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POLYSACCHARIDE AND OLIGOSACCHARIDE CHANGES IN GERMINATING LUPIN COTYLEDONS

N. K. MATHESON and HARPAL S. SAINI

Department of Agricultural Chemistry, University of Sydney, N.S.W. 2006 Australia

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Key Word Index—*Lupinus luteus*; Leguminosae; lupins; cotyledons; cell wall growth; arabinogalactan; pectic substances; polyuronide; raffinose; galactosylsucrose.

Abstract—In germinating lupin cotyledons, there was a rapid depletion of raffinose series oligosaccharides, a temporary increase in sucrose and constant low levels of reducing monosaccharides. The major polysaccharide fraction was extracted with hot NH_4 oxalate–EDTA solution and had the constitution of intercellular/cell wall polysaccharide. GLC examination of component sugars showed that as cotyledons expanded this fraction was depleted and that there was selective hydrolysis of arabinose and galactose, so that the uronic acid proportion increased. Gel and DEAE-cellulose chromatography showed that this fraction became more heterogeneous. The neutral and acidic fractions were separated and the component sugars, viscosities, gel chromatographic behaviour and sedimentation constants of these determined. The results indicated that in the later phase of plant cell wall expansion in germinating lupin cotyledons the arabinogalactan side chains of the pectic polysaccharide fraction are selectively hydrolysed leaving a primary wall with a high uronic acid content.

INTRODUCTION

Legume seeds contain galactosylsucrose oligosaccharides, galactomannan and starch as their carbohydrate reserves [1]. If present, galactomannan is found in the endosperm, starch in the cotyledons and the raffinose series oligosaccharides in both parts. Other neutral polysaccharides can be extracted from ungerminated legume seeds, e.g. a galactan has been prepared from white lupin seeds [2] and an arabinogalactan has been isolated from soybeans [3]. These polysaccharides are intercellular and primary cell wall material from the cotyledons. A number of neutral polysaccharides can be extracted from primary walls. Arabinan [4,5] galactan and arabinogalactans of two structural types (I and II) [6] that can have different arabinose:galactose ratios within each type have been found. In some cases the extraction of the polysaccharide may have involved the splitting of covalent bonds [6,7].

The cell wall fractions containing uronic acid vary widely, according to plant source, in the proportion of uronic acid to neutral sugars [6]. The latter are mainly arabinose, galactose and rhamnose with smaller amounts of other sugars. Structural aspects of a number of wall polysaccharides, including those from ungerminated soybean cotyledons have been described [8]. Acidic polysaccharides have been separated by several techniques into a number of fractions varying in uronic acid content [6]. The total pectic substances differ considerably between tissues, e.g. the rinds of citrus fruits contain about 30%, but woody tissues have only small amounts. Variations in the composition of pectic materials may reflect differences in the amounts of neutral sugars covalently bound in the acidic polysaccharide structure, different proportions of neutral to acidic polysaccharide in the extract or different extraction procedures, as pectic substances are readily depolymerized even near neutral pH values [7,9,10].

A number of theories have been proposed for the molecular changes involved in plant cell wall formation, cell division and expansion. Northcote [11] has proposed that increasing plasticity in primary wall development on cell differentiation may be regulated by branching of an acidic polysaccharide chain. Rees [5,12] has suggested that modification of the polysaccharides changes their capacity for entanglement thus altering plasticity. Lamport [13] has proposed that a labile glycosidic linkage between an hydroxyproline containing protein and arabinofuranoside groups is involved. Galactose serine linkages are also present [14]. Albersheim has suggested [15,16] that the making and breaking of hydrogen bonds between xyloglucan and cellulose microfibrils allows the wall to creep.

A preparation of a β1-4 galactan has been described from ungerminated lupin seeds (*Lupinus albus*) [2] but the method of isolation may have caused the removal of readily hydrolysable α-L-arabinofuransoyl side chains and the experimental data indicates this. This polysaccharide is probably a type I arabinogalactan [6].

Germinating lupin cotyledons are epigeal, remain viable for a considerable time and provide an organ in which cells are undergoing only expansion, allowing a

* All correspondence should be addressed to: Associate Professor N. K. Matheson, Department of Agricultural Chemistry, University of Sydney, N.S.W. 2006, Australia.

study of the polysaccharide changes accompanying this later stage of growth. In this paper the changes in carbohydrates, that occur after imbibition in the light and the dark, including the sucrose, raffinose series reserve oligosaccharides and the neutral and acidic polysaccharides from the cell wall, including intercellular material, have been followed and these related to cell expansion mechanisms.

RESULTS AND DISCUSSION

The seedlings were grown at constant temperature in perlite both in the light and the dark and the changes in wet and dry weights, leaf areas and chlorophyll contents are shown in Figs 1 and 2. Microscopic examination of

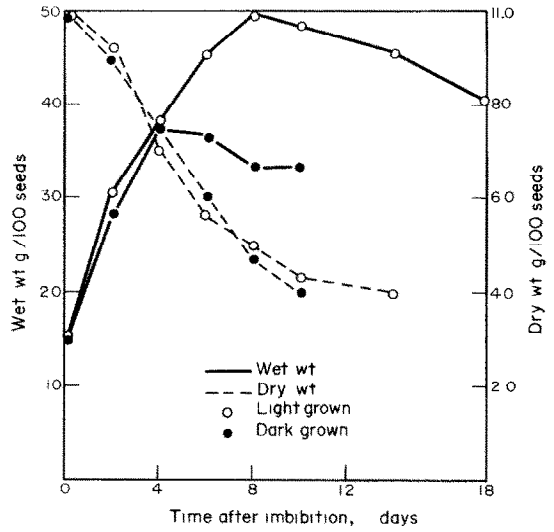

Fig. 1. Wet and dry wt. changes in germinating lupin cotyledons in the dark and light. Wet wt ——; dry wt – – –; light grown ○; dark grown ●.

sections showed that cells increased in size during the period studied.

The mono, di- and oligosaccharide fractions were extracted from the cotyledons and separated by gel filtration and the changes in these that accompanied germination in the dark are shown in Fig. 3. PC and TLC examination of the three fractions showed that the oligosaccharide fraction was composed of raffinose series oligosaccharides and that the disaccharide fraction was all sucrose, except for a trace of melibiose in the ungerminated seed, that rapidly disappeared after germination. The reducing monosaccharides were glucose, fructose and galactose with small amounts of arabinose and xylose at later stages of germination. *myo*-Inositol was also present but is not included in the assay of this fraction. Prior to germination lupin seeds lack any starch in the cotyledons and there are possibly trace amounts of galactomannan in the seed. The amount of raffinose series oligosaccharides is high and approximates the combined galactomannan plus galactosylsucrose oligosaccharide content of seeds like lucerne, guar and carob and the starch plus galactosylsucrose oligosaccharide content of seeds like peas. Germination was characterized by a rapid depletion of the raffinose oligosaccharides associated with an initial increase in sucrose which was then more slowly depleted.

Further extraction of the seeds with cold water, containing Hg^{2+}, and then hot water, containing NH_4^+ oxalate and Na_2 EDTA and brought to pH 5, produced fractions, that from their component monosaccharides

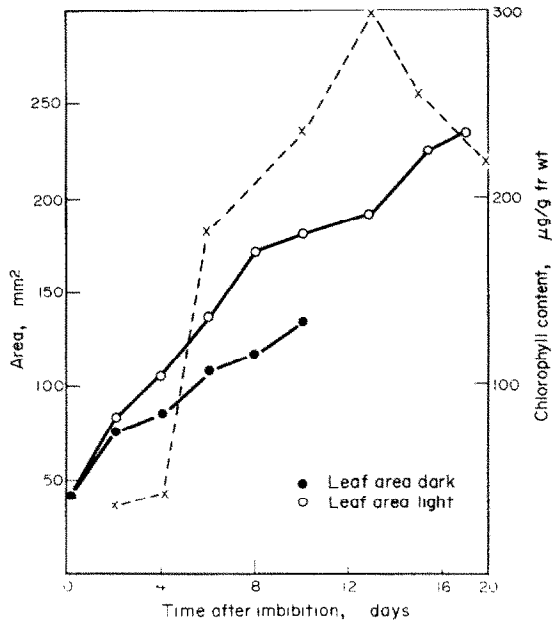

Fig. 2. Changes in cotyledon area and chlorophyll content of germinating lupins. Leaf area (in dark) ●; in light ○; chlorophyll content X.

and solubility correspond to intercellular and primary cell wall polysaccharides. Seeds were germinated in the dark and at 2 day intervals up to 10 days, cotyledons were extracted to give four fractions, cold water soluble, hot water soluble, alkali soluble and a residue. In the light, cold and hot water extractions were made. The conditions

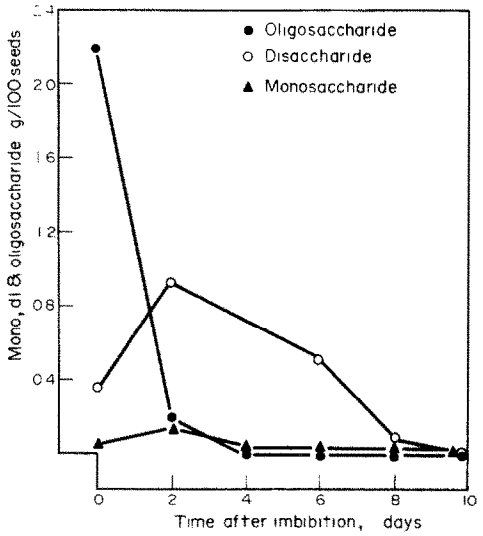

Fig. 3. Changes in the levels of mono, di, and oligosacchardies in cotyledons during germination in the dark.

Table 1. Amounts, and protein contents of extracts of lupin cotyledons during germination in the dark

Time after imbibition (days)	Fraction*	Wt (g/100 seeds)	Protein content (% dry wt)
0	CW	0.38	50
	HW	2.76	45
	AS	2.88	30
	Res	0.56	14
	Total	6.58	
2	CW	0.23	57
	HW	2.41	45
	AS	2.16	35
	Res	0.56	16
	Total	5.36	
4	CW	0.12	42
	HW	1.96	52
	AS	1.90	34
	Res	0.60	10
	Total	4.58	
6	CW	0.05	n.d.†
	HW	1.08	29
	AS	1.47	45
	Res	0.66	8
	Total	3.26	
8	CW	0.03	n.d.
	HW	0.76	32
	AS	1.27	25
	Res	0.61	13
	Total	2.67	
10	CW	0.01	n.d.
	HW	0.56	14
	AS	1.09	9
	Res	0.56	9
	Total	2.22	

* CW, cold water; HW, hot water; AS, alkali soluble; Res, residue.

† Not determined.

of extraction were standardized so that even if degradation products were produced fractions could still be compared. The amounts of each fraction and their protein contents are shown in Table 1 and are means from duplicate extractions. The values for the cold water and hot water extracts of light grown cotyledons are shown in Table 2. The cold water and alkali extracts also contained considerable amounts of ash.

Table 2. Amounts of extracts and protein contents of lupin cotyledons during germination in the light.
CW cold water; HW hot water

Time after imbibition (days)	Fraction	Wt (mg/100 seeds)	Protein content (% dry wt)
0	CW	0.35	54
	HW	2.76	45
2	CW	0.34	41
	HW	2.58	41
4	CW	0.19	37
	HW	1.68	48
6	CW	0.16	31
	HW	1.10	43
8	CW	0.20	n.d.
	HW	0.80	36
10	CW	0.14	n.d.
	HW	0.55	33

The extracts were hydrolysed and, after reduction and acetylation, the neutral component sugars were estimated by GLC. Uronic acid content was estimated by decarboxylation. The changes in the amounts of these constituent monosaccharides during germination are shown in Figs 4–6. At 0 days the largest polysaccharide fraction was the hot water soluble (1200 mg/100 seeds). The average dry wt of 100 seeds prior to germination was 11 g. The alkali soluble fraction was also of significant size (400 mg), but the residue was much smaller and

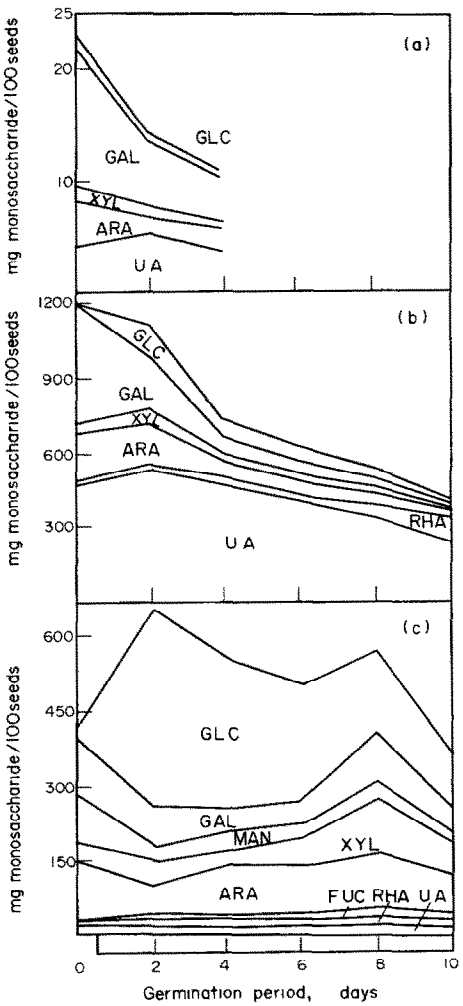

Fig. 4. Changes in constituent monosacchardies of cold water (a), hot water (b) and alkali (c) extracts of germinating lupin cotyledons in the dark.

the amount of cold water extract very low (24 mg). This was isolated by precipitation of the aqueous extract into 3 volumes of ethanol. Addition of a further 3 volumes to the supernatant after isolation of this fraction gave material, which contained only a small quantity of carbohydrate, that after hydrolysis, showed only very

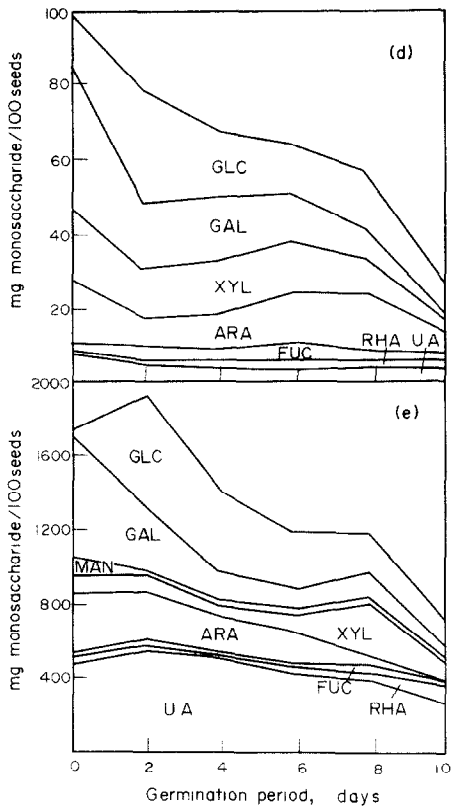

Fig. 5. Changes in constituent monosaccharides of residue (d) and total fractions (e) of extracts of germinating lupin cotyledons in the dark.

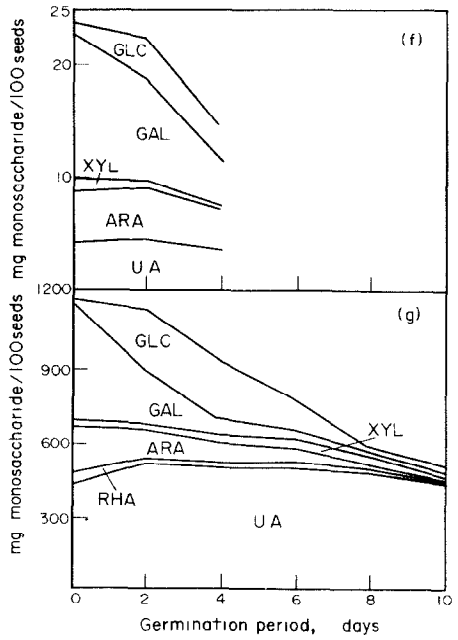

Fig. 6. Changes in constituent monosaccharides of cold water (f) and hot water (g) extracts of germinating lupin cotyledons in the light.

faint traces of arabinose on GLC, indicating that arabinan was not present. For hydrolysis of the cold, hot water and alkali extracts, M H_2SO_4 at 100° for 6 hr was used. Anthrone estimation indicated that about 80% recovery after GLC was obtained. The residual fraction was hydrolysed by initial dispersion in 72% H_2SO_4 for 1 hr at room temperature, followed by dilution to molar concentration and the heating for 6 hr at 100°.

The hot water extract at 0 days had a high neutral sugar to uronic acid ratio for a cell wall polysaccharide (uronic acid 39%). The cold water extract showed similarities to the hot water but contained a higher proportion of galactose and lower uronic acid. Considerable variation has been found in the ratio of neutral sugar to uronic acid in extracted pectin fractions [6], e.g. values of 87% uronic acid and 12% neutral sugar have been reported for apple pectin, but the polysaccharide from ungerminated soybean contains only 24% uronic acid. In the dark, as germination proceeded, a major change found in the hot water extract was a continuous reduction in the neutral sugar content combined with a smaller change in uronic content leaving a polysaccharide that at 8 days in the dark contained 69% uronic acid. Light grown cotyledons also gave a reduction in neutral sugar with limited uronic acid loss, but the increase in uronic acid proportion (to 82%) was more marked as the uronic acid change was less. Thus the cell expansion associated with lupin cotyledon growth is accompanied by a reduction in the amount of cell wall polysaccharide, but the loss in carbohydrate mainly involves the neutral sugars. A comparison of the pectin from cured and uncured tobacco leaves has shown that the former contained 20% and the latter 90% uronic acid [17]. Soluble polyuronide levels have been found to change in ripening apple fruits and the galactose levels to be reduced [18].

The initial increase in glucose in the hot water extract and at least some of the increase in the alkali extract are due to a temporary polysaccharide that gave a similar colour with iodine as starch does. The mannose may be due to small amounts of water insoluble galactomannan or galactoglucomannan [1]. The alkali extracts and the residues contained only small amounts of uronic acid, indicating that hot water removed the major portion of the uronic acid containing cell-wall polysaccharides. However, these fractions still contained some galactose, arabinose, fucose and rhamnose, monosaccharides that are found in cell wall polysaccharides. The alkali soluble extracts did not show a loss in neutral monosaccharides as seeds germinated but showed a slight increase at 6–8 days. This may have been due to changing solubilities of the polysaccharides at this time leading to a decrease in the residue. The alkali soluble + residual fractions remained relatively constant apart from a temporary increase in glucose. At 0, 8 and 10 days these combined fraction weights were 520, 630 and 420 mg per 100 seeds.

The hot water extracts were then chromatographed on DEAE-cellulose using an NaCl gradient and the elution patterns at selected times are shown for dark grown (Fig. 7) and light grown seedlings (Fig. 8). In all cases there was an unbound and bound fraction as is usually found with cell wall polysaccharides [6, 7, 11]. From ungerminated seeds, the optical rotation of the unbound fraction, after protein removal with pronase, was +22 (*c*, 0.72, H_2O) and for the bound fraction +23 (*c*, 0.65, H_2O). The bound fractions were heterogeneous and the

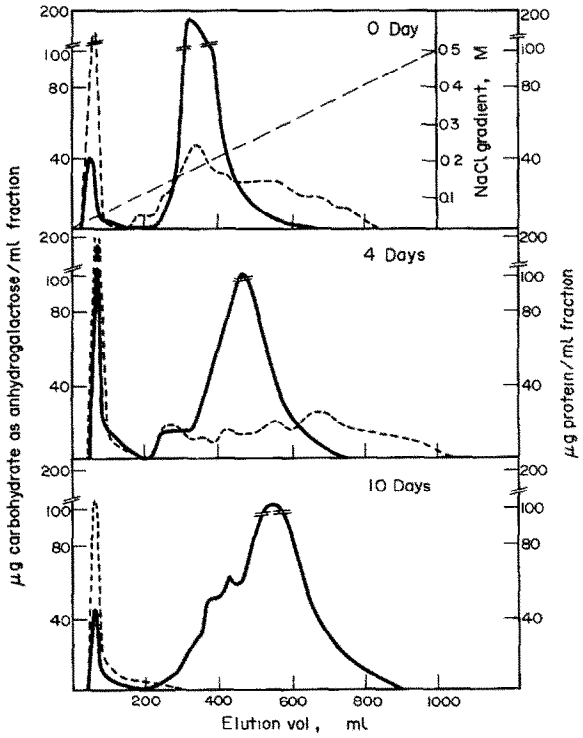

Fig. 7. DEAE cellulose chromatography of hot water soluble polysaccharide extracts at various times of germination in the dark. Carbohydrate ———; protein – – –.

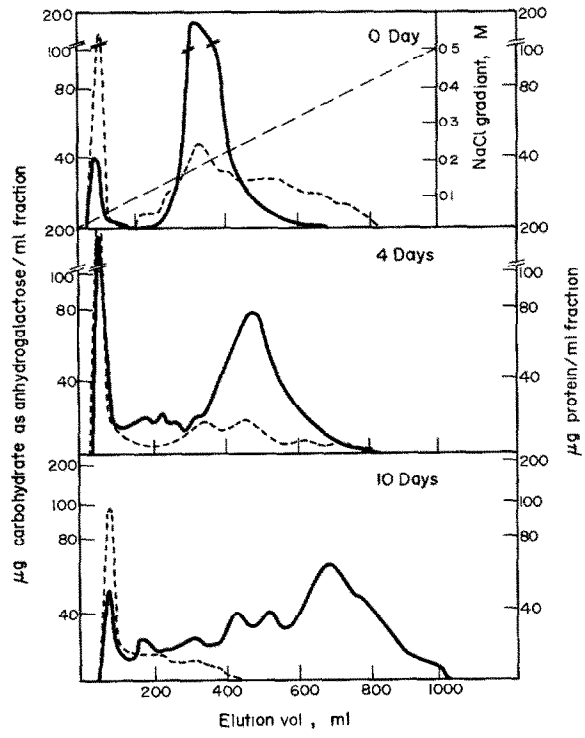

Fig. 8. DEAE cellulose chromatography of hot water soluble extracts at various times of germination in the light. Carbohydrate ———; protein – – –.

elution volume of the major peak increased with time, indicating increasing acidity. The hot water extracts from the light-grown plants differed in that the bound fraction became even more heterogeneous. All fractions were associated with protein but by 10 days only a small part of the protein was absorbed. The changes in the comparative amounts of the two fractions are shown in Fig. 9. These were measured by anthrone. The unbound

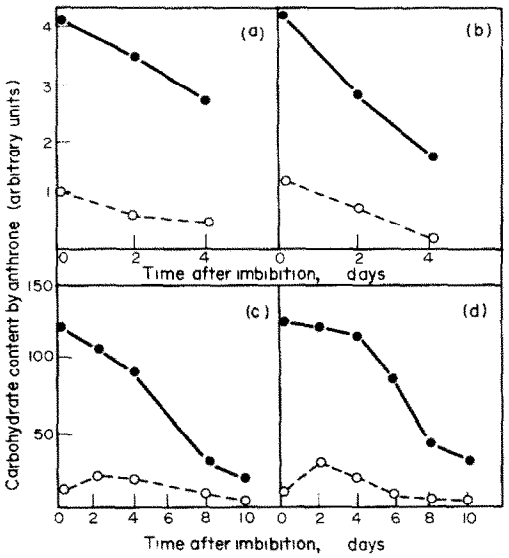

Fig. 9. Changes in the amounts of bound (———) and (unbound) (– – –) fractions from DEAE cellulose chromatography of extracts of lupin cotyledons. (a) cold water (light); (b) cold water (dark); (c) hot water (light); (d) hot water (dark).

was always much less than the bound and both decreased The hot water soluble unbound fraction increased from 0 to 2 days, suggesting that, unless new polysaccharide synthesis occurred, it derived from the acidic fraction present at 0 days. Conclusions about the hot water soluble fraction must consider the time of heating for extraction but the isolation of an unbound fraction in the cold water extract, where very gentle conditions were used, would support the possibility that at least part of the hot water unbound extract is not an extraction artifact. The amounts of unbound fraction decreased a little more rapidly in the dark than in the light.

When the cold water extracts were chromatographed on Sepharose 2B (Fig. 10a) the samples were shown to be heterogeneous and the average MW decreased as germination proceeded. The hot water extracts were incubated with salivary α-amylase and dialysed to remove starch. The α-amylase preparation contained no β-galactosidase, as shown by the lack of hydrolysis of nitrophenyl β-galactoside on long incubation. The hot water extract at 0 days had a higher MW than the cold water extract (Fig. 10b) and the MW distribution was skew. As germination proceeded, the MW decreased and a more retarded fraction appeared. Similar behaviour was shown by the extracts from both dark and light grown plants.

The hot water soluble fractions were separated by chromatography on DEAE-cellulose on a preparative scale into bound and unbound fractions. These were

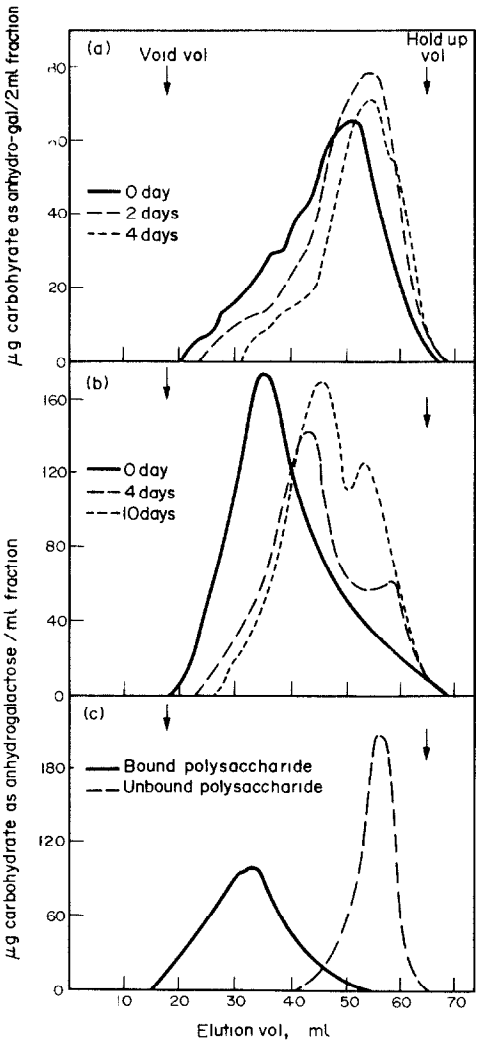

Fig. 10. Gel chromatography on Sepharose 2B of polysaccharides from lupin cotyledons. (a) cold water (dark); (b) hot water (dark); (c) hot water (dark) at 2 days after DEAE cellulose chromatography.

then treated with α-amylase and pronase and dialysed, which removed all the starch and most of the protein. Gel chromatography before and after treatment with pronase gave no significant difference in the elution pattern. Gel chromatography on Sepharose 2B showed that the bound fractions eluted ahead of the unbound fractions indicating a higher MW (Fig. 10c). Both fractions gave skew patterns but there was little overlap in MW. The elution volumes of the peaks of the unbound fractions increased as the cotyledons grew. On Sepharose 4B, at 0 days it was 57 ml (total volume of column 72 ml) and at 2 days in the dark it was at the hold up volume with some of the fraction not fully retarded. At 4 days the whole fraction was fully retarded. On Sepharose 2B the bound fractions showed a small increase in the elution volumes but the pattern became progressively more skew with a larger amount of low MW, more retarded material.

The viscosities of the fractions from DEAE-cellulose chromatography were determined (Table 3) and, after hydrolysis, reduction and acetylation, the monosaccharide ratios estimated using GLC (Table 3). The viscosities of the unbound fractions, which were similar to the bound before germination, rapidly dropped to very low values. In the bound fractions, on the other hand, the viscosities increased and then decreased, until at 10 days they returned to near the 0 days value. The unbound fractions contained insignificant amounts of uronic acid indicating that their neutrality was not due to carboxyl esterification. Their ratio of arabinose to galactose increased considerably. The bound fractions contained uronic acid which increased at later times. The uronic acid contents were estimated colorimetrically [19] and this method gave consistently higher values than those found by decarboxylation. An increase in the ratio of arabinose to galactose was also found up to 6 days.

Ultra-centrifugation (Table 4) showed that the unbound fractions consisted of slowly sedimenting material of a wide MW range as indicated by the rapid formation of a broad sedimentation pattern. The sedimentation constants decreased with time of germination. The bound fractions consisted mainly of a faster moving peak showing hyperfine sharpening and a much smaller amount of a

Table 3. Limiting viscosity and monosaccharide ratios of fractionated HW extracts (DEAE cellulose)

Growth conditions	Time (days)	Fraction*	Limiting viscosity (ml/g)	Ara:Gal ratio of U*	Ara:Gal:Rh:Xyl:UA ratio of B*
Dark	0	U	49	16:84	—
		B	57	—	8:34:3:2:53
	2	U	8	14:86	—
		B	60	—	14:20:3:2:61
	4	U	1	n.d.	—
		B	71	—	12:10:3:3:72
	6	U	n.d.	46:54	—
		B	101	—	11:10:3:3:73
	10	U	n.d.	47:53	—
		B	66	—	2:9:4:5:80.
Light	4	B	63	—	11:10:3:4:72
	10	B	65	—	4:6:6:5:79

* U, unbound; B, bound.

more slowly sedimenting fraction giving a broad peak. The sedimentation constants of the major, faster fractions were concentration dependent and appeared to decrease in the dark samples but not in the light.

Many studies of cell wall expansion have connected this process with cell wall polysaccharide synthesis [20,21], although in some cases depletion has been observed [13]. Synthesis is usually associated with the early stages of division and expansion, such as in coleoptile tip growth. Cotyledon growth after germination involves only expansion. In lupins, the increases in cotyledon area, wet wt and, in the light, chlorophyll content (Figs 1, 2) are accompanied by a loss of raffinose series oligosaccharides (2 g/100 seeds) (Fig. 3), a protein loss of about 4 g, estimated by Kjeldahl digestion of dried tissue and another loss of 0.9 g of intercellular and cell wall polysaccharides (Figs 4–6) in which the neutral sugars arabinose and galactose are the main components depleted. This suggests, with respect to plant cell wall polysaccharides, that cell growth may involve at least two phases. In the first, polysaccharides are synthesized and mechanisms have been proposed to explain the plasticity of the wall at this stage [5,12–16]. The second phase, which predominates in the later stages of growth, would involve polysaccharide depletion. However, there is not a uniform removal of polysaccharide. Current theories of the structure of acidic pectic substances depict a model of a chain of polygalacturonic acid in which rhamnose units are interposed. To this are attached arabinogalactan side chains [6,11,12]. The changes that occur on cotyledon development after germination are consistent with the removal of a considerable number of these side chains with perhaps some scission of the rhamnogalacturonan core if the cotyledons remain in the dark. The large amount of neutral sugar lost (Figs 4–6) and the low MW of the unbound fractions indicates that at least some of these may be produced from hydrolysis of the larger acidic polysaccharide. The increase in the arabinose:galactose ratio suggests that later unbound fractions are not products of slow hydrolysis of the original neutral fraction, as arabinogalactans of type I [3,6,22] contain a core of galactan to which is attached arabinose side chains. Lupin cotyledons efficiently utilize the products of cell wall hydrolysis as no accumulation of galactose and arabinose is found (Fig. 3). The cell wall polysaccharide fractions isolated in the early stages of germination co-chromatographed with protein but at later stages the bound fractions contained little protein (Figs 7, 8). The significance of this in a protein storing seed is limited but is not inconsistent with the polysaccharide portion of the wall being part of a glycoprotein structure [13,14,23]. The viscosity. sedimentation constant and gel filtration data indicate changes in structure and properties during germination. The initial increase in viscosity of the acidic fraction after incubation could be a consequence of the removal of side chains, producing a less branched polymer. This and the increase in uronic acid content may allow the development of a more rigid cell wall [12, 13].

Table 4. Sedimentation constants of polysacchardies of lupin cotyledons extracted by hot water and chromatographed on DEAE cellulose

Time and conditions of growth	$10^{13} \times S_{20}$					
	Unbound fraction concentration %			Bound fraction concentration %		
	1.0	0.7	0.4	1.0	0.7	0.4
Dark						
0 days	2.1	1.8	1.8	4.7	6.4	7.0
2 days	2.0	n.d.	n.d.	4.9	n.d.	n.d.
4 days	1.0	1.1	0.8	3.7	5.0	6.9
10 days	1.0	n.d.	n.d.	3.9	n.d.	5.5
Light						
4 days	1.1	n.d.	n.d.	4.1	n.d.	n.d.
10 days	n.d.	n.d.	n.d.	5.7	n.d.	n.d.

EXPERIMENTAL

Plant material. Lupin seeds (*Lupinus luteus*, cv. Weiko III) were sterilized by soaking in 0.5% NaOCl for 10 min followed by washing with H_2O. They were planted in trays of moistened perlite and grown at 30°. Panasand was added to the surface.

Growth properties. These were followed by collecting 30 seedlings at 2, 4, 6, 8 and 10 days after imbibition in the dark and light and at 14, 18 and 21 days in the light. Cotyledons were separated and dried between filter papers before recording wet wt. Cotyledon shapes were traced on graph paper and areas measured. Dry wts were obtained after drying in a force draught oven for 24 hr. Chlorophyll content was measured by extraction of a separate batch of seedlings with Me_2CO.

Estimation of N content. In dried cotyledons, N content was estimated, after grinding, by H_2SO_4 digestion and estimation with phenate–hypochlorite–nitroprusside [24]. In cold H_2O, hot H_2O and alkali soluble extracts protein content was determined by the Folin–Lowry method. In the insoluble residue, N content was determined after H_2SO_4 digestion by the phenate–hypochlorite method.

Extraction of mono, di and oligosaccharide fractions and the cold H_2O soluble polysaccharide fraction. Cotyledons from ungerminated seeds were ground in a cooled mill. Those from imbibed seeds were macerated in EtOH and the mixture boiled for 5 min. After cooling, the soln was filtered through sintered glass and residue washed with EtOH, Me_2CO and Et_2O and dried. Residue was macerated 3 × at room temp with 10 mM $HgCl_2$. Extracts were centrifuged (24000 *g*, 10 min) and supernatants poured into 3 vol EtOH. After 18 hr, the soln was centrifuged and the supernatant combined with solvent extracts of the cotyledons and the ppt. washed with EtOH, Me_2CO, Et_2O and dried. The dried polysaccharide was dispersed in H_2O and centrifuged (24000 *g*, 30 min). This removed some denatured protein. Supernatant was poured into 3 vol EtOH washed and dried to give the cold H_2O-soluble polysaccharide fraction. The combined solvent extracts were concentrated to 50 ml below 40° and extracted with $CHCl_3$ (3 × 100 ml). Final traces of $CHCl_3$ and denatured protein were removed by centrifugation. The aq soln was de-ionized (Amberlite IRA 410 and AG 50—W × 8). The eluate was concentrated to a suitable vol for chromatography.

Extraction with hot NH_4 oxalate–EDTA. Residue from cold $HgCl_2$ extraction was suspended in 0.5% NH_4 oxalate to which Na_2 EDTA 2 g/100 ml was added. The pH was adjusted to 5. The mixture was stirred at 100° for 4 hr. After centrifugation (24000 *g*, 15 min), the residue was re-extracted twice. Combined supernatants were dialysed for 24 hr and centrifuged (24000 *g*, 30 min). Supernatant was poured into 3 vol EtOH and collected, washed and dried.

Extraction with alkali. Residue from oxalate extraction was extracted 2 × with 5% NaOH under N_2. After centrifugation, the supernatants were adjusted to pH 5 with HOAc and centrifuged. The supernatant was added to 3 vol EtOH and the

ppt. collected, washed and dried. The residue from alkaline extraction was suspended in EtOH, acidified with HOAc and centrifuged. The residue was washed with EtOH, Me_2CO, Et_2O and dried.

Separation, estimation and identification of mono, di, and oligosaccharide fractions. Aliquots of the EtOH extract were separated into 3 fractions by gel filtration on polyacrylamide (Biogel P-2) and the amounts estimated with anthrone [25]. The fractions were examined by PC (solvent A, *n*-BuOH–Py–H_2O–C_6H_6, 5:3:3:1 upper phase, and B; EtOAc–HOAc–HCO_2H–H_2O, 18:3:1:4 using multiple irrigation when required. TLC with solvent A and a cellulose carrier was also used. Papers were developed with *p*-anisidine HCl and papers and TLC plates with $AgNO_3$.

Estimation of uronic acid content. Uronic acid was estimated by decarboxylation using the method and apparatus of ref [26] and also colorimetrically [19].

Gel filtration of cold and hot water soluble fractions. Solns (1%) of the extracts were prepared in 0.1M NaCl, 5mM Na_2 EDTA, 0.001% NaN_3 by heating at 100° for 10 min. The hot H_2O extracts were treated with salivary α-amylase after cooling and dialysed. Aliquots were applied to a Sepharose 2B column, maintained at 18° and eluted with the same solvent as used for soln. Fractions were assayed for total carbohydrate by anthrone, using a standard curve prepared from sugar solns in the elution solvent.

DEAE-cellulose chromatography of cold water and hot water soluble extracts. A 1% soln of the fraction, prepared by heating in 5mM NaCl at 100° was applied to a column of DEAE-cellulose Cl^- and eluted with a continuous linear NaCl gradient (5–500 mM NaCl). Fractions were assayed for total carbohydrate with anthrone. On a preparative scale, 1 g of fraction was separated and the fractions concentrated in dialysis bags in a current of air, pptd in EtOH, washed and dried. Starch was removed from the non-bound fraction by treatment with salivary α-amylase and protein from both fractions with pronase. Solns were dialysed prior to EtOH pption.

Estimation of aldose contents of extracts by GLC after hydrolysis, reduction and acetylation. Cold H_2O, hot H_2O and alkali extracts (50 mg) were hydrolysed in M H_2SO_4 (5 ml) at 100° for 6 hr. Residue was first treated with 72% H_2SO_4 at room temp for 1 hr and then the soln diluted to 1M and heated. Hydrolysates were neutralized with $BaCO_3$, centrifuged and the residue washed twice. Supernatants were combined and an aliquot examined by PC (solvent A). Papers were developed with *p*-anisidine HCl and $AgNO_3$. To another aliquot, *myo*-inositol soln was added as an internal standard and sugars reduced with KBH_4 (2 hr). After addition of excess HOAc, the soln was passed through a column of Dowex AG50WX8.H^+ and evaporated to dryness under red pres and the borate volatilized by 5 additions of MeOH and distillation. Equal vol of Py and Ac_2O were added and the mixture heated at 100° for 1 hr. Py and Ac_2O were removed by distillation under red pres followed by 5 additions of H_2O and distillation. After thorough drying samples were dissolved in CH_2Cl_2. A glass column (2 m × 3.5 mm) was packed with 1.7% silicone oil (XF-1150) and 1.3% ethylene glycol succinate. The oven was operated isothermally at 170° with injection at 235°. The carrier gas was N_2 and the internal standard *myo*-inositol hexa-acetate had a retention time of 60 min. FID was used.

Viscosity. Limiting viscosities were determined at 25° in an Ubbelohde suspended level viscometer with a reservoir large enough to allow *in situ* dilution. The solvent was 0.5M KCl. No kinetic energy or shear correction factors were applied.

Sedimentation velocities. These were determined in 0.5M KCl, 0.05M Na_2 EDTA in an An-D rotor at 20° and 60000 r.p.m.

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