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A galactomannan-hydrolysing α-galactosidase from jack fruit (*Artocarpus integrifolia*) seed: Affinity chromatographic purification and properties

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Abstract. An acid α-galactosidase from the seeds of the jack fruit seed (*Artocarpus integrifolia*) has been purified to homogeneity by affinity chromatography on a matrix formed by cross-linking the soluble α-galactose-bearing guar seed galactomannan. The 35kDa enzyme was a homotetramer of 9·5kDa subunits. Its carbohydrate part (5·5%) was composed of galactose and arabinose. The K_m with *p*-nitrophenyl α-D-galactoside as substrate was 0·35 mM. The K_i values indicated inhibition by galactose, 1-O-methyl α-galactose and melibiose in the decreasing order. Among α-galactosides, the enzyme liberated galactose from melibiose, but not from raffinose or stachyose at its pH optimum (5·2). The guar seed galactomannan was however efficiently degalactosidated; limited enzyme treatment abolished the precipitability of the polysaccharide by the α-galactose-specific jack fruit seed lectin, and complete hydrolysis yielded insoluble polysaccharide. Though similar in sugar specificity and subunit assembly, α-galactosidase and the lectin coexisting in the jack fruit seed gave no indication of immunological identity.

Keywords. α-Galactosidase; galactomannan; jack fruit seed; *Artocarpus integrifolia.*

Introduction

Storage polysaccharides in seeds are mostly starches or galactomannans (Dey, 1978) in which the mannan back bone is built of *ß* (l→4) linked mannose residues and single unit galactose side chains are attached α (l→6) to all or some of the mannose residues (Baker and Whistler, 1976). The extent and pattern of galactosidation on the mannan backbone varies among plant varieties (Dey, 1978). Galactomannan polysaccharides serve as water retainers for the seeds due to their swelling property, in addition to being food reserve for the germinating seeds. The latter process is initiated during germination by endogenous exo-acting α-galactosidase (EC 3·2·1·22) which hydrolyse the galactomannans to liberate galactose (Dey, 1980). α-Galactosidases have been characterized in many seeds including *Cyamopsis tetragonolobus* (guar), *Coffea sp* (Coffee beans), *Lens culinaris* (lentil) and *Phaseolus vulgaris* (pinto beans) (Dey, 1984).

We detected in the seeds of the tropical jack fruit (*Artocarpus integrifolia*) an α-galactosidase which readily liberates α-linked galactose from galactomannans. We had earlier reported the purification and characterization, interaction with polysaccharides, and sugar binding kinetics of an α-D-galactose-specific lectin (jack

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Abbreviations used: JSA, Jack fruit seed agglutinin; PNP α-Gal, para-nitrophenyl α-D-galactoside; BSA, bovine serum albumin; PBS, 20 mM sodium phosphate buffer pH 7·4 containing 150mM NaCl; SDS, sodium dodecyl sulphate; PAGE, Polyacrylamide gel electrophoresis.

fruit seed agglutinin, JSA) from this seed (Suresh kumar *et al.,* 1982; Appukuttan *et al.,* 1984; Appukuttan and Basu, 1985). In this communication the purification of jack fruit seed α-galactosidase by afinity chromatography on a cross-linked galactomannan (guar gum) gel, its properties, and action on various galactosides includeing soluble guar gum are described. Results show that though the enzyme differs from the lectin of the same seed in pH optimum of substrate binding and immunologically, the two proteins are remarkably similar in sugar specificity. While many seeds containing both α-galactosidases and lectins have been reported, none of these lectins are α-galactoside specific (Goldstein and Hayes, 1978; Dey, 1984). The jack fruit seed is unique in that its principal glycosidase as well as lectin are both specific to α-galactosides. In so far as the physiological function of lectins is not known, this seed therefore offers an excellent system to study the role of lectins *vis-a-vis* that of glycosidases in the processing, storage and utilization of glycoconjugates.

Materials and methods

Para-nitrophenyl α-D-galactoside (PNP-α-Gal), guar gum and 1-O-methyl α-galactoside and all molecular weight standards were purchased from Sigma Chemicals Company, St. Louis, Missouri, USA. Melibiose, raffinose and stachyose were purchased from Romali Chemicals, Bombay. JSA was purified by the method of Suresh kumar *et al.* (1982). Guar gum was cross-linked using epichlorohydrin in alkaline medium as described previously to obtain an insoluble matrix (Appukuttan *et al.,* 1977). To obtain purified guar gum for use as substrate, a saturated solution in water was mixed with an equal volume of 99·5% ethanol. The precipitate separated by filtration was washed with 50% ethanol and finally dried in vacuum. Estimation of neutral sugar was by the method of Dubois *et al.* (1956) and of protein by the method of Lowry *et al.* (1951). α-Galactosidase activity was assayed by incubating aliquots with 375 n mol of PNP α-Gal in the presence of 100 μg bovine serum albumin (BSA) in 0·5 ml 50 mM citrate-phosphate buffer, pH 5·2 for 30 min at 37°C. The tubes were heated at 100°C for 1 min, cooled and the liberated *p*-nitrophenol measured at 405 nm after adding 2·5 ml 0·5 M glycine-NaOH bufer, pH 10·5. One unit of activity was defined as that amount of enzyme which liberates 1 μ mol *p*-nitrophenol per min at 37°C.

Purification of α-galactosidase

The ammonium sulphate precipitate (at 65% saturation) of 20 mM sodium phosphate bufer pH 7·4, containing 150 mM NaCl (PBS)-soluble proteins from 50 g jack fruit seed was prepared as described earlier (Sureshkumar *et al.,* 1982). This was dissolved in 10 ml of 20 mM sodium acetate buffer, pH 5·0 and dialyzed against the same bufer for 18 h. The dissolved proteins were passed through a column of CM-Sephadex (2×15 cm) equilibrated with the above buffer. The unbound protein was dialyzed for 18 h against 20 mM citrate-phosphate buffer, pH 5·2 containing 150 mM NaCl and concentrated by ultrafiltration to 10 ml. This was applied to a column of cross-linked guar gum (2·5×15 cm) which was washed with the same buffer and 8 ml fractions were collected. The fractions were monitored for both protein concentration and enzyme activity. When the absorbance at 280 nm was below 0·01, the

bound α-galactosidase was eluted by PBS. The high specific activity fractions (18-30, figure 1) were pooled, dialyzed against PBS and concentrated by ultrafiltration.

Gel filtration and electrophoresis

Molecular weight was determined by gel filtration as described by Andrews (1965), on a Biogel P-100 column (2·3 × 85 cm) using BSA, ovalbumin, chymotrypsinogen and cytochrome C as standards. Gel electrophoresis at pH 8·3 was. done in 7·5% acrylamide as described by Davis (1964). Sodium dodecyl sulphate (SDS) Polyacrylamide gel electrophoresis (PAGE) with or without 2-mercaptoethanol was done according to Laemmli (1970), using ovalbumin, chymotrypsinogen, soybean trypsin inhibitor, cytochrome C and JSA as standards.

Kinetics

Michaelis-Menton constant (K_m) and the inhibition constants (K_i) of galactoside derivatives 1-O-methyl α-galactoside, melibiose and galactose using PNP α-Gal as substrate were determined according to Dickson and Webb (1979).

Hydrolysis of natural sugars

One ml, 80 mM solution, of melibiose, lactose, raffinose and stachyose and 3 ml of a saturated solution of purified guar gum, all in 0·5 M citrate-phosphate buffer, pH 5·2 and containing 0·09 unit of the purified enzyme per ml were incubated for 48 h at 37°C. The samples were then heated in boiling water bath for 1 min (to destroy enzyme activity) and neutralized with 6 N NaOH, before analysis by paper chromatography.

Bulk of this partially hydrolysed guar gum was purified by gel filtration of the reaction product on a Biogel P-60 column (1·5×60 cm) in PBS at 4°C. The polysaccharide emerged in the void volume, free from protein, as shown by absorbance at 280 nm. This guar gum sample was then compared with purified guar gum for precipitability with JSA, as described previously (Appukuttan *et al.,* 1984). Briefly, increasing amounts of the two guar gum samples were treated separately with 1 mg JSA in 3 ml PBS at 25°C and the turbidity produced measured after 15 min at 405 nm.

Enzyme hydrolysis of guar gum as above was partial as the product remained soluble. For complete hydrolysis leading to precipitation of the product, guar gum was incubated under the same conditions for 48 h but using 0·90 unit of enzyme per ml, one half of which was added 24 h after the other.

Component sugars and paper chromatography

Complete hydrolysis of oligosaccharide chains of α-galactosidase to individual sugars by 2 NHC1 and separation of amino and neutral sugars were carried out as described, by Spiro (1966). Neutral sugars from α-galactosidase molecule, as well as the product of enzyme treatment on galactoside sugars described above, and mono-

saccharide standards were analysed by paper chromatography using the solvent system butanol:ethanol:.water (10:1:2) for 120 h and visualized with $AgNO_3$ spray.

Results

Purification

The seed proteins that emerged unbound to the CM-Sephadex column contained most of the enzyme activity, but no lectin, as seen by haemagglutination and immunodiffusion against rabbit anti-JSA antibody. This strong basicity of JSA is in agreement with its high mobility and lack of mobility, respectively in acid and alkaline pH electrophoresis (Sureshkumar *et al*., 1982). Chromatography of the lectin-free proteins on cross-linked guar gum column (figure 1) facilitated specific binding of α-galactosidase activity on the column at pH 5·2 (at 4°C) and elution at pH 7·4, without change in ionic strength Elution of the column with 100 mM galactose at pH 5·2, instead of a pH change to 7·4 also showed a similar elution pattern (results not shown). The latter elution was not used for routine purification, since galactose inhibited assay of activity in fractions. Protein and activity level at each purification step (table 1) indicates 82-fold purification by affinity chromatography alone and an overall purification of 2350-fold.

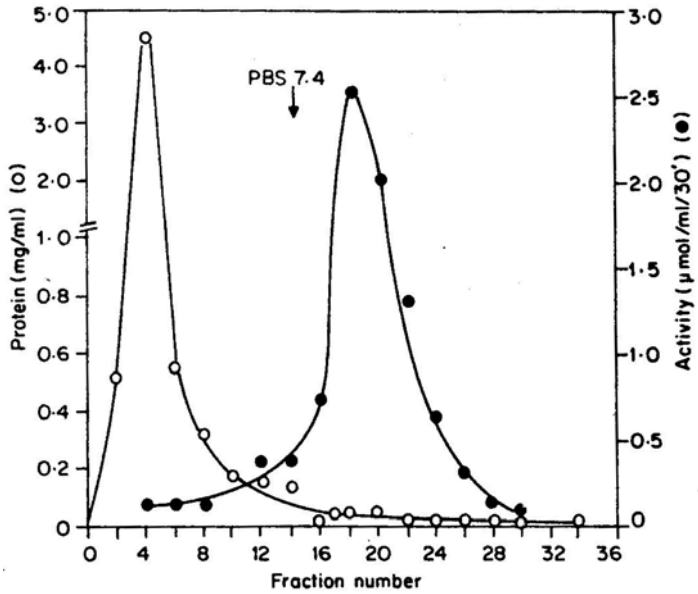

Figure 1. Chromatography on cross-linked guar galactomannan of jack fruit seed proteins unbound to CM-Sephadex. The proteins were applied at pH 5·2 and eluted with PBS, pH.7·4 (arrow). Details are in the text. (O), Protein (mg/ml); (●), enzyme activity (as μ mol of *p*-nitrophenol liberated by 1 ml in 30 min).

Structure

The enzyme purified as above showed a single band in PAGE (figure 2A). Molecular weight determined by gel filtration on Biogel P-100 was 35kDa. Only a single 9·5kDa

Table 1. Purification of jack fruit seed α-galactosidase.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units per mg × 10^3)	Fold purification
Homogenate supernatant	10·43	3300	3·16	1
Ammonium sulphate precipitate	9·76	2233	4·37	1·4
CM-Sephadex-ion exchange	7	76·49	91·51	29
Cross-linked guar-gum affinity chromatography	4·27	0·575	7430	2350

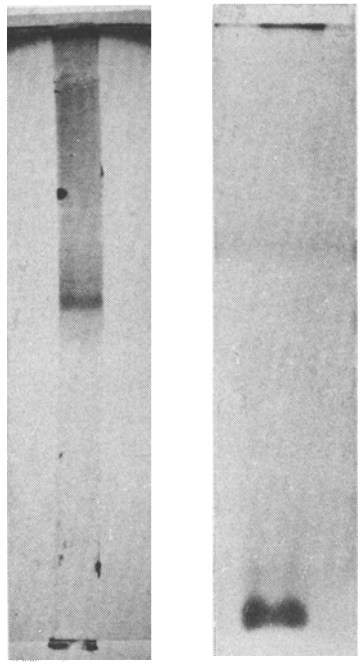

Figure 2. Electrophoresis of jack fruit seed α-galactosidase. (A), pH 8·3 in 7·5% Polyacrylamide gel (20 μg protein). (B), SDS·PAGE with or without 2-mercaptoethanol according to Laemmli (1970), using 10% acrylamide and 30 μg protein.

subunit was observed by SDS-PAGE with or without 2-mercaptoethanol (figures 2B and 3). These results suggest that the enzyme is a homotetramer of noncovalently linked 9·5kDa subunits. Notably the lectin from the same seed (JSA) is also a homotetramer with a molecular weight of 39·5kDa and subunit molecular weight of 10kDa (Sureshkumar *et al.,* 1982). However the purified enzyme neither agglutinated

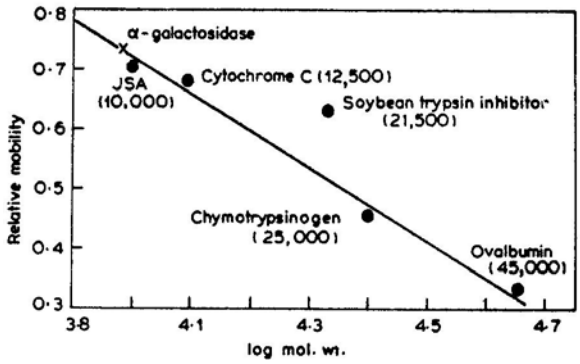

Figure 3. Estimation of subunit molecular weight of α-galactosidase by SDS-PAGE according to Laemmli .

native or trypsin treated rabbit or human erythrocytes, nor was it immunologically cross-reactive with JSA when tested in Ouchterlony double diffusion against rabbit anti-JSA serum (results not shown).

The enzyme contained 5·5% sugar as estimated with galactose as standard. Among individual sugars released by acid hydrolysis, none bound to the cation exchanger Dowex-50, indicating the absence of aminosugars. The neutral sugars detected by paper chromatography were galactose and arabinose (figure 4). This is in agreement with the behaviour of the enzyme on concanavalin A-Sepharose. The enzyme did not bind to this immobilized lectin which has no affinity for galactose or arabinose.

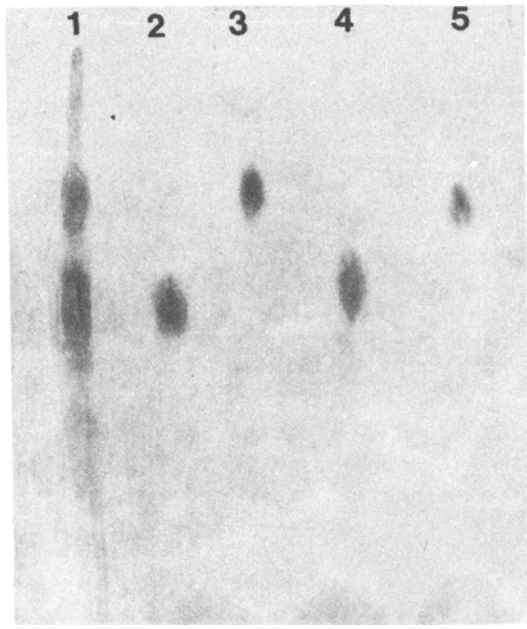

Figure 4.. Paper chromatography of neutral sugars of α-galaciosidase along with standards. (1); α-Galactosidase sugars; (2), arabinose; (3), galactose; (4), mannose and (5), glucose.

Kinetics and stability

Jack fruit seed α-galactosidase had a relatively sharp pH optimum of 5·2. with PNP α-Gal as substrate, K_m value obtained was 0·35 mM. The enzyme was competitively inhibited by galactose, 1-O-methyl α-galactoside and melibiose in the decreasing order, with inhibition constants (K_i) of 0·3, 2·2 and 4·2 mM, respectively.

The enzyme was quite stable; the activity of a solution in PBS was unaffected at 4° or –20°C for 2 months. After 7 months of storage at 4° and –20°C, the activity was reduced to 70 and 30%, respectively of the original. Glycerol (10%) could help retain 60% of the activity at –20°C. On treatment at 37° and 50°C the enzyme was completely inactivated within 6 days and 50 min, respectively.

Action on α-galactosides

Among the oligosaccharides treated with the enzyme for 48 h, galactose was liberated only from melibiose, and the larger α-galactosides raffinose and stachyose were not affected, as revealed by paper chromatogram (results not shown). However the enzyme did liberate galactose from purified guar gum, even after partial enzyme treatment. The resultant depletion of the galactomannan in galactose units led to a decrease in affinity towards JSA. Interaction of a lectin with a high molecular weight polysaccharide containing its specific sugars often produces turbidity. With increasing concentrations of guar gum and a fixed concentration of JSA, turbidity increased to a maximum corresponding to the equivalence point and then decreased (figure 5) (Appukuttan *et al.,* 1984). No turbidity was observed when the same amounts of partially hydrolysed guar gum were treated with JSA, showing considerable loss of galactose units. Complete hydrolysis of guar gum yielded a water-insoluble polysaccharide.

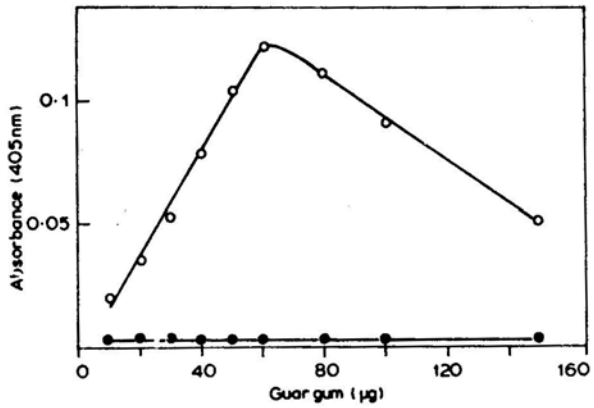

Figure 5. Lectin-precipitability of native (O) and partially α-galactosidase-treated (●) guar gum galactomannan. Turbidity produced by a fixed concentration of JSA with increasing amounts of the two samples were measured at 405 nm in 3 ml.

Discussion

α -Linked galactose units are the principal energy source during germination of a large number of seeds (Dey, 1980). These occur as galactose-containing polysaccharides, often galactomannan. The remarkable swelling and water retaining capacity of these polysaccharides also results from the hydrophilicity of their α-galactoside units, depending on the mannose: galactose ratio which varies from 1–7 among galactomannans (Dey, 1980). Seed galactomannans are located in the endosperm and contain, as mentioned, linearly *ß* (l→4) linked mannose units which occasionally contain α (l→6) linked galactose units. The primary enzyme involved in the utilization of galactomannans is α-galactosidase. Neutral or acid α-galactosidases occur in the aleurone layers of the seeds, adjacent to the endosperm (Dey, 1978). During germination these enzymes are mobilized to digest the galactomannans and to liberate galactose units which are utilized in the glycolytic pathway. In fenugreek seeds (*Trigonella foenum-graecum*), 24 h germination changes the mannose: galactose ratio from 1·05–7·15 (Sioufi *et al.,* 1970). A galactokinase that acts on the liberated galactose has also been detected in germinating seeds (Pridham *et al.,* 1969).

The specific binding of jack fruit seed galactosidase to the cross-linked galactomannan at its pH optimum, reversal of this binding by pH change or with galactose and the degalactosidation of the galactomannan by the enzyme observed here indicate that this enzyme has the same physiological role as detailed above. Notably the natural α-galactosidase for this galactomannan in the guar seeds is a 34kDa enzyme of pH optimum 5·0 (McCleary and Matheson, 1974). Despite similarity in subunit size, molecular weight and affinity towards galactomannan between jack fruit seed α-galactosidase and JSA, no antigenic similarity is indicated in the immunodiffusion experiment.

Inhibition studies indicate that the product of enzyme activity, galactose, is the best of the inhibitors tried, having the lowest K_i. This apparently explains the observed partial hydrolysis of guar gum though the enzyme used retained activity for several days hence, complete hydrolysis leading to precipitation required 10 times as much enzyme. Physiologically this product inhibition might keep α-galactosidase activity in the seed in abeyance until other enzymes for utilization of galactose have been synthesized during germination.

Inhibition and hydrolysis data suggest that among the sugars galactose, 1-O-methyl α-galactoside, melibiose, raffinose and stachyose, the affinity for α-galactosidase decreases with the increase in the chain length of the substrates. This is in sharp contrast to the high enzyme susceptibility of guar gum having a molecular weight of about 220,000 and an average distribution of one galactose unit for every two mannose units (Baker and Whistler, 1976). A close similarity in structure between guar gum and the natural substrate for α-galactosidase in the seed is suggested by this observation

Several α-galactosidase-containing plant seeds contain lectins but with different substrate specificity (Goldstein and Hayes, 1978; Dey, 1984). The jack fruit seed is apparently the first in which the major glycosidase enzyme and the lectin are found to be specific to α-galactosides. This tissue therefore offers a unique system to study the complementary roles if any of lectins and glycosidases in glycoconjugate transport, storage and utilization. In this regard it seems significant that both galactomannans and protein bodies containing the lectins reside in the endosperm of the

seeds. Recently Einhoff *et al.* (1986) observed that lectin-bound proteins isolated from leguminous seeds included glycosidases. The possible role of JSA in transport of galactopolymers and their storage until digestion by α-galactosidase is worth investigating. Towards such a goal, isolation and characterization of jack fruit seed α-galactoside polymers are under way.

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