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PHOSPHOMANNOISOMERASE AND PHOSPHOGLUCOISOMERASE IN SEEDS OF *CASSIA COLUTEOIDES* AND SOME OTHER LEGUMES THAT SYNTHESIZE GALACTOMANNAN

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Key Word Index—*Cassia coluteoides*; Leguminosae; galactomannan biosynthesis; phosphomannoisomerase; phosphoglucoisomerase.

Abstract—One phosphomannoisomerase and two phosphoglucoisomerases have been obtained from developing and germinating *Cassia coluteoides* seeds. The MW of phosphomannoisomerase and those from other legumes were about half those of phosphoglucoisomerases. Kinetic properties, including inhibition by 6-phosphogluconate and erythrose 4-phosphate have been studied.

INTRODUCTION

An early step in the biosynthesis of the (1 → 4) β mannan chain of galactomannan is the conversion of fructose-6-phosphate to mannose-6-phosphate by phosphomannoisomerase (PMI) [EC 5.3.1.8] [1]. This enzyme is also involved in the utilization of mannose released by hydrolysis of galactomannan on germination, after phosphorylation to Man-6-P. Reaction then proceeds in the reverse direction as Man-6-P is converted to Fru-6-P [2, 3]. PMI has been identified in extracts of konjac corms [2] and was separated from phosphoglucoisomerase (PGI) [EC 5.3.1.9] by DEAE cellulose and gel chromatography. PMI has also been detected in a number of legume seeds and the enzyme from lucerne was purified by chromatography on DEAE cellulose, initially with a Tris–HCl and then a phosphate gradient followed by gel chromatography to give a preparation free of PGI [3]. PGI, which catalyses a similar type of reaction to PMI has been prepared from a number of plant tissues, e.g. seeds of peas [4], maize [5], green gram [6] and lucerne [3], sweet potato tubers [7] and spinach [8] and oat [9] leaves. Multiple forms have been isolated from several sources and in leaves different forms have been located in the cytosol and chloroplasts [10]. 6-Phosphogluconate competitively inhibits PGI activity from pea seeds (K_i 13 μM) [4] and all three forms from maize were also inhibited. The enzyme from a number of plant sources is inhibited by μM amounts of erythrose-4-P [9], similar to the muscle enzyme. PMI from yeast was inhibited by mannitol-P, erythrose-4-P and 6-phosphogluconate [11].

This paper describes a study of the PMI and PGI activities in *Cassia coluteoides* seeds.

RESULTS AND DISCUSSION

In the extraction of these enzymes, the presence of high levels of galactomannan interfered with the initial steps, since it gave extracts with very high viscosities, was precipitated by ammonium sulphate and co-chromatographed with enzymic activity on gel chromatography. The addition, at 4°, of β-mannanase, free of interferring PMI, PGI or proteolytic activities [12] overcame these problems. The galactose content of *C. coluteoides* galactomannan is sufficiently low to allow depolymerization without added α-galactosidase.

When developing *C. coluteoides* pods were divided into cotyledons and the remainder of tissue inside the pod coat, and PMI and PGI activities measured, 95% of the PMI activity was detected in the cotyledons (0.43 nkat per g fr. wt) but one third of the PGI (0.77 nkat per g fr. wt) was found in the surrounding mucilaginous matrix. The changes in activity of PMI and PGI are shown in Fig. 1 and the changes in fresh and dry weights of the pods and seeds in Fig. 2. The activity of both enzymes showed an initial increase in activity per pod followed by a decrease and then a slight increase in the case of PGI but a large increase in PMI up to maturity (dehiscing pods). The first increase was concurrent with the maximum rate of increase in both dry weight and wet weight, when the endosperm was growing rapidly, consistent with the

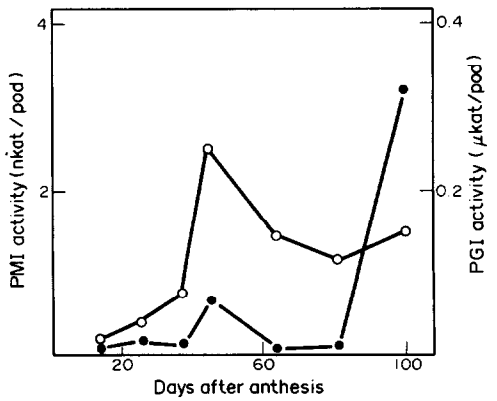

Fig. 1. Changes in phosphomannoisomerase (●) and phosphoglucose isomerase (○) in *Cassia* pods during development.

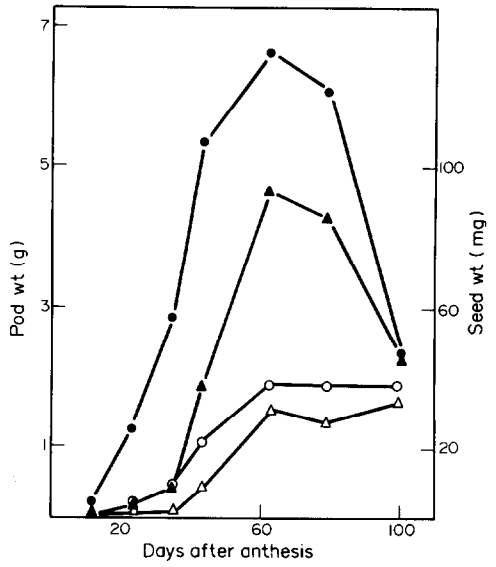

Fig. 2. Growth curves for *Cassia* pods and seeds. ●, Pod fresh weight; ○, Pod dry weight; ▲, Seed fresh weight; △, Seed dry weight.

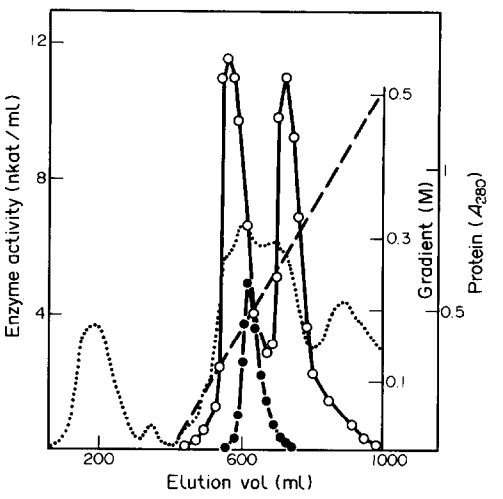

Fig. 3. DEAE-cellulose chromatography of developing *Cassia* pods. ●, Phosphomannose isomerase; ○, Phosphoglucose isomerase; . . ., Protein.

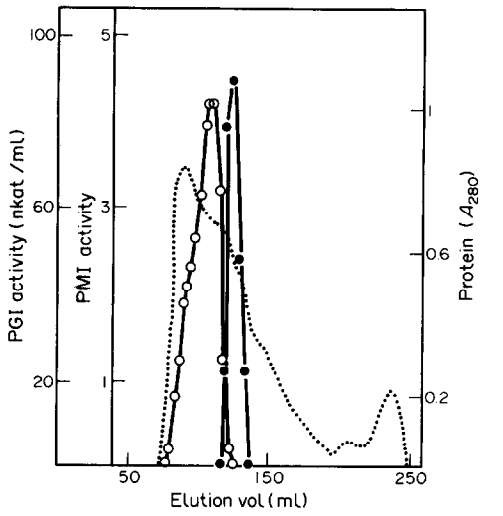

Fig. 4. Chromatography on Sephacryl S-200 of the mixed fraction of PGI I and PMI obtained from DEAE-cellulose chromatography. ●, Phosphomannoisomerase; ○, Phosphoglucoisomerase; . . ., Protein.

involvement of PMI in the synthesis of the $(1 \rightarrow 4)$ β mannan chain. The levels of another enzyme involved in $(1 \rightarrow 4)$ β mannan chain synthesis in developing seeds of fenugreek, mannan synthase, showed an initial increase, followed by a decrease and then a second increase. However, this was followed by a decrease to negligible activity and all these changes occurred during the period of galactomannan accumulation [13]. The second, greater increase in PMI activity in *Cassia* pods, that starts about 20 days after the dry weight increase stops, indicates a role in the utilization of galactomannan, with the high ultimate level reflecting the much shorter duration of depletion compared to accumulation.

DEAE cellulose chromatography of extracts of developing pods (Fig. 3) showed the presence of two fractions of PGI and one of PMI. PMI co-eluted with the first PGI fraction (PGI I). This mixture of PGI and PMI was not separated on hydroxylapatite as no binding occurred, on chromatofocussing, as no activity could be recovered, or on affinity elution chromatography, since there was no binding to phosphocellulose at pH 6–8 and hexose-P concentrations up to 10 mM did not elute these enzymes from DEAE cellulose. Elution with a phosphate gradient [3] gave separation but with *Cassia* PMI was accompanied by considerable loss of activity. Gel chromatography on Sephacryl S-200 superfine (Fig. 4), with rechromatography of the mixed intermediate fraction gave effective separation. PGI II was also further purified by gel chromatography, when it also gave a single peak of activity. Polyacrylamide gel electrophoresis in tubes or on slabs, with staining for PGI activity, indicated two bands in the crude extract and examination of the fractionated material showed that the more slowly moving band was PGI I. Although yeast PMI could be stained for activity after electrophoresis, attempts to stain *Cassia* PMI were unsuccessful.

The changes in PMI activity in germinating seeds of *C. coluteoides*, as activity per unit fresh weight (Fig. 5), initially increased about eight fold and then decreased as galactomannan was depleted. A curve with a similar shape was obtained if activity per seed was plotted. The wet weights of germinating seeds (relative to ungerminated seeds) were 2.2 fold at A, 2.8 at B, 3.1 at C, 3.5 at D and 4.7 at E. This is a much greater increase than was detected in lucerne seeds [3] and is consistent with PMI being involved in mannose utilization. DEAE cellulose chromatography of PGI and PMI from germinated seeds gave a very similar elution pattern to extracts from developing seeds, with two PGI fractions, one of which (eluted first) co-chromatographed partly with PMI. This mixed fraction was separated by chromatography on Sephacryl S-200 superfine. Germination of seeds in the dark reduced

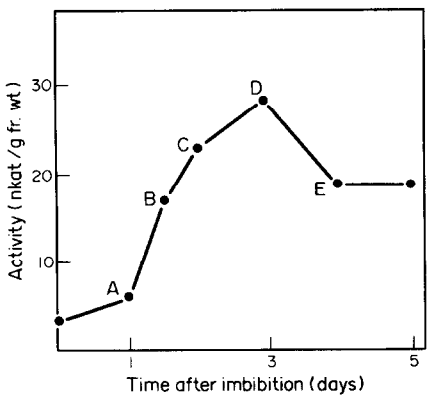

Fig. 5. Changes in phosphomannoisomerase activity in germinating *Cassia* seeds. A, No root emerged; B, root—5 mm; C, root—10 mm (some galactomannan depleted); D, root—25 mm (little galactomannan remaining); E, root—50 mm (all galactomannan depleted).

the amount of PGI II, the more anodic of the two fractions on electrophoresis, indicating that PGI II is chloroplastic and PGI I cytosolic [8, 10]. The electrophoretic mobilities of PGI I from developing and germinating seeds were the same as were also those of the PGI II fractions.

A number of properties of the PGI forms and the PMI from *C. coluteoides* and the MW of activities from several legume seeds were determined (Table 1). The values for PMI are all much less (about half) than those of PGI. This is in agreement with previous results for konjac corm PMI, 45000 [2], yeast PMI, 45000 [11], spinach leaf PGI, 120000 and *Clarkia xantiana* PGI 130000 [14]. The MW of PMI in developing and germinating seeds of *C. coluteoides* are similar as also are the PGI forms from both sources.

The pH optima in Tris–acetate buffer for PGI I from both developing and germinating *C. coluteoides* were broad (6–11) and for PGI II, 7–11. Maximal activity for both PMI enzymes occurred at pH 7.0, with half maximal activity at about 6.0, but both showed relatively high activity up to pH 11. The K_m values with Man-6-P, Glc-6-P and Fru-6-P, determined from Lineweaver–Burk plots, are given in Table 2. Substrate concentrations were varied between 0.4 and 80 mM for Man-6-P and 0.25 and 50 mM for Glc-6-P, with rates determined at 13–16 different concentrations at pH 7.5. The range of substrate concentrations for Fru-6-P with PGI was 0.05–2 mM. For Glc-6-P and Man-6-P, Fru-6-P appearance was assayed by the resorcinol method and for Fru-6-P with PGI, Glc-6-P production was assayed with Glc-6-P dehydrogenase.

A number of sugar phosphates at 2 mM concentration (ATP, 2-deoxy-Glc-6-P, 2-deoxy-Rib-5-P, Gal-1- and -6-P, gluconate, $GlcNH_2$-6-P, Glc-1,6-bis-P, Glc-6-SO_4, DL-glyceraldehyde-3-P, D-mannonate, Man-1- and -6-P, Rib-5-P, ribulose-1-P, xylulose-1-P, pyrophosphate) all produced negligible or slight (< 20%) inhibition of the two PGI and single PMI activities. Both PGI I and II from developing pods were significantly inhibited by 6-phosphogluconate, 6-phospho-2-deoxy-gluconate, erythrose-4-P, mannitol-1-P and 6-phosphomannonate when Glc-

Table 1. Molecular weights of phosphomannoisomerase and phosphoglucoisomerase

Plant source	Phosphomannose isomerase	Phosphoglucose isomerase	Method of determination
Developing *Cassia* pods	74500		Density gradient centrifugation
	68000		Gel chromatography
		PGI I, PGI II } 126000	Density gradient centrifugation
		PGI I, PGI II } 120000	Gel chromatography
Germinating *Cassia* seeds (cotyledons)	74500		Density gradient centrifugation
	67000		Gel chromatography
		PGI I, PGI II } 127000	Density gradient centrifugation
		PGI I, PGI II } 123000	Gel chromatography
Developing soybean pods	59000	128000	Gel chromatography
Developing guar pods	54000	96000	Gel chromatography
Developing fenugreek pods	63000	104000	Gel chromatography
Germinating lucerne seeds	46000	91000	Gel chromatography

Table 2. K_m values of PGI and PMI

	In developing seeds			In germinating seeds		
Enzyme	Man 6-P	Fru 6-P	Glc 6-P	Man 6-P	Fru 6-P	Glc 6-P
PMI	1.6 mM	n.d.*	—	1.9 mM	n.d.	—
PGI I	—	0.46 mM	1.5 mM	—	0.14 mM	1.1 mM
PGI II	—	0.23 mM	1.3 mM	—	0.35 mM	0.83 mM

*Not determined.

6-P was the substrate. 6-Phosphomannonate caused more inhibition than 6-phosphogluconate with both forms. Both enzymes were assayed in the direction of Fru-6-P formation and this was measured by the resorcinol method. Rabbit skeletal muscle PGI was found to be similarly inhibited in both directions of reaction by 6-phosphogluconate with K_i of 0.16 mM [15]. In contrast, 6-phosphogluconate gave only a slight inhibition of PMI from developing pods reacting with Man-6-P (10% at 2 mM). The other four compounds inhibited PMI competitively, with K_i values for D-erythrose-4-P of 0.98 mM; 6-phospho-2-deoxygluconate 0.66 mM, mannitol-1-P, 0.97 mM and 6-phosphomannonate 0.19 mM. Relatively insensitive competitive inhibitions of yeast PMI by erythrose-4-P (K_i, 8 mM) and 6-phosphogluconate (K_i 3.2 mM) have been reported [11]. D-Erythrose-4-P has been shown to inhibit PGI, from oat leaf with both Glc-6-P and Fru-6-P as substrates, as well as other plant enzymes, at the μM level and a proposal has been made for the possible significance of this potent inhibition [9]. The higher level required for the inhibition of *C. coluteoides* PMI, combined with the low estimated concentration of erythrose-4-P in plants would not suggest a direct physiological role with this enzyme. The sensitive inhibition of PGI by 6-phosphogluconate and D-erythrose-P, [9] in conjunction with the much less sensitive inhibition of PMI by D-erythrose-P and the very slight inhibition by 6-phosphogluconate may mean that in the cytoplasm of *C. coluteoides* an increase in the concentration of these compounds selectively reduces the activity of PGI relative to PMI, directing the flow of Fru-6-P, derived from translocated sucrose or from triose-P transported out of chloroplasts and surplus to the needs of glycolysis towards the production of galactomannan.

EXPERIMENTAL

Plant material. Cassia coluteoides pods were collected from shrubs in the Sydney region and plant samples identified at the Royal Botanic Gardens, Sydney. Lucerne (*Medicago sativa* cv Hunter Valley Broadleaf) and fenugreek (*Trigonella foenum-graecum*) were obtained commercially, soybean (*Glycine max* cv Lee) from the University of Sydney Farms and guar (*Cyamopsis tetragonolobus*) from the N.S.W. Department of Agriculture. *C. coluteoides* seeds were germinated in the dark at 30°, after prior treatment to enhance the germination rate by soaking in EtOH for 72 hr and washing thoroughly or nicking with a razor blade near the micropyle. Seeds were sterilized (0.5% NaOCl, 10 min) washed, soaked in H_2O and germinated on wet filter paper.

The samples for extraction and estimation of enzymic activity were bulk samples of pods or seeds with sufficient numbers to eliminate between-seed variation. The quantity was small enough to allow rapid disintegration and extraction. The values plotted in Figs 1 and 5 were then calculated and plotted per pod or per seed.

Preparation of 6-phospho-D-mannonate and D-mannonate solns [16]. D-Mannose or D-mannose-6-P (0.5 g) in H_2O (50 ml) and $BaCO_3$ (2 g) were stored with Br_2 (0.2 ml) in the dark overnight. Excess Br_2 was removed by aeration and the soln centrifuged. 1 M H_2SO_4 was added to ppt Ba^{2+}. After centrifugation the pH was adjusted to 5 and the soln stored overnight.

Assay of PMI and PGI (with resorcinol [17]). An aliquot was incubated with either Man-6-P or Glc-6-P in 100 mM Tris–HCl, pH 7.5 in a final vol. of 0.5 ml at 30° for 20 min. 27% HCl (2 ml) and 0.1% resorcinol in 95% EtOH (0.5 ml) was added and the mixture heated for 10 min at 80 ± 1° and then immersed in an ice bath and A_{530nm} compared with a standard curve for Fru-6-P.

Assay of PMI and PGI with (PGI)/Glc-6-P dehydrogenase [18]. 0.2 M NADP (50 μl), Glc-6-P dehydrogenase (1.7 nkat in 0.1 ml), 0.1 M Tris–HCl, pH 7.5 (2 ml) were mixed in a spectrophotometer cell, PMI (0.1 ml) added and A_{340nm} measured. In the assay of PGI, Man-6-P was replaced with Fru-6-P (20 mM) and PGI deleted. This method was only used in determining kinetic constants with purified preparations free of 6-phosphogluconate dehydrogenase activity and MW by density gradient ultracentrifugation.

Extraction and purification of PMI and PGI. Chilled, developing *C. coluteoides* pods (300 g) were homogenized for 2 min at 4° in 0.1 M Tris–HCl (1.2 l), pH 7.5, containing insoluble polyvinylpyrrolidone (300 g) and purified *Bacillus subtilis* β-mannanase (3.5 μkat) [12]. After centrifuging (20 000 *g*, 30 min, 4°), the supernatant was decanted through Miracloth. The fraction pptd by 40–65% $(NH_4)_2SO_4$ was re-dissolved in 10 mM Tris–HCl and, after dialysis, applied to a DEAE-cellulose column equilibrated with this buffer, washed with 3 vols of buffer and eluted with a linear gradient of buffer (10–500 mM). Active fractions were concd in a Diaflo cell with PM-10 membrane and applied to a column of Sephacryl S-200 superfine and eluted with 0.2 M Tris–HCl, pH 7.5. Germinated seeds were dissected and cotyledons extracted with no added β-mannanase. The crude extract of developing seeds had a sp. act. for PMI of 0.58 nkat per mg protein (total protein 1860 mg) and after Sephacryl S-200 chromatography the sp. act. was 11.6 nkats per mg protein (total protein 23 mg), a 19.9 fold purification. The sp. act. of PGI in the initial extract was 20 nkat per mg protein and the final activity for PGI I 107 nkat per mg (total protein 12 mg) and for PGI II 48 nkat per mg (total protein 26 mg).

Estimation of changes in PMI and PGI activities during development and germination. Developing *C. coluteoides* pods (5 g fresh wt.) were homogenized at 4° in 0.1 M Tris–HCl, pH 7.5 (25 ml) with polyvinylpyrrolidone (5 g). Mature seeds were milled in a Janke and Kunkel water-cooled mill and *B. subtilis* β-mannanase added to the meal during extraction. The homo-

genate was centrifuged (20 000 *g*, 30 min, 4°) and the supernatant dialysed against extraction buffer for 18 hr. On germination, seeds (60) were dissected and radicles and testa were removed prior to homogenization.

The enzymic assays on the extracts were replicated at least three times and individual values were always within 5%. The extraction procedure was made optimal following preliminary studies of activity extracted at various pH values and ionic strengths of buffer. Samples of both rapidly expanding and germinated seeds were studied and the extraction procedure modified until the enzymic values were reproducible. Different β-mannanase and PVP levels were also applied and the best levels used.

Polyacrylamide gel electrophoresis. The acrylamide concn was 10% and protein samples contained 1 μg per μl. PGI was detected [19] by incubating at 4° in the dark with Fru-6-P (80 mg), NADP (10 mg), phenazine methosulphate (1 mg), thiazolyl blue (10 mg), $MgCl_2$ (40 mg), 0.2 M Tris–HCl, pH 8, (25 ml), H_2O (25 ml) and glucose-6-P dehydrogenase (13 μkat). Yeast PMI was detected [20] by incubating with Man-6-P (50 mg), NADP (15 mg), nitro blue tetrazolium (15 mg), phenazine methosulphate (1 mg), $MgCl_2$ (50 mg), 0.2 M Tris–HCl, pH 8, (10 ml), H_2O (40 ml), PGI (1.7 μkat) and glucose-6-P dehydrogenase (1.3 μkat).

Determination of MW (gel chromatography). The column material was Sephacryl S-200 superfine and the standards cytochrome *c*, ovalbumin, glucose-6-P dehydrogenase, aldolase, alcohol dehydrogenase and malate dehydrogenase. *Density gradient ultracentrifugation* [21]. A gradient of 5–20% sucrose in 50 mM Tris–HCl (pH 7.5) was used with malate dehydrogenase, glyoxylate reductase, alcohol dehydrogenase and aldolase as standards. Samples were applied from band forming caps and centrifuged at 60 000 rpm for 6 hr at 5°.

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