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α-L-ARABINOFURANOSIDASES AND β-D-GALACTOSIDASES IN GERMINATING-LUPIN COTYLEDONS*

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ABSTRACT

Extraction of germinating-lupin cotyledons, followed by ion-exchange and gel chromatography, gave two α-L-arabinofuranosidases and three β-D-galactopyranosidases. Some fractions were further purified by using Concanavalin A–Sepharose. The changes in the activities of the enzymes during germination have been determined. Some kinetic and physical properties of these enzymes are described, and their role in the modification of cell-wall polysaccharides is discussed.

INTRODUCTION

On germination of lupin seeds, the growth of cotyledons is associated with a depletion of the intercellular and cell-wall polysaccharides. The degradation of these polysaccharides is not uniform, but the neutral sugars, arabinose and galactose, are preferentially hydrolysed, leaving, at the later stages of germination, a polysaccharide having a much higher content[1] of uronic acid. Present theories of cell-wall polysaccharide structure depict a rhamnogalacturonan chain to which are attached arabinogalactan side-chains, with much smaller amounts of other neutral monosaccharides, and a small proportion of D-glucuronic acid[2]. In lupins, the arabinogalactan structure is based on a β-(1→4)-linked D-galactose core[3,4], and the L-arabinose most probably has a furanoid structure. Enzymes that could be involved in the selective degradation of this structure are the exo-enzymes, α-L-arabinofuranosidase (EC 3.2.1.55) and β-D-galactosidase (EC 3.2.1.23), and possibly the endo-enzyme β-D-galactanase. In the naming of these enzymes, if the ring size of the substrate sugar is not specified, pyranose is understood.

β-D-Galactosidases have been found in many plant sources, including spinach leaves[5], *Ipomea* flowers[6], *Avena* coleoptiles[7], pea epicotyls[8], apple[9], plum[10], and tomato fruits[11,12], sweet-almond emulsin[13], *Vicia* seedlings[14], the seeds of clover[15], *Cajanus indicus*[16], jack beans[17], *Dolichos biflorus*[18], *Phaseolus vulgaris*[19], *Canavalia*

*Dedicated to the memory of Sir Edmund Hirst, C.B.E., F.R.S.

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ensiformis[20], wheat[21], and rice[22]. Multiple activities have been isolated from sweet-almond emulsin[13] and from seeds of *Dolichos biflorus*[18] and *Cajanus indicus*[16].

α-L-Arabinofuranosidase activity is produced by a number of plant pathogens, *e.g.*, *Corticum rolfsii*[23], and there is also a report of the endo-enzyme α-L-arabinanase (furanose) from lesion extracts of *Fusarium caeruleum* cultured on potatoes[24]. However, neither enzyme appears to have been described as an endogenous plant activity, although an extract of wheat flour has been shown to release arabinose from wheat-flour arabinoxylan[25]. (1→4)-β-D-Galactanases have not been reported from plant sources.

Changes in levels of activity of β-D-galactosidase have been examined. Total activity in germinating *Dolichos biflorus* seeds increased three-fold from 0–6 days, and then declined[18]; rice seeds[22] showed similar behaviour. In ripening apples, there was an increase in both soluble and bound fractions during ripening[9], and the activity in the corolla of *Ipomea tricolor* increased and then decreased slightly[6]. In tomato fruits[11,12], β-D-galactosidase activity was found to be high, relative to that of polygalacturonase, at the green stages, and the latter activity did not rise to significant levels until the fruit started to colour.

We now report studies of enzymes that hydrolyse α-L-arabinofuranosyl and β-D-galactosyl residues, and that could modify cell-wall and intercellular polysaccharides.

RESULTS AND DISCUSSION

The ability of extracts of cotyledons, at various times after germination in the dark, to hydrolyse the cell-wall polysaccharide that can be extracted from lupin cotyledons by hot water containing EDTA and oxalate is shown in Fig. 1. This

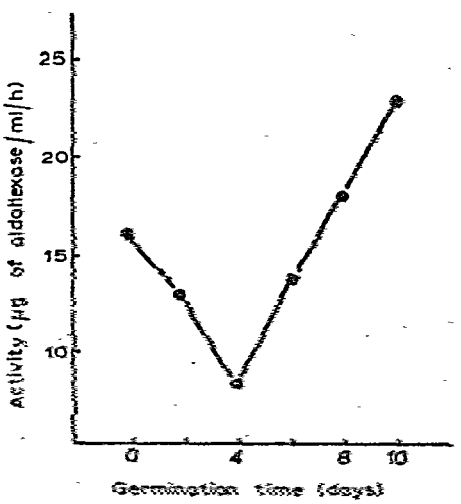

Fig. 1. Change in activity of polysaccharase on germination of lupin cotyledons.

polysaccharide is a mixture of a neutral arabinogalactan and an acidic polysaccharide containing arabinose, galactose, rhamnose, and traces of other neutral sugars[1–4]. Hydrolytic acitivity was always present in the extracts of cotyledons; after an initial decrease, it subsequently increased. Using nitrophenyl glycoside substrates, the changes in a number of soluble glycosidase activities were also measured. The β-D-galactosidase level was 0.2 μKatal per 100 seeds before imbibition, increased to 0.3 at 5 days, and then decreased to 0.22 by 12 days. α-D-Galactosidase activity was initially higher (0.6), rapidly increased to 0.8 by 2 days, and then decreased to 0.6 by 12 days. α-L-Arabinofuranosidase activity was low in the ungerminated seed (0.08) and changed little on germination. Seeds grown in the light and in the dark behaved similarly. When the activities of apparently cell-bound enzymes were assayed as seeds germinated in the light, the ratio of cell-bound to soluble β-D-galactosidase increased. At imbibition, the bound activity (0.03) was much less than the soluble, but by 12 days there was more bound (0.34) than unbound, and the increase continued up to 18 days when it was 0.5 μKatal per 100 seeds. In the dark, there was a small increase in bound enzyme, but there was always less of this than the soluble fraction.

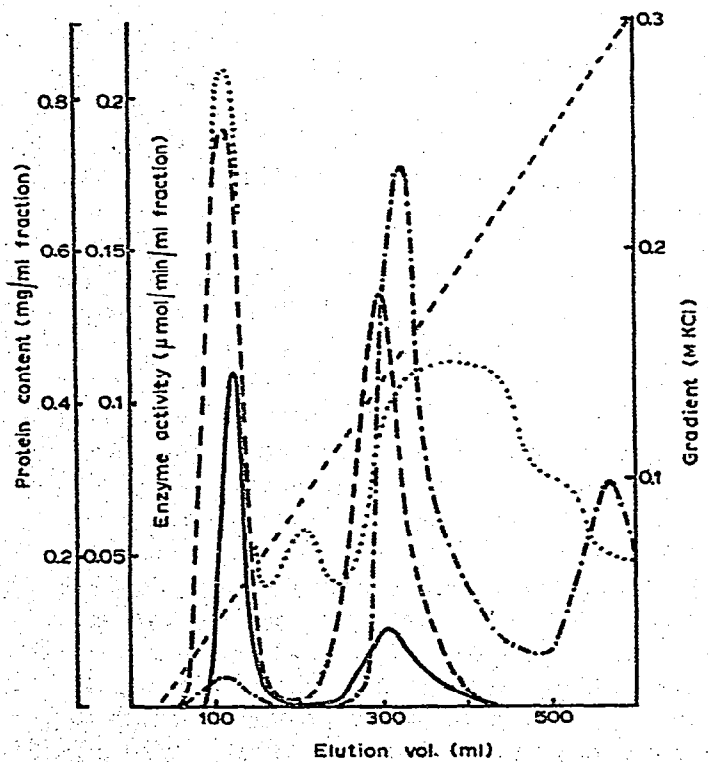

Fig. 2. Chromatography on DEAE-cellulose of extracts from lupin cotyledons after germination in the dark for 10 days: ———, α-L-arabinofuranosidase; — — —, β-D-galactosidase; ·····, protein; — · — ·, α-D-galactosidase.

The initial level of bound α-L-arabinofuranosidase was less than the soluble, but there was some increase, both in the dark and the light, as seedlings grew, so that by 12 days, the amount of bound fraction was slightly higher. Additional extraction of residues with buffer did not increase the levels of soluble enzyme significantly, and when cotyledons that had grown for 8 days in the dark were extracted with buffers of higher ionic strength (up to 0.2M), there was no increase in the soluble fraction. When the soluble fractions were chromatographed on DEAE-cellulose, two α-L-arabinofuranosidases, two β-D-galactosidases, and three α-D-galactosidase activities were obtained (Fig. 2). The α-L-arabinofuranosidases and β-D-galactosidases were labelled I and II from the order of elution, and the α-D-galactosidases *A*, *B*, and *C*, following the convention applied to other legume seeds[26]. Extracts of cotyledons grown in the dark were then prepared at 2-day intervals up to 12 days, and the changes in the levels of the multiple forms of the enzymes measured after separation on DEAE-cellulose. The level of β-D-galactosidase I increased from 0.04 Katal per 100 seeds to 0.12 at 6 days, and then remained constant, whereas II, after an initial increase from 0.06 to 0.11 at 2 days, decreased steadily to 0.05. α-L-Arabinofuranosidase I decreased initially and then became constant (0.05 to 0.03), but enzyme II was, at first, very low (0.01) and then increased to 0.025.

α-D-Galactosidase *A* decreased from 0.30 to very low values (0.01), and *C* increased from a trace to 0.13 in 12 days. Enzyme *B* rapidly increased from 0.13 to 0.34 in 4 days, and then decreased to 0.24 by 12 days. Two (*A* and *C*) or three (*A*, *B*, and *C*) activities have been reported from other legume seeds[26]. The changes in the levels of enzyme *A* in these seeds are relatively small, and the direction of change is variable. Enzyme *B* was detected only in soybean, and initially increased and then decreased, as did the lupin enzyme. The amounts of activity of *A* and *B* in lupins are of the same order as those of the other legume seeds. One of the probable functions of these forms is the hydrolysis of raffinose-series oligosaccharides, which are present in high amounts is lupins[1]. From the location, amount, and changes of enzyme *C*, it was concluded[26] that it was involved in the hydrolysis of galactomannan. In contrast to guar, lucerne, carob, and honey locust, which have endosperms of galactomannan, in soybeans, which contain only trace amounts of galactomannan, the increase in enzyme *C* was slight. The changes in lupin enzyme *C* are similar to those of soybean, and only trace amounts of this polysaccharide are present in lupin seeds.

After chromatography on DEAE-cellulose, the fraction containing β-D-galactosidase I and α-L-arabinofuranosidase I was chromatographed on Sephadex G-100 (Fig. 3). The β-D-galactosidase activity was separated into two fractions (Ia and Ib). The fraction of higher molecular weight co-chromatographed with α-L-arabinofuranosidase I, but was free of α-D-galactosidase. Fraction Ib still contained α-D-galactosidase *A*. When Ia was further chromatographed on sulphoethylcellulose, with a linear gradient of KCl up to 0.4M, it eluted as a single peak. On carboxymethylcellulose (CM), it also eluted as a single peak with partial separation from α-L-arabinofuranosidase I. These two activities were separated by adsorbing the mixture on a column of Concanavalin A–Sepharose, and eluting the α-L-arabino-

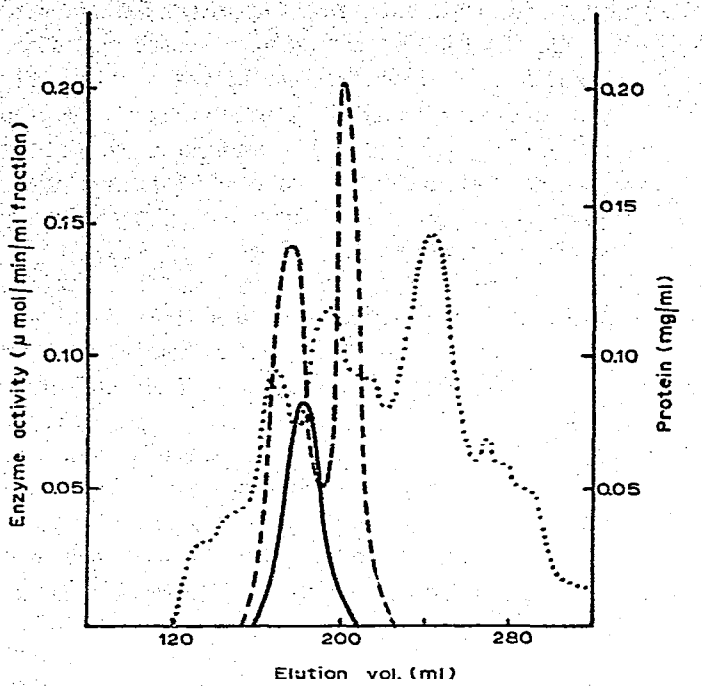

Fig. 3. Chromatography on Sephadex G-100 of the fraction from DEAE-cellulose containing α-L-arabinofuranosidase I and β-D-galactosidase I: ———, α-L-arabinofuranosidase; — — —, β-D-galactosidase; ·····, protein.

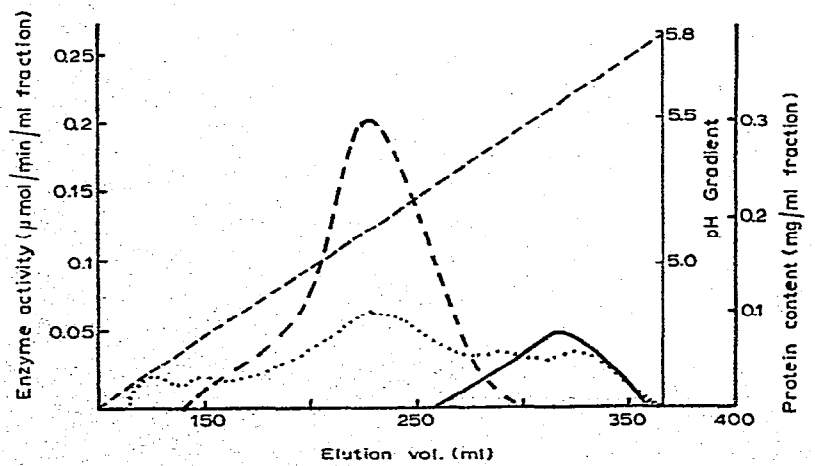

Fig. 4. Chromatography on CM-cellulose of β-D-galactosidase Ib, using a pH gradient (4.5–5.8): — — —, β-D-galactosidase Ib; ———, α-D-galactosidase; ·····, protein.

furanosidase I with methyl α-L-arabinofuranoside and the β-D-galactosidase Ia with *o*-nitrophenyl β-D-galactopyranoside. During the chromatography, there was some leakage of protein from the column material. β-D-Galactosidase Ia was only found in extracts prepared from cotyledons that had germinated for more than six days. On storage, its molecular weight decreased, and the product eluted on gel filtration at the same volume as Ib. This change in molecular weight took several weeks for completion. β-D-Galactosidase Ib could be almost completely separated from α-D-galactosidase A activity by chromatography on CM-cellulose with a linear pH-gradient (Fig. 4).

The second fraction from DEAE-cellulose chromatography, which contained β-D-galactosidase, α-L-arabinofuranosidase, and α-D-galactosidase activities, was chromatographed on Sephadex G-100, when the first and second activities were separated to give β-D-galactosidase II and α-L-arabinofuranosidase II (Fig. 5). One α-D-galactosidase fraction (*B*) was present, and its elution position overlapped that of β-D-galactosidase II. The latter enzyme chromatographed as a single activity on SP-Sephadex C-50, when it co-chromatographed with α-D-galactosidase *B*. When the mixture was applied in 5mM acetate buffer to a column of Sepharose 4B linked directly to 4-aminophenyl 1-thio-β-D-galactoside[27], the enzyme was bound. However, activity was eluted (with a KCl gradient up to 0.5M at pH 5.0) at a KCl concentration of 0.2M, and α-D-galactosidase co-chromatographed.

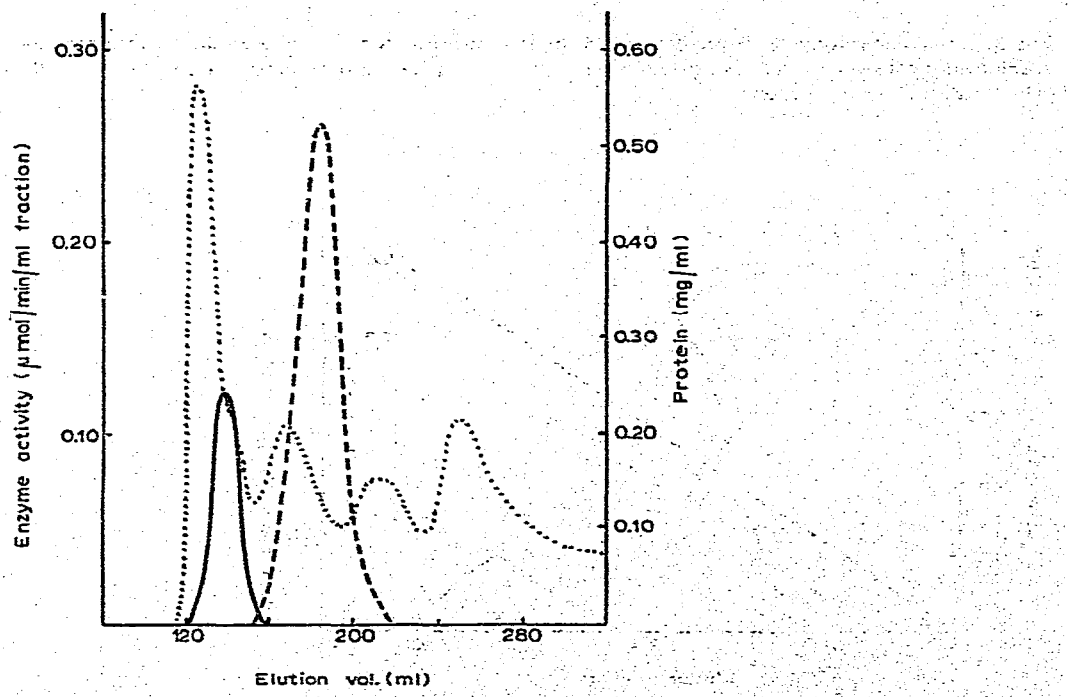

Fig. 5. Chromatography on Sephadex G-100 of the fraction from DEAE-cellulose containing α-L-arabinofuranosidase II and β-D-galactosidase II: ———, α-L-arabinofuranosidase; — — —, β-D-galactosidase; ·····, protein.

All the fractions from DEAE-cellulose chromatography of 0-, 6-, and 8-day extracts were tested for their ability to hydrolyse lupin cell-wall polysaccharide. Released carbohydrate was estimated by reaction with ferricyanide[28]. Only fractions containing the two peaks of β-D-galactosidase and α-L-arabinofuranosidase activities produced reducing sugar. Whole extracts from 0, 4, and 10 days after imbibition were assayed for their ability to degrade the galacto-galacturonan produced by partial hydrolysis of lupin cell-wall polysaccharide with dilute acid. The viscosity decreased very slowly. Examination of the hydrolysate by t.l.c. showed only galactose, and no oligosaccharides were produced. These results indicate an absence of a readily detected β-D-galactanase.

The purified α-L-arabinofuranosidases and β-D-galactosidases were tested for other glycosidase activities. The results are shown in Table I. Both α-L-arabinofuranosidases were free of other activities that were assayed, but the β-D-galactosidases hydrolysed a number of other substrates. The ability of β-D-galactosidase preparations to hydrolyse β-glycosides that have the 5-hydroxymethyl group of D-galactose modified to a smaller size[29] (*e.g.*, by change to H as in α-L-arabinosides, to CH_3 as in β-D-fucosides, or to $-CH{=}CH_2$) has been noted. Some preparations have also been found to hydrolyse substrates in which the configuration at C-4 has been changed (*i.e.*, β-D-glucosides)[13,30]. When β-D-galactosidases I and II from lupin cotyledons were further purified by chromatography on Concanavalin A–Sepharose and the activities eluted with *o*-nitrophenyl β-D-galactoside, enzyme Ia lost α-D-galactosidase, β-D-glucosidase, β-D-xylosidase, and α-D-mannosidase activities, and enzyme II lost all but β-D-galactosidase activity.

TABLE I

GLYCOSIDE SPECIFICITY[a] OF α-L-ARABINOFURANOSIDASES AND β-D-GALACTOSIDASES FROM LUPIN COTYLEDONS

Substrate	α-L-*Arabinofuranosidase*		β-D-*Galactosidase*		
	I[b]	*II*[c]	*Ia*[c]	*Ib*[c]	*II*[c]
p-NO_2-phenyl α-L-Ara*f*	100[a]	100	28	9	0
o-NO_2-phenyl β-D-Gal*p*	0	0	100	100	100
p-NO_2-phenyl α-L-Ara*p*	0	0	17	3	7
p-NO_2-phenyl β-D-Fuc*p*	0	0	15	9	50
o-NO_2-phenyl α-D-Gal*p*	0	0	0	3	61
p-NO_2-phenyl β-D-Glc*p*	0	0	33	2	0
p-NO_2-phenyl α-D-Glc*p*	0	0	0	0	0
o-NO_2-phenyl β-D-Xyl*p*	0	0	9	5	0
p-NO_2-phenyl α-D-Man*p*	N.d.[d]	N.d.	37	2	14
o-NO_2-phenyl β-D-Man*p*	N.d.	N.d.	0	0	0
p-NO_2-phenyl α-L-Fuc*p*	0	0	0	0	0
p-NO_2-phenyl β-D-GlcA*p*	0	0	0	0	0

[a]Nitrophenol (μg) released in the same period that the enzyme releases 100 μg from *p*-nitrophenyl α-L-arabinofuranoside or *o*-nitrophenyl β-D-galactopyranoside. [b]After Con-A–Sepharose chromatography. [c]Before Con-A–Sepharose chromatography. [d]N.d., not determined.

TABLE II

PROPERTIES OF α-L-ARABINOFURANOSIDASES AND β-D-GALACTOSIDASES FROM LUPIN COTYLEDONS

Property	*α-L-Arabinofuranosidase*		*β-D-Galactosidase*		
	I	*II*	*Ia*	*Ib*	*II*
K_m					
p-NO_2-phenyl α-L-Ara*f* (mM)	16.6	1.6	—	—	—
Methyl α-L-Ara*f* (mM)	[a]	11.1	—	—	—
Neutral cell-wall polysaccharide (g/l)	2.9	0.14	—	—	—
Acidic cell-wall polysaccharide (g/l)	0.19	0.14	—	—	—
o-NO_2-phenyl β-D-Gal*p* (mM)	—	—	2.3	2.4	1.0
Lactose (mM)	—	—	26	33	12.5
Galactan[b] (g/l)	—	—	N.d.	0.17	0.19
Relative V_{max}					
Methyl α-L-Ara*f*	[a]	21	—	—	—
Neutral cell-wall polysaccharide	4.6	7.7	—	—	—
Acidic cell-wall polysaccharide	1.3	11.5	—	—	—
Lactose	—	—	13.3	16.7	221
Galactan[b]	—	—	N.d.	17.4	19.2
pH vs. activity					
Optimum	4.4	3.5	3.2	3.9	3.1
50% of max. values	2.8	2.7	<2.5	2.5	<2.5
	5.6	5.0	6.6	5.5	5.1
Mol. wt. (gel filtration)	7.0×10^4	12.0×10^4	7.4×10^4	5.4×10^4	6.5×10^4
E_A (kjoule)	41	10.4	3.5	12.6	58

[a]See text. [b]From partial hydrolysis of cell-wall polysaccharide. [c]Relative to *p*-NO_2-phenyl α-L-Ara*f* or *o*-NO_2-phenyl β-D-Gal*p* as 100.

Some of the properties of the purified enzymes are shown in Table II. The values for Ia were determined as soon as possible after separation, but some Ib may have been present. The high, relative V_{max} of II, using lactose as substrate, suggests a possible role in oligosaccharide hydrolysis. All three enzymes had similar pH maxima, and the 50% maximum values on the high-pH side lie near to plant-cell pH values, so that a slight variation in cell pH would cause a considerable change in rate of hydrolysis. All were stable up to at least pH 6. All the β-D-galactosidase activities were stable to freezing for up to 14 days followed by thawing, and were stable, when stored at 4°, for at least 8 weeks.

Common neutral sugars and uronic acids at 33mM concentration had no inhibitory effect, apart from D-galactose which gave 80% inhibition. D-Galactal at 0.7mM, and D-galactono-1,5-lactone at 3mM, gave almost complete inhibition. D-Glucono-1,5-lactone and L-arabinonolactone at the same concentration had no effect. 3mM 4-Aminophenyl 1-thio-β-D-galactoside slightly decreased the activity of Ib, but not that of II. 0.3mM Hg^{2+} and Cd^{2+} gave almost complete inhibition, and Pb^{2+} and Cu^{2+} at the same concentration gave 50–70% inhibition. Zn^{2+}, Fe^{2+}, Ni^{2+}, molybdate, Na^+, Co^{2+}, Ca^{2+}, and Mg^{2+} at 0.3mM concentration had no significant effect, as did also 3mM EDTA.

α-L-Arabinofuranosidase I had a relatively high K_m with *p*-nitrophenyl α-L-arabinofuranoside (16.6mM). The hydrolysis rate with methyl α-L-arabinofuranoside was too low to determine accurate kinetic constants using the ferricyanide assay for reducing sugar[28]. The K_m with the acidic cell-wall polysaccharide was low (0.10 g/l), suggesting a high affinity, but the relative V_{max} was also low. This enzyme was very stable to heat and could be held at 70° for 15 min with some decrease in activity, and this property gave an alternative method of removing other activities, as all other β-D-galactosidases and α-L-arabinofuranosidase II were inactivated when held for this time at 70°. α-L-Arabinofuranosidase II was also distinguished from I by its apparently high molecular weight. In view of the binding to Concanavalin A–Sepharose, the enzymes may be glycoproteins, which can behave abnormally on gel chromatography. Both enzymes, like the β-D-galactosidases, had 50% pH-maximum values near pH 5 and were stable up to at least pH 6. Both enzymes were stable to freezing for 14 days followed by thawing; on storage at 4°, they lost about a quarter of their activity in four weeks. They were not significantly inhibited by neutral monosaccharides, including L-arabinose and uronic acids at 33mM. At 0.3mM, enzyme I was slightly inhibited by Hg^{2+}, Cd^{2+}, Zn^{2+}, Pb^{2+}, Ni^{2+}, Co^{2+}, and Fe^{2+}. All of these metals at the same concentration gave an unusual enhancement of activity of enzyme II, although 3mM dithiothreitol had no effect, and neither enzyme was affected by 3mM EDTA. Mg^{2+} and Ca^{2+} (0.3mM) had no effect on either enzyme. A mM solution of L-arabinonolactone, prepared by bromine oxidation of L-arabinose, inhibited enzyme I completely and enzyme II to 80%. D-Galactono-1,5-lactone, D-galactal, and 4-aminophenyl 1-thio-β-D-galactoside at 0.3mM concentration caused slight inhibition of I, but were without effect on II, and L-ascorbate at the same concentration slightly inhibited both enzymes.

Extracts of cotyledons that had germinated in the dark were assayed for polygalacturonase activity. Solutions, prepared at 0, 4, and 10 days, on incubation with sodium polygalacturonate solution for 3 h, decreased the specific viscosity by 1.0, 4.5, and 4.5%, respectively. This effect was accompanied by a 4.8, 6.4, and 7.2% release of reducing sugar. The sample extracted from 4-day plants, on incubation for 36 h with polygalacturonate, decreased the viscosity to low values. These results can be interpreted as indicating low levels of polygalacturonase activity, and similar results have been found for soybeans[31].

Overnight incubation of lupin cell-wall arabino-(1→4)-β-D-galactan (a type I arabinogalactan[2]), the acidic cell-wall fraction (a rhamnogalacturonan with arabinogalactan side-chains[1]), and an arabino-(1→3),(1→6)-β-D-galactan (Type II, Serva) with the β-D-galactosidases gave only several percent release of reducing sugar. When incubated with the products of partial acidic hydrolysis of lupin cell-wall polysaccharide and of gum arabic [(1→3),(1→6)-β-linkages], there was a large increase in the reducing sugar released, although hydrolysis was not complete. These polysaccharides had lost most of their arabinose. G.l.c.[1] of alditol acetates, formed after borohydride reduction, showed arabinose contents of 4% for both, and galactose contents of 64 and 36%, respectively. The uronic acid contents (determined colori-

metrically[32]) were 25 and 60%. α-L-Arabinofuranosidases released amounts of reducing sugar from lupin arabinogalactan and the acidic cell-wall polysaccharide of the same order as their arabinose contents, and mixtures of α-L-arabinofuranosidase and β-D-galactosidase gave much increased amounts, but hydrolysis was still not complete.

The isolation of α-L-arabinofuranosidase from germinating-lupin cotyledons is the first report of a preparation of this enzyme from an endogenous plant source. It has been suggested[1] that, in the cotyledons of germinating lupins (where only cell expansion occurs), there is a second phase of chemical structural modification of the primary-wall polysaccharide. This differs from the first phase, associated with differentiation and early expansion when the wall is highly plastic, and in which there is extensive synthesis of pectic polymers. In this second phase, the neutral arabinogalactan side-chains are removed, and the exo-enzymes α-L-arabinofuranosidase and β-D-galactosidase described in this paper could cause part of the observed pattern of polysaccharide modification. The pH-activity curves of all the enzymes would cause them to be very sensitive to changes in cell pH. In some organs, *e.g.*, tomato fruits[11,12], a third phase involving polygalacturonase activity, that is accompanied by hydrolysis of the polyuronic acid chain, is evident. In ripening tomato fruits, it has been suggested[11] that β-D-galactosidase and other glycosidases contribute to cell modification that leads to fruit softening, prior to the action of polygalacturonase. The three phases may overlap, and the last may be associated with particular organs. Although some loss of polyuronic acid was detected in dark-grown lupin cotyledons at 10 days, there was no significant loss in light-grown plants[1].

EXPERIMENTAL

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

Enzyme substrates. — *p*-Nitrophenyl α-L-arabinopyranoside was prepared by the method of Feier and Westphal[33], and *p*-nitrophenyl α-L-arabinofuranoside by the method of Fielding and Hough[34]. Other nitrophenyl glycosides were supplied by Sigma. Methyl α-L-arabinofuranoside was prepared by alkaline hydrolysis of methyl 2,3,5-tri-*O*-benzoyl-α-L-arabinofuranoside[35].

Lupin cell-wall polysaccharide was prepared from lupin seeds that had been converted into flour, extracted with boiling ethanol, and dried. This flour was extracted with aqueous 0.5% ammonium oxalate, to which Na_2EDTA (2 g/100 ml) had been added, in a boiling water-bath for 4 h. After cooling and centrifugation, the supernatant was added to 4 vol. of ethanol. The precipitate was dispersed in water (1 g/25 ml), heated in a boiling water-bath for 10 min, cooled, and centrifuged (24,000 *g*, 15 min, 25°). The supernatant was incubated with purified pronase (Sigma, 4 mg/100 ml) at pH 5 and 37° in calcium acetate for 18 h. After centrifugation (24,000 *g*, 15 min, 25°), the supernatant was dialysed against water, centrifuged, and

poured into 4 vol. of ethanol. The precipitate was collected, washed with ethanol, acetone, and ether, and dried.

The neutral fraction (arabinogalactan) and the acidic polysaccharide fraction were prepared by chromatography on DEAE-cellulose (Cl^-), and the bound fraction was eluted with 0.5M NaCl[1].

Lupin galactogalacturonan was prepared from the cell-wall polysaccharide extracted by hot EDTA–oxalate solution. This was heated at 1% concentration in 5mM oxalic acid[3], until the rate of release of reducing sugar was constant. After cooling, neutralization with 0.1M NaOH, dialysis, and centrifugation, the polysaccharide in the supernatant was precipitated in 3 vol. of ethanol, collected by centrifugation, re-washed with ethanol, acetone, and ether, and dried. Arabino-(1→3),(1→6)-β-D-galactan was obtained from Serva. The partial hydrolysis product of gum arabic was obtained from Calbiochem (Galactan 345211). The arabinonolactone solution was prepared by treating L-arabinose (0.5 g) in water (50 ml) and barium carbonate (2.0 g) with bromine (0.2 ml). The bromine was dissolved by shaking, and the mixture stored for 36 h in the dark. Excess of bromine was removed by aeration, and the solution centrifuged. H_2SO_4 (0.1M) was added to the supernatant to pH 2, followed by conc. aqueous Na_2SO_4 until all the barium was precipitated. The solution was centrifuged again, brought to pH 5, and stored overnight before use.

Assay of glycosidase activities in cotyledon extracts. — Cotyledons from 30 seeds were macerated in 0.05M acetate buffer (pH 5.0), and then further ground in a glass Tenn-Brock homogenizer and centrifuged (24,000 *g*, 15 min, 2°). Ammonium sulphate was added to 90% saturation to the supernatant, and the precipitated protein was redissolved in acetate buffer and dialysed for 18 h against the same buffer. After centrifugation, the supernatant was suitably diluted, and an aliquot (0.1 ml) was incubated with nitrophenyl glycoside (25mM in 0.05M acetate, 0.1 ml) at 30° for 15 min. Reaction was stopped by the addition of 2% Na_2CO_3 (2.8 ml), and the amount of nitrophenol released was measured spectrophotometrically at 420 nm. To measure cell-bound activities, the residue was washed once with acetate buffer, and a portion was gently shaken in a water bath during incubation, and filtered before assay.

Assay of polygalacturonase activity. — An extract of cotyledons from 30 seeds was prepared in 0.05M acetate buffer (pH 5.5, 10 ml). An aliquot (1.0 ml) was incubated with 0.5% sodium polygalacturonate (Sigma, 10 ml). A solution of the commercial sample in water was centrifuged, the supernatant poured into ethanol, and the precipitate washed and dried before use. Viscosity change was measured in an Ubbelohde viscometer, and reducing sugar released, after suitable dilution, was determined with ferricyanide[28].

Purification of β-D-galactosidases and α-L-arabinofuranosidases. — Cotyledons from 200 lupins that had germinated for 10 days in the dark were washed with distilled water, and macerated, using an Ultra-Turrax, in 0.05M acetate buffer (pH 5.0). The homogenate was centrifuged (24,000 *g*, 30 min, 2°) and washed. Ammonium sulphate was added to the supernatant to 90% saturation, and the precipitate was centrifuged (24,000 *g*, 15 min, 2°). The pellet was re-dissolved in acetate buffer and equilibrated

against 5mM KCl by dialysis for 18 h. The solution was centrifuged (24,000 *g*, 10 min, 2°). The supernatant had a specific activity for β-D-galactosidase of 2.71 nKat/mg of protein, and for α-L-arabinofuranosidase of 1.32 nKat/mg. It was chromatographed on a column (20 × 3 cm) of DEAE-cellulose (Cl^-) with a 0.005–0.3M linear gradient of KCl in mM EDTA. Two fractions, that each contained both β-D-galactosidase and α-L-arabinofuranosidase activities, were obtained, and were concentrated by the addition of ammonium sulphate. β-D-Galactosidases I and II had specific activities of 15.2 and 10.1 nKat/mg, with recoveries of 84 and 12%, and α-L-arabinofuranosidases I and II had specific activities of 2.4 and 1.9 nKat/mg, with recoveries of 48 and 19%.

The first active-fraction from DEAE-cellulose was chromatographed on a column (80 × 2 cm) of Sephadex G-100 with 0.05M acetate buffer (pH 5.0, mM EDTA) for elution. Two active fractions were obtained. The one that eluted first contained α-L-arabinofuranosidase I (sp. act., 3.1 nKat/mg, recovery 69%) and β-D-galactosidase Ia (sp. act., 18.3 nKat/mg, recovery 20%); and the second, β-D-galactosidase Ib (sp. act., 17.1 nKat/mg, recovery 76%). These fractions were concentrated by ultrafiltration with a Diaflo PM-10 membrane. β-D-Galactosidase Ia was chromatographed on a column (10 × 1.3 cm) of sulphoethylcellulose (Cellex-SE, Bio-Rad) with a 0.005→0.4M KCl gradient in 5mM acetate buffer (pH 5.0). There was no further separation, but the specific activity of β-D-galactosidase Ia was increased to 40 nKat/mg, with 20% recovery. β-D-Galactosidase Ib was chromatographed on a column (10 × 1.3 cm) of CM-cellulose using a linear pH gradient (4.5→5.8) of acetate buffer, which gave a single peak of β-D-galactosidase Ib activity (sp. act., 79.8 nKat/mg, recovery 80%) and which separated most of the α-D-galactosidase. The mixture of α-L-arabinofuranosidase I and β-D-galactosidase Ia from gel chromatography was separated on a column (10 × 1.3 cm) of Concanavalin A–Sepharose (Pharmacia) in 25mM acetate buffer (pH 5.0) containing mM Mn^{2+} and mM Ca^{2+}. The column was washed with 4 column-volumes of this solution, followed by a solution of methyl α-L-arabinofuranoside in the same solvent, when α-L-arabinofuranosidase I was eluted. The column was then washed with 4 column-volumes of the acetate buffer, followed by a solution of *p*-nitrophenyl β-D-galactoside, when β-D-galactosidase Ia was recovered.

The second active-fraction from DEAE-cellulose was chromatographed on a column (80 × 2 cm) of Sephadex G-100 with 0.05M acetate buffer (pH 5.0), mM EDTA. Two active fractions were obtained. α-L-Arabinofuranosidase II (sp. act., 7.2 nKat/mg, recovery 69%) was eluted first, free of β-D-galactosidase, followed by β-D-galactosidase II (sp. act., 14.4 nKat/mg, recovery 60%) free of α-L-arabinofuranosidase. Chromatography of β-D-galactosidase II on a column (10 × 1.3 cm) of SP-Sephadex C-50 with a pH gradient (4.5→5.8) in 25mM acetate buffer gave a single fraction (sp. act., 63.8 nKat/mg, recovery 59%).

Protein concentration in solutions for estimating activities, and protein concentrations in fractions from DEAE-cellulose, were estimated by the Folin–Lowry method. Protein concentrations in fractions from CM-cellulose, Cellex-SE, and gel-filtration columns were measured by the absorbance at 280 nm.

Measurement of enzyme properties. — K_m and V_{max} values were determined from Lineweaver–Burk plots using the substrate ranges: *o*-nitrophenyl β-D-galactopyranoside, 0.5–12.5mM; *p*-nitrophenyl α-L-arabinofuranoside, 0.5–12.5mM; polysaccharides, 0.2–1.0 g/l; and lactose, 20–100mM. pH-Activity curves were determined in 0.1M citrate–phosphate buffer (pH 2.2–8.0). Reaction mixtures containing buffer (0.1 ml) and nitrophenyl glycoside (0.1 ml) were incubated at 30° for 15 min. The pH stability was determined in the same buffer, and enzyme was stored for up to 2 h at room temperature. Energies of activation were determined at pH 5.0 in acetate buffer in the temperature range 20–50°. To determine the temperature of inactivation, enzymes were heated in the same buffer. Molecular weights were determined by gel chromatography with Sephadex G-100, and the standards were myoglobin (mol. wt., 18,000), ovalbumin (45,000), bovine serum albumin (67,000), calf-intestinal alkaline phosphatase (115,000), and lactic dehydrogenase (140,000). The regression equation of the calibration line was log mol. wt. $= -0.00595 \times$ elution vol. (ml) $+ 5.917$, with a correlation coefficient of 0.999 and the following elution volumes: α-L-arabinofuranosidase I, 181 ml; II, 142 ml; β-D-galactosidase Ia, 177 ml; Ib, 199 ml; and II, 188 ml.

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