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ENZYMIC INTERACTIONS IN THE HYDROLYSIS OF GALACTOMANNAN IN GERMINATING GUAR: THE ROLE OF EXO-β-MANNANASE

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Key Word Index—*Cyamopsis tetragonolobus*; Leguminosae; guar; galactomannan; exo-β-mannanase; α-galactosidase; β-mannanase; endosperms; cotyledons; sugar uptake.

Abstract—Hydrolysis of galactomannan in endosperms of germinating guar is due to the combined action of three enzymes, α-galactosidase, β-mannanase and exo-β-mannanase. α-Galactosidase and exo-β-mannanase activities occur both in endosperm and cotyledon tissue but β-mannanase occurs only in endosperms. On seed germination, β-mannanase and endospermic α-galactosidase are synthesized and activity changes parallel galactomannan degradation. Galactomannan degradation and synthesis of these two enzymes are inhibited by cycloheximide. In contrast, endospermic exo-β-mannanase is not synthesized on seed germination, but rather is already present throughout endosperm tissue. It has no action on native galactomannan. α-Galactosidase, β-mannanase and exo-β-mannanase have been purified to homogeneity and their separate and combined action in the hydrolysis of galactomannan and effect on the rate of uptake of carbohydrate by cotyledons, studied. Results obtained indicated that these three activities are sufficient to account for galactomannan degradation *in vivo* and, further, that all three are required. Cotyledons contain an active exo-β-mannanase and sugar-uptake experiments have shown that cotyledons can absorb mannobiose intact, indicating that this enzyme is involved in the complete degradation of galactomannan on seed germination.

INTRODUCTION

On germination of a range of legume seeds, the endospermic reserve carbohydrate, galactomannan, is degraded and utilized [1]. Hydrolysis of galactomannan is thought to require the presence of at least three enzymes: α-galactosidase (EC 3.2.1.22) for removal of the (1 → 6)-α-D-galactose side chains; β-mannanase (EC 3.2.1.78) for hydrolysis of the (1 → 4)-β-D-mannan backbone into oligosaccharides; and β-mannoside mannohydrolase (EC 3.2.1.25) for complete hydrolysis of β-D-manno-oligosaccharides to D-mannose [2, 3]. The β-mannoside mannohydrolase may be β-mannosidase or exo-β-mannanase [4].

The role of α-galactosidase and β-mannanase in the mobilization of reserve galactomannans has been established [2, 3, 5–8]. Galactomannan degradation is paralleled by an increase in activity of these two enzymes in endosperm tissue and on incubation of galactomannan with a mixture of purified α-galactosidase and β-mannanase, galactose, mannobiose, mannotriose and traces of D-mannose are obtained as the final reaction products. Hydrolysis of β-D-manno-oligosaccharides to D-mannose requires the presence of a β-mannoside mannohydrolase [9]. However, attempts to demonstrate the presence of this enzyme in extracts of germinating legume seeds have met with varying degrees of success. The inability to detect the enzyme in extracts of seeds of a range of legumes, employing either phenyl-β-D-mannoside or β-D-mannobiose as substrate, led to the conclusion that β-mannosidase may not play an important role in the mobilization of galactomannan [10, 11]. It was suggested that phosphorolysis was a possible alternative pathway [10]. A phosphorolytic enzyme capable of catalysing the depolymerization of manno-oligosaccharides, namely oligo-β-D-mannosyl-(1 → 4)-phosphorylase, was subsequently detected in extracts of germinating seeds of fenugreek [12]. However, the possible role of phosphorylases in the degradation of β-D-manno-oligosaccharides in the non-living galactomannan-containing storage tissue of fenugreek seed has been discounted by Reid and Meier [2]. These authors successfully demonstrated the presence of β-mannosidase in the endosperm, and cotyledon tissue of fenugreek seed and found that, during seed germination, the levels of this enzyme in the seed endosperm increased, paralleling galactomannan breakdown. Similar results were reported by Seiler for seeds of *Ceratonia siliqua* (carob) [7].

McCleary and Matheson reported the presence of β-mannosidase in a range of legume seeds, but activity changes on seed germination did not parallel galactomannan breakdown [3]. However, it was considered that the level of enzyme activity present was sufficient to account for the hydrolysis of the β-D-manno-oligosaccharides released on the *in vivo* degradation of galactomannan by α-galactosidase and β-mannanase [3].

An enzyme, tentatively termed exo-β-mannanase, was partially purified from extracts of germinating guar seeds by Lee [13]. This enzyme released D-mannose from the non-reducing end of reduced ivory-nut β-D-mannan (degree of polymerization; d.p. 10–13), and the possibility that the enzyme was, in fact, a β-mannosidase was not excluded. This enzyme has recently been purified to homogeneity and its physico-chemical properties studied [4].

The results of Reid and colleagues [2, 6] and McCleary and Matheson [3] has provided strong circumstantial evidence that β-mannoside mannohydrolase (β-mannosidase or exo-β-mannanase) plays a role in the mobilization of galactomannan in germinating legume seeds. The

aim of the current work was to clearly demonstrate the key role played by this enzyme. The results currently reported, together with those reported in a preceding paper, also provide some insight into the problems associated with the detection of this enzyme in seed extracts and in the quantitation of activity in endosperm and cotyledonary tissue on seed germination.

RESULTS AND DISCUSSION

Extraction of exo-β-mannanase

In the extraction of α-galactosidase, exo-β-mannanase and β-mannanase from guar seeds at various stages of germination, two separate problems were experienced. Extraction of whole seed or cotyledon–embryo material with buffers of low pH resulted in only partial solubilization of exo-β-mannanase whereas α-galactosidase and β-mannanase were solubilized quantitatively (Table 1). In the presence of buffers of low pH and high salt concentration (0.5 M NaCl) the exo-β-mannanase was effectively solubilized, but under these conditions was quite unstable. Quantitative extraction of exo-β-mannanase could be achieved using buffers of high pH (pH 8.0) in the presence of high salt concentrations (0.5 M NaCl) [4]. Under these conditions the enzyme was quite stable. The inability to extract exo-β-mannanase with buffers of low pH and low salt concentration (0.1 M) appeared to be due to the interaction of the enzyme protein with other insoluble protein material in the seed extract [4].

Extraction of exo-β-mannanase from endosperm tissue posed a second problem. Unlike α-galactosidase and β-mannanase, endosperm exo-β-mannanase is not synthesized on seed germination (see Fig. 1) but, rather, is already present in the dormant seed and is distributed throughout the endosperm. Thus effective extraction of the enzyme required complete dissolution of the endospermic galactomannan. This in practice could only be achieved for dormant seeds by allowing the homogenized seed material to slowly hydrate over *ca* 20 hr. However, where seeds had germinated with concomitant synthesis of β-mannanase, extraction of exo-β-mannanase was greatly simplified due to the galactomannan-solubilizing action of the β-mannanase. This effect of β-mannanase on the ease of extraction of exo-β-mannanase and the increase in β-mannanase on seed germination could lead to an erroneous conclusion that exo-β-mannanase (β-mannosidase) is synthesized on germination. In the current studies, exogenous *Irpex lacteus* β-mannanase was added to extracts of endosperms and of whole seeds to catalyse galactomannan solubilization and release of exo-β-mannanase.

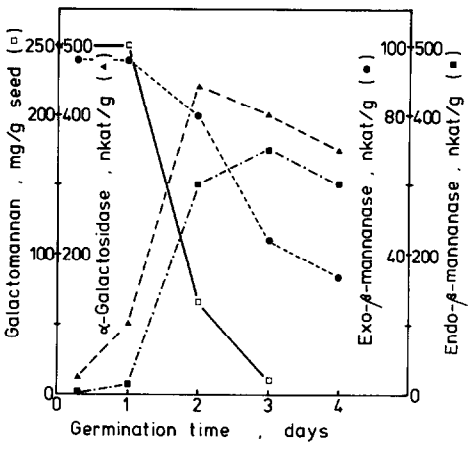

Fig. 1. Changes in the levels of galactomannan and of endospermic α-galactosidase, β-mannanase and exo-β-mannanase on germination of guar.

Enzyme activity and galactomannan changes on seed germination

Galactomannan depletion on germination of guar is accompanied by a parallel increase in activity of endospermic α-galactosidase and β-mannanase (Fig. 1). In similar studies previously reported, a correlation between galactomannan breakdown and increase in activity of α-galactosidase and β-mannanase was also found. However, the seeds used in that study were less vigorous than those currently used and thus the time required for the induction of galactomannan mobilization was significantly different. The seed used in the current study was obtained from the previous years harvest stored over desiccant at 4°.

Unlike α-galactosidase and β-mannanase, the levels of endospermic β-mannoside mannohydrolase (exo-β-mannanase) did not increase on seed germination. Rather, the level remained constant until galactomannan breakdown commenced and then there was a decrease in activity. This is in agreement with results previously obtained by us [3] with a range of legume seeds, but contrasts with results obtained by other researchers with fenugreek [2] and carob [7] seeds. A reinvestigation of the changes in activity of carob β-mannoside mannohydrolase on seed germination, using the current techniques, confirmed results previously obtained, i.e. that the activity of this enzyme in endosperm tissue did not increase on seed germination, but rather remained constant at *ca* 10 nkat/endosperms from 1 g of seed (original dry wt).

Changes in the activity of α-galactosidase and exo-β-

Table 1. Extraction of exo-β-D-mannanase (β-D-mannoside mannohydrolase), α-D-galactosidase and β-D-mannanase from whole guar seeds germinated for 2.5 days at 25°*

Extraction solution	Exo-β-D-mannanase (nkat/g)	α-D-Galactosidase (nkat/g)	β-D-Mannanase (nkat/g)
100 mM acetate buffer (pH 5.0)	30	420	320
200 mM NaCl in 50 mM Tris buffer (pH 8)	160	435	350

*Activity is expressed as nkat/g original dry wt of seed (i.e. per 30 seeds).

mannanase in cotyledon tissue of guar seed on germination are shown in Fig. 2. The levels of both enzymes increased slightly on seed germination and then decreased. In extracts of thoroughly washed cotyledons (to remove adhering endosperm tissue) β-mannanase activity could not be detected at any stage of germination (cf. ref. [6]).

On incubation of whole guar seed or of isolated endosperms in the presence of cycloheximide, there was no increase in endospermic α-galactosidase (Fig. 3) or β-mannanase activity nor was there any detectable galactomannan degradation. Furthermore, there was no significant change in the level of exo-β-mannanase, whereas in seeds incubated in the absence of cycloheximide the level of activity decreased sharply, paralleling galactomannan degradation (Fig. 3). The decrease in endospermic exo-β-mannanase activity on seed germination may be due to degradation of the enzyme by proteolytic enzymes synthesized on seed germination or, alternatively, it may simply be due to a greater physical instability of the enzyme in solution than in the undegraded endosperm tissue. The enzyme is very unstable in aqueous solutions

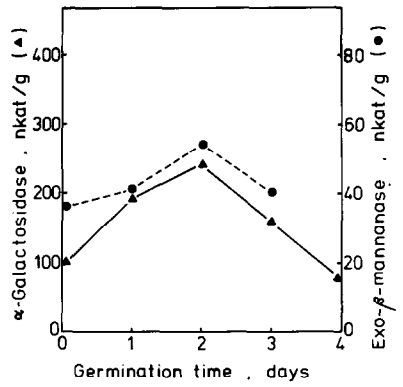

Fig. 2. Changes in the levels of α-galactosidase and exo-β-mannanase in cotyledons of guar on seed germination.

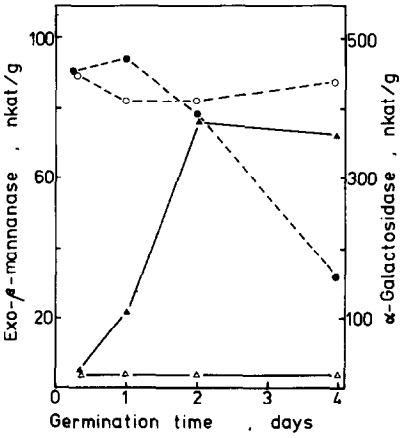

Fig. 3. Changes in the levels of endospermic α-galactosidase and exo-β-mannanase on germination of guar in the presence or absence of cycloheximide (10^{-5} M). α-Galactosidase in the absence of cycloheximide (▲), in the presence of cycloheximide (△); exo-β-mannanase in the absence of cycloheximide (●), in the presence of cycloheximide (○).

below pH 5 and is also unstable to isoelectric focusing [4].

Experiments in which exo-β-mannanase activity was specifically stained with naphthyl-β-D-mannopyranoside–Fast Blue BB showed that the enzyme is located throughout the endosperm tissue. Furthermore, no difference was found in the staining pattern or rate of staining of endosperms from seed allowed to imbibe in cycloheximide solution for 6 hr compared to endosperms from seed germinated in the absence of cycloheximide for 1.5 days. This result further indicates that endospermic exo-β-mannanase is not synthesized on seed germination, but rather is already present in an active form in the dormant seed.

Purification and properties of galactomannan degrading enzymes

The three enzymes thought to be involved in galactomannan degradation in endosperms of germinating guar seed, namely α-galactosidase, β-mannanase and exo-β-mannanase, have been purified to homogeneity and isoelectric focusing patterns are shown in Fig. 4. The properties of the purified exo-β-mannanase have already been discussed in detail [4]. On chromatography of guar extract (from seeds germinated 3 days at 25°) on DEAE-cellulose, two peaks of α-galactosidase activity, termed I (formerly called A) and II (formerly C), were separated. These represented 11% (I) and 89% (II) of the total α-galactosidase activity. In the seed, α-galactosidase I occurs in the cotyledon–embryo and α-galactosidase II occurs in the endosperm. α-Galactosidase I, purified by affinity chromatography (sp. act. 752 nkat/mg), appeared as several protein bands on isoelectric focusing. The protein bands had p*I*s in the range 3.9–5.5 and each displayed α-galactosidase activity (Fig. 4). Affinity-purified α-galactosidase II, in contrast, appeared as a single band on isoelectric focusing (p*I* 3.7) and on SDS-gel electrophoresis (MW 40 500). A much lower MW of 24 000 had previously been obtained for this enzyme by gel-permeation chromatography [8]. The enzyme purified in the current studies had a sp. act. of 870 nkat/mg on *p*-nitrophenyl α-D-galactopyranoside at pH 4.5 and 40° and displayed optimal activity at pH 4.5–5.0 and at 45°. On extended incubation the enzyme was unstable at temperatures above 40°.

Guar β-mannanase, purified by substrate affinity chromatography, appeared as a single protein band on SDS-gel electrophoresis (MW 41 700), but on isoelectric focusing the preparation was fractionated into two major protein bands (p*I* 5.35 and 6.1) and three minor protein bands (p*I* 4.8, 5.8 and 6.2) each of which appeared to have enzyme activity. Furthermore, guar β-mannanase purified on several different occasions and at different times of seed germination (2–4 days) always gave the same isoelectric focusing pattern. The two major protein bands and one of the minor components (p*I* 5.8) were recovered separately by slicing isoelectric focusing gels and their properties were studied. These enzymes have also been separated by chromatofocusing. The enzymes had very similar pH activity curves showing optimal activity at pH 4–5. They were stable at temperatures up to 50° (15 min incubation at pH 5). The sp. act. of the whole preparation was 2020 nkat/mg protein on carob galactomannan (0.2%) at pH 4.5 and 40°, however, the sp. act. of the individual components could not be determined due to insufficient enzyme protein. With carob galactomannan as substrate a

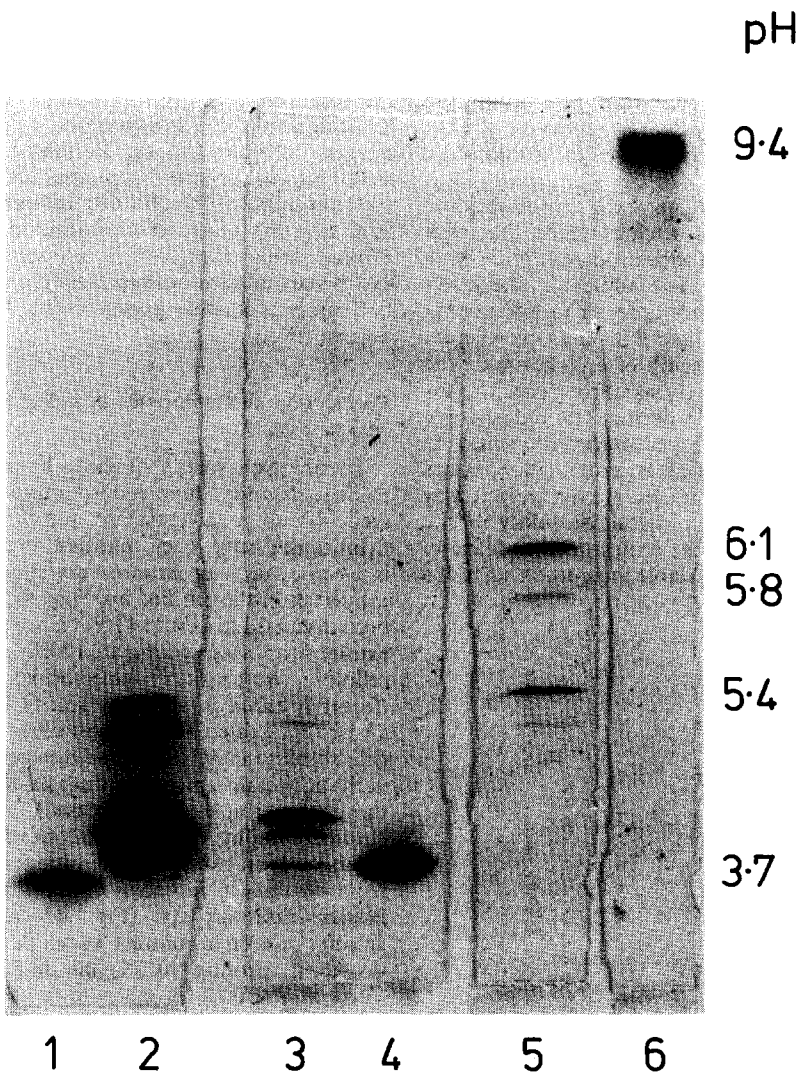

Fig. 4. Isoelectric focusing patterns of purified guar seed galactomannan-degrading enzymes: (1) and (2) stained for activity; (3)–(6) stained for protein. (1) α-Galactosidase II, (2) α-galactosidase I, (3) α-galactosidase I, (4) α-galactosidase II, (5) β-mannanase, (6) exo-β-mannanase.

K_m of 0.03 % (w/v) was obtained for β-mannanase (p*I* 6.1) and a value of *ca* 0.05 % was obtained for both β-mannanases (p*I* 5.35) and (p*I* 5.8).

The action of guar α-galactosidase II and β-mannanase (total) on galactomannan solutions is shown in Figs. 5 and 6. α-Galactosidase II is very effective in the removal of galactose from galactomannan polymers. After 90 min, over 78 % of the galactosyl residues had been removed with only a slight decrease in solution viscosity. The subsequent viscosity decrease was due to an alignment of mannan polymers which eventually precipitated from solution [17]. Hydrolysis by β-mannanase resulted in a rapid decrease in solution viscosity. Endo-hydrolysis by β-mannanase proceeds more rapidly with galactomannans lightly substituted by D-galactose. Thus carob galactomannan (23 % D-galactose) was hydrolysed more rapidly than guar galactomannan (38 % D-galactose), which in turn was hydrolysed more rapidly than lucerne galactomannan (47 % D-galactose). The relative initial rate of hydrolysis of *Leucaena leucocephala* galactomannan compared to carob galactomannan was 14 %. This, together with the fact that the products of hydrolysis of carob galactomannan by guar β-mannanases of p*I* 5.35, 6.1 and 5.8, and by the whole guar β-mannanase preparation, are identical, and are similar to those produced by β-mannanases from germinated seeds of honey locust, lucerne (B) and *Leucaena leucocephala*, indicates that these enzymes have similar action patterns [23]. One major difference is that the guar β-mannanase hydrolysate of carob galactomannan contained essentially none of the tetrasaccharide 6^1-α-D-galactosyl β-D-mannotriose [22], (see also Fig. 2 in ref. [23]) and lesser amounts of mannobiose.

Purified exo-β-mannanase from guar seeds had no detectable activity on carob or guar galactomannan measured either viscometrically or by estimation of mannose reducing sugar levels.

Incubation of guaran β-mannanase limit galactoman-

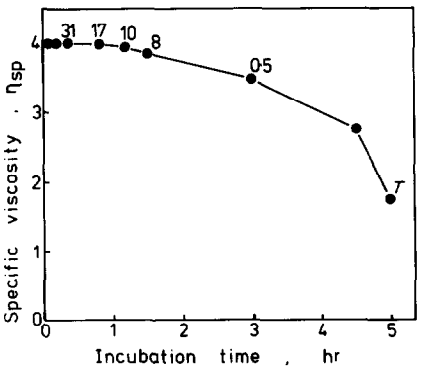

Fig. 5. Hydrolysis of guar galactomannan by guar α-galactosidase II. Galactomannan solution (17 ml, 0.12%) in 0.07 M sodium acetate (pH 4.5) was incubated with guar α-galactosidase II (0.3 ml, 668 nkat/ml on *p*-nitrophenyl α-D-galactopyranoside) in an Ubbelohde suspended-level viscometer at 40°. Samples were removed for determination of released galactose by the *p*-hydroxybenzohydrazide reducing sugar method. Numbers represent the galactose content of the remaining polysaccharide. At point *T*, the solution was very turbid.

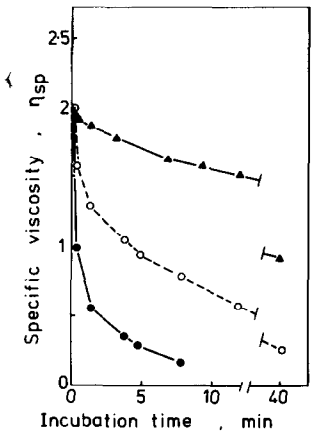

Fig. 6. Hydrolysis of lucerne (▲), guar (○) and carob (●) galactomannans by guar β-mannanase. Galactomannan solution (15 ml, 0.09%) in 0.1 M sodium acetate (pH 4.5) was incubated with total guar β-mannanase (5.7 nkat on 0.2% carob galactomannan) in an Ubbelohde suspended-level viscometer at 40°. In previous experiments [23] the level of β-mannanase used was incorrectly reported as 0.5 nkat. The activity actually employed was 5.0 nkat.

nan with α-galactosidase plus β-mannanase (see Experimental) resulted in hydrolysis to galactose, mannobiose, mannotriose and traces of mannose. If exo-β-mannanase was also included the final hydrolysis products were galactose, mannose and traces of mannobiose.

Effect of α-galactosidase, β-mannanase and exo-β-mannanase on the rate of galactomannan uptake by cotyledons

To determine the relative importance of these three enzymes in galactomannan hydrolysis and uptake by guar cotyledons, endosperm tissue was removed from five germinated seeds, the seeds thoroughly washed and the cotyledons immersed in a solution of galactomannan in the presence of various combinations of the three enzymes. The incubation conditions were chosen to simulate conditions used in the germination studies. The amount of galactomannan employed was that present in five dormant guar seeds [i.e. 240 mg/g seed (1 g ≃ 30 seeds)] and the level of added enzyme was that present in endosperms of seeds germinated at 25° for 2.5 days, the time of maximum rate of galactomannan breakdown. The concentration of galactomannan in imbibed guar seed endosperms is *ca* 16% (w/v). Since native galactomannan is extremely viscous, even at concentrations of 0.4% (w/v), it was necessary to use guar galactomannan partially degraded by β-mannanase, which was considerably less viscous. The material employed has been termed guaran β-mannanase limit galactomannan and is the polymeric material remaining on exhaustive hydrolysis of guar galactomannan by *Irpex lacteus* β-mannanase [14] (degree of hydrolysis *ca* 7%). Guaran β-mannanase limit galactomannan has the same galactose content (38%) as native guar galactomannan [14]. The results of these experiments are summarized in Fig. 7. Carbohydrate uptake is plotted both as mg/g whole seed (i.e. per 30 seeds), and as a percentage of that originally present. The guar cotyledons rapidly absorbed free mannose. The amount of monosaccharide uptake in a given time is greatly dependent on the concentration of the sugar in the external solution. In the current experiments this concentration was 80 mg/ml whereas, in previous experiments [24], a concentration of only 3.75 mg/ml was employed which gave *ca* a seven-fold difference in the amount of mannose absorbed in the first 8 hr. In endosperms from imbibed seeds the concentration of galactomannan is *ca* 160 mg/ml (16% w/v) but in the currently described experiments it was not technically possible to employ solutions of this concentration.

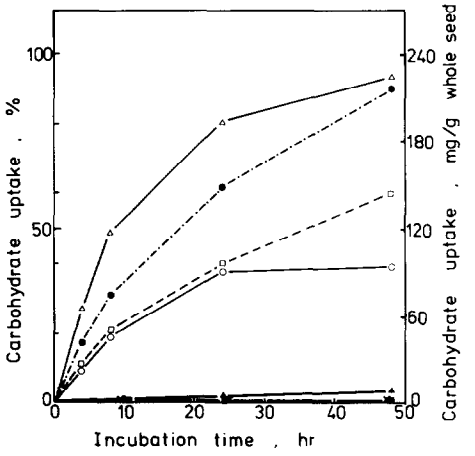

Fig. 7. Hydrolysis of galactomannan and uptake of carbohydrate by guar cotyledons in the presence or absence of galactomannan-degrading enzymes. Cotyledons from five guar seeds (germinated for 2 days) were incubated in solutions of mannose (△), or of guaran-β-mannanase limit galactomannan in the absence of added enzymes (×) or in the presence of: exo-β-mannanase (■); β-mannanase (▲); α-galactosidase (○); α-galactosidase plus β-mannanase (□); α-galactosidase plus β-mannanase plus exo-β-mannanase (●). After various time intervals the carbohydrate in the external solution was determined.

Cotyledons were incubated in solutions of galactomannan such that the amount of galactomannan in solution per cotyledon–embryo was that present in one seed. At a concentration of 160 mg/ml this would have required the use of a volume of only 0.25 ml for the cotyledon–embryos from five seeds. The volume actually employed in the described experiments was 0.5 ml which was the smallest volume which allowed effective immersion of the cotyledons in the solutions of galactomannan plus enzymes.

When guar cotyledons were incubated in the presence of galactomannan there was no apparent uptake of carbohydrate. The guaran β-mannanase limit galactomannan was resistant to hydrolysis by both exo-β-mannanase and β-mannanase and, consequently, in the presence of either of these two enzymes there was little uptake of carbohydrate. α-Galactosidase gave a quantitative removal of galactose from the polymer and this galactose was rapidly absorbed and further utilized. The mannan polymer remaining on removal of the galactose [17] interacted to form a rigid, rubber-like gel which encased the cotyledons. When a combination of α-galactosidase and β-mannanase were employed the galactomannan was rapidly hydrolysed to galactose and manno-oligosaccharides of degree of polymerization (d.p.) 2–5. The galactose was rapidly absorbed but uptake of manno-oligosaccharides occurred more slowly (Fig. 8). After 48 hr there was only 60% uptake of carbohydrate, whereas in whole seeds in this period of time and under these incubation conditions (25°), the galactomannan was completely hydrolysed and quantitatively absorbed by the cotyledons. However, when the *in vitro* incubations employed the three enzymes α-galactosidase, β-mannanase and exo-β-mannanase, galactomannan degradation was complete and carbohydrate uptake essentially quantitative within 48 hr. This result clearly de-

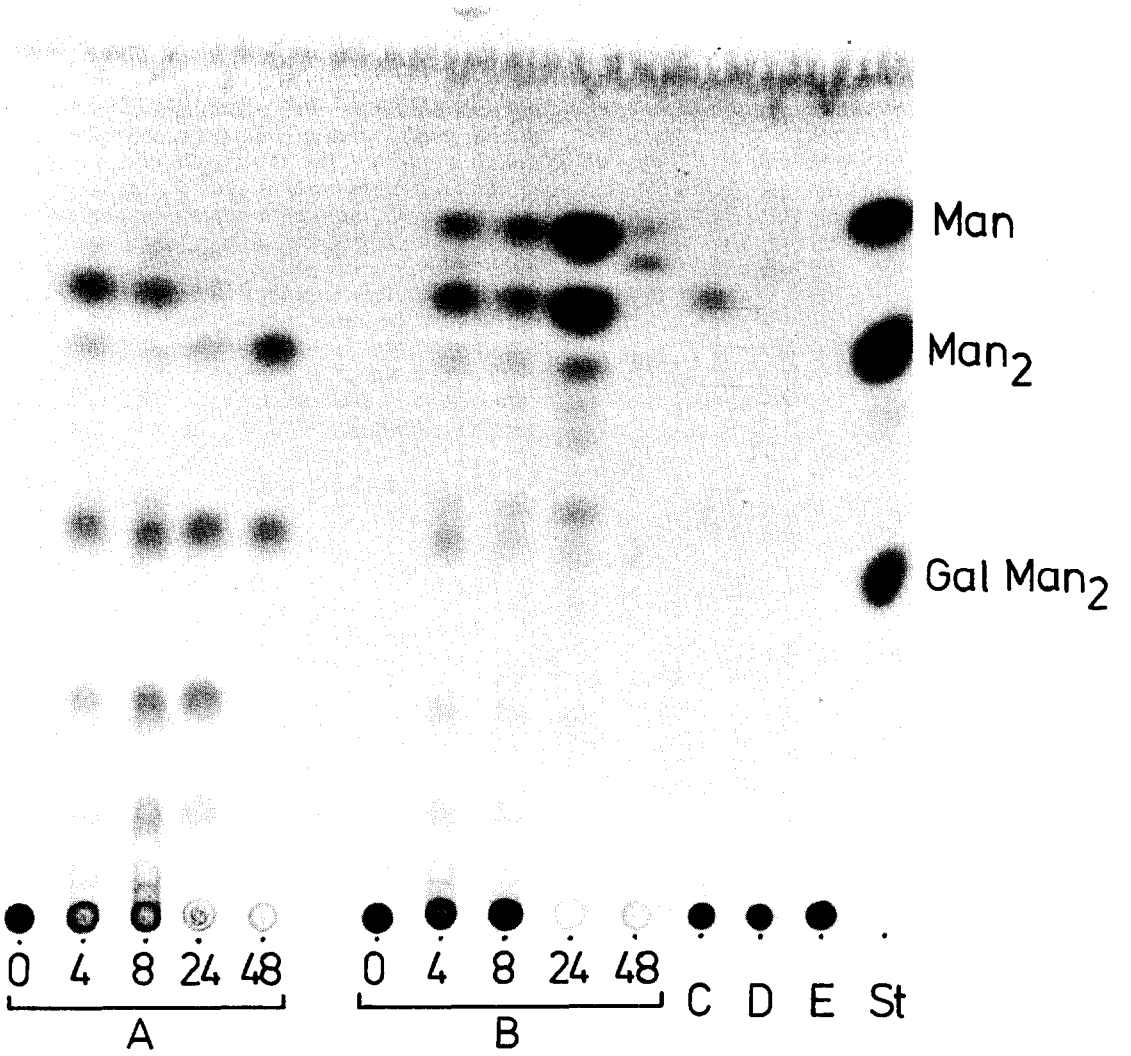

Fig. 8. TLC of the oligosaccharides remaining in the external solution on incubation of guar seed cotyledons in solutions of guaran β-mannanase-limit galactomannan plus enzymes for up to 48 hr. (A) α-Galactosidase plus β-mannanase; (B) α-galactosidase plus β-mannanase plus exo-β-mannanase; (C) α-galactosidase (48 hr); (D) β-mannanase (48 hr); and (E) exo-β-mannanase (48 hr); (St) standard. After incubation the reaction was terminated by heating to 100° for 2 min, the solutions were deionized and concentrated to 2 ml and the aliquots (20 μl) examined by TLC (solvent b).

monstrates the key role played by exo-β-mannanase in the hydrolysis of galactomannan in germinating guar seeds and also indicates that the above three enzymes are sufficient to account for galactomannan mobilization in these seeds.

It was previously mentioned that an active exo-β-mannanase, is also present in guar cotyledons. This enzyme, which appears to be identical to the endosperm form [4], may participate in the complete degradation of galactomannans to monosaccharides by hydrolysing manno-oligosaccharides absorbed by the cotyledons. The uptake of mannose, manno-oligosaccharides and reduced manno-oligosaccharides by guar cotyledons is shown in Fig. 9. Mannose, mannobiose and mannotriose are all rapidly absorbed. Mannitol is absorbed less rapidly and there is no apparent uptake of mannobiitol or mannotriitol. In fact, in the presence of the latter two sugar alcohols the cotyledons exude sugar to the external solution giving negative carbohydrate uptake values. The sugar exuded was mainly glucose and the reason for this phenomenon is at present unclear.

An interpretation of the relative rates of uptake of manno-oligosaccharides by cotyledons is complicated by the presence of an active exo-β-mannanase on the external surface of the cotyledons. *Ca* 0.2 nkat of activity on *p*-nitrophenyl β-D-mannopyranoside at 25° was present on the cotyledons of one seed. This enzyme could not be removed by washing the cotyledons with salt, buffer or detergent (0.1 % Triton X-100) solutions. The presence of this enzyme introduced the possibility that the manno-oligosaccharides might be hydrolysed just prior to uptake. However, studies on the rates of uptake of mannopentaose and mannopentaitol provide some information on the relative significance of uptake of intact manno-oligosaccharides compared to hydrolysis just prior to uptake. The apparent rates of uptake of mannopentaose and mannopentaitol are the same, but it is unlikely that mannopentaitol is absorbed intact as mannobiitol and mannotriitol are not. Rather, this rate of uptake more likely reflects the rate of hydrolysis of these oligosaccharides by the exo-β-mannanase attached to the external surface of the cotyledons. Evidence supporting this proposal has been obtained by chromatographic examination of the carbohydrate remaining in the external solution after various times of incubation of mannopentaose and mannopentaitol in the presence of cotyledons. It was found that the mannopentaose and mannopentaitol were hydrolysed to a series of lower d.p. oligosaccharides and mannose.

Purified exo-β-mannanase hydrolyses mannobiose at *ca* 12 % and mannotriose at *ca* 20 % the rate of mannopentaose, yet the rate of absorption of these two oligomers by guar cotyledons is *ca* three-times the rate of uptake of mannopentaose. Thus, most of the mannobiose and mannotriose must be absorbed intact, indicating that cotyledonary exo-β-mannanase is involved in the complete hydrolysis of galactomannan to monosaccharides on the germination of guar.

The results of the foregoing studies are summarized in Fig. 10. On seed germination, endospermic α-galactosidase and β-mannanase are synthesized (most probably in the aleurone layer) and catalyse galactomannan degradation to D-galactose and β-D-manno-oligosaccharides of d.p. 2–5. These manno-oligosaccharides are further hydrolysed to mannose by an exo-β-mannanase already present in the seed endosperms. Some of the mannobiose and mannotriose, produced on the combined action of these enzymes on galactomannan, is absorbed by the cotyledons and further hydrolysed by a cotyledonary exo-β-mannanase. The monosaccharides are phosphorylated and further metabolized by the developing cotyledon–embryo.

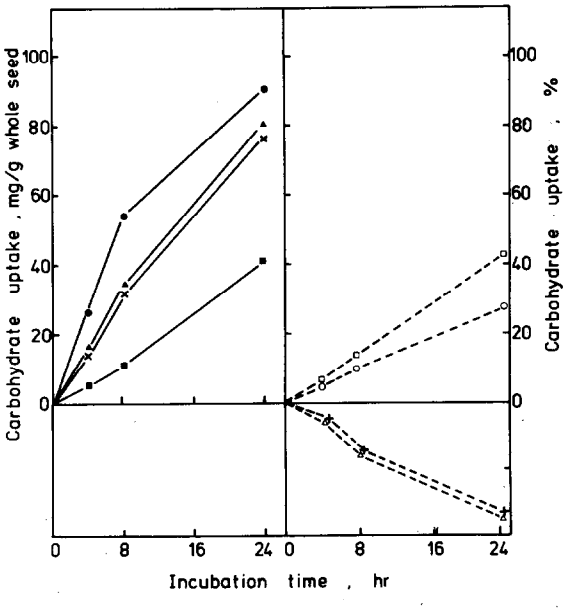

Fig. 9. Rate of uptake of mannose, mannitol, manno-oligomers and reduced manno-oligomers by guar seed cotyledons. Experimental conditions are described in the text. Mannose (●), mannobiose (▲), mannotriose (×), mannopentaose (■), mannitol (○), mannobiitol (△), mannotriitol (+) and mannopentaitol (□).

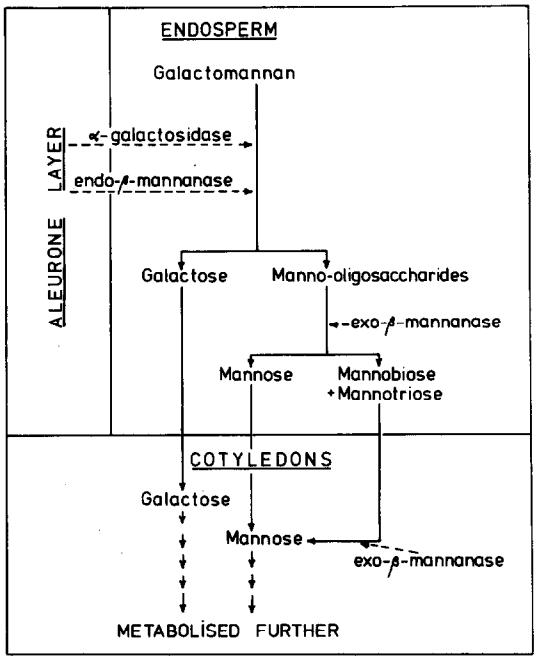

Fig. 10. A proposed scheme for the enzymic interaction in the degradation of endosperm galactomannan to monosaccharides.

EXPERIMENTAL

Plant material. Seeds of guar (*Cyamopsis tetragonolobus* (L) Taub cv CP 177) were obtained from Mr. I. Parkin, Fielder-Gillespie, Albion, Queensland. The seeds (1980 harvest) were stored over desiccant at 4°. Seeds were surface sterilized and germinated as previously described [8]. In excess of 95% of the seeds were viable and germinated under these conditions.

TLC. This was performed on Merck DC-Alufolien Kieselgel 60 (0.2 mm) plates, which were developed with either (a) *n*-PrOH–$MeNO_2$–H_2O (5:2:3) or (b) *n*-PrOH–EtOH–H_2O (7:1:2) (twice). Spots were detected by spraying with 5% H_2SO_4 in EtOH and heating at 110° for 5–10 min.

Isoelectric focusing. This was performed as previously described [4].

Staining exo-β-mannanase activity in polyacrylamide gels and in seed sections [14]. The substrate soln was prepared by dissolving naphthyl β-D-mannopyranoside [4] (250 mg) in a mixture of Me_2CO (12.5 ml) plus 0.5 M acetate buffer (pH 5.5, 12.5 ml) and it was stored at 4° between uses. The enzyme stain was prepared immediately before use by adding Fast Blue BB salt (20 mg) to a soln containing the substrate (0.6 ml), 0.5 M acetate buffer (pH 5, 5 ml), and H_2O (15 ml). The gels or seed sections were incubated in the enzyme stain soln at 40° for 5–20 min.

Assay of α-galactosidase [4], *exo-β-mannanase* [4] *and β-mannanase* [15]. Assays were performed as previously described [4, 15]. Activity of α-galactosidase and exo-β-mannanase (nkat) is defined as the amount of enzyme required to release 1 nmol *p*-nitrophenol from *p*-nitrophenyl α-D-galactopyranoside (at pH 4.5) or from *p*-nitrophenyl β-D-mannopyranoside (at pH 5.5) in 1 sec at 40°. 1 nkat β-mannanase is the amount of enzyme releasing 1 nmol mannose reducing sugar equivalents from carob galactomannan (0.2%) at pH 4.5 and 40° in 1 sec. Unless otherwise stated, the activity of α-galactosidase reported throughout this paper is that on *p*-nitrophenyl α-D-galactopyranoside; of exo-β-mannanase is that on *p*-nitrophenyl-β-D-mannopyranoside; and of β-mannanase is that on 0.2% (w/v) carob galactomannan.

Changes in activity of α-galactosidase, β-mannanase and exo-β-mannanase on seed germination. Seeds were germinated at 25° for up to 4 days. At various time intervals the seeds were dissected and cotyledon–embryo and endosperm tissues separated. Cotyledons were suspended in a soln (25 ml) of 0.2M NaCl plus bovine serum albumin (1 mg/ml) in 50 mM Tris–HCl buffer (pH 8) and gently agitated to remove adhering endosperm-galactomannan material (present in significant amounts at later stages of germination). This soln was added to the dissected endosperms which were then blended with an Ultraturrax apparatus and incubated with purified Driselase (*Irpex lacteus*) β-mannanase (100 nkat/sample) for 4 hr at 30° (to solubilize galactomannan). The samples were then re-blended, adjusted to vol. (50 ml) and centrifuged (14 000 g, 10 min) before assaying. Cotyledons were washed with a further two aliquots of Tris–HCl buffer (pH 8) and then suspended in the same buffer and homogenized using a mortar and pestle. The vol. of the homogenate was adjusted to 25 ml, the suspension centrifuged (14 000 g, 10 min) and assayed for α-galactosidase and exo-β-mannanase activities using *p*-nitrophenyl-α-D-galactopyranoside and *p*-nitrophenyl-β-D-mannopyranoside as substrates. To determine enzyme activity in essentially ungerminated seeds, the seeds were allowed to imbibe in a soln of cycloheximide (10^{-5} M) for 6 hr before dissection and extraction.

The levels of β-mannanase in endosperms and cotyledonary tissue of guar seeds at various stages of germination were determined by extracting plant material with NaOAc buffer (0.1 M, pH 5). The endosperm fractions were blended using an Ultraturrax apparatus and incubated at 30° for 2 hr (to solubilize galactomannan). The homogenate was reblended, centrifuged (14 000 g, 10 min) and dialysed against 20 mM NaOAc buffer (pH 5) for 16 hr. On centrifugation (14 000 g, 10 min) the vol. was adjusted (50 ml) and aliquots removed for the assay of β-D-mannanase and α-D-galactosidase. Cotyledonary tissue was washed and extracted exactly as described for the preparation of extracts for assay of exo-β-mannanase except that the Tris–HCl buffer (pH 8) was replaced by NaOAc buffer (100 mM, pH 5). The extracts were assayed, before and after dialysis, for β-mannanase (using Remazol Brilliant Blue-dyed carob galactomannan) and for α-galactosidase (using *p*-nitrophenyl-α-D-galactopyranoside).

The effect of cycloheximide on enzyme activities in endosperms and cotyledons of germinating guar was studied by allowing the seeds to imbibe in, and then incubating the seeds in, the presence of cycloheximide soln (10^{-5} M) in place of H_2O.

Determination of the level of exo-β-mannanase activity attached to the external surface of cotyledons. After careful removal of endosperm tissue from one guar seed, the seed was thoroughly washed with one of the following solns; 0.1 M NaCl, 0.5 M NaCl, 0.5 M NaCl plus 0.05 M Tris–HCl (pH 8.0) or 0.1% Triton X-100. The seed was then rinsed with H_2O and the cotyledons immersed in a soln of 10 mM *p*-nitrophenyl-β-D-mannopyranoside (1.0 ml, pH 5.5) and incubated at 25° for 0–15 min. The reaction was terminated by the addition of Na_2CO_3 soln (2.0 ml, 2% w/v) and the cotyledons removed immediately. Control expts in which cotyledons were immersed and incubated in solns of *p*-nitrophenol indicated that only limited amounts of this compound were absorbed over a 15 min incubation period. Control expts in which cotyledons were immersed in buffer soln were also performed.

Purification of α-galactosidase II and β-mannanase. Surface sterilized guar seed [8] (500 g) was germinated at 25° for 3 days, homogenized in acetate buffer (0.1 M, pH 4.5, 3l.) in a Waring Blendor, and incubated at 30° for 1 hr. The slurry was re-homogenized and squeezed through fine nylon mesh. The filtrate was centrifuged (3500 g, 30 min) and the supernatant dialysed against three changes of 20 mM acetate buffer (10 l., pH 4.5) during 48 hr. After dialysis the soln was centrifuged (4000 g, 20 min). Tris–HCl buffer (pH 8), to give a final concn of 30 mM, was added to the supernatant and the pH adjusted to 8.0. This soln was applied to a column (3.7 × 17 cm) of DEAE-cellulose pre-equilibrated with 30 mM Tris–HCl buffer (pH 8). After washing with 30 mM Tris buffer (500 ml, pH 8), protein was eluted with a linear NaCl gradient (0 → 0.4 M) in the same buffer. The fractions active in either β-mannanase or α-galactosidase II (previously termed α-galactosidase C) were pooled separately, concd by $(NH_4)_2SO_4$ pptn (80% satn) and dialysed against H_2O for 16 hr. After centrifugation (4000 g, 10 min) solid NaCl was added to each to a final concn of 0.5 M, and 2 M acetate buffer (pH 4.5) to a final concn of 0.1 M. α-Galactosidase II was then purified to homogeneity by chromatography on a column of *N*-ε-aminocaproyl-α-D-galactopyranosylamine–Sepharose 4B conjugate (2.5 × 35 cm) [16, 17], α-Galactosidase I, which partially overlapped β-mannanase on DEAE-cellulose chromatography, was purified by the same technique.

β-Mannanase was purified by chromatography on a glucomannan-AH–Sepharose affinity column (1.5 × 15 cm) [18]. On isoelectric focusing of the purified enzyme several protein bands were detected, each of which had β-mannanase activity.

The purified α-galactosidases and β-mannanase on concn and dialysis, were stored frozen.

Chromatofocusing of guar β-mannanases [19]. Guar β-mannanase recovered from the glucomannan-AH–Sepharose 4B affinity column was concd by dialysis against polyethylene glycol 4000 and then dialysed against H_2O. Before application to a

tightly packed column (1.2 × 6 cm, pre-equilibrated with 25 mM imidazole buffer, pH 7.4) of Polybuffer Exchange PBE 94, the pH was adjusted to 7.4 by addition of imidazole buffer to a final concn of 25 mM. Proteins were eluted with Polybuffer 74–HCl® (diluted 1:8 and adjusted to pH 4). Three peaks of β-mannanase activity eluted and these corresponded approximately to the two major protein bands (p*I* 5.35 and 6.1) and one of the minor bands (p*I* 4.8) obtained in isoelectric focusing.

Properties of guar β-mannanases and α-galactosidase II. The MW was determined by SDS-gel slab electrophoresis as previously described [4]. pH optima were determined for α-galactosidase with *p*-nitrophenyl-α-D-galactopyranoside (10 mM) with 0.1 M acetate–Pi buffer (pH 3.5–8.0); and for β-mannanase with 0.2% (w/v) carob galactomannan with 0.1 M acetate buffer (pH 2.9–6.0). Hydrolysis of carob galactomannan by β-mannanase was assayed by the *p*-hydroxybenzohydrazide method [20]. Temp. stability was determined by incubating 1.0 ml aliquots of enzyme at 30–65° for 15 min. The solns were cooled and aliquots removed for assay of enzyme activity.

Preparation of guaran β-mannanase limit galactomannan. Guar galactomannan (5 g, 0.5% w/v) in acetate buffer (20 mM, pH 4.5) was incubated with pure *Irpex lacteus* β-mannanase (2 μkat) at 40° for 24 hr, at which time the galactomannan had been hydrolysed to its limit (*ca* 7%) by this enzyme. β-Mannanase was inactivated by incubation of the reaction mixture at 100° for 20 min. On cooling, the remaining polysaccharide material was ppted by the addition of EtOH (two vol.), and then washed with EtOH, Me_2CO and hexane and dried *in vacuo*. The polysaccharide material had the same galactose content (within experimental error) as the native galactomannan (Gal–Man = 38:62), but could be readily dissolved in warm H_2O to a concn of up to 200 mg/ml. This material was termed guaran-β-mannanase limit galactomannan.

Hydrolysis of galactomannan by α-galactosidase, β-mannanase and/or exo-β-mannanase. To a soln of guaran β-mannanase limit galactomannan (250 mg, 10.8 ml) was added α-galactosidase (0.26 ml, 437 nkat on *p*-nitrophenyl-α-D-galactopyranoside) plus β-mannanase (0.28 ml, 246 nkat) plus exo-β-mannanase (1.0 ml, 68.5 nkat) and the mixture incubated at 40°. Aliquots were removed between 0 and 48 hr, heated (100°, 5 min) to inactivate enzymes and aliquots removed for TLC (solvent b). Parallel expts in which exo-β-mannanase was replaced by an equal vol. of H_2O were also performed. In these expts the level of galactomannan used was that present in 1 g of ungerminated guar seed, and the levels of enzymes employed were *ca* equal to those present in endosperms of guar seed germinated for 2.5 days at 25° (assayed at 40°) and the pH of the incubation mixture (unbuffered) was *ca* 5.5.

Enzymic hydrolysis of galactomannan and uptake of released sugars by cotyledons. To solns of guaran β-mannanase limit galactomannan (0.22 ml, 40 mg) in small snap-cap polypropylene containers (with holes in the cap for ventilation) was added a mixture (0.28 ml) of various combinations of the enzymes: α-galactosidase (47 nkat); β-mannanase (42 nkat); exo-β-mannanase (12 nkat). To each of these containers (except the controls) was added thoroughly washed cotyledon–embryos from five guar seeds (which had been germinated for 2 days at 25°). The containers were then incubated in the dark for up to 48 hr. After various time intervals, containers from each incubation expt were taken and cotyledon–embryos removed and thoroughly washed. The washings plus the remaining soln in the polypropylene containers were combined, incubated at 100° for 2 min to inactivate enzymes, centrifuged (14 000 g, 10 min) and the vol. adjusted to 25 ml. Aliquots (20–50 μl) were removed for the determination of total carbohydrate by the anthrone procedure [21]. In some cases the carbohydrate solns were deionized [8], concd at red. pres. and adjusted to 2 ml. Aliquots (20 μl) were examined by TLC (solvent b).

Carbohydrate uptake by guar cotyledon–embryos. To a soln of mannose, manno-oligosaccharide or reduced manno-oligosaccharide (0.5 ml, 16.7 mg) was added exhaustively washed (H_2O) cotyledon–embryos from five guar seeds (germinated at 25° for 2 days). The containers were incubated in the dark at 25° for 0–24 hr. The carbohydrate remaining after various times of incubation, and the oligosaccharides remaining in these mixtures were determined as for the incubations with guaran-β-mannanase limit galactomannan. Carbohydrate uptake (as mg/g of whole seed, or as a percentage of the total carbohydrate originally present) was calculated by subtracting the amount of carbohydrate remaining from that present originally.

Hydrolysis of carob galactomannan by guar β-mannanases. Carob galactomannan (5 ml, 0.5% w/v) in acetate buffer (5 mM, pH 5) was incubated with guar β-mannanase I (p*I* 5.35), II (p*I* 5.8), III (p*I* 6.1) or total guar β-mannanase (0.2 μkat on carob galactomannan) at 40° for 18 hr and then heated at 100° for 5 min to denature β-mannanase. The soln was deionized (IRA 410 and AG 50 W-X8), centrifuged to remove denatured protein (14 000 g, 10 min), lyophilized and readjusted to 2% (w/v) carbohydrate. Aliquots (20 μl) were applied to Merck DC-Alufolien Kieselgel 50 (0.2 mm) plates which were developed twice with solvent b. Spots were visualized by spraying with 5% H_2SO_4 in EtOH and heating at 110° for *ca* 10 min. Parallel expts were performed with pure β-mannanases from several other sources [14, 18].

Hydrolysis of guar galactomannan by guar α-galactosidase II. Guar galactomannan (17 ml, 0.12%) in 0.07 M acetate buffer (pH 4.5) was incubated with guar α-galactosidase II (0.3 ml; 200 nkat on *p*-nitrophenyl-α-D-galactopyranoside; 9.1 nkat on this substrate) in a Ubbelohde suspended level viscometer at 40°. Viscosity was measured after various time intervals and aliquots were removed for determination of released galactose by the *p*-hydroxybenzohydrazide method [20]. The galactose content of the remaining polysaccharide was determined by difference.

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