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GALACTOMANNAN UTILIZATION IN GERMINATING LEGUME SEEDS

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Key Word Index—*Ceratonia siliqua*; carob; *Medicago sativa*; lucerne; *Cyamopsis tetragonolobus*; guar; *Gleditsia triacanthos*; honey locust; Leguminosae; seeds; germination; galactomannan; galactose; mannose; phosphomannoisomerase.

Abstract—Galactose and mannose, released on hydrolysis of galactomannan in the endosperm of germinating seeds of carob, guar, honey locust and lucerne were absorbed by the cotyledons and further metabolized. In guar, the distribution of ^{14}C from [U-^{14}C]-D-glucose, D-mannose and D-galactose into various cotyledon fractions did not provide evidence for preferential channelling of D-galactose into cell wall fractions and D-mannose into glycolysis. Phosphomannoisomerase, which has previously been reported in animals and microorganisms was detected in a number of legume seeds. In honey locust it was located in the cotyledons and its level declined after galactomannan was depleted. This enzyme from lucerne was purified until free of phosphoglucoisomerase and some of its properties are described.

INTRODUCTION

A number of legume seeds contain galactomannan in the endosperm, which is hydrolysed after imbibition. In germinating fenugreek [1] the galactose and mannose produced on hydrolysis are rapidly utilized in whole seeds, as only traces of these monosaccharides are found at any stage of germination. Low levels of galactose and mannose, as well as manno-oligosaccharides have also been reported in germinating *Gleditsia* species [2]. However, in incubated endosperms of fenugreek, clover and lucerne [1] much larger amounts of galactose and mannose appeared, indicating that the products of galactomannan hydrolysis are taken up by the cotyledon/embryo and further metabolised. Their incorporation probably involves an initial phosphorylation to D-Gal-1-P and D-Man-6-P [3,4]. The conversion of D-Man-6-P to D-Fru-6-P, allowing entry to glycolysis, requires phosphomannoisomerase (PMI, E.C.5.3.1.8). This enzyme has previously been reported from animal [5,7] and microbial [6,8] sources and purified [8]. Unlike D-mannose, which can be converted to a glycolytic intermediate by 2 enzymes, D-galactose requires 4 (galactokinase, Gal-1-P uridyl transferase, UDP-Gal epimerase and Glc-1-P uridyl transferase). Also, the second and third products in this sequence UDP-Gal and UDP-Glc are precursors of pectic substances, the latter via UDP-Glc UA and UDP-Gal UA. It appeared possible that the 2 monosaccharides derived from galactomannan might be used by germinating legume seedlings in different ways; galactose for cell wall synthesis and mannose in glycolysis. Legumes do contain small amounts of galactoglucomannan in stems and leaves [9], but in insufficient quantities to require significant amounts of the mannose released. The preferential channelling of *myo*-inositol into cell-wall polysaccharide constituents occurs in a number of plant tissues [10].

The aim of this paper has been to compare the quantitative changes in mono- and oligosaccharides in the cotyledons and endosperms of whole and dissected seeds after imbibition, to follow the utilization of galactose and mannose, to examine some legume seeds for the presence of phosphomannoisomerase and to study its properties.

RESULTS AND DISCUSSION

Seeds of carob, honey locust, guar and lucerne, during germination, all contained low amounts of free galactose and mannose, very much less than the quantities calculated to be released by the hydrolysis of galactomannan. Figure 1 shows the results for carob. Only traces of manno-oligosaccharides were detected. The quantitative changes in a number of mono and oligosaccharides in both the endosperm and cotyledon–embryo* sections of

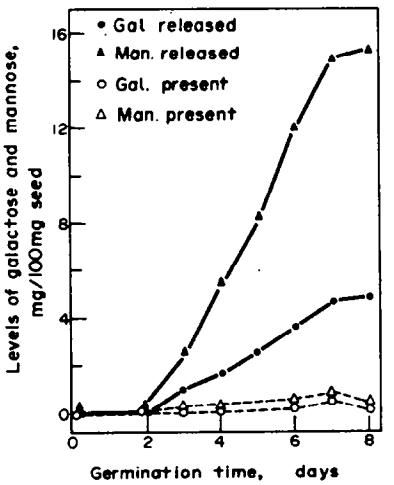

Fig. 1. Differences between the amounts of galactose and mannose released on hydrolysis of galactomannan and raffinose series oligosaccharides and the levels of these monosaccharides in seeds of carob.

* Whole seed with endosperm removed.

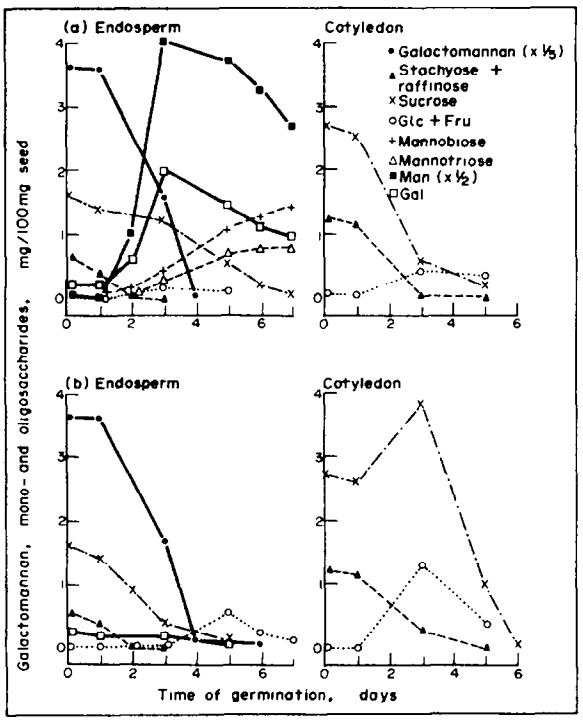

Fig. 2. Changes in the sugar levels in endosperm and cotyledons of honey locust on seed germination. (a) Seeds dissected after imbibition (1 day) and separated cotyledons and endosperms incubated under germination conditions; (b) seeds germinated whole and dissected immediately before extraction.

whole seeds of honey locust, as well as seeds which had been separated after imbibition for one day were measured by anthrone estimation of eluted fractions from thick PC (Fig. 2). Galactose and mannose accumulated in isolated endosperms similarly to fenugreek [1]. Unlike fenugreek, the depletion of galactomannan in both whole and separated endosperms followed the same pattern and mannobiose and triose continued to increase after this depletion was complete. Smaller amounts of higher oligomers, some of which contained both galactose and mannose, also appeared. Isolated endosperms depleted sucrose less rapidly than the endosperms of whole seeds and in isolated cotyledons sucrose content immediately decreased whereas the cotyledons in whole seeds showed an increase in sucrose content. Higher amounts of glucose plus fructose appeared in the non-dissected cotyledons. The results are consistent with the mechanism proposed for fenugreek, that most of the galactose and mannose produced in the endosperm is absorbed by the cotyledons and further metabolised [1].

The rates of uptake of D-glucose, D-galactose and D-mannose by isolated cotyledon–embryos of guar were followed by half-immersing dissected seedlings in a solution of monosaccharide in the dark and estimating residual hexose in solution. All three were absorbed (Fig. 3) readily and more rapidly than the calculated release of hexose from galactomannan during its depletion on the germination of guar seeds. The non-accumulation of D-galactose and D-mannose in cotyledons of germinating seeds indicates that once in this tissue they are rapidly metabolised further.

The relative conversion of D-glucose, D-mannose and D-galactose into various fractions was estimated using

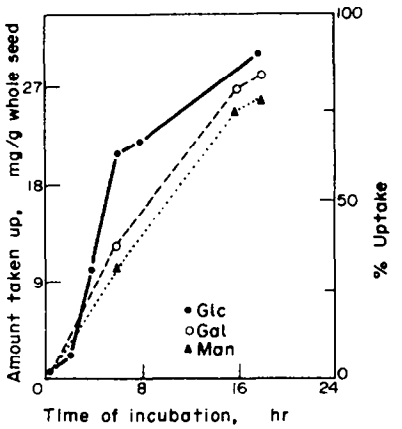

Fig. 3. Uptake of monoccharides by guar cotyledons.

[U-^{14}C] monosaccharides and guar seedlings minus endosperms, at 5 days after intitial imbibition. At this time the maximum rate of galactomannan depletion was occurring. The amount of monosaccharide supplied was similar to that released by galactomannan depletion during a 24 hr period. The fractions collected were (i) CO_2 released (ii) water-soluble (divided into EtOH-soluble and insoluble) (iii) $CHCl_3$–MeOH soluble (iv) water-soluble after crude pectinase treatment (v) water-soluble after 72% H_2SO_4 hydrolysis. The largest ^{14}C containing fraction was the EtOH-soluble portion of the water-soluble (Table 1) and for all three sugars supplied more than 60% of this label was found in glucose, fructose and sucrose. The amounts of label in the CO_2, pectinase hydrolysate and cellulose-hemicellulose fractions were similar for all three sugars (Table 1). In another incubation, D-galactose [U-^{14}C] was supplied along with abundant unlabelled D-glucose, but there was little change in the $^{14}CO_2$ or pectinase hydrolysate fractions. The results do not indicate any major difference in the utilization of D-mannose and D-galactose released by galactomannan hydrolysis. Since so much label was found in CO_2, glucose, fructose and sucrose, whichever sugar was supplied, it appears that both D-mannose and D-galactose are readily metabolised in isolated cotyledons after absorption.

In the pathway of conversion of D-mannose to the glycolytic intermediate D-Fru-6-P, the second enzyme involved is PMI, which reversibly converts D-Man-6-P and

Table 1. U-^{14}C Monosaccharide incorporation into guar

	Cotyledon/embryos (% incorporation)			
Labelled monosaccharide	D-Glc	D-Man	D-Gal	D-Gal
Amount of radioactivity (μCi)	1	1	1	1
Unlabelled monosaccharide	D-Glc	D-Man	D-Gal	D-Glc
Amount (mg)	15	15	15	15
Uptake (%)	90	73	75	75
	Label in fraction / label taken up %			
CO_2	25	21	23	26
water-soluble				
(i) ethanol-soluble	36	30	42	26
(ii) ethanol-insoluble	2	4	4	4
$CHCl_3$–MeOH soluble	3	3	3	3
Crude pectinase hydrolysate	12	9	12	12
72% H_2SO_4 hydrolysate	1	1	1	1

Table 2. PMI and PGI Levles in ungerminated seeds

Plant Material	PMI (kat × 10^8/g seed)	PGI (kat × 10^8/g seed)
Legumes		
Lucerne	1·6	8·0
Guar	0·8	5·4
Carob	0·6	6·6
Honey locust	3·1	9·1
Soybean	2·2	12·5
Mung bean	3·1	40·2
Broad bean	0·7	20·2
French bean	0·2	nd*
Peas	0·3	22·3
Lupins	0·5	15·0
Non-legumes		
Wheat	ud†	nd
Barley	0·1	nd
Sorghum	ud	nd
Safflower	ud	nd
Rape	0·03	4·0
Sesame	0·02	nd
Sweet corn	ud	nd

* Not determined. † undetectable.

D-Fru-6-P. Significant levels of PMI activity were found in a number of leguminous seeds and the values of these and phosphoglucoisomerase activity (PGI) in ungerminated seeds when assayed with PGI/Glc-6-P dehydrogenase/6 phosphogluconate dehydrogenase are shown in Table 2. Non-leguminous seeds had very low or undetectable amounts of PMI, but significant PGI activity, which would be required for glycolysis, was present in the one of these that was examined, rape. Any PMI present was located in the cotyledons with negligible amounts in the endosperms. The latter contained PGI.

On germination of honey locust seeds (Fig. 4), the levels of PMI assayed with PGI/Glc-6-P dehydrogenase/6-phosphogluconate dehydrogenase declined as the galactomannan reserve was depleted. In separated cotyledons, under germination conditions but without a supply of D-mannose, this decline started earlier. PGI levels in whole seeds increased initially and were still higher than the initial value at 5 days. In the dissected cotyledons PGI showed similar behaviour until 4 days, at which stage seedling growth in the dark was restricted and plants developed an abnormal appearance. These results

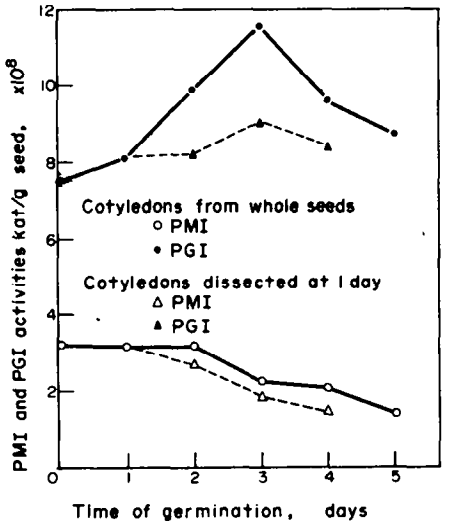

Fig. 4. Changes in the activity of PMI and PGI on the germination of honey locust seeds; ---- activity in cotyledons dissected before germination; —— activity in cotyledons from whole germinated seeds.

Table 3. Purification of lucerne PMI

Stage	Total protein (mg)	Total activity (kat × 10^8)	Specific activity (kat × 10^8/mg protein)	Recovery per step (%)	Purification (fold)
1. Crude	15400	813	0·05	100	1
2. Bentonite	4013	683	0·17	84	3·3
3 $(NH_4)_2SO_4$ (30–60%)	945	583	0·62	72	12·4
4. DEAE cellulose (pH 7, Tris 5–400 mM)	106	173	1·63	30	33
5. DEAE cellulose (pH 7, phosphate 5–50 mM, 50–100 mM)	15·8	67	4·24	38	83
6. Sephadex G200	5	16·6	3·32	25	67

are consistent with the PMI being primarily involved in the conversion of D-mannose, released by galactomannan hydrolysis, into a glycolytic intermediate. It is found only in the cotyledons where any further metabolism of D-mannose takes place and it declines as the amount of D-mannose released from galactomannan hydrolysis decreases.

PMI activity was detected in a number of legume seeds (Table 2). In general, higher levels were found in seeds that store galactomannan. Much lower levels or undetectable amounts were found in non-legume seeds not known to contain any mannan or substituted mannan as polysaccharide reserve e.g. barley, safflower. In these seeds PMI could be required to catalyse the reverse reaction i.e. the conversion of Fru-6-P to Man-6-P which would then be converted via Man-1-P to GDP-Man, a polysaccharide pre-cursor. The incorporation of D-mannose into cell wall polysaccharides of corn root tips has been demonstrated [11], but no PMI activity could be detected in tomato roots [12]. Mannose containing polysaccharides such as glucomannan and galactoglucomannan [13] occur widely in plant tissues as structural support material, but the small amounts present would not require high levels of PMI.

The purification of lucerne PMI is summarized in Table 3. Bentonite was useful in the preliminary stage of purification. Optimum addition was 4 mg bentonite/mg protein which gave 74% recovery with a 2·8 fold purification. At 2 and 8 mg, 1·7 and 1·2 fold purifications were obtained with 80 and 35% recoveries. In contrast to a yeast enzyme [8], it lost activity on DEAE-cellulose and Sephadex G-100 and the recovery was not improved by using 5 mM dithiothreitol or 20% glycerol. The enzyme did not bind to CM-cellulose above pH 5 and was unstable below this pH. On polyacrylamide gel-disc electrophoresis there was complete inactivation, unlike PGI which was quite stable. Chromatography on DEAE-cellulose in tris–HCl buffer gave PGI free of PMI but not the inverse. PMI, free of PGI, suitable for a study of its properties was obtained by chromatography on DEAE-cellulose with a phosphate gradient (Fig. 5).

The K_m value for the purified PMI was 0·77 mM Man-6-P and the equilibrium between Man-6-P and Fru-6-P was 40:60. Assay by both resorcinol and PGI/Glc-6-P dehydrogenase methods gave this value. Similar K_m values (0·8 mM) and equilibrium ratios have been reported for the yeast enzyme. The elution volumes on Sephadex G-200 of lucerne PMI and PGI were both identical with the value for the PGI from pea seeds which has previously been shown to have a MW of

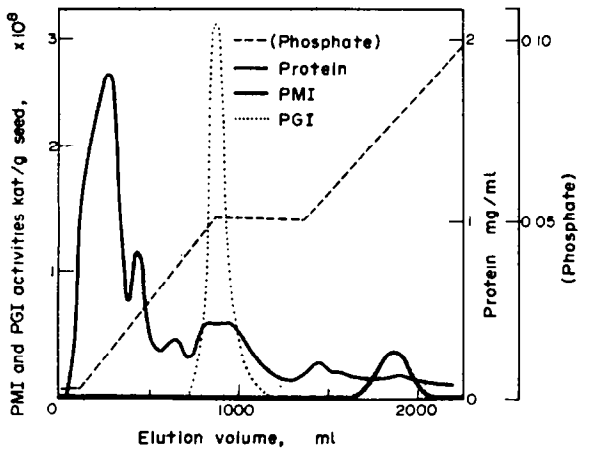

Fig. 5. Chromatography of PMI and PGI of lucerne on DEAE-cellulose using a phosphate gradient.

110000 [14]. Both enzymes were stable for 60 min at 30° over the pH range of 6–11 but were completely inactivated at pH 5. Lucerne PMI showed a very broad pH optimum between 6 and 9 with half maximal values of 5·2 and >11. Lucerne PGI showed optimal activity at pH 9 with half maximal activity at pH 6·5 and >11. The energy of activation for lucerne PMI was 14·7 kJ/mol and the energy of activation for denaturation was 25·5 kJ/mol. Both PMI and PGI were rapidly denatured at 55°.

PMI from mammalian cells is a metal dependent enzyme [7]. One mM EDTA caused slow inactivation of lucerne PMI at 30° at pH 7 but there was 88% loss of activity after 30 min at 40° and complete loss at 50°. Solutions without EDTA at 40° were stable for this time. Similar results were obtained at both pH 5·5 and 7, unlike the enzyme of pig red cells [15] in which inactivation was affected by pH. Lucerne PMI was completely inactivated by Hg^{2+} at very low concentrations (1–10 μM). PGI was not completely inactivated until the concentration reached 1 mM. PMI from mammalian cells was found to be an SH dependent enzyme [7]. Other metals (Mn^{2+}, Mg^{2+}, Ca^{2+}, NH_4^+, Ba^{2+}, Rb^+ and Co^{2+}) had no significant effect at 1 mM concentration. These effects in purified PMI and PGI were assayed by the resorcinol method in case any of the enzymes in the enzyme-linked assay were susceptible to treatment with EDTA or metal ions.

EXPERIMENTAL

Details of the plant material have been previously described [16,17].

Extraction and estimation of galactomannan, mono- and oligosaccharides. Galactomannan was extracted and estimated as described in ref. [16]. Mono and oligosaccharides were extracted as described previously [16] separated by PC (solvent EtOAc–Py–H_2O, 2:1:2) on 3MM paper, eluted and filtered through very fine sintered glass and carbohydrate estimated by the anthrone method [18]. Galactose and mannose contents were also estimated after PC (*n*-BuOH–Py–C_6H_6–H_2O, 9:3:1:3 with double irrigation) by the method of Pridham [19]. Galactose was also measured using galactose dehydrogenase [20,16].

Supply of $U^{14}C$ labelled sugars and fractionation of seedlings. 10 guar seedlings, 5 days after imbibition (epicotyl 4 cm), less endosperm were washed well with sterile soln and incubated at 30° with $U^{14}C$ labelled monosaccharide (15 mg, 1 μCi) in a sterile soln for 24 hr. Respired CO_2 was collected after washing well with sterile soln. Seedlings were fractionated as previously described [21]. Samples of fractions applied to filter paper were counted in a scintillation spectrophotometer. Efficiency was determined by the channels ratio method.

Extraction and purification of PMI from lucerne. Germinated lucerne seeds (3 days) were macerated in tris HCl buffer (100 mM, pH 7·5) at 2° in a blendor followed by an Ultra-turrax. After centrifugation (20000 *g*, 30 min, 2°), $(NH_4)_2SO_4$ was added to the supernatant soln, the fraction precipitating between 30–60% satn collected and dialysed against tris–HCl buffer (pH 7·5, 10 mM) for 24 hr at 2°. This soln was then chromatographed on DEAE-cellulose (Cl) and after washing with tris–HCl buffer (10 mM, pH 7·5, 3 column vols) was then eluted with a linear gradient of the same buffer (10–400 mM). The active fraction was precipitated by the addition of $(NH_4)_2SO_4$, dialysed against 5 mM PO_4 buffer pH 7·5 and chromatographed on DEAE-cellulose (PO_4). The column was eluted with a linear PO_4 gradient 5–50 mM and with 50 mM buffer to remove PGI activity. PMI was eluted with a 50–100 mM gradient. The active fraction was concentrated by dialysis against polyethylene glycol. This was then chromatographed on Sephadex G-200 with tris–HCl buffer (100 mM).

Assay of PMI. (i) with resorcinol [5]. Enzyme soln 0·1 ml, (0·1–0·5 U) was incubated with Man-6-P (1 ml, 4 mM) in tris–HCl buffer (0·1 M, pH 7·5) for 5–20 min. Aliquots (0·1 ml) were added to 0·1% resorcinol in EtOH (0·5 ml). H_2O (0·4 ml) and conc. HCl (2 ml) were added and the soln held at 80° for 10 min. Tubes were cooled in ice-H_2O and the *A* read at 530 nm. (ii) with PGI/Glc-6-P dehydrogenase. A reaction mixture containing 0·02 M NADP (25 μl) Glc-6-P dehydrogenase (0·2–0·5 U, 25 μl), Man-6-P (0·02 M, 25 μl) PGI (0·1 U, 25 μl) and tris–HCl buffer (pH 7·5, 0·1 M, 1 ml) was made. An aliquot of enzyme (0·1 ml, 1–10 mU) was added and the reaction followed at 340 nm when assaying unpurified preparations, M $MgCl_2$ (25 μl) and 0·5 U 6-phosphogluconate dehydrogenase (20 μl) was added and the final values halved.

Assay of PGI. Similar assay mixtures were used, except that in the resorcinol method Glc-6-P replaced Man-6-P and in the Glc-6-P dehydrogenase method Fru-6-P replaced Man-6-P and no PGI was added.

Estimation of changes in levels of PMI and PGI on germination. Germinated seeds (2 g original dry wt) as whole seeds or as dissected cotyledons or endosperms were ground in tris HCl buffer at 2° (0·1 M, pH 7·5) in a mortar followed by a glass Tenn Broeck. After centrifugation (24000 *g*, 15 min, 2°) the supernatant was made up to 20 ml. Aliquots (0·1 ml) were assayed by the 2 methods described.

Characterization of PMI and PGI. The K_m value for lucerne PMI was determined using Man-6-P conc 0·1–2 mM and assaying with PGI/Glc-6-P dehydrogenase. pH optima of PMI and PGI were determined by incubating enzyme (0·1 ml, 200 mU) in 1 mM KCl with either Man 6-P or Fru 6-P and tris–acetate buffer (0·2 ml, 100 mM, pH 3–11) for 10 min. Reaction was stopped in a boiling H_2O bath and the amount of conversion to Fru-6-P for PMI or to Glc-6-P measured for PGI. The pH stability for both enzymes was determined by incubating enzyme (0·1 ml) with tris–acetate buffer (0·2 ml, 100 mM, pH 3 to 11) for 1 hr at 30°. Aliquots (0·1 ml) were removed for assay. To measure energy of activation and activation for denaturation, enzyme (0·1 ml) was incubated with tris–HCl buffer (0·2 ml, 100 mM, pH 7·5) at temp from 30° to 60°. Residual activity was assayed by the Glc-6-P dehydrogenase method. To determine the effect of cations, enzyme (0·1 ml) in 10 mM buffer was incubated with the chlorides. Residual activity was assayed by the resorcinol method. The effect of EDTA was estimated in enzyme soln (1 ml) containing 1 mM EDTA at 30°–50° for 30 min to 2 hr. Aliquots (0·1 ml) were assayed. Molecular sizes were compared on Sephadex G-200 in tris–HCl buffer (pH 7·5, 0·1 M).

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