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PHOSPHOMANNOMUTASE AND PHOSPHOGLUCOMUTASE IN DEVELOPING *CASSIA CORYMBOSA* SEEDS

DARRYL M. SMALL and N. K. MATHESON
Department of Agricultural Chemistry, The University of Sydney, N.S.W. 2006, Australia

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Key Word Index—*Cassia corymbosa*; Leguminosae; galactomannan biosynthesis; phosphomannomutase; phosphoglucomutase.

Abstract—Phosphomannomutase and phosphoglucomutase in developing *Cassia corymbosa* seeds have been completely separated from each other and from glucose phosphate and mannose phosphate isomerases by a series of chromatographic procedures that included affinity elution chromatography. Some properties, including the K_m for D-mannose 1,6-biphosphate with phosphomannomutase, are described. The activities of phosphoglucomutase and phosphomannomutase in some other plant tissues are also compared. The significance of these enzymes and the pathway of galactomannan synthesis are discussed.

INTRODUCTION

The synthesis of D-mannose containing polysaccharides, such as galactomannans in the endosperm of legume seeds, could involve conversion of sugars of the gluco configuration, formed from photosynthesis, to the manno configuration, as sugar phosphates, nucleoside diphospho sugars or as free sugars. No evidence has been found for the conversion of fructose to mannose in plants as the unsubstituted sugars [1]. Epimerization of GDP-mannose to GDP-glucose may be catalysed by particulate preparations from mung bean seedlings, since D-Glc-[^{14}C] was released on hydrolysis of the glucomannan synthesized from GDP-Man-[^{14}C] [2] and it has been suggested that the conversion in plants follows this pathway [3]. However, this epimerization was not observed with particulate fractions from orchids [4]. The interconversion of nucleoside diphospho-D-glucose and D-mannose has been found in microorganisms [5]. Evidence that mannose synthesis in plants occurs at the sugar phosphate level comes from the detection of GTP: Man 1-P guanylyl transferase (EC 2.7.7.13) in developing cotyledons of *Gleditsia* [6] and Man-P isomerase (EC 5.3.1.8) in konjac corms [1] and germinating legume seeds [7]. The occurrence of this latter enzyme in seeds that were rapidly hydrolysing galactomannan was related to the presence of a pathway for D-Man utilization by conversion to Fru 6-P via Man 6-P. It is possible that Man-P isomerase in konjac corms also functions in mannan utilization. However, the detection of phosphomannomutase (PMM) in konjac corms [8] and in developing *Cassia corymbosa* seeds [9] provides more definite evidence for the pathway, Fru 6-P $\rightleftharpoons$ Man 6-P $\rightleftharpoons$ Man 1-P $\rightleftharpoons$ GDP-Man $\rightarrow$ (galacto)mannan.

Phosphomannomutase was first described in yeast [10] when it was partly separated from phosphoglucomutase (PGM, EC 2.7.5.1) by $(NH_4)_2SO_4$ fractionation. More recently Murata [8] has described its incomplete separation by chromatography from PGM in konjac corm extracts.

This paper describes the complete separation of *Cassia corymbosa* PMM from PGM, Man-P isomerase and Glc-P isomerase and gives some of its properties. A preliminary account has been published [9]. Also, although the properties of PGM from a number of animals and microorganisms have been described, there are limited details of this enzyme from plant sources. The PGM of *Cassia corymbosa* has also been purified and some properties measured.

RESULTS AND DISCUSSION

Several plant sources were examined for PGM and PMM activities. Extracts were assayed by estimating the decrease in acid hydrolysable phosphate when either D-Glc 1-P or D-Man 1-P, respectively, was added. Phosphatase was also assayed at the same pH by the increase in inorganic phosphate (Fiske–Subbarow method), but this was negligible in all extracts (Table 1). No PMM was found in mung bean cotyledons, consistent with the absence of galactomannan and the presence of starch as storage polysaccharide. Low PMM activity relative to PGM was found in the growing parts of mung bean

Table 1. PGM and PMM activities in some plant tissues

Plant		Enzyme activity nkat/g fr. wt	
		PGM	PMM
Mung beans, germinated at 30°, 2.5 days (6–8 cm high)	cotyledons	230	nd*
	remainder	210	5.5
Peas, germinated at 25°, 2.5 days (radicle just emerged)		1520	9.8
Cymbidium tuber		7.7	1.1
Developing *Cassia corymbosa* seeds		248	42

* nd—Not detected.

seedlings and whole peas. Although these do not contain storage galactomannan, PMM may be required for the synthesis of mannose containing structural polysaccharide [11]. Developing *Cassia corymbosa* seeds had the highest PMM activity and the highest proportion relative to PGM.

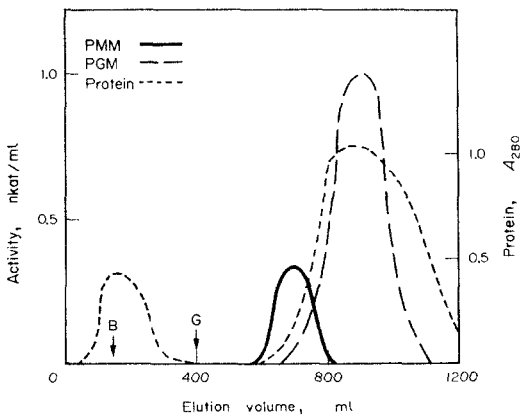

Fig. 1. Chromatography of PGM and PMM from *Cassia corymbosa* on DEAE-cellulose. B, buffer wash applied; G, gradient applied.

These seeds were used for the extraction and purification of both mutase activities. Pi concentration in enzyme assays was estimated by the Bartlett method. Seeds were macerated in buffer (pH 6.2) that contained 40 mM histidine and 5 mM $Mg(OAc)_2$. PVP was also present. The fraction precipitated by 30–80% $(NH_4)_2SO_4$ was collected and chromatographed on DEAE-cellulose with a KCl gradient in Tris buffer pH 8 (Fig. 1). This gave an incomplete separation of single peaks of activity of PGM and PMM, as was found with the konjac enzymes [8]. The fractions were also assayed for hexosephosphate isomerase activity by measuring the reduction in colour with resorcinol, on incubation of an extract with Fru 6-P [12]. This activity was found to elute as a single peak between PMM and PGM, so that most of the tube fractions of these enzymes contained it. PGM and PMM were separated by chromatography on phosphocellulose (Fig. 2). When the active portion of the eluate from DEAE-cellulose was applied in Tris buffer (pH 8), PGM, hexosephosphate isomerase and most of the protein was unbound but PMM was bound. PMM could be eluted with high concentrations of salt but more conveniently as a single peak with 0.5 mM Fru 1,6-biP. This appears to be an example of affinity elution chromatography which has recently been described for the purification of glycolytic enzymes from animal muscle [13]. The Fru 1,6-biP may mimic a substrate structure or, since it was prepared by cellular extraction, it may contain a suffiently high concentration of Glc 1,6-biP to effect elution. Later, it was found that the two activities could be completely separated on DEAE-cellulose using a SO_4^{2-} gradient. The affinity chromatography procedure, however, had the advantage of not introducing high levels of anions into the eluant, some of which (e.g. SO_4^{2-}, Cl^-) were found to be inhibitory. Polyacrylamide gel electrophoresis of the PMM at this stage showed one major protein band with a faster moving trace band. If a subsequent gel chromatography step with Sephadex G-100 was added, two bands were still obtained. The overall purification was 262-fold and the recovery 16%.

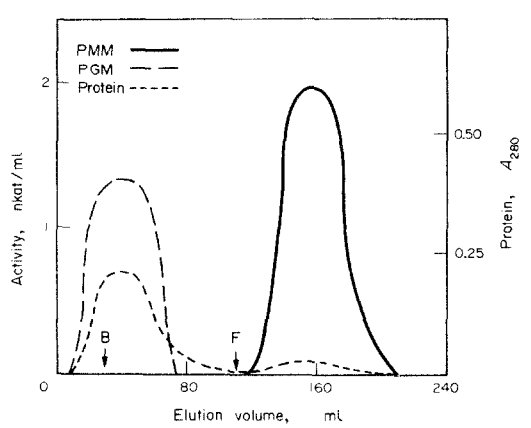

Fig. 2. Chromatography of PGM and PMM from *Cassia corymbosa* on phosphocellulose. B, buffer wash applied; F, Fru 1,6-biP applied

Attempts to purify the PGM using phosphocellulose were not successful, as, even when the pH was as low as 6, no binding occurred, although the enzyme from rabbit muscle can be purified by this technique [13]. The procedure used for purification of PGM was passage through a column of phosphocellulose after DEAE-cellulose chromatography, to remove PMM, followed by gel chromatography on Sephadex G-100, when PGM was retarded and most of the protein, including hexosephosphate isomerase, was excluded. The overall purification was 20-fold and the recovery 10%.

The products of reaction of PMM were examined by PC. After removal of cations with ion exchange resin, chromatographing in a solvent that separated Man 1-P, Man 6-P, Fru 6-P, Glc 1-P and Glc 6-P showed that a time series of Man 1-P incubations with purified enzyme produced increasing amounts of Man 6-P. These were the only two sugar phosphates that were detected on the chromatogram.

A number of properties of the purified PMM and

Table 2. Properties of PMM and PGM from developing *Cassia corymbosa* seeds

Property	PMM	PGM
MW		
Gel chromatography	58000	58000
Density gradient *ultra-centrifugation*	41000	60000
Equilibrium, 1-P: 6-P	19:81	5:95
Concentration for optimum activation		
by histidine	10 mM	nd*
by Mg^{2+}	2.5 mM	nd
pH optimum	6.5–7.0	7.5
pH for half maximal activity	5.5, 7.6	6.8, 8.3
K_m values		
D-Man 1-P	0.15 mM	—
D-Man 6-P	0.3–0.5 mM	—
D-Glc 1,6-biP	0.87 μM	0.50 μM
D-Man 1,6-biP	2.5 μM	—
D-Glc 1-P		0.065 mM

* nd—Not determined.

PGM were determined and these are shown in Table 2. The MWs of both enzymes were estimated as 58000 by gel chromatography and they could not be separated by this technique. PGM from potato tubers had a MW of 63000 [14] and PMM from konjac corms a value of 62000 [8] determined by gel chromatography. More recently, two PGM activities have been separated on DEAE-cellulose from potato [15]. However, the two *Cassia* activities sedimented at different rates on sucrose density gradient ultra-centrifugation and the MW of PMM was estimated as 41000 and of PGM as 60000. The equilibrium for Man 1-P: Man 6-P with PMM was 19:81 at pH 7.5. Murata [8] found this value to be 11:89 at pH 7 for the konjac enzyme. For activity, both *Cassia* PGM and PMM required the presence of histidine, presumably to bind some divalent cations which have been found to inhibit rabbit muscle PGM [16]. Added Mg^{2+} and low levels of hexosebiphosphate were also required. Either D-Glc 1,6-biP or D-Man 1,6-biP were utilized by PMM and the K_m values for both of these were similar, indicating that activity with D-Glc 1,6-biP was not due to D-Man 1,6-biP contamination. Konjac PMM also reacted with Glc 1,6-biP. A phosphopentomutase from *E. coli* has been found to use as co-substrates D-Glc 1,6-biP or D-Rib 1,5-biP or 2 deoxy D-Rib 1,5-biP [17]. Rabbit muscle PGM may also react with a range of biphosphate substrates [18] including Fru 1,6-biP under appropriate conditions [19].

In this study, the D-Man 1,6-biP was prepared by heating tetra-acetyl D-Man 6-P with crystalline phosphoric acid under diminished pressure [20]. Man 1,6-biP was prepared by elution after thick PC.

The apparent K_m for Man 1-P with *Cassia* PMM was similar to that found for the konjac enzyme. The K_m for Man 6-P is difficult to determine accurately as it is low and the equilibrium lies well towards Man 6-P. The value determined for *Cassia* PMM was *ca* 2–3 times that for Man 1-P. Both these K_m values are *ca* 10-fold the value for rabbit muscle PGM with Glc 1-P as substrate [21]. Other K_m values for Glc 1-P with PGM from other animal and microbial species [21, 22] are similar to the K_m (Man 1-P) with *Cassia* PMM and the K_m (Glc 1-P) with *Cassia* PGM. For PGM from both animal and microbial sources, the K_m values determined for the co-substrate D-Glc 1,6-biP have varied but have always been very low [18, 21, 22]. The hexosebiphosphate values with *Cassia* PMM and PGM lie at the higher end of the range. The value for D-Glc 1,6-biP with *Cassia* PMM was similar to that determined for konjac PMM. The $(NH_4)_2SO_4$ fraction from yeast, showing PMM activity, gave much higher values than any that have been reported for other mutases [10].

The isolation of PMM activity from konjac and its complete separation from PGM in developing *Cassia* seeds indicate that the conversion of sugars with the D-gluco configuration, produced from photosynthesis, glycolysis or transport, into the mannan portion of plant storage polysaccharides, probably involves hexose phosphates.

EXPERIMENTAL

Plant material. Seeds were sterilized (0.5% NaOCl, 10 min), washed, soaked in H_2O and germinated in rolled filter paper sheets. *Pisum sativum* (cv Alaska) seeds were grown at 25° and *Vigna radiata* cv aureus (L.) Wilczek (mung beans) at 30°, both in the dark for 2.5 days. Seed coats were discarded and, for mung beans, cotyledons were separated from the remainder before enzyme extraction. Bulb material of a garden orchid (*Cymbidium* sp.) was used freshly and the outer layers discarded. Developing pods of *Cassia corymbosa* were collected while still green and expanding. Seeds were removed and macerated immediately or pods were frozen in liquid N_2 and stored at −15°.

Enzyme substrates. Sugar monophosphates were K or Na salts from Sigma. Glc 1-P (Sigma Grade V) was used for the determination of kinetic parameters of PGM. Glc 1,6-biP was also from Sigma. D-Mannose 1,6-biphosphate was prepared from D-Man 6-P (Sigma, Ba salt) by heating the tetra-acetate with crystalline phosphoric acid. The previously described procedure [20] was modified. After removal of Pi by the addition of LiOH, cations were exchanged for cyclohexylammonium ion by passage through Dowex 50W in the cyclohexylammonium form. PC (*n*-PrOH–0.01% EDTA–conc NH_3, 7:2:1), drying and dipping in molybdate reagent (12.5% NH_4 molybdate (8 ml), $HClO_4$ (70%, 2 ml), 2 M HCl (4 ml) and Me_2CO (86 ml), partly dried, heated at 100° for 1 min, and irradiated) showed two fractions that migrated more slowly than Pi and hexosemonophosphate. There was a major fraction R_{Pi} 0.23 with a mobility similar to that of Glc 1,6-biP and Fru 1,6-biP and a faster minor spot at R_{Pi} 0.35. The mixture was chromatographed on Whatman 3 MM and the slower, major fraction eluted. Mannose 1,6-biP content was estimated from acid hydrolysable Pi released.

Preparation of extracts for assay of mutase level. Fresh tissue (*ca* 1 g) was homogenized in a blender and an Ultra-turrax with insoluble PVP (acid washed, 1 g per g fr. wt) and buffer (30–50 ml—40 mM histidine HCl, 10 mM Tris, 5 mM $MgSO_4$, pH 8.2 at 0°). The extract was filtered through Miracloth and centrifuged (20000 *g*, 15 min, 2°). Before assay, an aliquot of the supernatant was suitably diluted (10–50-fold) with buffer (10 mM histidine, 10 mM Tris, 5 mM $MgSO_4$, adjusted to pH 7.5 at 30°).

Assay of PMM and PGM activities. Histidine (10 mM) and Mg^{2+} (5 mM) were present in the buffered enzymic solns and, when dilution was necessary before assay, this supplemented buffer was used. For assay, enzyme soln (0.1 ml) was preincubated at 30° for 10 min, after which substrate (0.1 ml) was added. Man 1-P was used for assays of PMM and Glc 1-P for PGM, both with added biphosphate and Pi estimated by method A or B. (A) Assay by the Fiske–Subbarow method [23]. Enzymic reaction was stopped by the addition of H_2SO_4 (4 M, 0.5 ml) and the tubes heated for 5 min at 100°. H_2O (5 ml) was added, followed by molybdate (2.5%, 1 ml) and aminonaphthol sulphonic acid reagent (0.5 ml). After 10 min, A_{670} was measured. The substrate contained 10 mM monophosphate and 0.1 mM Glc 1,6-biP and the presence of phosphatase activity in extracts was checked by also assaying tubes which had not been heated. (B) Assay by the Bartlett heating method [24]. Reaction was stopped by the addition of H_2SO_4 (0.74 M, 1.7 ml) and then molybdate (1%, 0.5 ml) and aminonaphthol sulphonic acid reagent (0.1 ml) were added. Tubes were heated (100° for 7 min), cooled and A_{830} measured. For this assay, substrate contained 1 mM monophosphate and 0.02 mM Glc 1,6-biP.

Extraction and purification of PMM and PGM. Developing pods of *Cassia corymbosa* were macerated at 2° with PVP (50 g) and buffer (500 ml) (40 mM histidine, 5 mM $Mg(OAc)_2$, pH 6.2). After filtration through Miracloth, a further quantity of PVP (50 g) was added. After filtering and centrifuging, the supernatant was adjusted to pH 8 with Tris (1 M, pH 9). Solid $(NH_4)_2SO_4$ was added to 30% satn and the ppt. discarded. The 30–80% fraction was collected, redissolved and dialysed for 18 hr against

buffer (Tris 10 mM, histidine 10 mM, $Mg(OAc)_2$ 5 mM, pH 8). This buffer was present in all solns at all later steps. A DEAE-cellulose column (150 ml) was pre-equilibrated with buffer, the dialysed extract applied and protein eluted with buffer (250 ml) followed by a linear KCl gradient (0–300 mM). All tubes showing mutase activity were pooled, dialysed, and applied to a phosphocellulose column (15 ml). Protein was eluted with buffer (100 ml). The PGM and hexosephosphate isomerase activities were eluted. The column was then eluted with buffer containing 0.5 mM Fru 1,6-biP when PMM was eluted. The two fractions were concd in dialysis tubing under red. pres. and then dialysed. Each fraction was chromatographed on Sephadex G-100 in buffer containing 100 mM NaOAc. The sp. act. of PMM in the unfractionated extract was 170 pkat per mg protein. At each fractionation step the degrees of purification and the sp. act. (nkat) were: $(NH_4)_2SO_4$ ppt., ×3.3 and 0.57; DEAE-cellulose, ×5.3 and 3.0; phosphocellulose, ×15 and 45. Sephadex G-100 chromatography did not change the degree of purification or sp. act. The sp. act. of PGM in the unfractionated extract was 890 pkat per mg of protein. At each fractionation step the degrees of purification and the sp. act, (n kat) were: $(NH_4)_2SO_4$ ppt., ×1.7 and 1.5; DEAE-cellulose, ×4.7 and 7.0; phosphocellulose, no change and Sephadex G-100, ×2.5 and 17.6.

PC of sugar phosphates from PMM reaction. Purified PMM (0.1 ml) was incubated with 20 mM Man 1-P (0.1 ml) and 0.1 mM Glc 1,6-biP (0.1 ml) at 30°. Samples (30 μl) were withdrawn at intervals from 0 to 3 hr and cations removed by adding a few beads of cation exchange resin. Whatman No. 1 papers were prepared by irrigating twice with HCO_2H (1 M) and drying before sample application. The temp. was 16° ± 1 and the irrigation solvent EtOAc–formamide–Py (4:4:1) [25]. Chromatograms were dried, dipped in molybdate reagent, partly dried, heated at 100° (1 min) and irradiated.

Determination of MW. Purified enzymes were chromatographed on Sephadex G-100 and Biogel P150 in buffer (Tris 10 mM, histidine 10 mM, $Mg(OAc)_2$ 5 mM) containing 0.1 M NaOAc. The standards were *E. coli* alkaline phosphatase (78000), BSA (68000), ovalbumin (45000) and myoglobin (17000). MW was also estimated by sucrose density gradient ultracentrifugation [26]. Gradients were prepared from 1 ml each of 20, 16, 12, 8 and 4% sucrose in buffer and equilibrated for 18 hr at 2°. Samples (0.1 ml) were applied from band forming caps and centrifuged at 64000 rpm at 10° for 8 hr in a Beckman SW 65 K rotor. Standards were *E. coli* alkaline phosphatase (6.3 S), pig heart malate dehydrogenase (4.3 S), pig pancreas α-amylase (4.5 S) and myoglobin (2.0 S).

Determination of kinetic properties. All initial velocities were measured by mixing equal vols. of enzyme and substrate solns and sampling (0.2 ml unless specified) at various times. For the estimation of pH optima a concd soln of enzyme (5 or 10 μl) was diluted in buffer (5 ml of Tris 20 mM, histidine 10 mM and $Mg(OAc)_2$ 10 mM) adjusted at 30° to pH values of 5 to 9 before preincubation and assay. The effects of varying Mg^{2+} and histidine were studied by the same dilution method. One was varied between 0–10 mM and the other kept constant at 5 mM at pH 6.8. Equilibria were determined by incubating purified enzyme with either substrate (1-P or 6-P, 2 mM) and Glc 1,6-biP (10 μM) at pH 7.5 and 30°. For the determinations of apparent Michaelis constants, both enzymes and substrates were prepared in buffer (2.5 mM $Mg(OAc)_2$ and 10 mM histidine, pH 6.8 at 30°). The K_m of PMM for Glc 1,6-biP was determined using a concn range 0.75–50 μM at 1.0 mM Man 1-P. The K_m for Man 1-P was determined by taking samples (0.2–1.5 ml as appropriate), stopping the reaction with 3.13 M H_2SO_4 (0.4 ml) and adjusting the vol. to 1.9 ml before assaying Pi. The concn range was 0.025–1.0 mM with Glc 1,6-biP constant at 5 μM. For Man 6-P, the concn range was 0.1 1.0 mM, the aliquot vols. were 0.2–1.0 ml and the biphosphate concn was 5 μM. For PGM, similar concn ranges and conditions were used but the buffer also contained Tris–HCl (10 mM) and incubations were adjusted to pH 7.5 and 30°.

Assay of hexosephosphate isomerase activity. Enzyme soln (0.1 ml) was incubated with Fru 6-P (0.1 ml, 3.3 mM in 10 mM Tris, pH 7.5). The reaction was stopped by the addition of resorcinol and colour developed as described previously [7].

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