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α-GALACTOSIDASES OF *VIGNA UNGUICULATA*

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Key Word Index—*Vigna unguiculata*; Leguminosae; cowpea; α-galactosidases; characterization; multiple forms.

Abstract—Three forms of α-galactosidase, I, II^1 and II^2, were isolated from dormant cowpeas by means of gel filtration and ion exchange chromatography. Enzymes I, II^1 and II^2 exhibited optimum activity at pH 5.0, 5.9 and 5.3, respectively. The activation energies for the hydrolysis of *p*-nitrophenyl α-D-galactoside (PNPG) were 5.1, 3.5 and 3.3 kcal/mol for enzymes I, II^1 and II^2, respectively. The M_rs were *ca* 111 000, 29 000 and 30 000, respectively. Two column chromatographic separations and SDS-PAGE indicated that enzyme I is a tetramer of enzyme II^2. The K_m and V_{max} values of the three enzymes were different for one artificial and two natural substrates. The enzymes also differed in their stabilities during storage for 24 hr at four different temperatures and various pH values.

INTRODUCTION

α-Galactosidases (α-D-galactoside galactohydrolase, E.C. 3.2.1.22) have been shown to be widely distributed in plants, animals and microorganisms. As a rule, these enzymes are present in all tissues containing α-D-galactosides [1–3]. The galactosyl oligosaccharides rank next to sucrose in their abundance in the plant kingdom [4]. The enzymes have multiple significance. They may be used in molecular structure studies of sugars, in plant and animal physiology and in applied enzymology (removal of flatulence from legume seeds, improvement of sucrose extraction from beets and conversion of type-B human erythrocytes to type-O).

Petek and Dong [5] were the first to report multimolecular forms of α-galactosidase. Using alumina CC, they isolated two separate forms of this enzyme. Dey and Pridham [6] showed that dormant seeds of *V. faba* have two forms of α-galactosidase (I and II) with different M_rS. In later work enzyme II was eluted as two active fractions (II^1 and II^2) using ion exchange chromatography [7]. Multiple forms of α-galactosidase showed different pH optima and K_m and V_{max} values with artificial and natural substrates, and also differed in their thermal stabilities [8].

French *et al.* [9] reported that almond α-galactosidase was able to split the internal galactosidic linkage of stachyose, forming galactobiose and sucrose. Coffee α-galactosidase cleaves stachyose in a stepwise fashion starting from the nonreducing end. α-Galactosidase of *Streptococcus bovis* hydrolyses verbascose in a similar stepwise mode [10].

The ionic strength of α-galactosidase solutions may also be an important factor in their stability [11]. The pH optima of α-galactosidases vary. Most show single broad pH optima, but in several cases the enzymes exhibit two peaks. The tetrameric enzyme from mung bean was converted predominantly into the monomeric form on storage at −10° for two years in pH 7 buffer. However, on dialysis at pH 4 the monomeric enzyme was reconverted to the tetramer [12].

In higher plant tissues, galactose-containing oligo- and polysaccharides are common carbohydrate reserves, particularly in seeds. There is now little doubt that α-galactosidases function as hydrolytic agents in the utilization of these compounds [13]. These enzymes may also play an important role in the metabolism of galactolipids [14] and in the function of chloroplast membranes [15, 16]. Few observations appear to have been made on the role of α-galactosidase in the animal kingdom. The enzyme does occur in brain tissues; *in vivo* it may be involved in the hydrolysis of digalactosyl diglycerides [17].

RESULTS AND DISCUSSION

Enzyme purification

The purification of cowpea α-galactosidases is summarized in Table 1. In preparing a crude extract the pH which resulted in the maximum enzyme activity and the clearest extract was 5.5. Precipitation with citric acid almost tripled the specific activity of the extract with very little loss of total activity. The addition of citric acid also increased the specific activity obtained after ammonium sulphate precipitation. The optimum pH for citric acid precipitation of the inactive protein was 3.5. Ammonium sulphate fractionation was used rather than −10° acetone precipitation, as suggested by ref.[8], because acetone inactivated the enzyme. The cut points in ammonium sulphate precipitation for maximizing the activity of the precipitate were 25 to 55%. In stage 4, the enzyme solution was dialysed against McIlvaine buffer, pH 5.5, and then passed through a Sephadex G-100 column. The specific activity after this stage increased considerably. Two protein fractions with α-galactosidase activity were observed. Figure 1 shows the elution profiles of enzymes I and II from this column. Recycling fractions I and II removed some inactive protein and raised the specific activity. Enzymes I and II were fractionated on CM-cellulose using a 0.05–0.4 M sodium chloride gradient.

Table 1. Purification of cowpea α-galactosidases

Stage	Volume (ml)	Activity (nkat/ml)	Protein (mg/ml)	Sp. activity (nkat/mg)	Recovery* (%)
1. Crude extract	1575	63	174	0.4	—
2. Citric acid precipitation	2060	42	37	1.1	87
3. $(NH_4)_2SO_4$ 25–55%	154	324	220	1.5	50
4. Sephadex G-100					
α-galactosidase I	152	180	4	45.0	28
α-galactosidase II	113	77	40	1.9	8.8
5. CM-cellulose chromatography					
I	18.5	395	0.1	3950	7.4
II^1	15.5	368	10.0	37	5.8
II^2	13.5	5.3	0.2	27	0.1

*% recovery was calculated relative to the first stage, i.e. crude extract. Seed powder wt taken = 1 kg.

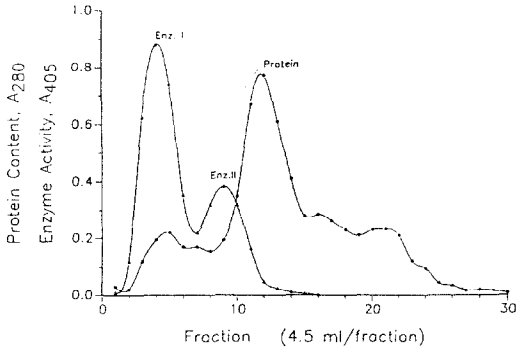

Fig. 1. Elution profiles of α-galactosidases I and II from a G-100 gel filtration column.

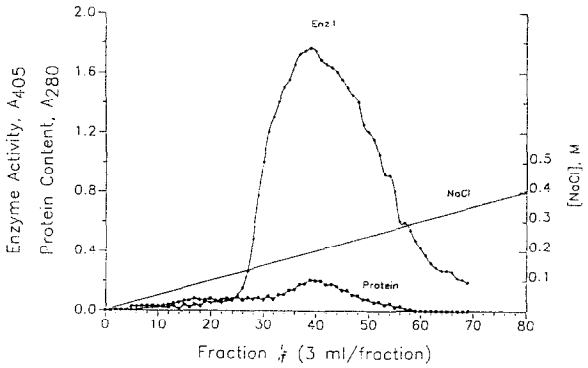

Fig. 2. Elution profile of α-galactosidase I from a CM-cellulose column eluted with a sodium chloride gradient.

Enzyme I eluted as one peak (Fig. 2), but enzyme II gave rise to two peaks (Fig. 3). When the same two fractions were passed through an Accell-CM-cation (Waters Assoc., Inc.) column using a pH gradient (3–7), fraction II was again separated into two active peaks, II^1 and II^2. The specific activity of fraction I was greatly enhanced.

The specific activity of enzyme II^1 was higher than that of II^2 and the proportions of the two total activities

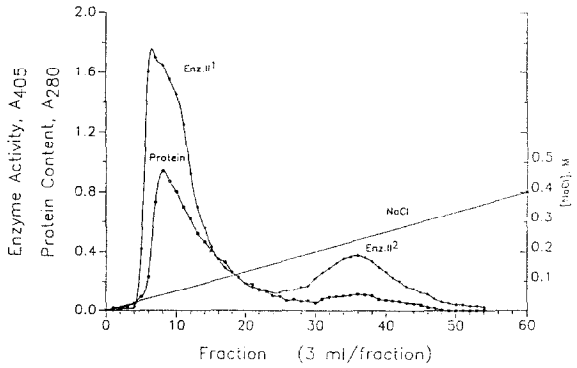

Fig. 3. Elution profile of α-galactosidase II from a CM-cellulose column eluted with a sodium chloride gradient.

varied with different batches of seeds. Enzymes I and II^2 were eluted from CM-cellulose and Accell-CM-cation columns at similar sodium chloride concentrations and pH values, respectively, which indicated that they have similar net electric charges.

Polyacrylamide gel electrophoresis (PAGE)

Enzymes I, II^1 and II^2, subjected to PAGE at pH 3.5, migrated equal distances as shown by enzyme activity testing and Coomassie staining. Apparently, all three enzymes have equal net electric charges. Interestingly, PAGE at pH 8.3 resulted also in equally fast moving bands, as shown by Coomassie staining, although this pH inactivated the enzymes.

Molecular weight determination

Two methods, gel filtration chromatography and sodium dodecyl sulphate-PAGE (SDS-PAGE), were used to determine the M_r of the enzymes. When gel filtration chromatography was used, enzymes I, II^1 and II^2 displayed M_r of *ca* 111 000, 29 000 and 30 000, respectively. Using SDS-PAGE, the mobilities observed for the three α-galactosidases gave the following M_r estimates for enzymes I, II^1 and II^2: 31 500, 30 000 and 31 000, respectively. Weak bands observed with the main enzyme bands probably indicate the presence of minor polypeptides or

contaminating proteins. Various M_r have been reported in the literature for α-galactosidases from different plant sources [7].

The following observations suggest that enzyme I is a tetramer of enzyme II²: (i) gel filtration indicated that the M_r of enzyme I is 111 000 vs 30 000 for enzyme II², (ii) SDS-PAGE, which depolymerized enzyme I, resulted in almost equal M_r estimates of the two enzymes: 31 500 for enzyme I and 31 000 for enzymes II² and (iii) the two enzymes were eluted at the same pH value (4.4–4.8) in Accell-CM chromatography and at the same sodium chloride concentration value (*ca* 0.24 M) in CM-cellulose chromatography. These methods separate compounds on the basis of charge only.

K_m *for enzymes I, II¹ and II² using various substrates*

PNPG. Lineweaver–Burk plots were used to calculate K_m values for the enzymes. The K_m value, 5.3 mM, of enzyme II² with PNPG as substrate was more than twice that of enzyme II¹, 2.2 mM, and *ca* four times as high as the K_m of enzyme I, 1.5 mM.

Raffinose. When raffinose was the substrate, the activity of enzymes I, II¹ and II² was measured by the amount of liberated galactose. Originally, it was attempted to determine the freed galactose using HPLC, but the method was not sensitive enough to measure the small quantities of galactose present. An enzymic method capable of determining microquantities of galactose was then used. The K_m values of enzymes I, 4.6 mM, and II¹, 5.0 mM, were close to each other and *ca* three times as high as that of the K_m of enzyme II², 1.6 mM. Apparently, enzyme II² has higher affinity ($1/K_m$) for raffinose than enzymes I and II¹.

Stachyose. Theoretically, two moles of galactose will be produced from the hydrolysis of one mole of stachyose by the action of α-galactosidases. While the reaction is in progress, it is impossible to assume a definite ratio of freed galactose to hydrolysed stachyose. Therefore, K_m and V_{max} values were determined using HPLC to measure the disappearance of stachyose. The K_m value for enzyme I was 11 mM and for II¹ 15 mM. No value for enzyme II² was obtained as this fraction was not available in sufficient quantity for testing.

Optimum pH and optimum temperature for enzyme activity

The pH optima for the α-galactosidases I, II¹ and II² were determined at 30° and found to be 5.0, 5.9 and 5.3, respectively. (The pH values for half-maximum activity were 2.8 and 6.6 for enzyme I; 4.2 and 7.2 for enzyme II¹; and 2.7 and 6.3 for enzyme II².) The activity decreased rapidly on either side of the optimum pH.

The rate of PNPG hydrolysis by the three α-galactosidases was determined as a function of temperature at the optimum pH. The activation energies for the hydrolysis of PNPG were 5.1, 3.5 and 3.3 kcal/mol for enzymes I, II¹ and II², respectively. The activity decreased rapidly at temperatures higher than the optimum temperature, indicating heat denaturation.

Stability

The stability of enzymes I, II¹ and II² was measured during storage for 24 hr at four different temperatures, 4, 22, 37 and 45°, and at pH varying from 2 to 8. Enzyme I maintained its full activity in the pH range 4.5–5.0 during 24 hr at 4° (Fig. 4). The enzyme lost almost 35% of its original activity in the pH range 4.5–6.0 at 22°. In general, enzyme I was very unstable at 37°, while the minimum loss of activity occurred at pH 5.8. Figure 5 shows the effect of pH and temperature on the stability of enzyme II¹. At 4 and 22°, enzyme II¹ was more stable than enzymes I and II² (Fig. 5). Enzyme II¹ maintained 100% activity in the pH range of 3.5 to 7.0 for 24 hr. At 37° almost 90% of the original activity was preserved for 24 hr, at pH 4.5–5.5. At 4°, enzyme II² maintained 75–80% of its original activity for 24 hr in a large pH range, 3.5 to 7.5 (Fig. 6).

In general, all three enzymes showed their greatest stability at 4° and at intermediate pH values (3.5–7.0). Enzyme II¹ was more stable than enzyme II²; enzyme I was the least stable under the conditions of this experiment. Enzyme II¹ was the only form active after 24 hr at 45°, pH 4.5–7.5.

EXPERIMENTAL

Enzyme assay. α-Galactosidase activity was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl α-D-galactoside as described in ref. [8]. Activity was expressed either as A_{405}/g dry wt or kat. Sp. act. were expressed as nkat/mg protein. Protein determinations were made by the method of ref.

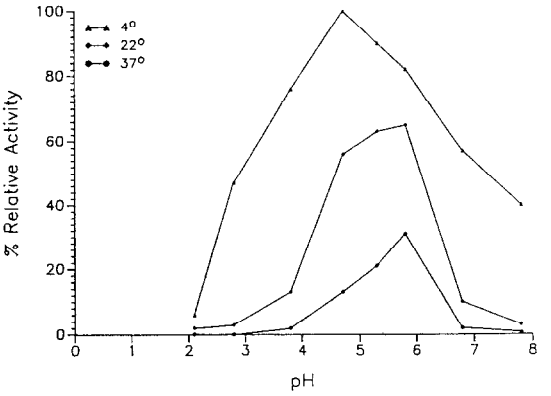

Fig. 4. Effect of pH and temperature on the stability of enzyme I after 24 hr of storage.

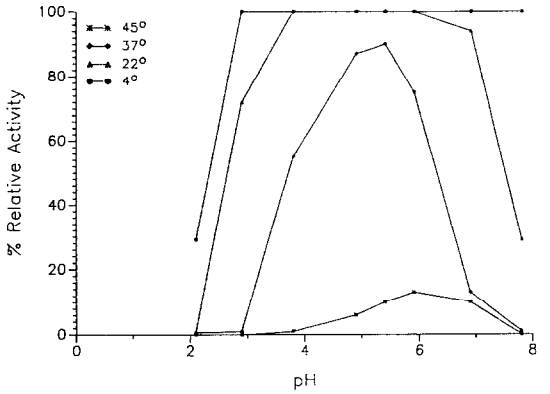

Fig. 5. Effect of pH and temperature on the stability of enzyme II¹ after 24 hr of storage.

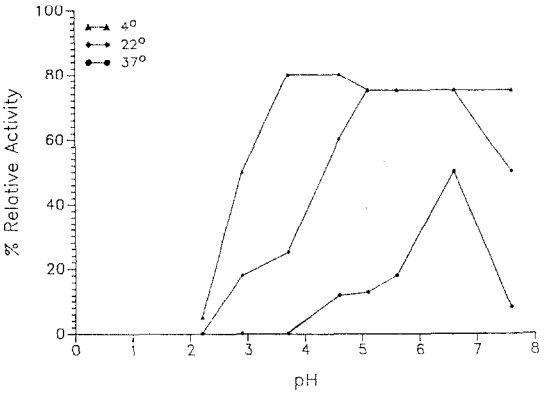

Fig. 6. Effect of pH and temperature on the stability of enzyme II^2 after 24 hr of storage.

[18] as modified in ref. [19] with crystalline bovine serum albumin as std or by UV measurement at 280 nm.

Enzyme isolation and purification. Cowpeas were purchased from California. They were ground in a Wiley Mill and extracted with 2 vols of McIlvaine buffer, pH 5.5, for 1 hr at 4°. The slurry was centrifuged at 20000 *g* for 40 min and the residue discarded. The pH of the supernatant was lowered to 3.5 by gradual addition of 1 M citric acid. The pptd protein was discarded after centrifugation at 16000 *g* for 40 min. The pH of the supernatant was raised to 5.5 with Na_2HPO_4 soln and brought to 25% satn with $(NH_4)_2SO_4$. The pptd protein was discarded and the supernatant was made 55% satd with $(NH_4)_2SO_4$. The pptd protein was collected by centrifugation, resuspended in McIlvaine buffer, pH 5.5, and dialysed overnight against the same buffer (2 l). This dialysed fr. was applied to a 1.9 × 70 cm Sephadex G-100 column and eluted with McIlvaine buffer, pH 5.5, containing 0.1 M KCl. The fractions for each peak were pooled and concd separately by ultrafiltration under N_2 using an Amicon YM_{10} membrane. Two different purification procedures were used after this step.

Procedure A. Enzymes I and II were dialysed against McIlvaine buffer, pH 4 and 3, respectively. The dialysed samples were applied to a 0.9 × 10 cm Accell-CM-cation exchange column and eluted with McIlvaine buffer containing 1 M NaCl at a flow rate of 20 ml/hr. The pH gradient used to elute enzymes I and II ranged between 4–7, and 3–6, respectively. Elution resulted in the resolution of α-galactosidase II into two enzyme fractions, II^1 and II^2.

Procedure B. Enzymes I and II obtained from the gel filtration on G-100 were dialyzed against McIlvaine buffer, pH 3.5 and applied to a 0.9 × 14 cm, CM-cellulose column equilibrated with the same buffer. The flow rate was 25 ml/hr. Following sample application, the column was washed with buffer and elution was carried out with a linear NaCl gradient (0.05–0.4 M) in the same buffer. Elution resulted in the resolution of α-galactosidase II into two enzyme fractions, II^1 and II^2. All three enzymes I, II^1 and II^2 were recycled through the CM-cellulose column. Samples were stored at −20° in 1 ml fractions until used.

Electrophoresis. PAGE was carried out under cationic conditions according to ref. [20] on slab gel with a 7.5% acrylamide resolving gel, pH 4.3, and 4% acrylamide stacking gel, pH 6.8. The gels were run in β-alanine-acetate buffer, pH 3.5. For SDS-PAGE, samples were heated to 100° in buffer containing 1.25% SDS and subjected to electrophoresis through 4% acrylamide stacking gels and 11% acrylamide resolving gels [21]. Gels were stained with Coomassie brilliant blue-R 250 (0.4% w/v) in 40% HOAc acid and 10% MeOH for 4 hr at room temp. The gel was destained with a 7.5% (v/v) HOAc and 25% (v/v) MeOH soln. For detection of enzyme activity on polyacrylamide gels, 0.3% 6-bromo-2-naphthyl-α-D-galactoside in DMF was used as substrate [22].

Gel filtration chromatography. A Sephacryl S-200 column 1.45 cm × 87 cm was prepd as described in refs [19, 23]. The column was eluted with McIlvaine buffer, pH 7 (enzymes II^1 and II^2) and pH 5 (enzyme I) containing 0.1 M KCl at a flow rate of 23 ml/hr and 2.3 ml frs were collected. The column was calibrated at 4° by determination of the elution vols of a number of ref. protein of known M_r. Results were expressed in terms of distribution coefficient (K_{av}) and relative elution volume (REV).

Galactose determination. The procedure in ref. [24] was used to determine the amount of liberated galactose using D-galactose dehydrogenase (25 U/ml).

K_m determination using stachyose as substrate. The K_m values for enzymes I and II^1 were determined using stachyose as substrate. The disappearance of stachyose was measured by HPLC. An aliquot of the soln was passed through a 0.22 μm membrane filter and a Sep-Pak C_{18} cartridge. A 20 μl sample was injected into an HPLC system composed of a guard-pack precolumn, an μ-Bondapak/carbohydrate analysis column (30 × 0.39 cm) and an RI-401 refractometric detector. The solvent (MeCN–H_2O, 13:7) flow rate was 2 ml/min.

Enzyme I. The following concns of stachyose in McIlvaine buffer, pH 5.5, were prepd: 5, 8, 13, 20 and 30 mM. Enzyme I soln, 0.1 ml, was added to 1 ml of each stachyose concn. The reaction was carried out at 30° for 45 min. After stopping the reaction by boiling for 3 min, each mixt. was injected onto an HPLC column. The area of the stachyose peak was measured and compared to the peak area of standard stachyose solns subjected to the same HPLC sepn. The disappearance of stachyose was proportional to reaction time up to 60 min at 30°.

Enzyme II^1. A 0.1 ml aliquot of the enzyme was added to 1 ml of each of the following concns of stachyose in McIlvaine buffer (pH 5.5): 3, 5, 8, 13 and 20 mM. The reaction was carried out at 30° for 30 min. The reaction progress was linear up to 45 min at 30°.

Stability. The stability of enzymes I, II^1 and II^2 during 24 hr storage at four different temp, 4, 22, 37 and 45°, and at various pH values from 2–8 were studied. Enzyme activity was assayed at time zero and after 24 hr using PNPG as substrate, as previously described. Activity at time zero was considered 100% in order to calculate the rel. act.

pH and temperature optima. The effect of pH on the rate of PNPG hydrolysis at 30° was studied with a series of McIlvaine buffers ranging between pH 2 and 8. The pH of the reaction mixt. was measured before the initiation of the reaction. The optimum temp. for activity was determined between 0 and 70° at the optimum pH for each enzyme. PNPG was used as substrate to assay the enzyme.

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