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α-D-GALACTOSIDASE ACTIVITY AND GALACTOMANNAN AND GALACTOSYLSUCROSE OLIGOSACCHARIDE DEPLETION IN GERMINATING LEGUME SEEDS

B. V. McCLEARY and N. K. MATHESON

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

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Abstract—Germinating seeds of lucerne, guar, carob and soybean initially depleted raffinose series oligosaccharides and then galactomannan. This depletion was accompanied by a rapid increase and then a decrease in α-galactosidase levels. Lucerne and guar contained two α-galactosidase activities, carob three and soybean four. One of these in each plant, from its location in the endosperm, time of appearance and kinetic behaviour, appeared to be primarily involved in galactomannan hydrolysis. This enzyme in lucerne had MW of 23000 and could not be separated from β-mannanase by $(NH_4)_2SO_4$ fractionation, DEAE, CM or SE-cellulose chromatography or gel filtration, but only by polyacrylamide gel electrophoresis. In guar, carob and soybean, it could be separated by ion-exchange chromatography and gel filtration. In lucerne, carob and guar most of the total increase in activity was due to this enzyme. The other α-galactosidases had MWs of about 35000 and could be separated from β-mannanase by dissection, ion exchange cellulose chromatography and gel filtration. They were located in the cotyledon-embryo and appeared to be primarily involved in galactosylsucrose oligosaccharide hydrolysis.

INTRODUCTION

GALACTOMANNAN occurs in varying amounts in the endosperm of a wide range of leguminous seeds.[1] The polysaccharides from different species have different proportions of D-galactose and D-mannose, but always consist of a β(1–4) mannan backbone with single D-galactose branches linked α(1–6).[2] α(1–6) Galactosylsucrose oligosaccharides, e.g. raffinose, are also found.[3] On germination both oligosaccharides and polysaccharides are quickly metabolized by α-D-galactosidase[4] (E.C. 3.2.1.22) and a β-mannanase[5] and possibly a β-mannosidase. Using specific inhibitors and seed dissection, Reid and Maher have shown that in fenugreek the enzymes hydrolysing galactomannan are produced in the aleurone layer.[6] Recently they have shown that in this seed increases in endosperm α-galactosidase and β-mannosidase activities parallel galactomannan depletion.[7]

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Two fractions of α-galactosidase which showed similar properties have been obtained from an extract of a *Coffea* sp., by stepwise pH elution on alumina.[8] However, Dey and Pridham[9] separated two activities by gel filtration of extracts of ungerminated *Vicia faba* seeds which showed different kinetic properties. They also obtained evidence for a monomer–oligomer interconversion and reported that the lower MW form could be further fractionated on CM-cellulose using stepwise pH elution.[10]

The relative hydrolysis rates of α(1–6) galactosylsucrose oligosaccharides and galactomannan show differences. Some fungal α-galactosidases could not hydrolyse galactomannan.[11] A preparation from *Phaseolus vulgaris* hydrolysed galactomannan at a rate of only 1–2% of that of raffinose.[12] In contrast, guar α-galactosidase hydrolysed polysaccharide more readily than oligosaccharide.[13]

During the development of a number of seeds both α-galactosidase activity and α(1–6) galactosylsucrose content increase, suggesting compartmentation or endogenous inhibition.[14] Dey and Pridham, studying changes in α-galactosidases during maturation and germination of *V. faba* seeds, proposed a relationship between the physiological state of the seeds and the predominance of one of the MW forms of the enzyme.[15]

The aim of the present work has been to examine the changes in the levels of α-galactosidase activity in four legume seeds after imbibition, to attempt to fractionate this activity, to compare the activities at different locations in the seeds, to find the substrate specificity and determine physiological function.

RESULTS AND DISCUSSION

Figure 1 shows the pattern of depletion of galactomannan in the germinating seeds, and the changes in the levels of mono- and oligosaccharides. These were separated by gel filtration on polyacrylamide (Bio-gel P-2) and characterized by TLC, PC and hydrolysis to constituent monosaccharides.

The disaccharides found were sucrose and melibiose and traces of mannobiose. The higher oligosaccharides were raffinose, stachyose, verbascose and traces of mannotriose. The total concentration of (α-1) linked anhydro D-galactose in galactomannan and galactosyl-sucrose oligosaccharides before germination was about the same in lucerne, guar and carob.

On imbibition there was an immediate and rapid depletion of the raffinose type oligosaccharides. In lucerne and carob, where stachyose was the major reserve oligomer, its decrease was accompanied by a transient increase in sucrose. In guar, raffinose was present as the main oligosaccharide and was initially hydrolysed to melibiose. In soybean, raffinose and sucrose were present. The raffinose content decreased but sucrose showed an initial increase followed by a decrease. There was some verbascose in lucerne and carob and it was rapidly hydrolysed. In all seeds the major monosaccharides were glucose and fructose with only traces of mannose and galactose.

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If seeds were macerated adequately, all the galactomannan could be extracted by cold aqueous mercuric chloride solution. Residues were checked for any residual galactomannan by hot water and alkali extraction, the extracts hydrolysed by acid and examined by PC. Hydrolysis of the cold aqueous extracts after precipitation into ethanol (with 0·75 M H_2SO_4 for 3 hr at 100°) showed that in the early stages of germination no other polysaccharides were detectable, but in the later stages these did appear. When sugars, other than mannose and galactose were found after acidic hydrolysis, the galactomannan was purified via the copper complex[27] before anthrone estimation.

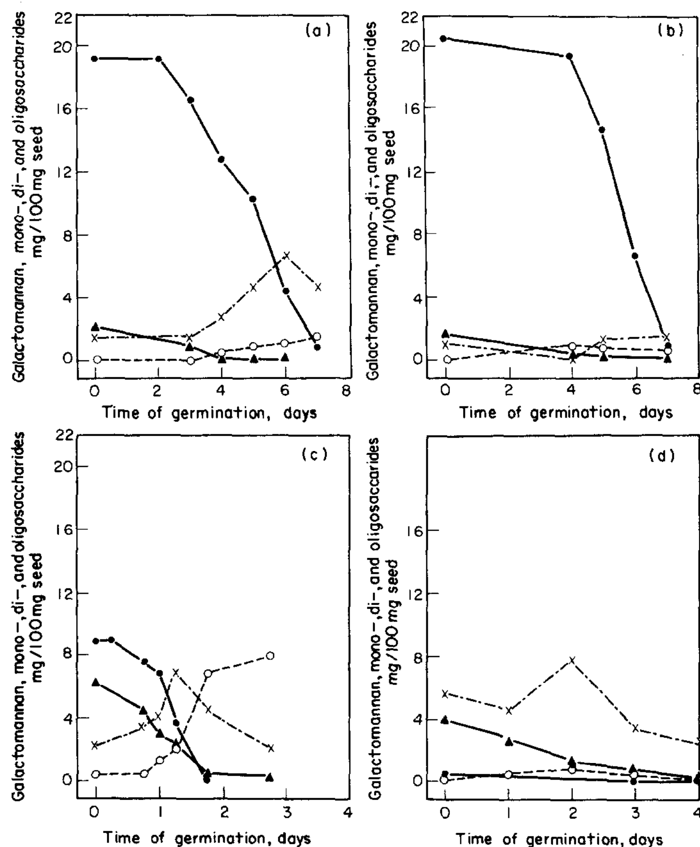

FIG. 1. DEPLETION OF GALACTOMANNAN, OLIGOSACCHARIDES AND MONOSACCHARIDES ON THE GERMINATION OF: (a) CAROB; (b) GUAR; (c) LUCERNE; AND (d) SOYBEAN.
●, Galactomannan; ▲, oligosaccharides; ×, disaccharides; ○, monosaccharides.

There was an initial period during which no detectable hydrolysis of galactomannan was found. This varied from 16 hr in lucerne to 3–4 days in guar. This lag was followed by a rapid and complete depletion which varied from 20 hr for lucerne to 4–5 days for carob and guar (cf. fenugreek seed[6]). The levels of galactomannan hydrolysing activity were measured during germination of lucerne by following the rate of reducing sugar release from lucerne galactomannan substrate incubated with extracts of the germinating seeds (Fig. 2). No measurable activity was found in the flour and during a lag period corresponding to the time when galactomannan was not hydrolysed. There was then a rapid increase that

corresponded with the period of galactomannan depletion in the seed followed by a decline after all the galactomannan had been hydrolysed.

One of the activities involved in the breakdown of galactomannan and raffinose type oligosaccharides is α-D-galactosidase, which can be readily assayed with *o*-nitrophenyl α-D-galactopyranoside (*o*-NPGal). When this activity was measured in germinating seeds from all four species, the patterns shown in Fig. 3 were obtained. Low activities were present in the flours; these increased on germination until the depletion of galactose oligomers and polymers and then decreased.

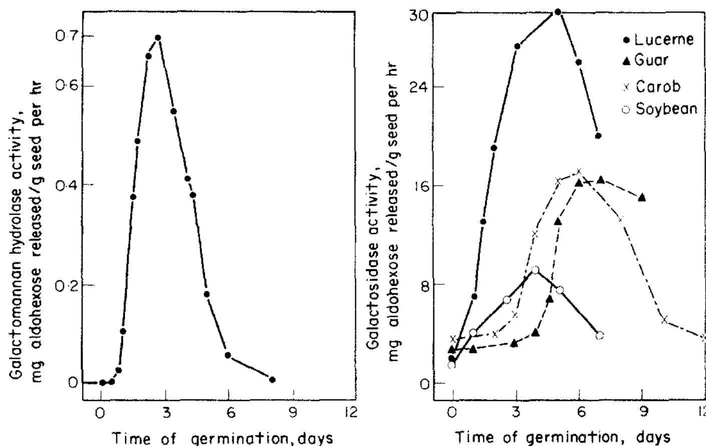

Fig. 2. Changes in galactomannan hydrolysing activity on germination of lucerne.

Fig. 3. Changes in total α-galactosidase activity on germination.

Fractionation and purification of the α-galactosidase activities, using $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography on DEAE, CM- and SE-celluloses and gel filtration, showed that at least four enzymes were involved.

In lucerne seed, extracted 3 days after imbibition, all α-galactosidase activity was found in the fraction precipitating between 20–60% $(NH_4)_2SO_4$ saturation. After dialysis, chromatography on DEAE-cellulose (with an unbuffered KCl gradient) gave two α-galactosidase fractions. One of these was not adsorbed (α-galactosidase A) and the other was eluted at a KCl concentration of 0·25 M (α-galactosidase C) (Fig. 4). Re-chromatography of each peak gave single fractions with the same elution behaviour. Further chromatography of each of these on Sephadex G-100, CM-cellulose or SE-cellulose gave no additional separation. α-Galactosidase A was firmly bound to CM-cellulose and only 20–40% activity was recovered with a 0·005–0·5 M acetate (pH 5) gradient. Stepwise increases in buffer concentration up to 2 M gave peaks of activity at each concentration change. However, SE-cellulose (0·005–0·3 M gradient, acetate buffer pH 5) produced a single peak of activity and 55–80% recovery. Extracts from guar seedlings at 6 days of germination also gave two peaks of α-galactosidase activity on DEAE-cellulose chromatography and these had the same elution characteristics as the lucerne enzymes. Lee[13] detected only one α-galactosidase in guar corresponding to α-galactosidase C. However, he used Somogyi estimation of galactose released from guaran to assay fractions. This method has a low sensitivity and, in fact, diagrams of his chromatograms do show a fraction of low activity that corresponds to α-galactosidase A.

Chromatography on DEAE-cellulose of extracts from soybean seeds that had germinated for 3 days gave three active fractions. Two of these were similar in elution behaviour to α-galactosidases A and C of lucerne and the third eluted at a KCl concentration of 0·05 M (α-galactosidase B) (Fig. 4). α-Galactosidase C could be separated into two activities (C_I and C_{II}) on Sephadex G-200. Dey and Pridham found that an α-galactosidase from *Vicia faba* could be separated into two activities on Sephadex.[9] This result was confirmed by us. However, chromatography of the broad bean extract on DEAE-cellulose showed that its elution profile resembled α-galactosidase A. Carob extracts also gave three fractions on DEAE-cellulose chromatography, and these resembled α-galactosidases A and C of lucerne and B of soybean. α-Galactosidase B was not always present in seeds harvested at different times. A different species of guar (*Cyamopsis psoraloides*) also contained α-galactosidase B.

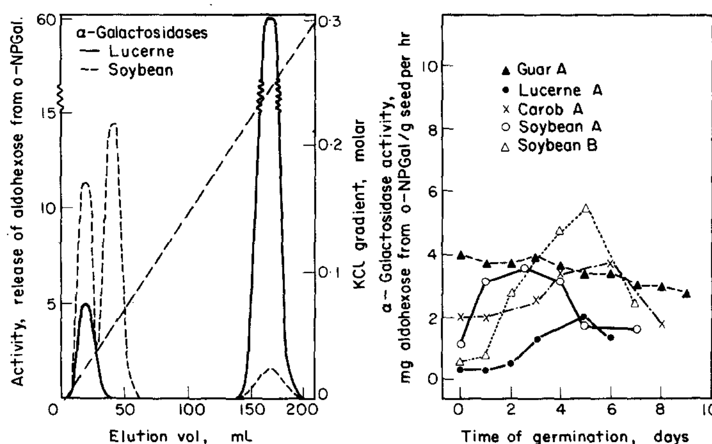

FIG. 4. DEAE-CELLULOSE CHROMATOGRAPHY OF LUCERNE AND SOYBEAN α-GALACTOSIDASES AT 3 DAYS OF GERMINATION.

FIG. 5. CHANGES IN α-GALACTOSIDASES A AND B ACTIVITIES ON GERMINATION.

If seeds were separated into endosperm and cotyledons plus embryo, α-galactosidases A and B were always found in the cotyledon-embryo fraction. In the endosperms, only α-galactosidase C was present. However, in carob seeds some α-galactosidase C was found in the cotyledons as well as the endosperm. On germination, the changes in the levels of activities varied. α-Galactosidase A of lucerne, carob and soybean were present before germination (Fig. 5) and showed a slight increase and decrease on germination, whereas the enzyme from guar decreased gradually. The changes were in the order of 2 scale units of activity. Soybean α-galactosidase B increased more than 4 scale units and then fell. Changes in the levels of α-galactosidase C were much larger except for soybean and the cotyledon-embryo C enzyme of carob (Fig. 6).

In lucerne, carob and guar, seeds that contain significant amounts of galactomannan, the major part of the total α-galactosidase increase on germination is due to α-galactosidase C. This increase in α-galactosidase C at the time of galactomannan depletion and its decrease after depletion, as well as its localization in the endosperm, where all the galactomannan is found, indicates that it is primarily involved in galactomannan hydrolysis. However, since galactosylsucrose oligosaccharides are found in the endosperm of these

three seeds, (cf. fenugreek[6]) it would also be involved in their hydrolysis. In soybean, there is only a slight increase in α-galactosidase C activity. This is consistent with the low galactomannan content. The presence of enzymes A and B in the cotyledon-embryos suggests these are responsible for hydrolysis of the raffinose type oligosaccharides they contain. The changes in levels of activities of these two enzymes parallel the depletion of the oligosaccharides.

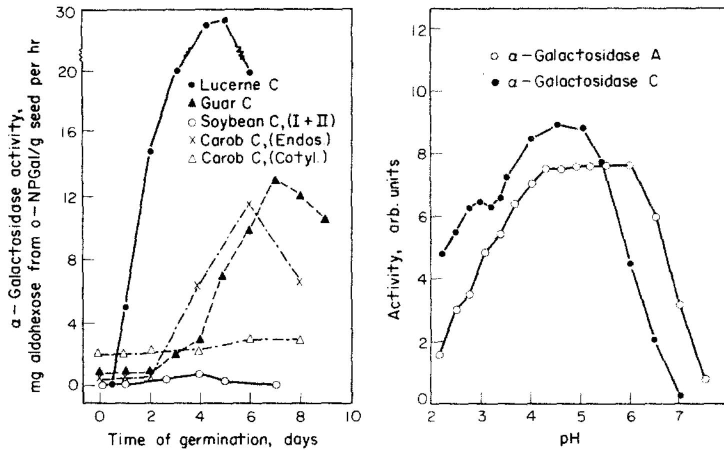

Fig. 6. Changes in α-galactosidase C activity on germination.

Fig. 7. Optimal pH for activity of lucerne α-galactosidases.

The isolation and purification of samples for the studies of enzymic properties was achieved by $(NH_4)_2SO_4$ precipitation, cellulose ion-exchange chromatography, gel filtration and polyacrylamide gel electrophoresis. The preparations for the lucerne enzymes are summarized in Tables 1–3.

Table 1. Purification and separation of lucerne α-galactosidases A and C

Stage of purification	Total protein (mg)	Total activity (μmol/min)	Recovery (%)	Specific activity (μmol/min/mg protein)	Purification
Crude extract	7510	1700	—	0·223	1·0
0–90% $(NH_4)_2SO_4$	6890	1700	100	0·252	1·13
40–60% $(NH_4)_2SO_4$	2260	1020	60	0·450	2·02
DEAE cellulose (pH 5·5)					
Galactosidase A	446	323	80	0·720	3·2
Galactosidase C	130	486		3·75	16·8

α-Galactosidase A from lucerne was free of β-mannosidase and β-mannanase after ion-exchange chromatography and gel filtration. However, α-Galactosidase C needed an extra procedure, gel electrophoresis, to remove β-mannanase. Used preparatively, when the gel was disintegrated and extracted, there was considerable loss of activity (Table 3). For very active preparations, α-galactosidase could be detected on the gel by dipping in M/80 *o*-NPGal in acetate buffer (0·5 M, pH 5·0).

TABLE 2. FURTHER PURIFICATION OF LUCERNE α-GALACTOSIDASE A

Stage of purification	Total protein (mg)	Total activity (μmol/min)	Recovery (%)	Specific activity (μmol/min/ mg protein)	Purification
DEAE-cellulose (pH 6)	53·3	260	81	4·90	21·9
Cellex-SE (pH 5)	15·6	147	56	9·4	42·3
Sephadex G-100	4·14	114	77	27·5	123
DEAE-cellulose (pH 6)	1·68	85·8	69	46·8	210

α-Galactosidases A and B of guar, carob and soybean were obtained free of β-mannanase by extraction from dissected cotyledons soon after imbibition. α-Galactosidase C of guar was obtained substantially free of β-mannanase and carob completely free by DEAE- and CM-cellulose chromatography. Soybean α-galactosidase C required only DEAE-cellulose chromatography. β-Mannosidases were removed by chromatography on Sephadex G-100 and ion-exchange celluloses.

TABLE 3. FURTHER PURIFICATION OF LUCERNE α-GALACTOSIDASE C

Stage of purification	Total protein (mg)	Total activity (μmol/min)	Recovery (%)	Specific activity (μmol/min/ mg protein)	Purification
Sephadex G-100	24·7	360	74	14·5	65·2
CM-cellulose (pH 5)	3·9	305	85	77·0	345
Polyacrylamide gel electrophoresis	3·10	122	40	39·0	175*

* Introduced to remove β-mannanase activity.

Contamination with β-mannosidase was detected using a p-nitro-phenyl β-mannoside substrate. Separation from β-mannanase was followed by the appearance of discrete β-mannanase fractions on chromatography. These were assayed using carob galactomannan.

TABLE 4. KINETIC PROPERTIES OF α-GALACTOSIDASES

	Substrate				
	Raffinose	Lucerne galactomannan		o-NPGal	
α-Galactosidase	K_m (mM)	K_m* (mM)	V_{max}†	K_m (mM)	V_{max}†
Lucerne A	67	25·7	125	0·49	186
Lucerne C	11·8	19·1	790	2·36	7850
Guar A	7·9	8·8	2·6	0·42	122
Guar C	22·2	25·8	186	0·63	294
Carob A	12·1	21·9	1·5	0·42	64
Carob B	9·8	33	25·1	0·42	43
Carob C (endosperm)	5·3	25·4	11·0	0·45	66
Carob C (cotyledon)	3·6	20·9	22·0	0·50	68
Soybean A	11·8	11·0	25·5	0·50	44
Soybean B	11·8	11·0	100	0·50	30
Soybean C (1)	22·2	19·7	23·2	0·55	462
Soybean C (2)	67	8·8	17·2	0·50	141

* Determined as mM anhydro galactose (galactomannan contained 43·8% galactose).
† V_{max} values are expressed relative to raffinose equalling 100.

Any contamination of α-galactosidase preparations was detected by comparing the rate of release, from galactomannan, of reducing sugar (by ferricyanide) with the rate of release of galactose (with galactose dehydrogenase). Also, β-mannanase could be sensitively detected viscometrically using carob galactomannan.

Pure α-galactosidase gave a very slight initial decrease in viscosity, whereas even traces of β-mannanase caused a very rapid initial reduction. In some cases the products of hydrolysis were examined chromatographically.

The K_m and V_{max} values for the various enzymes, using as substrates lucerne galactomannan, raffinose, melibiose, stachyose and o-NPGal are shown in Tables 4 and 5. Using the first four substrates, hydrolysis was estimated as galactose released, assayed with galactose dehydrogenase. This value was compared with reducing sugar released, assayed with ferricyanide, to detect any other carbohydrate hydrolases. Carob and guar galactomannans were hydrolysed at rates comparable to lucerne galactomannan. Lucerne and guar α-galactosidases C have noticeably high V_{max} values for galactomannan, in accord with their role in galactomanan hydrolysis. However, this does not hold for carob and soybean. Carob galactomannan has a low galactose content (23%) and the main function of the α-galactosidase C could be to hydrolyse galactose–mannose oligomers produced by the prior action of β-mannanase. The low galactomannan content of soybean (0·1%) would not require an enzyme with high hydrolysis rates.

Table 5. Kinetic properties of α-galactosidases

	Substrate			
	Melibiose		Stachyose	
α-Galactosidase	K_m (mM)	V_{max}†	K_m (mM)	V_{max}†
Lucerne A	8·7	9·5	67	10·0
Lucerne C	20·0	33	n.d.	n.d.
Guar A	4·5	20	18·8	13·6
Guar C	28·6	40	100	17·0
Carob A	2·5	7·8	17·9	16·7
Carob B	4·0	33	10·0	40
Carob C (endosperm)	5·4	31	67	30
Carob C (cotyledon)	10·0	28	n.d.	n.d.
Soybean A	n.d.*	n.d.	8·3	22·1
Soybean B	5·6	15·2	30	25·0
Soybean C (**1**)	3·6	20·3	100	11·7
Soybean C (**2**)	4·0	35	100	7·1

* Not determined.
† V_{max} values are expressed relative to raffinose equalling 100.

The affinities of lucerne and guar α-galactosidases C are of the same order for both raffinose and galactomannan. The much higher V_{max} for galactomannan suggests a multiple attack on galactose side chains by a single enzyme molecule on a single galactomannan molecule. The V_{max} for lucerne α-galactosidase C with o-NPGal is very high but this is complemented by its much lower affinity for this substrate than the other enzymes.

The pH activity curves for lucerne enzymes are shown in Fig. 7. α-Galactosidase A has a broad pH optimum in the acidic range characteristic of many hydrolases. α-Galactosidase C has a double pH optimum. This has been previously observed for α-galactosidases from a number of sources and Dey and Pridham[14] have discussed the significance of this pattern.

All enzymes were stable at room temperature for extended periods. Lucerne α-galactosidase C was inhibited 75% after heating at 60° for 30 min.

MWs were determined by gel filtration[16] and SDS polyacrylamide gel disc electrophoresis[17] and in one case by ultra-centrifugation using the sedimentation equilibrium method.[18] The results are shown in Table 6. Endosperm α-galactosidases C had MWs of about 23000 except the large MW form from soybean. The cotyledon-embryo enzymes had higher values of about 35000.

TABLE 6. MOLECULAR WEIGHTS OF α-GALACTOSIDASES

α-Galactosidase	Molecular weight (daltons)	Method of determination
Lucerne A	34000	Gel filtration
	33000	SDS-polyacrylamide electrophoresis
Lucerne C	23000	Gel filtration
	21000	SDS-polyacrylamide electrophoresis
	23000	Sedimentation equilibrium
Guar A	34000	Gel filtration
Guar C	24000	Gel filtration
Carob A	37000	Gel filtration
Carob B	37000	Gel filtration
Carob C (endosperm)	23000	Gel filtration
Carob C (cotyledon)	23000	Gel filtration
Soybean A	40000	Gel filtration
Soybean B	40000	Gel filtration
Soybean C (**1**)	130000 to 150000	Gel filtration
Soybean C (**2**)	25000	Gel filtration

EXPERIMENTAL

Plant material. Legumes used were lucerne (*Medicago sativa* cv. Hunter River), guar (*Cyamopsis tetragonolobus*), carob (*Ceratonia siliqua*) and soybean (*Glycine max* cv. Lee). Seeds were germinated in the dark. After soaking in 0·5% NaOCl for 10 min and washing, lucerne was germinated on filter paper in Petri dishes at 25°. Guar and soybean were soaked for 2 hr and carob was scarified and soaked for 18 hr and each planted in perlite wetted with 9 ppm of 5,5'-dichlor 2,2'-dihydroxy diphenyl and then incubated at 30°.

PC and TLC. Solvents used were A, *n*-BuOH:pyridine:H_2O:C_6H_6 (5:3:3:1); B, EtOAc–HOAc–HCOOH–H_2O (18:3:1:4) and C, *n*-PrOH:$MeNO_2$:H_2O (5:2:3). Compounds were detected with *p*-anisidine HCl[19] and $AgNO_3$.[20] Silica gel-Kieselguhr (3:1) was used for TLC and detection was with 50% H_2SO_4.[21]

Maceration. Wet mixtures were macerated in an ultra-turrax and flours were made in a water-cooled mill. Solns were evaporated below 40°.

Extraction of galactomannan. Ungerminated seeds, as flour, or whole germinated seeds were macerated in EtOH, the mixture boiled and macerated again. After filtration, the residue was washed with EtOH, Me_2CO and Et_2O. The dry residue was macerated in 0·01 M $HgCl_2$ soln. After 18 hr it was again macerated and centrifuged (24000 *g* for 10 min at 25°). Extraction of the residue was repeated 2×. The combined supernatants were precipitated in EtOH (4 vol.) and polysaccharide collected by centrifugation. The supernatant was combined with the solvent extract. the ppt. was dispersed in H_2O by maceration and centrifuged (24000 *g* for 30 min at 25°). The supernatant was added to EtOH (4 vol.), the ppt. collected by centrifugation and washed with EtOH, Me_2CO and Et_2O and dried.

Extraction of mono- and oligosaccharides. The solvent extract and the aq. EtOH supernatant from the preparation of galactomannan were combined and reduced in vol. to 50 ml and extracted 3× with $CHCl_3$. The aq.

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phase was deionized (IRA410 and AG50W-X8). After concn, EtOH was added to ppt. any traces of polysaccharide. The supernatant after centrifugation was concn to 3 ml/g of seed. This soln was used directly for PC (solvents A and B) and TLC (solvent C) and diluted 6 × for chromatography on Bio-Gel P-2. Carbohydrates were eluted with 0·1 M NaCl containing 1 mM EDTA and estimated by the anthrone method.[22] Fractions were examined by PC and TLC (solvents A, B and C).

Purification of galactomannan via the copper complex.[27] Fehlings soln (16 ml) was added to galactomannan (1 g) dissolved in H_2O (750 ml) in centrifuge tubes. After vigorous stirring and centrifugation (2000 *g* for 10 min at 25°) the ppt. was dispersed in H_2O (360 ml) in the same tubes. using a macerator. M HCl was added dropwise, with stirring, until the ppt. dissolved and the soln was dialysed. After centrifugation (14000 *g* for 30 min at 25°) the supernatant was poured into EtOH (3 vol.) the ppt. collected, washed and dried.

Preparation of extracts for assay of changes of galactomannan-hydrolyase and α-galactosidase activities on germination. Lucerne, guar, carob or soybean (original whole seed weight 5 g) as a flour at 0 hr and as seeds at later times were crushed in 0·1 M acetate buffer (pH 5) and dialysed against buffer at room temp. for 6 hr. The mixture was then finely ground in an all-glass Tenn–Broeck homogenizer and dialysed a further 18 hr (lucerne and soybean) or 40 hr (guar and carob) at room temp. to complete hydrolysis of galactomannan. After centrifugation (24000 *g* for 30 min at 2°). $(NH_4)_2SO_4$ was added to the supernatant and the ppt. produced between 20–60% saturation, which contained all the activity, was collected and re-dissolved in 0·01 M acetate buffer (pH 5·0) and dialysed against buffer for 20 hr at 2°. After centrifugation, the supernatant was made up to a vol. of 4 ml per g original seed weight and assayed using the release of *o*-nitrophenyl from *o*-NPGal and the increase in reducing power on hydrolysis of carob galactomannan. Carob galactomannan (Sigma) was purified by dissolving in H_2O using maceration, centrifuging (24000 *g* for 30 min at 25°), precipitating into EtOH, re-dissolving, centrifuging again, re-precipitating into EtOH, washing with EtOH, Me_2CO and Et_2O and drying. Aliquots of the enzyme extracts were chromatographed on DEAE-cellulose (Cl-form) with 0·01–0·3 M KCl elution both with and without 0·01 M acetate buffer (pH 6·0). Other aliquots were chromatographed on Sephadex G-100 using 0·025 M acetate buffer (pH 5·0) for elution. Fractions were assayed for ability to hydrolyse *o*-NPGal and to produce reducing activity with carob galactomannan.

Assay of α-galactosidase activity with o-NPGal. Nitrophenyl release was measured by incubating enzyme preparation (0·1 ml) with M/80 *o*-NPGal (Sigma) (0·1 ml) in 0·05 M acetate buffer (pH 5) at 30°. Reaction was stopped with 2% Na_2CO_3 (2·8 ml) and the A at 420 nm determined.[23]

Assay of α-galactosidase activity by measurement of D-galactose released using D-galactose dehydrogenase.[24] Enzyme preparation (0·1 ml) was incubated with substrate solution (1 ml) in 0·025 M acetate buffer (pH 5) at 30°. Reaction was stopped by heating to 100° for 2 min. An aliquot (0·5 ml) was transferred to a 1 cm quartz spectrophotometer tube containing Tris–HCl buffer (pH 8·5, 0·1 M–2·4 ml) and 0·013 M NAD (0·1 ml). D-Galactose dehydrogenase was added and D-galactose present was determined from NAD reduced.

Assay of β-mannosidase activity. Enzyme preparation (0·1 ml) was incubated with M/160 *p*-nitro-phenyl *β*-mannoside (0·1 ml) in 0·1 M acetate buffer (pH 5) at 30°. 2% Na_2CO_3 (2·8 ml) was added and the A at 420 nm determined.

Assay for β-mannanase activity. Enzyme preparation (0·1 ml) was incubated with 0·1% carob galactomannan solution (1·0 ml) at 30° at pH 5. Aliquots (0·1 ml) were assayed for reducing activity by reaction with ferricyanide by the method of Park and Johnson.[25] Viscosity changes were measured by incubation of enzyme preparation (1 ml) with 0·1% carob galactomannan solution (20 ml) in an Ubbelohde viscometer.

Extraction and purification of α-galactosidases. Lucerne seed (300 g) was germinated for 3 days. After maceration in 0·1 M acetate buffer (pH 5) the homogenate was filtered through muslin and centrifuged (24000 *g* for 15 min at 2°). $(NH_4)_2SO_4$ was added to 90% saturation. the ppt. dialysed for 24 hr against 0·1 M acetate buffer. After centrifugation, $(NH_4)_2SO_4$ was added and the fractions at 20% intervals of saturation collected. The fraction with the highest sp. act. (40–60%) was equilibrated against 5 mM KCl by dialysis. This was chromatographed on DEAE cellulose (Cl^- form) using a 0·005–0·3 M unbuffered KCl gradient. The active fractions obtained were concentrated with $(NH_4)_2SO_4$ and dialysed overnight against 0·01 M acetate buffer (pH 5). These fractions were then chromatographed on CM cellulose, Na^+ form or SE-cellulose, Na+ form. They were applied in 0·01 M acetate (pH 5) and eluted with a gradient of the same buffer (0·01–0·3 M). Active fractions were concn using a Diaflo-ultrafiltration cell with a PM10 membrane. α-Galactosidase A was then re-chromatographed on DEAE cellulose, Cl^- form, at pH 6 in acetate buffer using a 0·01–0·3 M acetate buffer gradient and the activity concentrated by ultra-filtration. The various fractions were then chromatographed on Sephadex G-100 using a 0·1 M acetate buffer (pH 5) for elution and the enzymes concn by ultra-filtration. The sample was then subjected to polyacrylamide gel disc electrophoresis by the method of Wrigley.[26] Enzyme preparations were concentrated to 1 mg protein per ml by dialysis against 30% polyethylene glycol for 2–6 hr. Sucrose was added to a final concn

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of 40% and aliquots (100 μl = 100 μg protein) were applied to a series of gel discs (10% cross-linked). Electrophoresis was performed in Tris–glycine buffer (pH 8·5) with a current of 125 mA for 2–3 hr. Brom-thymol blue was used as the indicator of the front. Some tubes were stained with amido black and the remainder cut into 5 mm segments and crushed in a glass Tenn–Broeck homogenizer, centrifuged and the supernatant assayed for activity. In the extraction of α-galactosidases from guar, carob and soybean, to reduce the amount of contamination by protein and especially different α-galactosidase activities, cotyledons were separated from endosperm in the early stages of imbibition as soon as seeds had swelled sufficiently.

Properties of the α-galactosidases. K_m and V_{max} values were determined using as substrate, o-NPGal, melibiose, raffinose, stachyose and lucerne galactomannan, by o-nitrophenyl release, and by reducing sugar release as measured by ferricyanide, and D-galactose released measured by D-galactose dehydrogenase. Lucerne galactomannan was prepared by extraction from lucerne flour that had been boiled in EtOH. No Hg^{2+} was present in the extraction solution. pH Optima were determined for o-NPGal with a 0·1 M citrate–phosphate buffer (pH 2·2–8·0). Inhibition by Hg^{2+} was determined by storing the enzyme for 1 hr in $HgCl_2$ soln at 30° and then assaying with o-NPGal. The molecular sizes of the α-galactosidases were determined by chromatography on Sephadex G-100 and G-200 as described by Andrews.[16] Bovine serum albumin, pepsin, myoglobin, lysozyme, yeast hexokinase and cytochrome C were used as standards in either 0·1 M sodium acetate (pH 5) or 0·1 M Tris–HCl (pH 8·5) buffers. MW was estimated by the method of Shapiro[17] using SDS-polyacrylamide gel disc electrophoresis in Tris-glycine buffer (pH 8·5) on a 10% cross-linked gel and also by ultracentrifugation using the sedimentation equilibrium method[18] (52 hr at 18520 rpm in 0·005 M acetate buffer (pH 5) using a Beckman An-D rotor).

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