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GALACTOMANNAN STRUCTURE AND β-MANNANASE AND β-MANNOSIDASE ACTIVITY IN GERMINATING LEGUME SEEDS

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Key Word Index—Leguminosae; lucerne; carob; honey locust; guar; soybean; β-mannanase; β-mannosidase; galactomannan; seed germination.

Abstract—Structural changes in galactomannan on germination of lucerne, carob, honey locust, guar and soybean seeds, as measured by viscosity, elution volumes on gel filtration and ultra-centrifugation were slight, consistent with a rapid and complete hydrolysis of a molecule once hydrolysis of the mannan chain starts. β-Mannanase activity increased and then decreased, paralleling galactomannan depletion. Multiple forms of β-mannanase were isolated and these were located in the endosperm. β-Mannanase had limited ability to hydrolyse galactomannans with high galactose contents. Seeds containing these galactomannans had very active α-galactosidases. β-Mannosidases were present in both endosperm and cotyledon-embryo and could be separated chromatographically. The level of activity was just sufficient to account for mannose production from manno-oligosaccharides.

INTRODUCTION

Galactomannans are found as a major component of the endosperm in the seeds of a number of the *Leguminosae*, *Palmae*, *Anonaceae*, *Rubiaceae* and *Convolvulaceae*. The D-galactose content of these polysaccharides varies from 10 to 50% according to the species [1,2] and MW determinations have given values in the range of 200000 to 300000 [3,4], although values as low as 50000 have been found [5]. Germination is accompanied by depletion of galactomannan. Low MW products from hydrolysis do not accumulate but appear to be rapidly utilised [6,7]. In the germination of *Gleditsia ferox* the galactose content in the residual polysaccharide has been reported as decreasing to very low levels as germination proceeded [8]. In contrast, no significant change in galactose content was found in fenugreek galactomannan [7].

The known enzymic activities in seeds containing galactomannan are sufficient to explain its complete hydrolysis. α-D-Galactosidase is an exo-polysaccharase [9], β-D-mannanase an endo-enzyme that can hydrolyse mannose oligomers with a DP > 3 [10,11] and β-D-mannosidase is a glycosidase that can hydrolyse mannobiose and mannotriose. It is possibly also an exo-mannopolysaccharase. α-Galactosidase and β-mannanase activities increase on germination concurrently with galactomannan depletion [6,7 12–15]. Different α-galactosidases are present in endosperm and non-endosperm regions and some of these have multiple forms [6]. At least two β-mannanases have been separated in germinating guar seeds [13]. β-Mannosidase activity has been detected at low levels in a number of seeds [14, 16]. In germinating fenugreek a six-fold increase in activity was found during 3 days [14] but no increases were found in seeds of other legumes [16, 17]. A number of these enzymes from fungal sources have also been studied [11]. Most reports of plant β-mannosidases have used unfractionated

extracts. A preparation from germinating guar released mannose from a mannan and was described as an exo-mannanase [13].

In this paper the structural changes in galactomannan during depletion in lucerne, carob, honey locust, soybean and guar seeds, as well as the fractionation, purification and properties of β-mannanases and β-mannosidases have been studied. The properties and changes in the activities of these enzymes are discussed in relation to the changes in the structure of the galactomannan.

RESULTS AND DISCUSSION

Germination was characterized by the depletion of raffinose series oligosaccharides and galactomannan [6]. Residual galactomannans were isolated by cold water extraction, following adequate maceration, at various times after imbibition from all seeds except soybean, where the initial galactomannan content was low. Seeds were extracted with alkali after cold water but only trace amounts of galactomannan were detected. The samples were purified via copper complexing, to remove another water-soluble polysaccharide fraction containing galactose, arabinose and xylose. At the later stages of galactomannan depletion the proportion of this polysaccharide in the water soluble extract was high enough to give significantly different galactose contents of this extract before and after purification. Structural changes were investigated by ultra-centrifugation, gel filtration, viscometry and determination of hexose composition.

Ultra-centrifugation of lucerne galactomannan prior to germination, both before and after purification via the copper complex, and 30 hr after imbibition, showed no differences in sedimentation values. However, the significance of these results was limited by the extremely high dependence of the sedimentation values on concentration. The sedimentation coefficient ($S_{20} \times 10^{13}$) ranged from 10 at a concentration of 0·1% to 4 at 0·4% concn. The Schlieren patterns showed hyperfine sharpening even at low concentrations.

Despite the known MW range of galactomannans, all samples were excluded on Sepharose 2B and controlled pore glass beads up to a pore size of 2000 Å. Retardation was obtained on Merckogel SI 5000 Å and elution volumes for the samples are shown in Table 1. Molecular sizes of residual galactomannans in a particular species did not change as germination proceeded. The one exception found was from carob at 7 days when the MW decreased. However, at this stage very little polysaccharide (about 5% of the original amount) remained. The elution volumes showed only small differences between species, indicating a similarity of molecular size for the galactomannans from the

Table 1. Galactomannan changes on germination

Time from imbibition (days)	Galactomannan in seed (%)	Merckogel SI 5000 Å elution volume (ml)*	Limiting viscosity (ml/g)	Galactose in galactomannan (%)
Lucerne				
0	8·8	48	1250	45
3/4	7·5	48	1300	43
1 1/4	3·5	48	1000	37
0†	8·8	48	1250	n.d.‡
Guar				
0	20·6	48	1320	33
5	14·5	48	1330	28
6	6·5	48	1440	25
Carob				
0	19·0	51	1000	24
5	10·0	51	950	24
7	1·0	60	n.d.	n.d.
Honey locust				
0	18·5	48	1380	23
3	8·0	48	n.d.	23

* Total vol 62 ml. † Before copper fractionation. ‡ Not determined.

various sources. Guar galactomannan has a MW of 2×10^5 to $2{\cdot}5 \times 10^5$ [4] and 2/3 of the molecule has a mannan backbone, that is, there is a mannan chain of about 1000 anhydro-mannose units. Since retardation requires a pore size of 5000 Å and a β1-4 linked anhydromannose unit has a length of 5·2 Å [18], this indicates that the polymer in aqueous solution has the mannan backbone in a fully extended rod conformation. Theoretical calculations have also predicted this [18].

On Sepharose 2B some of the galactomannan was irreversibly adsorbed and the amount was inversely proportional to the galactose content. With lucerne 60% was eluted, with guar 42% and with carob, none. When the galactose content of lucerne galactomannan was reduced to 26% by limited incubation with α-galactosidase free of β-mannanase, the amount of bound material increased to 85%. The lucerne and guar fractions that were eluted showed similar galactose–mannose ratios to the material applied, so differential adsorption of a sample was not due to a separation of molecules with differing amounts of galactose substitution. Possibly the bound molecules have a different distribution of galactose giving unsubstituted sections. Viscometry, a sensitive method of detecting structural differences in rod-like molecules indicated only minor changes in the residual galactomannans as depletion occurred (Table 1). It also showed that copper fractionation caused no depolymerisation. The very high viscosities (*ca* 1000 ml/g) are in agreement with an extended shape in aqueous solution.

The absence of molecular size change in residual galactomannans indicates that hydrolysis of the polymer proceeds rapidly to monomer units once main chain scission is initiated, rather than by a partial depolymerisation of all molecules. However, in lucerne and guar the initial process is a removal of some of the galactose branches from the mannan backbone (Table 1) as the galactose percentages decreased on germination.

β-Mannanase, an endopolysaccharase, reduces β-1,4 mannans to mannotriose and mannobiose. The smallest substrate is the tetrasaccharide. Changes in the total β-mannanase activities on germination of lucerne, carob and soybean are shown in Fig. 1. Like α-galactosidase [6], these first increased and then decreased, paralleling galactomannan depletion. Honey locust and guar

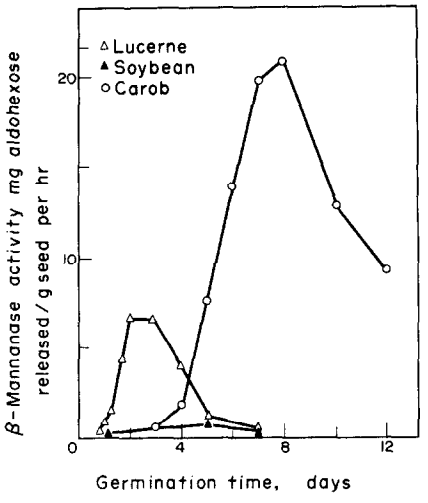


Fig. 1. Changes in total β-mannanase activity on germination.

showed similar patterns. Soybean with a low initial galactomannan content had correspondingly low levels of β-mannanase. If a soluble galactomannan with a low galactose content (carob galactomannan) was used in the assay, similar values were found, after allowing for galactose released by galactosidase. Assay by rate of reduction of viscosity of galactomannan also showed an increase then a decrease in activity, paralleling the reducing sugar values.

The β-mannanase activities in each species were fractionated and purified using cellulose ion-exchange chromatography and gel filtration. For preparative purposes, large seeds were dissected into endosperm and cotyledon-embryo. β-Mannanase activity was precipitated at $(NH_4)_2SO_4$ concentrations of 20–60%. Only one β-mannanase was separated from honey locust. However, polyacrylamide gel electrophoresis showed two very close protein bands of about equal intensity. Extraction of segments of the gel showed that all the β-mannanase activity was in the section containing this double band. The bands were too close to extract separately to determine if both had activity. Honey locust endosperm was the best source for preparative scale isolation of β-mannanase and α-galactosidase, the main reason being, that in comparison to the other seeds, a good separation of α-galactosidase and β-mannanase was obtained using DEAE cellulose chromatography. The α-galactosidase C had sp. act. of 25·2, 17·0 and 1·2 μmol hexose/min/mg protein respectively with *o*-

nitrophenyl α-D galactopyranoside, raffinose and lucerne galactomannan.

Carob seeds contained three β-mannanases and two of them (A and B) were separated on DEAE cellulose. A eluted first and was further divided by CM-cellulose into A2α and A2β. Prior dissection of the seed into endosperm and cotyledon-embryo gave complete separation of α-galactosidase and β-mannosidase from the two forms of β-mannanase A.

Lucerne contained four β-mannanases. Three corresponded to those from carob but the A form separated on Sephadex G-100 into a high MW (A1) and a low MW (A2) fraction. Because it was difficult to dissect a reasonable weight of lucerne seeds on a preparative scale, β-mannanases A1, A2α and A2β still contained α-galactosidase A and β-mannosidase A at the DEAE-cellulose chromatography stage. β-Mannanase A1 was obtained free of α-galactosidase by gel filtration on Sephadex G-100. A2α and A2β were only partially separated from α-galactosidase by this method and CM-cellulose chromatography. β-Mannosidase was removed in all cases by CM-cellulose chromatography. β-Mannanase B could be separated from α-galactosidase C on CM-cellulose with a combined salt–pH gradient (0·04–0·2 M acetate and pH 3·5–4·6 acetate buffer). These had been previously separated by polyacrylamide gel electrophoresis [6]. The final sp act of A1, A2α, A2β and B on carob galactomannan were 0·59, 0·55, 0·65 and 16·5 μmol hexose equivalent released per mg protein per min respectively.

Soybean contained three β-mannanases whose chromatographic behaviour was similar to the three enzymes in carob. The single β-mannanase in honey locust corresponds to the B enzyme in other species and, with the exception of soybean, the B enzyme is at least 70% of the total activity.

All of the β-mannanase preparations showed more than one protein band on polyacrylamide gel electrophoresis. The properties of the various enzymes are shown in Table 2. All the β-mannanases were found only in the endosperm and were endohydrolases, as indicated by the rate of reduction of the viscosity of a galactomannan solution relative to the release of reducing sugar, and examination by PC of the hydrolysis products. They showed sharp pH optima ranging from 4·6 to 4·9 and half maximal activity values for A1 of 4·35 and 5·4; for A2α 4·1 and 5·8; for A2β 3·6 and 5·7; and for B 3·8 and 6·0. There were minor differences in their inhibition by Hg^{2+}. The concentrations required for complete inhibition of the β-mannanases were much higher than those required for α-galactosidase inactivation. Lucerne α-galactosidases A and C are inhibited 95 and 96% at Hg^{2+} concentrations of 10^{-6} M. With one exception, the β-mannanases had similar and low MW. There

Table 2. Properties of β-mannanases

β-Mannanase	% of total activity	Hg^{2+} Inhibition, % [Hg^{2+}]M	MW (gel filtration)	K_m* (mM)
Lucerne				
A1	6	86 (10^{-3})	100000	4·6
A2-α	7	93 (10^{-3})	23000	2·4
A2-β	11	100 (10^{-3}) 71 (10^{-4})	23000	0·7
B	76	90 (10^{-3}) 0 (10^{-4})	28000	4·4
Soybean				
A2-α	24	n.d.	23000	10·0
A2-β	21	n.d.	23000	0·3
B	45	n.d.	27000	3·3
Carob				
A2-α	13	n.d.	22000	10·0
A2-β	17	n.d.	22000	0·5
B	70	n.d.	20000	0·4
Honey locust				
B	100	n.d.	22000	0·6

* Using carob galactomannan as substrate.

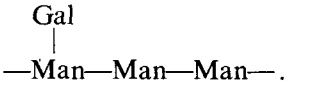
Table 3. Hydrolysis of galactomannans by β-mannanases

Source of galactomannan	Percentage galactose in polymer	V_{max}*	K_m (mM)
Carob β-mannanase B			
Bangalow Palm	0	22	0·6
Carob	24	23	0·3
Gleditsia	23	22	0·5
Kennedia	24	19	0·4
Guar	33	2	1·2
Lucerne	45	1	1·5
Lucerne β-mannanase B			
Bangalow Palm	0	23	6·5
Carob	24	23	4·4
Guar	33	1	4·0
Lucerne	45	1	5·9
Lucerne†	26	23	n.d.

* Relative to a rate of 1 with lucerne galactomannan. † Pretreated with purified α-galactosidase.

were differences in the K_m with a galactomannan substrate.

The hydrolysis of galactomannan was strongly influenced by the amount of galactose branching on the mannan backbone. This is illustrated in Table 3 where the V_{max} values for two purified β-mannanases with a mannan and a range of galactomannans containing increasing amounts of galactose branching are compared. These β-mannanases were free of any α-galactosidase or β-mannosidase. When galactose substitution was low the rate was high but it dropped abruptly when the percentage was more than 25–30. This shows that in lucerne and guar, before rapid depolymerization of the mannan backbone can take place some of the galactose side chains must be removed. The physiological significance of this observation is indicated by the presence of α-galactosidases, capable of high rates of removal of galactose from a galactomannan substrate, in the endosperms of lucerne and guar, seeds that contain galactomannans with high galactose contents. These rates can be compared with the lower rates of the equivalent enzymes from seeds such as carob and honey locust that contain polysaccharides with less galactose substitution [6]. Also, the residual galactomannans of lucerne and guar showed a decrease in galactose content on germination, unaccompanied by depolymerization of the mannan backbone (Table 1), indicating an initial partial removal of galactose side chains prior to scission by β-mannanase. The results also suggest that the minimum possible number of contiguous, unsubstituted mannose units in galactomannan for effective hydrolysis by β-mannanase is two. In carob galactomannan, with about 25% galactose content, if the distribution of galactose along the mannan chain is uniform the repeating unit would be:



Any other arrangement would give some longer sections of neighbouring units free of galactose indicating the minimum requirement for rapid β-mannanase attack is two unsubstituted mannoses (cf Ref. [19]).

A comparison of the effects of α-galactosidase and β-mannanase, on the viscosity of lucerne and carob galactomannans respectively (Fig. 2) showed that β-mannanase caused an immediate and rapid drop in the specific viscosity, whereas the removal of galactose side chains caused no change until the galactose content was somewhere between 26 and 12%. At 12% the viscosity of the solution was much lower but this was due to precipitation of the galactomannan from solution. Thus the removal of some galactose from lucerne and guar galactomannans during germination can be reconciled with the observed limited change in the physical properties found for the residual galactomannans of these two species (Table 1).

The combined actions of α-galactosidase and β-mannanase hydrolyse galactomannans to galactose, mannobiose and mannotriose. Several mechanisms for the production of mannose can be

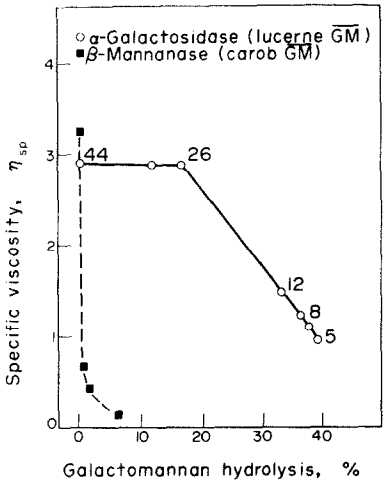


Fig. 2. Viscosity changes on treatment of galactomannan with purified β-mannanase and α-galactosidase.

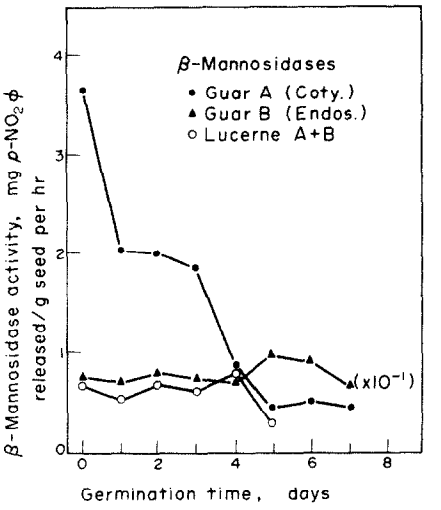


Fig. 3. Changes in activity of lucerne and guar β-mannosidases on germination.

proposed. Among these, one is reversion of manno-di- and trisaccharides and rehydrolysis at the ultimate glycosidic bond. For this process to be effective, high concentrations of oligosaccharides are required. However, only trace amounts of mannobiose and -triose were found at all stages of germination [6].

There is one report of an exo-mannopolysaccharase [13]. In the species examined, no fraction was detected that was unable to hydrolyse manno-oligosaccharides but able to release mannose from a mannan. The β-mannosidases isolated, slowly increased the reducing power of a suspension of mannan.

Another pathway of hydrolysis is by β-mannosidase action after preliminary β-mannanase depolymerization. This mechanism is involved in the fungal breakdown of mannan [11]. β-Mannosidase activity was precipitated at $(NH_4)_2SO_4$ concentrations of 40–80%. Two were separated from all seeds by DEAE cellulose chromatography. Seed dissection showed that one was located in the cotyledonembryo (β-mannosidase A) and the other in the endosperm (B). Enzyme A was not bound on DEAE-cellulose at pH 5·5, B eluted at a concentration near 0·2 M KCl. The levels of activity of β-mannosidase A decreased on germination (Fig. 3 and 4). The endosperm enzyme, unlike the other hydrolases involved in galactomannan depletion, had a constant level of activity.

Further chromatography of the lucerne enzymes on Sephadex and CM and SE-cellulose gave only a 14-fold (A) and 6-fold (B) purification and no further fractionation. The carob enzymes showed similar behaviour. The low levels of activity and losses during purification caused difficulties. The MW of the endosperm enzymes were significantly higher than the other endosperm hydrolases (Table 4). The hydrolysis rate of mannotriitol, a substrate similar in structure to the native substrate [11], would indicate that although the levels of activity are low they are of an order just sufficient to convert the manno-oligomers produced in the germinating seeds to mannose.

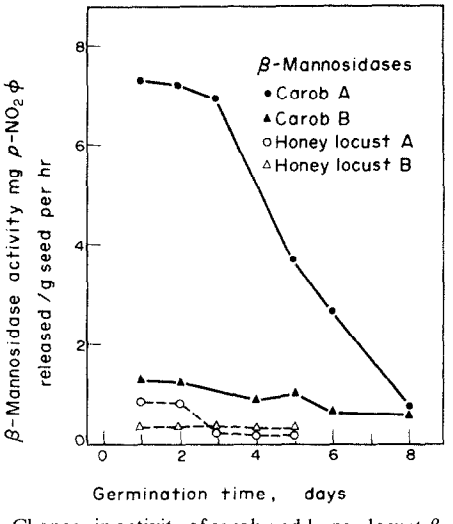


Fig. 4. Changes in activity of carob and honey locust β-mannosidases on germination.

Table 4. Properties of β-mannosidases

Location and form	MW (daltons)	Inhibition 10^{-3}M Hg^{2+} (%)	p-NO_2 Øβ-Man K_m	p-NO_2 Øβ-Man V_{max}	Mannotriitol K_m	Mannotriitol V_{max}*
Lucerne						
Cotyledon A	54000	45	0·29	100	10·0	61
Endosperm B	100000	42	0·83	100	1·2	134
Carob						
Cotyledon A	54000	n.d.	0·29	n.d.	n.d.	n.d.
Endosperm B	100000	n.d.	0·29	n.d.	n.d.	n.d.

* Relative to a rate with p-nitrophenyl β-D-mannoside of 100.

EXPERIMENTAL

Details of the plant materials have been described previously [6] except for honey locust (*Gleditsia triacanthos*), which was treated similarly to guar and soybean. PC and TLC methods previously described were used [6].

Extraction and purification of galactomannan. Powdered or macerated seeds were extracted with cold $HgCl_2$ and purified as previously described [6]. To test if extraction had been complete the residue was stirred in 17% NaOH–3% borate for 18 hr in N_2 and the supernatant, after centrifugation, treated with Cu^{2+}.

Determination of galactose–mannose ratios. After hydrolysis (0·75 M H_2SO_4 at 100° for 3 hr) and neutralisation, total carbohydrate was determined by the anthrone method [20] and galactose, using NAD dependent D-galactose dehydrogenase [21]. Only galactose and mannose were detected on PC.

Protein content. The Folin–Lowry method was used.

Viscosity. Limiting viscosities were determined at 25° in an Ubbelohde suspended level viscometer with a resevoir large enough to allow *in situ* dilution. The solvent was 0·5 M KCl. No kinetic energy or shear correction factors were applied.

Sedimentation velocities. These were determined in 0·5 M KCl in an An-D rotor at 20° and 50000 rpm.

Gel chromatography of galactomannans. Galactomannan soln (0·1% in 0·1 M NaCl and 0·5 mM Na_2 EDTA) was chromatographed on Sepharose 2B, controlled pore glass of nominal pore dia. 200 nm and Merckogel SI of nominal pore dia. 100 and 500 nm. Carbohydrate was measured by the anthrone method [20] and no azide was used in the solvent as it interfered with colour development.

Preparation of water-insoluble mannan from seeds of Bangalow Palm (Archontophoenix cunninghamiana). The seeds were macerated in 10 mM $HgCl_2$ to give chips that were washed with H_2O in a fine sieve. These were extracted with hot EtOH and washed with EtOH, Me_2CO and Et_2O. They were then ground in a H_2O cooled mill. Coarse powder was extracted 2× with 17% NaOH in N_2 and the extract purified by 3 precipitations as the Cu^{2+} complex. After re-dissolving the first complex by acidifying with HCl, the dark insoluble residue was removed by centrifuging (20000 *g*, 15 min). Hydrolysis of the product (0·75 M H_2SO_4 at 100° for 6 hr) and PC examination showed only mannose. The polysaccharide had $[\alpha]_D^{20}$ − 45·5 (c, 0·6, 1 M NaOH).

Preparation of mannobiitol and mannotriitol. Carob galactomannan was incubated with an extract from lucerne of α-galactosidase and β-mannanase [6], that was free of β-mannosidase. The products found by PC and characterized by R_G values and hydrolysis to constituent sugars were Gal, mannobiose and mannotriose. The hydrolysate was boiled, pptd protein removed by centrifugation and $NaBH_4$ added. After 1 hr, excess $NaBH_4$ was removed by adding MeCHO and the soln passed through a cation exchange resin. The eluate was concentrated below 40° until nearly dry and MeOH added and distilled × 4. The reduced sugars were separated by PC on thick paper. They co-chromatographed with the products formed by small scale reduction of previously isolated mannobiose and mannotriose and were non-reducing oligosaccharides.

Assay of β-mannanase and β-mannosidase activities on germination. Lucerne, guar, carob, honey locust or soybean (dry 5 g) as whole seeds, dissected endosperms or cotyledons were extracted as previously described [6]. After pptn by $(NH_4)_2SO_4$ and dialysis, preparations were assayed for β-mannanase activity by incubation, in a shaking bath, with a suspn (0·1%) of reduced bangalow palm mannan and estimation of the increase in reducing action by reaction with ferricyanide [22]. This activity was also measured by decrease in viscosity of a 0·1% soln of galactomannan. β-Mannosidase activity was assayed by incubation with p-nitrophenyl β-mannopyranoside and estimation of the nitrophenyl released as previously described for α-galactosidase [6].

Extraction and purification of β-mannanase and β-mannosidase. The method described is that used for lucerne, where the maximum number of enzymes were found. Similar methods were applied to the other seeds after dissection. Lucerne seed (300 g) was germinated for 3 days and extracted as previously described for α-galactosidase preparation [6]. An $(NH_4)_2SO_4$ fraction (20–80%) was dissolved in 0·01 M acetate buffer (pH 6) and chromatographed on DEAE cellulose (Cl^-) and eluted with 0·01–0·4 M KCl in 0·01 M OAc^- buffer, pH 6. Two active fractions, each of which had both β-mannanase and β-mannosidase activities, were obtained. In lucerne both also contained α-galactosidase. The retarded fraction separated on Sephadex G-100 into a β-mannosidase (B) and a β-mannanase (B). Both showed some α-galactosidase activity. β-Mannosidase B was chromatographed on CM-cellulose (0·01–0·3 M OAc^-, pH 5) without removal of the α-galactosidase. β-Mannanase B was chromatographed on CM-cellulose using a pH gradient (3·5–5·0, 10 mM OAc^-) to determine the pH of elution of α-galactosidase C [6] and β-mannanase (4·5 and 5·0 respectively). The sample was then applied to a column at 0·1 pH units above the elution pH for the galactosidase and after it passed through the column β-mannanase B was eluted with a salt gradient (0·01–0·3 M, pH 4·6). The first fraction from DEAE-cellulose gave two fractions on Sephadex G-100. One at the void vol, contained β-mannanase and β-mannosidase activities. The second, retarded fraction contained β-mannanase (A2) and no β-mannosidase, but α-galactosidase. The first fraction on CM-cellulose (0·01–2·00 OAc^-, pH 5) separated into β-mannanase A1 (free of α-galactosidase) and β-mannosidase A. β-Mannanase A2 on CM-

cellulose (0·01–0·3 M OAc^-, pH 5) gave two β-mannanases (A2α and A2β), both of which contained α-galactosidase activity.

In the fractionation and purification of β-mannanase and α-galactosidase C from honey locust, seeds that had imbibed for 3½ days were dissected. The cotyledons contained α-galactosidase A and β-mannosidase A. The endosperm contained β-mannanase B (sp. act. 0·1 μmol/min/mg protein), α-galactosidase C (sp. act. 0·5) and β-mannosidase B (sp. act. $2·4 \times 10^{-4}$). From this extract, the 20–60% $(NH_4)_2SO_4$ ppt was collected and chromatographed 2× on DEAE cellulose at pH 5·5 in a 0·01–0·4 M KCl gradient. At 0·24 M KCl, α-galactosidase C (sp. act. 10·5, 70% recovery) with a small amount of β-mannanase B (1% recovery) was eluted. On Sephadex G-100 the sp. act. was increased to 18·6 (61% recovery of original activity) but it still contained a small amount of β-mannanase B. It eluted at 2·25 times the elution vol. of bovine serum albumin. On CMC, using a salt gradient (pH 4, 0·01–0·3 M OAc^-), when α-galactosidase C eluted at 0·09 M, this β-mannanase activity was still not removed. On CMC, with a pH gradient (pH 4·2–5, 0·01 M OAc^-), the α-galactosidase C eluted, free of β-mannanase, at pH 4·65.

On the DEAE column, at 0·34 M, β-mannanase B (sp. act. 1·1, 72% recovery), β-mannosidase B and a small amount of α-galactosidase C (1% of original activity) eluted. On Sephadex G-100 these separated into β-mannosidase B (elution vol. relative to BSA, 0·68) and β-mannanase B (elution vol. 2·6, sp. act. 2·7, 65% recovery) which still contained a small amount of α-galactosidase C (1% of original activity). On CMC chromatography with a salt gradient (pH 4, 0·01–0·3 M OAc^-) the β-mannanase B eluted at 0·06 M with an increased sp. act. (8, 52% recovery) but it still contained a small amount of α-galactosidase C. On CMC chromatography, using a pH gradient (pH 4·2–5, 0·01 M OAc^-), β-mannanase B eluted at pH 4·4, free of α-galactosidase, and with a sp. act. of 11 in a 46% recovery.

In the fractionation and purification of β-mannanases from carob, seeds that had imbibed for 8 days were dissected. The cotyledons contained α-galactosidases A, B and C (1) and β-mannosidase A. The endosperm contained β-mannanases A and B with a combined sp. act. of 0·05, α-galactosidase C (2) and β-mannosidase B. The 20–60% $(NH_4)_2SO_4$ ppt was collected and chromatographed on DEAE cellulose with a KCl gradient (pH 5·5, 0·01–0·4 M). At a salt concn of 0·05 M β-mannanase A eluted (sp. act. 0·58, 11% recovery), at 0·15 M β-mannosidase B eluted and at 0·26 M β-mannanase B (sp. act. 2·3, recovery 82%) plus all the α-galactosidase C (2) activity. On Sephadex G-100 the sp. act. of β-mannanase A was increased to 1·1 with a 9% recovery. It was called A(2) at this stage by comparison with the elution vol. (2·57) of the same enzyme from lucerne. On CMC with a salt gradient (pH 5, 0·01–0·3 M OAc^-) it separated into β-mannanase A 2α (0·05 M, sp. act. 2·7, 3% recovery) and β-mannanase A 2β (0·1 M, sp. act. 11, 3·5% recovery).

In the fraction from DEAE cellulose containing β-mannanase B, on Sephadex G-100 the β-mannanase B eluted at 2·7 times the elution vol. of BSA (sp. act. 9·6, recovery 70%) but still contained 21% of the α-galactosidase C(2) activity. Chromatography on CMC with a pH gradient (pH 4·2–5, 0·01 M OAc^-), when most of the α-galactosidase eluted at pH 4·5 and the β-mannanase at pH 5·0, reduced this to 2·1%. The sp. act. of the β-mannanase was 12·2 and the recovery 65%. On chromatography on CMC with a salt gradient (pH 4·6, 0·01–0·2 M OAc^-) the remaining α-galactosidase C(2) eluted at 0·01 M and the β-mannanase at 0·08 M (sp. act. 21, recovery 63%).

Polyacrylamide gel electrophoresis. This was carried out at pH 8·3 on 10% cross-linked gel.

Properties of β-mannanases and β-mannosidases. K_m values were determined for the β-mannanases at pH 5 using galactomannans from carob, honey locust, *Kennedia rubicunda*, guar and lucerne and the mannan from bangalow palm. The values for the β-mannosidases were determined on mannotriitol and *p*-nitrophenyl β-D-mannopyranoside at pH 5. pH optima were found using carob galactomannan as a substrate for β-mannanases and *p*-nitrophenyl β-D-mannopyranoside as a substrate for β-mannosidases, in 10 mM citrate Pi buffer (pH 2·2–8·0). Molecular sizes were estimated by gel chromatography on Sephadex G-100 using as standards lysozyme, myoglobin, pepsin, hexokinase and bovine serum albumin.

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