, although Cleland[12a] found that hydroxy proline recently incorporated into *Avena* coleoptile was alkali soluble, the hydroxy proline containing polymer is often at best only partially solubilized by alkali.[9,11]

While the proposed model fits much of the evidence referred to above and could represent part of a scheme for the control of cell wall extensibility, we find that it is not completely reconcilable with evidence obtained from lupin hypocotyl cell walls. In this plant tissue polymer hydroxyproline can be easily extracted from the cell walls with "dilute" alkali into non-dialysable molecules which are also associated with part of the conventional cell wall hemicellulose. The present paper considers this and other evidence in relation to possible primary cell wall protein-polysaccharide complexes and their role in controlling cell wall extensibility.

RESULTS AND DISCUSSION

Solubility of cell wall bound hydroxyproline in various neutral extractants

In cell wall preparations, hydroxyproline is measured as an index for the presence of some compound such as protein or glycoprotein. In extracts, non-dialysable hydroxyproline is likewise used as an index of these polymers extracted from the cell walls. Such compounds could be, *in vivo*, simply mixed with other cell wall constituents or attached to them by either hydrogen bonds or covalent links. In agreement with studies in other plant tissues, it seems that in lupin a simple mixture of the polymer with other cell wall polymers is unlikely since all the plant hydroxyproline remains with the cell wall preparations prepared by grinding the hypocotyls with buffer (0·5 M PO_4; pH 7·0) to remove cytoplasmic protein. Extraction of these cell walls with either boiling neutral detergent or boiling water removes less than 10% of the hydroxyproline. Rees[13] has suggested non-covalent bonding as the main type of linkage holding the polymers together in primary plant cell walls. Results summarized in Table 1 show that the hydroxyproline could not be extracted from cell wall preparations with 6 M guanidine thiocyanate (GTC) which is a powerful chaotropic agent[14] and should remove hydrogen bonded protein or glycoprotein. Treatment of the cell walls with boiling neutral detergent to remove pectic substances likewise did not permit any extraction of hydroxyproline by guanidine thiocyanate. Similar experiments showed that 90–95% of the wall hydroxyproline was not extracted by other neutral solvents such as 8 M urea or concentrated $CaCl_2$ which are able to disrupt hydrogen bonds.

Solubility of cell wall bound hydroxyproline in alkali

When neutral detergent-extracted cell walls were extracted by stirring at 35° successively for 24 hr with dilute and strong alkali (1 gm cell wall preparation/100 ml alkali), almost

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TABLE 1. EXTRACTION OF CELL WALL BOUND HYDROXYPROLINE WITH GUANIDINE THIOCYANATE (GTC)

Wall preparation	Hydroxyproline (mg/g of original wall preparation) Cell wall	Dialysed extract	% Unextracted from wall
(a) Initial buffer extracted cell walls	5·4	—	—
After extraction with 6 M GTC	4·68	0·49	86·6
(b) Initial detergent extracted cell walls	3·70	—	—
After extraction with 6 M GTC	3·43	0·12	92·7

all the hydroxyproline was dissolved as shown in Table 2. These results are in marked contrast to those from sycamore (*Acer pseudoplatanus*) suspension culture cell walls in which either partial solubility[9] of hydroxyproline in 10% alkali or complete insolubility[11] in strong alkali (17%) + borate (4%) has been reported. Either the tissue used, i.e. hypocotyl compared with suspension cultured cells, or the different species may explain this difference in alkali solubility.

TABLE 2. EXTRACTION OF CELL WALL BOUND HYDROXYPROLINE WITH ALKALI

Cell wall fraction or extract*	Hydroxyproline (mg/g of original cell wall preparation)	% of initial cell wall hydroxyproline
Original cell wall preparation	2·14	100
10% KOH extract	1·80	84·1
24% KOH extract	0·24	11·2
Residue	0·10	4·7

* Cell walls extracted sequentially with 10 and 24% KOH by stirring overnight at 35°.

On acidification of the 10% alkaline extract, only a slight hemicellulose-A precipitate was obtained. The acidified solution after dialysis yielded on freeze-drying a hemicellulose-B fraction containing 70% of the extracted hydroxyproline. Further fractionation also involving dialysis showed that most of this hydroxyproline remained non-dialysable. Much of the hydroxyproline-rich glycoprotein has, therefore, been extracted as non-dialysable material and not in small dialysable fragments.

It has been shown that the large loss of cell wall arabinose and galactose[10] during delignification of lupin hypocotyl cell walls was due partly to the acid conditions (pH 2·0–3·9) used in the process because of the cleavage of acid labile furanoside glycosidic links. If, as postulated,[4] the peptides containing hydroxyproline are linked to the cell walls through such acid labile arabino-furanoside links then this mild acid treatment might be expected to render the protein portion extractable with GTC. However, results (Table 3) showed that mild acid treatment (acetic acid, pH 2·9, 5 hr at 100°) did not solubilize the hydroxyproline or render it extractable with GTC. Treatment of the cell walls with N acid at 100° for 1 hr on the other hand completely removed the hydroxyproline along with the hemicellulose sugars.

When dilute acid treated walls were extracted with dilute alkali to dissolve the hydroxyproline polymer (Table 3) the results showed that this polymer was still non-dialysable in spite of any cleavage of arabino furanoside links.

Table 3. Effect of dilute acid treatment on extraction of cell wall hydroxyproline

Acid treatment	Extraction	Hydroxyproline (mg/g original buffer extracted cell walls) Cell wall residue	Extract	Per cent of extracted hydroxyproline non-dialysable
Batch A detergent extracted walls as in Table 1				
Cell walls		3·7		—
Refluxed 0·1 N acetic (pH 2·9 5 hr)		3·55 (96*)	0·01	—
	GTC extracted	3·73 (100)	0·01	—
Batch B detergent extracted walls				
Refluxed in oxalic acid (pH 2·0 5 hr)		4·19		
	(1) 10% KOH 1 hr followed by			
	10% KOH 5 hr	1·23 (29)	1·76	58·9
	(2) 10% KOH 9 hr	0·93 (22)	4·34	86·2

* Values in parentheses are % of hydroxyproline remaining in cell walls after each extraction.
† Calculated by difference.

The results with the acid treated walls (pH 2–5 hr) indicate linkage of the hydroxyproline glycoprotein to the insoluble part of the cell wall complex through sugars other than those involved in acid labile links as suggested in the hydroxyproline-arabinose sequence.

Rates of extraction of cell wall polymers with alkali

Extraction of the cell walls at room temperature (18–20°) for 18–24 hr with alkali gave a similar almost complete extraction of the hydroxyproline but there was still some (20–30%) loss of hydroxyproline as dialysable material. As this loss could have been due to alkaline degradation of the polymer extractions at lower temperatures (2°) and for shorter times (1–2 hr) were investigated but only small amounts of the hydroxyproline were extracted. Extraction with 10% alkali, usually overnight at room temperature after delignification, is also used to dissolve the bulk of the hemicellulose polysaccharides (principally xylans) from plant cell walls. The alkali extractions at various temperatures and for short and long times were therefore further investigated in terms of both hydroxyproline and the hemicellulose polysaccharides. From the results of two alkali extraction sequences (Table 4), it is evident that the bulk of the hemicellulose xylan is much more readily removed by the alkali than the glycoprotein. These results suggest that the glycoprotein is linked to the alkali insoluble portion of the cell walls by links which are slowly broken by alkali and is either not linked to the bulk of the hemicellulose or only bound to it by very alkali-labile links.

Relationship of Hydroxyproline Glycoprotein to Hemicellulose Polysaccharides

The non-cellulosic matrix polysaccharides extracted from plant cell walls with alkali are commonly separated into hemicellulose A (xylans precipitated by acidification of the alka-

line extract) and hemicellulose-B (which remains in the acid solution). Hemicellulose-B may be further fractionated[15] by dissolving it in concentrated $CaCl_2$ solution and adding iodine solution when hemicellulose-B arabino-xylan is precipitated; the polymer remaining in solution (heteroglycan-B) is rich in arabinose and galactose. Total hemicellulose from several of the alkaline extracts from sequences A and B (Table 4) were separated into

TABLE 4. EFFECT OF TEMPERATURE AND TIME ON EXTRACTION OF CELL WALL POLYMERS BY 10% ALKALI

	Extractions in sequence	Time (hr)	Temp. (°C)	Hydroxyproline (mg extracted/g NDR cell walls)	Hemicellulose
Detergent extracted cell wall batch A					
	1	4	2	0·172	188
	2	19	2	0·675	58
	3	13	18–20	2·55	83
Detergent extracted cell wall batch B					
	1	4	18–20	1·80	188
	2	16	18–20	3·13	89

these fractions which were analysed for hydroxyproline and monosaccharides after hydrolysis. Results from hemicellulose-B arabino-xylan and heteroglycan-B are given in Table 5; the small amounts of hemicellulose-A were discarded. Although the sequential extraction studies (Table 4) showed that most of the hydroxyproline was extracted after the removal of the bulk of the hemicellulose, it is evident that when the glycoprotein is extracted it is largely associated with the hemicellulose-B arabino-xylan-B fraction. This

TABLE 5. POLY- AND MONO-SACCHARIDE COMPOSITION OF ALKALI EXTRACTS FROM LUPIN HYPOCOTYL CELL WALLS

Sequence	Total hemicellulose	Hemicellulose-B Arabino-xylan-B	Hemicellulose-B Hetero-glycan-B	Monosaccharide composition of arabino-xylan-B (Xyl:Ara:Gluc:Gal)
	(mg/g of initial NDR cell walls)			
A 1*	188	135 (0·162)†	7·3 (0·013)†	1:0·34:0·39:0·47
3	83	46·5 (2·26)	8·6 (0·29)	1:0·51:0·95:2·38
B 1*	188	155 (1·60)	10·3 (0·12)	1:0·46:0·29:0·52
2	89	46·5 (1·7)	9·2 (0·14)	1:2·64:0·51:1·17

* Extracts from sequence A and B of Table 4.

† Figures in parentheses hydroxyproline content of hemicellulose fractions in mg.

association is further emphasized by the monosaccharide compositions of the arabino-xylan fractions with the hydroxyproline rich fraction higher in arabinose and galactose than the more rapidly extracted polymer. The much smaller amounts of heteroglycan-B contain about the same proportion of hydroxyproline to polysaccharide as the hemicellulose-B-arabino-xylan. The amount of hydroxyproline associated with this heteroglycan-B fraction

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increases when the extraction is done at a higher temperature; e.g. 35°. It is possible, therefore, that this heteroglycan-B fraction represents alkali degraded glycoprotein whose yield is increased by extraction at higher temperatures.

TABLE 6. COMPOSITION OF POLYSACCHARIDE FRACTIONS EXTRACTED FROM DELIGNIFIED AND NON-DELIGNIFIED LUPIN HYPOCOTYL CELL WALLS*

Polysaccharide fraction	Yield (mg/g cell wall)	Hydroxyproline content (%)	Monosacchardie composition (Xyl:Ara:Gluc:Gal)
Hemicellulose-A			
Delignified	20	0	1:0·04:0·18:0·04
Non-delignified	32	0·6	1:0·28:0·27:0·24
Hemicellulose-B arabino-xylan-B			
Delignified	69	0	1:0·12:0·49:0·29
Non-delignified	158	3·78	1:0·84:0·40:0·77
Hemicellulose-B heteroglycan-B			
Delignified	7	0	1:0·85:0·62:0·64
Non-delignified	17	2·15	1:18·8:0·84:6·26

* Cell walls extracted overnight at room temperature with 10% KOH.

Delignification has been shown to remove large amounts of lupin hypocotyl cell wall arabinose and galactose.[10] Lupin hypocotyl cell walls with and without this delignification were extracted with alkali and the extracts separated into the three hemicellulose fractions. Analyses of the fractions for hydroxyproline and monosaccharides (Table 6) clearly show the effect of delignification on the composition of the polymers. The complete removal of the hydroxyproline from the walls during delignification presumably results from the action of chlorine on the protein.

While it might not normally be considered necessary to delignify primary walls low in lignin before extraction of hemicellulose, the results of Tables 5 and 6 taken together do suggest that polysaccharide fractionations of plant tissues after delignification may be only of limited meaning in terms of the *in vivo* matrix polymers.

Polymer composition in relation to cell wall extension in lupin hypocotyls

Although previous studies[10] had shown that 70% of the potential elongation of a 6 cm lupin hypocotyl was in its upper 2 cm, no apparent differences in levels of hemicellulose or cellulose between the elongating (top 2 cm) and non-elongating (bottom 2 cm) regions were observed. A detailed fractionation using delignified cell walls showed that the conventional hemicellulose fractions from both regions had the same monosaccharide content. Analysis of non-delignified cell wall acid hydrolysates did, however, suggest more cell wall arabinose in the lower (least extensible) regions of the hypocotyl.[10]

Results from an analysis of the fractions extracted with alkali from non-delignified cell walls of top 2 cm and bottom 2 cm sections of 6 cm lupin hypocotyl are presented in Table 7. They are in agreement with a greater association of the glycoprotein with the other wall constituents in the lower section of the hypocotyl in accordance with the concept of the control of extensibility by this compound. Thus there is more hydroxyproline in the walls of this non-elongating region and higher hydroxyproline levels in its polysaccharide frac-

tions. A further indication of some closer or tighter linkage of the glycoprotein in wall polymeric material in the non-elongating lower region, compared with the elongating upper region, is indicated by dialysis losses of hydroxyproline. Both the fractionation to hemicellulose-A and -B and the subsequent fractionation of the hemicellulose-B involve dialysis of the fractions. The extent of loss of hydroxyproline during these dialyses may, therefore, be considered as an index of levels of low molecular weight material, containing this amino acid, released by the alkali. This dialysis loss is very much higher for the upper hypocotyl sections suggesting either the presence in the wall of lower molecular weight glycopeptide fragments not yet extensively linked to wall galactan, or greater susceptibility to alkaline degradation as a result of this lower linkage. In this connection it has been shown[16] that carrot cell walls contain an acid soluble precursor of cell wall glycoprotein containing arabinose but not galactose.

TABLE 7. EXTRACTION OF CELL WALL COMPONENTS FROM ELONGATING AND NON-ELONGATING SECTIONS OF LUPIN HYPOCOTYL

Component* in cell walls	Top 2 cm (elongating)	Bottom 2 cm (non-elongating)
Hydroxyproline†	3·21	5·34
Hemicellulose-A†	18·5 (0·12)‡	32·5 (0·48)‡
Hemicellulose-B arabino-xylan-B†	147 (0·41)	138 (1·60)
Hemicellulose-B heteroglycan-B†	26 (1·18)	52 (3·07)
Extracted hydroxyproline lost on dialysis (%)	69·6	26·4
Monosaccharide composition of hemicellulose-B arabinoxylan-B (Xyl:Ara:Gluc:Gal)	1:0·41:0·76:0·61	1:0·89:0·84:0·66

* Cell walls extracted with 10% KOH at room temperature overnight.
† mg/g of neutral detergent extracted cell walls.
‡ Values in parentheses hydroxyproline as % of polysaccharide fraction.

The polymers described in Table 7 are, however, a combination of the two fractions extracted sequentially at 2° and 20° in Table 4. The cell walls from a further batch of top and bottom hypocotyl sections were extracted sequentially with alkali at 2° and 22°. In this experiment the 22° extract yielded, after centrifuging the $CaCl_2$ solution at 70000 *g* for 90 min, an iodine precipitated hemicellulose-B-arabino-xylan of more markedly different monosaccharide composition with xylose:arabinose:glucose:galactose ratios of top, 1:0·6:0·4:0·6 and bottom, 1:1·9:0·4:1·07 together with four times as much hydroxyproline (1·9%) in the bottom fraction. In these 22° extracts a greater proportion of the total hemicellulose-B fraction appeared to be extracted from the bottom (50%) compared with the top (38%) cell walls.

Application of the alkali extraction procedure to cell walls from the upper and lower sections of lupin hypocotyls does, therefore, provide evidence for a polysaccharide composition difference between elongating and non-elongating regions. It seems significant that this difference is in a fraction with which most of the extracted hydroxyproline is associated. Whether this association involves direct linkage remains to be seen.

Since this work was completed a model of the primary cell wall of *Acer pseudoplatanus* suspension culture cells has been proposed.[17] In this case glycoprotein linkage to insoluble

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cell wall polysaccharide is postulated to be through serine-galactose units to a glucosyl-xylan associated with the cellulose. It should be noted that *A. pseudoplatanus* cell walls appear to differ from lupin hypocotyl cell walls in that their hydroxyproline containing polymer is much less readily extracted with alkali.[9,11]

Present results confirm and extend the suggestion that a glycoprotein containing hydroxyproline is involved in the control of cell wall extensibility and that the compound is covalently linked to other wall polysaccharides. Linkage of the glycoprotein also appears to be to the alkali insoluble portion of the cell wall (e.g. cellulose) rather than to the bulk of the non-cellulosic wall polymers (xylans) as these latter can be preferentially removed by alkali extraction at low temperature. The present results support the suggestion that such linkage is not through the acid labile arabinofuranose units joined to the hydroxyproline[4] but rather through other sugar molecules. In this latter connection also it seems likely that galactosylserine links are not the sole ones joining the amino acids to the cell walls as those links should be broken by β-elimination by dilute alkali at 0°[18] to permit extraction of the hydroxyproline. In conclusion, we believe that the role of a hydroxyproline glycoprotein in controlling extensibility involves the formation of more than one kind of link with the cell wall polysaccharides and may even involve amino acid to cellulose links.

EXPERIMENTAL

Plant tissue. Lupin hypocotyls, 6 cm (entire unless otherwise stated), were grown and prepared as described.[10]

Cell walls. Total cell walls were prepared by griniding the hypocotyls in iced water, filtering and extracting the residue, either for 2 hr with boiling neutral detergent[19] or by further grinding with buffer (0·5 M phosphate, pH 7·0) for a few min at room temp.

Extractions. Cell walls were extracted with 6 M urea or guanidine thiocyanate (GTC) by stirring with a magnetic stirrer in a closed flask overnight at room temperature using 0·5 g cell walls to 100 ml solution. Alkali extractions involved similar extraction of the tissue, 1 g per 100 ml alkali, in alkali in a sealed flask under N_2 for a specified time, or overnight (20 hr) at room temp. (18–22°), or at the specified temperature. Dilute acid treatments involved refluxing for 5 hr in the specified acid.

Analyses. Details of the polysaccharide fractionations, monosaccharide analyses of the polysaccharides and of the delignification are given in the previous paper.[10] All polysaccharide fractions were dialysed for 24 hr at 0° against distilled water and freeze dried. Hydroxyproline was measured by the method of Switzer and Summer[20] after hydrolysis for 16 hr in 6 N HCl.

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