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α-Galactosidase from germinating guar (*Cyamopsis tetragonolobus*) seeds

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Abstract. The changes in α-galactosidase activity in guar (*Cyamopsis tetragonolobus*) seeds was followed during seven days of germination. The enzyme activity was maximal on the first day of germination and gradually decreased during subsequent days. On the second day of germination the partially purified enzyme upon ion-exchange chromatography on CM-Sephadex C-50 was resolved into α-galactosidase-A (anionic), α-galactosidase-C_1 (cationic) and α-galactosidase-C_2 (cationic) and their relative proportions were 28,12 and 60%, respectively. The combined α-galactosidase C_1 and C_2 activities increased in the first two days of germination followed by significant decrease after the 3rd day onwards, whereas α-galactosidase-A remained fairly constant throughout the germination period, α-Galactosidase-A and C_2 had different K_m and V_{max} values with *p*-nitrophenyl α-D-galactopyranoside, raffinose and melibiose as substrates and also differed in their thermal stabilities.

Keywords. Guar; *Cyamopsis tetragonolobus;* Leguminoseae, germination; α-galactosidase.

Introduction

α-Galactosidase (α-D-galactoside galactohydrolase EC 3.2.1.22) catalyses the hydrolysis of α-galactosyl linkages present in oligosaccharides, galactomannans and glycolproteins and is found in many leguminous seeds, microorganisms and some animal tissues (Dey and Pridham, 1972). Galactose linked oligo- and polysaccharides occur in a number of leguminous seeds. Especially, galactomannans occur as a major storage polysaccharide in varying amounts in the endosperm of a wide range of leguminous seeds (Anderson, 1949). Galactose content of galactomannans varies depending on the plant source. Not all α-galactosidases hydrolyse galactomannans and those that do, appear to remove the terminal galactose residue only. The mature seeds of guar contain a huge reserve of galactomannan and to a lesser extent other galacto-oligosaccharides (Anderson, 1949). On germination the endospermic reserve carbohydrate is degraded and utilized (Dey, 1978). The role of α-galactosidase in the mobilization of reserve galactomannan has been established (Reid and Meier, 1973; McCleary and Matheson, 1974; Williams *et al.*, 1978).

The occurrence of multiple forms of α-galactosidases was first reported from coffee

Abbreviations used: PNPG, Paranitrophenyl α-D-galactopyranoside; PMSF, phenyl methyl sulphonyl fluoride; PVP, polyvinyl pyrrolidine.

beans by chromatography on alumina columns (Petek and Dong, 1961). Since then a number of reports from plant and microbial sources have indicated the existence of multiple forms of α-galactosidase (Dey and Pridham, 1968; Pridham and Dey, 1974; Barham *et al*., 1971; Williams *et al*., 1978; Lee and Wacek, 1970; Suzuki *et al*., 1970)

The present work demonstrates the existence of multiple forms of α-galactosidase from 2-day germinated guar seeds and their relative proportions during different stages of germination and describes some of the kinetic properties.

Materials and methods

Guar (*Cyamopsis tetragonolobus*) seeds were obtained from Haryana Seed Corporation, Hissar. CM-Sephadex C-50, melibiose, raffinose, paranitrophenyl α-D-galactopyranoside (PNPG), phenyl methyl sulphonyl flouride (PMSF), insoluble polyvinyl pyrrolidine (PVP) were products of Sigma Chemical Co., St. Louis, Missouri, USA. All other chemicals and solvents used were of analytical grade. Unless and otherwise mentioned all the isolation and fractionation steps were carried out at 4°C.

Isolation and fractionation of α-galactosidase

Guar seeds were surface sterilized with 0·1 % $HgCl_2$ solution, washed and imbibed with distilled water for 4 h. Germination was carried out in the dark at 25°C for 1–7 days on filter paper moistened with distilled water. At the end of germination period, the seeds were dehusked and chilled. The enzyme was extracted from the chilled seedlings by homogenizing with 5 volumes of 50 mM sodium acetate buffer pH 5·5 containing 2 mM EDTA, 2 mM *ß* -mercaptoethanol, 1 mM PMSF, 200 mM NaCl and 1% insoluble PVP. The slurry was filtered through a mull cloth and the filtrate was centrifuged at 5000 *g* for 20 min. The crude extract which had been brought to 40% $(NH_4)_2SO_4$ saturation was kept for 4 h and centrifuged at 12,000 *g* for 20 min. The supernatant which had all the α-galactosidase activity was adjusted to 70 % $(NH_4)_2SO_4$ saturation and kept for 4 h and centrifuged at 12,0000 *g* for 20 min. The precipitate was dissolved in a minimum volume of 50 mM acetate buffer pH 5·5 and dialysed against 2 litres of 5 mM acetate buffer (pH 5·5) overnight with two changes. The dialysed enzyme was adjusted to 50% (v/v) acetone saturation and kept for 30 min at 4°C and centrifuged at 12,000 *g* for 20 min. The precipitate was dissolved in a minimum volume of 50 mM acetate buffer (pH 5·5) and dialysed against 50 mM acetate buffer (pH 5·0) overnight with two changes. The enzyme thus obtained had a specific activity of 2·8.

Enzyme assay

The enzyme activity was determined according to the procedure of Suzuki *et al*. (1970) with minor modifications. The enzyme was assayed by incubating 2 *μ*mol of PNPG, 25 *μ*mol of acetate buffer pH 5·5 and 0·1 ml of suitably diluted enzyme in a total volume of 1 ml at 60°C for 5 min. The reaction was terminated by the addition of 2 ml of 2% Na_2CO_3 solution and the liberated *p*-nitrophenol was measured at 405 nm in a Backman Model-26 Spectrophotometer. Heated enzyme control and substrate blanks were included in all the cases. One unit of enzyme was defined as that amount of enzyme

which hydrolyses 1 μmol of PNPG/min. Protein was estimated according to Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

Results and discussion

Changes in total α-galactosidase activity during germination

A germination profile was carried out for 7 days to study the changes in the total α-galactosidase activity in the crude extracts of 100 uniformally germinated seedlings on each day under identical conditions. The change in total α-galactosidase activity is shown in figure 1. The levels of total α-galactosidase activity reached a maximum after 24 h of germination and decreased gradually upto 4th day and there was a rapid decrease in the activity on the 5th and 6th day of germination. Since the level of α–galactosidase activity was low in dry seeds, it was clear that α-galactosidase activity increased on germination until the depletion of galactomannan, which is a major storage polysaccharide in guar, and then decreased.

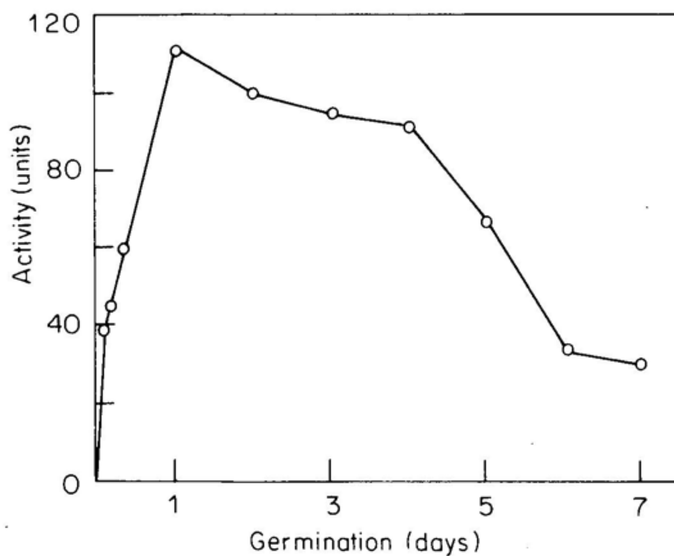

Figure 1. Changes in total α-galactosidase activity during germination.

Multiple forms of α-galactosidases

Acetone precipitated enzyme from 2-day old germinated seedlings was subjected to further fractionation by ion-exchange chromatography on CM-Sephadex C-50 column (3 × 70 cm) which had been equilibrated with 50 mM acetate buffer, pH 5·0. The flow ate was adjusted to 40 ml/h and 8 ml fractions were collected. After loading the enzyme, the column was washed with equilibration buffer and subjected to a step-wise elution with 0·2 M and 1·0 M NaCl in equilibration buffer. As shown in figure 2,

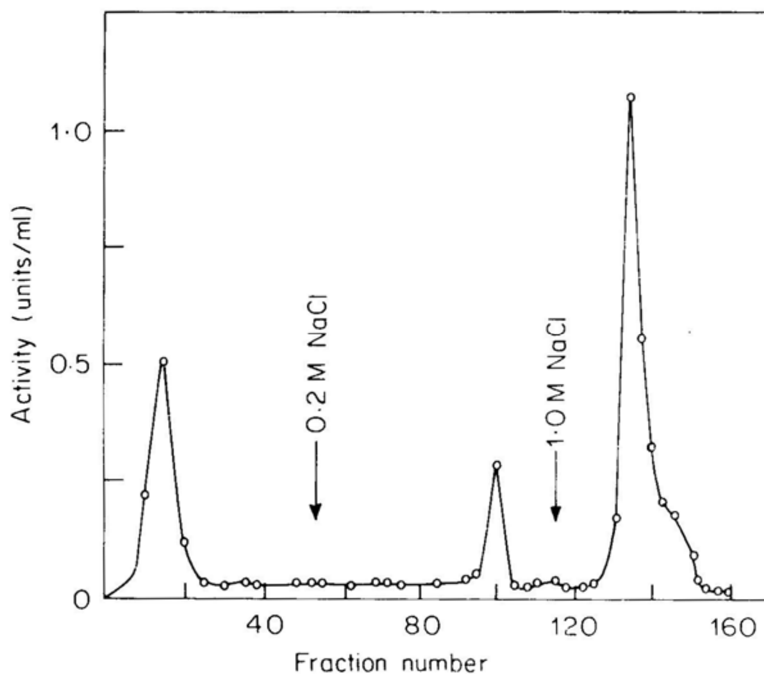

Figure 2. Elution profile of α-galactosidase on CM-Sephadex C-50 column.

chromatography of α-galactosidase on CM-Sephadex C-50 gave three distinct active fractions termed α-galactosidase-A (anionic, 28%) which comes out with buffer washings and strongly binds to DEAE-Sephadex A-50 at pH 7·0, α-galactosidase C_1 (cationic, 12 %) eluted at 0·2 M NaCl concentration and α-galactosidase C_2 (cationic, 60%) eluted at 1·0 M NaCl concentration.

McCleary and Matheson (1974) reported the presence of only two forms of α–galactosidases-A and C from extracts of six days old germinated guar seedlings by chromatography on DEAE-Cellulose column. The reason for their failure to detect three forms of α-galactosidases may be due to the fact that the enzyme was isolated after six days of germination when the enzyme was at its minimum level. Further in the same study they have also indicated that a different species of guar (*Cyamopsis psoraloides*) contained three forms of α-galactosidase, although no detailed information was given. Eventhough there are a good number of reports on the existence of multimolecular forms of α-galactosidase in a variety of leguminous seeds with different physico-chemical properties, their specific role during germination is not clearly understood. However, some evidence for the inter-conversion of multimolecular forms of α-galactosidase *in vitro* has been demonstrated in the case of *Viciafaba* enzyme by Dey *et al*. (1971).

Kinetic properties of α-galactosidases

Some of the kinetic properties of multimeric forms of α-galactosidase are shown in table 1. The pH and temperature optima for the different forms of α-galactosidase were

Table 1. Kinetic properties of multimolecular forms of α-galactosidase from germinated guar seeds.

	pH optimum	Temperature optimum (°C)	K_m (mM) and V_{max} (μmol/min/mg)					
			PNPG		Melibiose		raffinose	
			k_m	V_{max}	K_m	V_{max}	K_m	V_{max}
α-Galactosidase-A	5·5	60	1·20	5·75	4·6	0·08	10·0	0·32
α-Galactosidase C_1	5·5	70	0·25	9·25	11·1	1·42	10·0	3·75
α-Galactosidase C_2	5·5	70	0·50	4·76	5·6	1·31	14·3	25·35

determined using PNPG as substrate under standard assay conditions. All the three forms of α-galactosidase had a sharp pH optima of 5·5, similar to the enzymes from coffee beans (Petek and Dong, 1961), *S. oleracea* (Gatt and Baker, 1970), *Stacks offinis* (Ueno *et al*., 1980) and *Saccharum afficinarum* (Chinen *et al*., 1981). It was observed that α-galactosidases from guar exhibited a very high temperature optimum, α-galactosidase-A had an optimum temperature of 60°C whereas α-galactosidase C_1 and C_2 had an optimum temperature of 70°C similar to the enzyme from *Saccharum officinarum.*

The effect of substrate concentration on the enzyme activity was studied using *p*-nitrophenyl a-D-galactopyranoside (0·1–10 mM), melibiose and raffinose (0·5–40 mM). The reaction was carried out for 5 min at 60°C in 50 mM acetate buffer pH 5·5, in the case of PNPG the rate of hydrolysis was determined as described under materials and methods. In the case of melibiose and raffinose the reaction was carried out for 30 min at 60°C (pH 5·5) and followed by determining the liberated glucose by modified glucostat reagent method (Dahlqvist, 1951) and the liberated galactose by the method of Nelson (1944). There was no inhibition by any of these substrates at the above mentioned concentrations. The K_m and V_{max} values calculated from Lineweaver-Burk plot are given in table 1.

Thermal stability

α-Gaiactosidases exhibit varying degree of stability to heat depending on their origin (Dey and Pridham, 1972). Thermal stabilities were carried out in 50 mM acetate buffer pH 5·5 at temperatures ranging from 40–70°C over a period of 30 min and the residual activity was determined using PNPG as substrate. The pattern of thermal stability of α galactosidase-A, C_1 and C_2 are shown in figure 3. Guar α-galactosidases exhibited fairly high degree of thermal stability. All the three forms of enzymes were heat stable upto 45°C α-Galactosidases C_1 and C_2 were more heat stable compared to α-galactosidase A. α-Galactosidase-A lost about 28, 58 and 94 % of its activity at 50°, 60° and 70°C respectively in 30 min.

Changes in the relative proportions of α-galactosidase A, C_1 and C_2 during germination

Known aliquots of crude enzyme obtained during different days of germination (figure 1) were further resolved on identical CM- Sephadex C-50 columns. The column

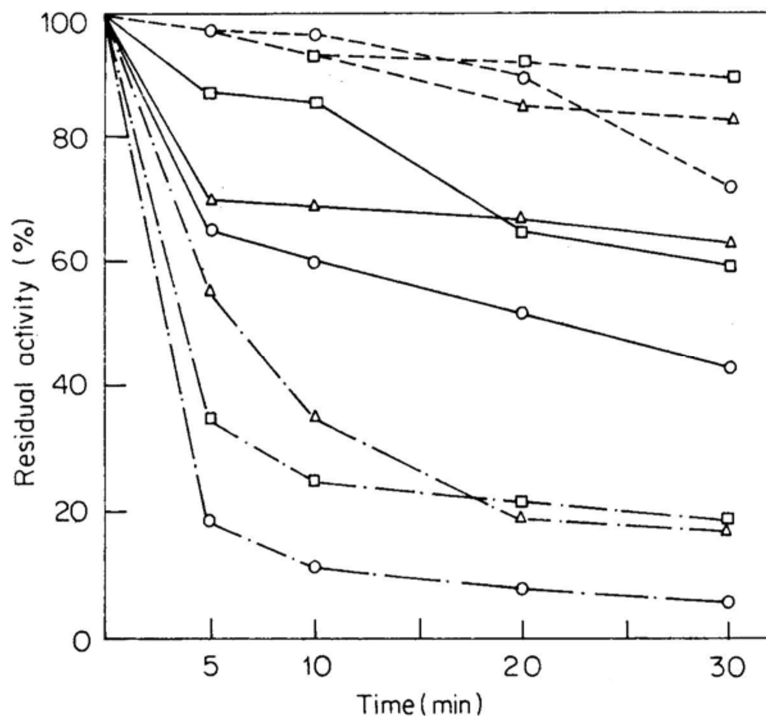

Figure 3. Thermal stability of α-galactosidases. (o), α-Galactosidase-A, (50°C); (□), α-Galactosidase C_1 (60°C); (Δ) α-Galactosidase C_2 (70°C).

was washed with equilibration buffer to remove α-galactosidase-A and then eluted with 1·0 M NaCl in the same buffer to fractionate α-galactosidase C_1 and C_2. The activity profiles of α-galactosidase-A, C_1 and C_2 during germination are shown in figure 4. The combined activities of C1 and C_2 decreased significantly during germination period whereas the activity of α-galactosidase-A remained fairly constant throughout the germination period.

It can be seen from figure 4 that the total recovery of enzymes from the CM-Sephadex C-50 column on 3rd and 4th day of germination is rather low compared to the level of total enzyme present in the extract (figure 1). The reason for this low recovery is not clear at the moment, however, it is possible that 3rd and 4th day extract contains some labile α-galactosidase C_1 and C_2 and may represent the onset of rapid change in activity after the 3rd day. The fact that the level of α-galactosidase-A is more or less similar on all the seven days of germination clearly suggests that only the α-galactosidase C_1 and C_2 change rather dramatically. Interestingly it has been observed that the galactomannan reserve in guar seed is rapidly reduced between 1st and 2nd day of germination (McCleary, 1983). The α-galactosidase activity is maximal during this period of germination (figure 1). Taken together these results suggest that α-galactosidase C_1 and C_2 are probably involved in the mobilisation of galactomannan reserve in guar seed.

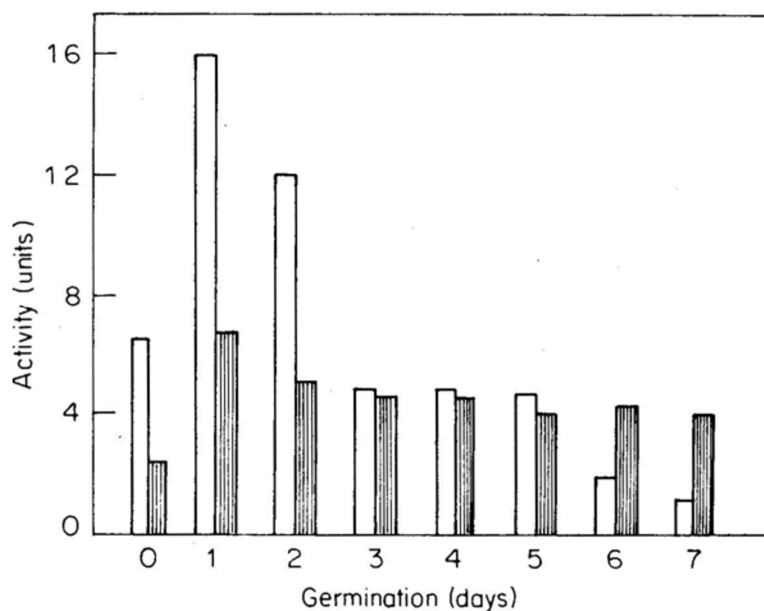

Figure 4. Changes in the α-Galactosidase-A, C_1 and C_2 during germination., (□) α-Galactosidase C_1 and C_2; ▥, α-Galactosidase-A.

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