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MULTIPLE FORMS OF α-GALACTOSIDASE IN MATURE LEAVES OF *CUCURBITA PEPO*

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Abstract—α-D-Galactosidase has been purified from mature leaves of *Cucurbita pepo* using pH and ammonium sulphate fractionation, Sephadex gel filtration and DEAE Sephadex gel chromatography. Gel filtration produced one peak of α-galactosidase activity from which three distinct enzyme forms were resolved on DEAE Sephadex and designated LI, LII and LIII. Purifications obtained were *ca* 75, 120 and 30 fold for LI, LII and LIII respectively. LI was slightly contaminated with β-galactosidase and LII with β-fructosidase activity. All forms hydrolysed the α-galactosyl linkages of raffinose and stachyose. Differences between each form were found in their pH optima, reactivity toward metal ions, thermal stability and K_m values using either *p*-nitrophenyl-α-D-galactoside (NPG) or raffinose as substrates. All forms were inhibited by NPG at high concentrations and by α-D-galactose. It is proposed that α-galactosidases may be components of a lysosomal system in plant cells.

INTRODUCTION

The α-galactosidases (α-D-galactosidase galactohydrolase (EC 3.2.1.22)) of plants have been mostly studied in seeds where they are reported to be involved in the degradation of galactomannans and the reserve oligosaccharides raffinose and stachyose during germination [1–4]. Different molecular forms of the enzyme occur in several plants [2, 3, 5] and the relative amounts of the various forms in *Vicia faba* seeds change during maturation and germination [1]. α-Galactosidases are also found in leaves [6, 7] where they may function in galactolipid metabolism [1, 7]. Gatt and Baker [6] extracted and partially purified an α-galactosidase from spinach leaves but they were unable to demonstrate galactolipid hydrolysis; neither did they report any multimolecular forms. Furthermore, Roughan [8] and more recently Williams *et al.* [9], reported no appreciable turnover of galactolipids in intact leaves of either *Vicia faba* or *Cucurbita pepo*. It is possible that α-galactosidases in leaves may play a role in the regulation of carbohydrate metabolism during leaf development. In the mature leaves of many plant species, stachyose and raffinose are among the principal sugars synthesized and transported to the growing regions of the plant, [10, 11]. Young, importing leaves of *Cucurbita* rapidly degrade raffinose and stachyose [10] possibly through α-galactosidase activity. At a certain stage of leaf development a net synthesis and accumulation of these sugars occurs and this is closely followed by a transition of the leaf from an importing to an exporting organ [12]. Our preliminary observations have shown that exporting leaves have appreciable levels of α-galactosidase activity suggesting that a high degree of compartmentation develops between this enzyme and the sites of sugar synthesis.

As part of a continuing study of this problem we report here three molecular forms of α-galactosidase isolated from fully expanded mature *Cucurbita* leaves and discuss their location.

RESULTS

Enzyme extraction and purification

At the commencement of the extraction procedure the pellet remaining after centrifugation of the leaf homogenate did not contain any detectable α-galactosidase activity and was discarded. Lowering of the pH of the leaf supernatant was a useful step in the purification process but below pH 3.5 appreciable losses of α-galactosidase activity occurred. The incorporation of a protamine sulphate precipitation step prior to the ammonium sulphate fractionation removed much of the nucleic acid material and improved the effectiveness of subsequent stages of purification.

By assaying the 5 ml fractions eluted from the Sephadex G-100 column, it was found that α-galactosidase emerged as a single peak of activity in fractions 10–17. These fractions were pooled and reduced to 1 ml by ultrafiltration for further purification. It should be emphasized that only one major peak of α-galactosidase activity was eluted from the Sephadex column. Most reports of α-galactosidase isolation from seeds show a resolution of at least two major molecular forms after gel filtration [15]. However α-galactosidase activity isolated from seeds of *Coffea canephora* and *Canjanus indicus* [3] and from the mould *Aspergillus niger* [13], have also yielded a single peak of activity. However, Courtois and Petek [5], using alumina columns, resolved 2 peaks of α-galactosidase activity from coffee beans. A very small peak of α-galactosidase activity eluting prior to the main peak was observed when crude extracts from *Cucurbita* leaves were run on Sephadex G-100. Gatt and Baker [6] observed a similar peak when preparing their enzyme from spinach leaves but ascribed

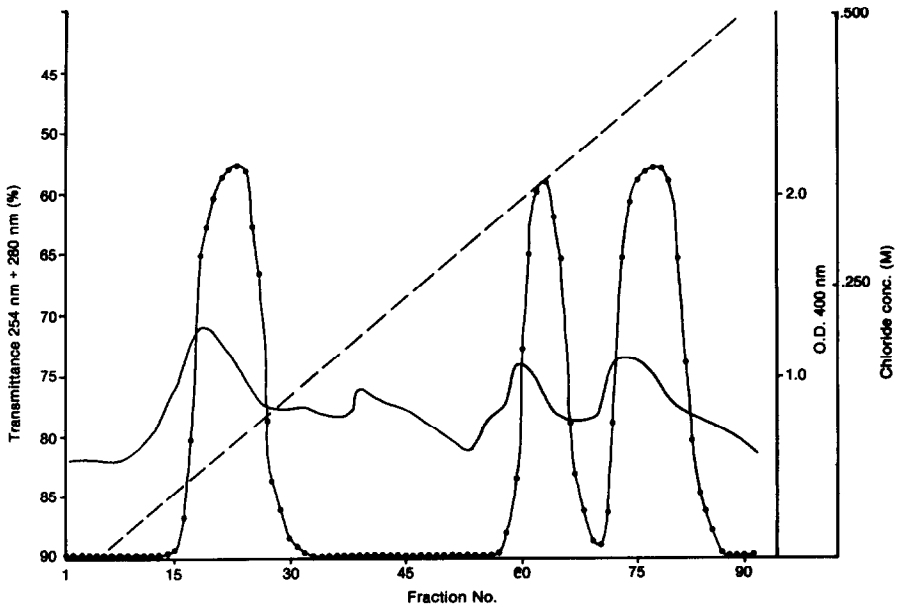

Fig. 1. Separation of *C. pepo* α-galactosidases on DEAE Sephadex. —●—● — α-galactosidase; ——— protein; ----- chloride ion concentration.

it to column overload. We found this peak to be present even if small quantities of sample were used and think it more likely to represent a trace of bound enzyme or possibly a higher MW enzyme, analogous to Dey and Pridham's enzyme 1 [14].

The separation obtained with the DEAE Sephadex A-50 column is shown in Fig. 1. Three peaks of α-galactosidase activity were observed and designated in order of elution LI, LII and LIII. The fractions within each peak were pooled and reduced by ultrafiltration. This behaviour of an apparently homogeneous molecular form on Sephadex G-100 to split into two or three forms showing different elution patterns on ion exchange gels is a typical observation for the α-galactosidases [15]. We have designated these different forms LI, LII and LIII in order to distinguish them from the three forms of α-galactosidase I, II and III isolated from *Vicia faba* seeds and seedlings [1, 15], with which our enzymic forms have similar but not identical properties.

The stages of the purification procedure are summarized in Table 1. These results represent an improvement on the 14.7 fold purification obtained by Gatt and Baker [6] for spinach leaf α-galactosidase. The enzymes were partially purified, LI showed traces of β-fructosidase activity when incubated with stachyose, raffinose and sucrose while LII showed traces of β-galactosidase activity. LIII showed no trace of either of these contaminating activities. β-galactosidase eluted just prior to α-galactosidase on Sephadex G-100 and its trace activity in LII probably represents a small degree of elution overlap.

Enzyme properties

Preincubation of LI, LII and LIII at 36° for 30 min in

Table 1. Purification sequence for 100 g mature leaves.

Purification step	Volume (ml)	Total enzyme activity (mu)	Total protein (mg)	Specific activity (mu/mg)	Recovery* (%)	Purification (fold)
Extraction in phosphate buffer	390	58500	4210	13.9	—	—
pH variation	424	55800	2370	23.8	95.4	1.7
Protamine sulphate	425	47400	2130	22.3	84.8	1.6
Ammonium sulphate	45	41100	612	67.9	86.6	4.8
Dialysis and ultrafiltration	0.9	19000	46	412·6	46.2	29.6
Sephadex G-100	25	12200	16.8	724.4	64.1	52.2
DEAE Sephadex LI	6.5	927	0.87	1070	—	76.6
LII	13.5	3780	2.30	1640	—	118.2
LIII	19.5	2640	6.24	430	—	30.9

* Recovery is calculated relative to the previous purification step. Overall recovery = 12.6%

100 mM Na–Pi buffer, pH 7, produced *ca* 50% loss of activity of LI, *ca* 15% loss of LII while LIII remained stable. At 45°, LI and LII were almost completely inactivated while LIII showed *ca* 10% inactivation. Using an enhanced protein concentration at 10 mg/ml, through the addition of BSA, thermal stability at 36° was restored for both LI and LII. However at 45°, in the presence of BSA, LI and LII were inactivated while LIII retained most of its activity. A 30 min preincubation at 55° was necessary to inactivate LIII.

The pH optimum of LI for the hydrolysis of *p*-nitrophenyl-α-D-galactopyranoside occurred at pH 5.7 with *ca* 50% reduction in activity at pH 4.2 and 6.8. For LII, the pH optimum occurred at pH 4.6 with *ca* 50% reduction in activity at pH 3.5 and 5.8. Both forms showed little activity either below pH 3 or above pH 7. LIII showed optimum activity at pH 5.6, but it has appreciable activity over a wide range with *ca* 50% activity still present at pH 2.5 and 7.0.

Metal ions K^+, Mg^{2+}, Ca^{2+} and Zn^{2+} at both 0.5 mM and 2.0 mM had no effect on the activity of LI, LII or LIII. LI and LII were both inhibited by Cu^{2+}. At 0.5 mM Cu^{2+} LI was inhibited *ca* 20%, LII *ca* 40%. At 2.0 mM Cu^{2+}, LI was inhibited 50% and LII 90%. LIII was uninhibited at either Cu^{2+} concentrations. Mn^{2+} appeared to activate LII *ca* 20% at 0.5 mM but there was no significant activation of either LI or LIII.

K_m values obtained from Lineweaver–Burk plots for the hydrolysis of *p*-nitrophenyl-α-D-galactopyranoside (substrate range from 0.1 to *ca* 1 mM) were 0.37 mM for LI, 0.57 mM for LII and 0.042 mM for LIII. At higher substrate concentrations the substrate became highly inhibitory. Inhibition began at *ca* 0.7 mM for LII, 1.3 mM for LI and 2 mM for LIII. The inhibiting effect of high concentrations of *p*-nitrophenyl-α-D-galactoside has been demonstrated for α-galactosidases obtained from several sources [14, 16]. Carchon and DeBruyne [16] have investigated this inhibition in some detail suggesting that an additional substrate molecule binds with the enzyme substrate complex forming an inhibitory complex ES'S. Substrate concentrations of raffinose of up to 50 mM were not inhibitory with LI, LII or LIII, and similar results have been reported for α-galactosidases obtained from a variety of sources [15, 16]. K_m values derived from Lineweaver–Burk plots for the hydrolysis of raffinose were 11.1 mM for LI, 12.5 mM for LII and 5.6 mM for LIII.

The hydrolysis of *p*-nitrophenyl-α-D-galactopyranoside in all three enzymic forms was inhibited by α-D-galactose. Activity linearly decreased with increasing galactose concentration. At 5 mM galactose, LI was inhibited *ca* 50%, LII *ca* 65% and LIII *ca* 30%. A similar degree of galactose inhibition has been reported for α-galactosidases obtained from other sources [15].

The *in vitro* properties reported here for the isolated forms of α-galactosidase from mature *Cucurbita* leaves lie well within the range of properties displayed by α-D-galactosidases obtained from alternative sources, e.g. [1] and [5]. However, each form of the enzyme shows distinct properties. LIII is more stable than either LI or LII, is active over a wider pH range and is less susceptible to metal ion inhibition. LI and LII have similar properties but can be distinguished by their pH optima and K_m values. It is not possible to say how significant these property differences are in terms of the role of these molecular forms in mature *Cucurbita* leaves.

The mature leaves of *Cucurbita* synthesize large amounts of raffinose and stachyose during periods of photosynthesis and these sugars are transported into the vascular system without any apparent hydrolysis. Furthermore, if the export of these sugars is inhibited through a blockage of the vascular system in the petiole (e.g. by localized exposure to 0°), raffinose and stachyose have been reported to accumulate in the blade [17]. We have not found any evidence of the accumulation of free galactose in mature leaves. Analyses of the hydrolysis products when LI, LII or LIII were incubated with stachyose and raffinose showed that free galactose was released in all cases. The apparent lack of *in vivo* activity in mature leaves therefore suggests a spatial separation of the α-galactosidases from both the sites of synthesis of raffinose and stachyose and their transport routes into the minor veins. This compartmentation could be either at the cellular or subcellular level. There is evidence that many acid hydrolases, including glycosidases, are associated with vacuoles or vacuolar membranes [18], thus resembling a lysosomal apparatus.

EXPERIMENTAL

Plant material. Seeds of *Cucurbita pepo* L. var. *melopepo* f. *torticollis* Bailey, (Early Prolific Straight-Neck squash, from W. A. Burpee, Seed Growers, Philadelphia, PA, U.S.A.) were germinated in perlite and grown in a controlled environment cabinet as described in ref [19]. Mature leaf blades from plants 3–5 weeks old were used for all enzyme extractions.

Extraction and enzyme fractionation. All extraction and fractionation steps were carried at 0–2°. Leaves, *ca* 100 g fr. wt, were homogenized in a blender for 1 min with 400 ml of 100 mM NaPi buffer pH 7. The homogenate was filtered through 4 layers of cheesecloth and centrifuged (10000 *g*, 10 min). The supernatant was recentrifuged (19000 *g*, 30 min) and the pellet discarded. The supernatant was adjusted to pH 3.5 with 1 M $H_3PO_4^-$ and allowed to stand 18 hr, then centrifuged (10000 *g*, 10 min). The pellet was discarded, and the supernatant readjusted to pH 6.5 with 1 M Na_2HPO_4. Protamine sulphate was added to the supernatant to give final conc of 10 mg/ml and the soln allowed to stand for 24 hr, then centrifuged (10000 *g*, 10 min). The pellet was discarded and to the supernatant, solid $(NH_4)_2SO_4$ was added to 30% saturation and the precipitated protein removed by centrifugation. More solid $(NH_4)_2SO_4$ was added to 60% saturation. The supernatant was discarded and the precipitated protein removed, redissolved in 45 ml 100 mM NaPi buffer, pH 7.0 and clarified by centrifugation (10000 *g*, 20 min). The protein soln was dialysed against 2 l. H_2O, 24 hr. followed by dialysis against 2 l. NaPi buffer pH 7, 24 hr. The ppt was removed by centrifugation, (10000 *g*, 10 min) and the supernatant reduced in vol by ultrafiltration (Amicon PM10 filter). At *ca* 1 ml, 10 ml 100 mM NaPi buffer, pH 7 was added and the vol again reduced to 1 ml. The ppt was removed by centrifugation (20000 *g*, 20 min) and discarded. The supernatant was applied to a Sephadex G-100 column (85 × 1.5 cm) and eluted with 100 mM NaPi buffer pH 7, flow rate 0.25 ml/min. Fractions (5 ml) were collected and each assayed for α-galactosidase activity (see below). Active fractions were pooled and the vol reduced to 1 ml by ultrafiltration. The total active fraction was applied to DEAE Sephadex A-50 column (30 × 1.5 cm) previously equilibrated with 100 mM NaPi buffer, pH 7, and eluted with a linear gradient 0–0.5 M NaCl in 100 mM NaPi buffer pH 7. The total vol eluted was 150 ml, flow rate 0.15 ml/min; 2 ml fractions were collected and each assayed for α-galactosidase activity. The active fractions were pooled, and BSA added to bring the final protein conc up to 10 mg/ml. The resultant solns were dialysed 18 hr against 100 mM NaPi buffer, pH 7. The dialysed solns were stored at 2° and, except where stated, used as the enzyme source for all subsequent reactions.

Enzyme assay. To 0.3 ml suitably diluted α-galactosidase solution in 100 mM NaPi buffer pH 7, was added 0.3 ml, 200 mM Na acetate buffer pH 5 containing 0.3% *p*-nitrophenyl-α-D-galactopyranoside. Reaction time 20 min (unless otherwise stated) at 37°. Reaction terminated with 3 ml 5% Na_2CO_3 and the *p*-NO_2 phenol released determined at 400 nm. The unit of enzyme activity was defined as the quantity that hydrolyzes 1 μmol substrate/min under above conditions. Sp acts are expressed in mu/mg protein. Protein determinations were made using method of ref [20] using crystalline BSA as the standard. Assay of β-galactosidase activity was determined as above but substituting *o*-nitrophenyl-β-D-galactopyranoside as substrate and determining *o*-NO_2 phenol at 420 nm. Using a range of raffinose concentrations as substrates the activity of α-galactosidase was determined by measuring galactose released with galactose dehydrogenose: 0.2 ml of a suitable diluted α-galactosidase preparation in 100 mM NaPi buffer pH 7 was added to 0.2 ml 200 mM NaOAc buffer pH 5 containing raffinose at the required concentration. After 20 min incubation at 37°, 3 ml Tris–HCl buffer pH 8.7 containing 4 mM glutathione (red.) and 0.15 mM NAD was added followed by 5 μl β-galactose dehydrogenase in 3.2 M $(NH_4)_2SO_4$, (12.6 u/ml). Amount of galactose released was determined by change in OD at 340 nm after 1 hr at 37°. The end products of α-galactosidase hydrolysis of stachyose, raffinose and sucrose (hydrolysed under conditions described above for raffinose) were assayed by PC of the neutral fraction from the reaction mixture. Hydrolysis of these sugars was terminated after 20 min at 37° by passing the reaction mixture through 1 ml strong cation (H^+) resin (Rexyn 101, Fisher) followed by passage through 1 ml strong anion ($acetate^-$) resin (Rexyn 201, Fisher). The mixture was washed through with 5 ml H_2O to obtain the neutral fraction which was reduced to dryness in an air stream, redissolved in 0.1 ml H_2O and the reaction products separated by descending PC using Whatman No. 1, double development, each for 24 hr, with *n*-PrOH–EtOAc–H_2O (7:1:2). Sugars were visualized with $AgNO_3$–NaOH [21]. The pH activity curve was determined using 0.1 ml α-galactosidase soln in 0.4 ml McIlvaines buffer of appropriate pH and 0.1 ml *p*-nitrophenyl-α-galactoside in H_2O (9 mg/ml). The reaction and measurement of *p*-NO_2–phenol released were carried out as described above. The effect of metal ions on α-galactosidase activity was determined with appropriate concs of the metal sulphates dissolved in 200 mM Na–acetate buffer, pH 5. 0.15 ml of this soln was added to 0.15 ml of *p*-nitrophenyl-α-D-galactoside (6 mg/ml) dissolved in the same buffer and 0.1 ml of the enzyme soln. The reaction and measurement of *p*-NO_2–phenol released were carried out as described above.

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