Plant Physiol. (1983) 71, 662–668
0032-0889/83/71/0662/07/$00.50/0

Partial Purification and Properties of an Alkaline α-Galactosidase from Mature Leaves of *Cucurbita pepo*[1]

Received for publication August 18, 1982 and in revised form October 26, 1982

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ABSTRACT

A fourth molecular form of α-galactosidase, designated L_{IV}, an alkaline α-galactosidase, was isolated from leaves of *Cucurbita pepo* and purified 165-fold. It was active over a narrow pH range with optimal hydrolysis of *p*-nitrophenyl-α-D-galactoside and stachyose at pH 7.5. The rate of stachyose hydrolysis was 10 times that of raffinose. K_m determinations in McIlvaine buffer (200 millimolar Na_2-phosphate, 100 millimolar citric acid, pH 7.5) for *p*-nitrophenyl-α-D-galactoside, stachyose, and raffinose were 1.40, 4.5, and 36.4 millimolar, respectively. L_{IV} was partially inhibited by Ca^{2+}, Mg^{2+}, and Mn^{2+}, more so by Ni^{2+}, Zn^{2+}, and Co^{2+}, and highly so by Cu^{2+}, Ag^{2+}, Hg^{2+} and by *p*-chloromercuribenzoate. It was not inhibited by high concentrations of the substrate *p*-nitrophenyl-α-D-galactoside or by *myo*-inositol, but α-D-galactose was a strong inhibitor. As observed for most other forms of α-galactosidase, L_{IV} only catalyzed the hydrolysis of glycosides possessing the α-D-galactose configuration at C_1, C_2, and C_4, and did not hydrolyze *p*-nitrophenyl-α-D-fucoside (α-D-galactose substituted at C_6). The enzyme was highly sensitive to buffers and chelating agents. Maximum hydrolytic activity for *p*-nitrophenyl-α-D-galactoside was obtained in McIlvaine buffer (pH 7.5). In 10 millimolar triethanolamine-hydrochloride-NaOH (pH 7.5) or 10 millimolar Hepes-NaOH (pH 7.5), hydrolytic activity was virtually eliminated, but the addition of low concentrations of either ethylenediaminetetraacetate or citrate to these buffers restored activity almost completely. Partial restoration of activity was also observed, but at higher concentrations, with pyruvate and malate. Similar effects were found for stachyose hydrolysis, but in addition some inhibition of L_{IV} in McIlvaine buffer, possibly due to the high phosphate concentration, was observed with this substrate. It is questionable whether the organic acid anions possess any regulatory control of L_{IV} *in vivo*. It was possible that the results reflected the ability of these anions, and ethylenediaminetetraacetate, to restore L_{IV} activity through coordination with some toxic cation introduced as a buffer contaminant.

The raffinose family of D-galactose-containing oligosaccharides (5) constitute a major portion of the soluble sugar translocated in a number of plant families (24) and they occur even more widely distributed in storage organs such as seeds, roots, and tubers (8). It is generally acknowledged that α-galactosidases (EC 3.2.1.22), function as hydrolytic agents in the metabolic utilization of these compounds. The α-galactosidases occur in multimolecular forms in many plant tissues (3). Three typical forms, which we have designated L_I, L_{II}, and L_{III}, have been isolated from leaves and other tissues of *Cucurbita pepo* (16), possessing properties corresponding closely with the many other forms reported. They are active over a broad acid range (17), rapidly hydrolyze the synthetic substrate *p*-nitrophenyl-α-D-galactoside, but hydrolyze melibiose and members of the raffinose family comparatively slowly, the rate decreasing with increasing mol wt of the substrate. Recently, we discovered another molecular variant (L_{IV}) of α-galactosidase in leaves of *C. pepo* (6). It was unique in so far as its activity was restricted to a relatively narrow pH range with optimal activity at pH 7.5 and it hydrolyzed the tetrasaccharide stachyose more rapidly than either of the two lower mol wt substrates raffinose or melibiose. We have termed this variant an alkaline α-galactosidase to distinguish it from other forms of the enzyme which operate optimally in an acid medium. In this report, we describe an improved purification of L_{IV} and some additional properties which further distinguish it from all other forms of α-galactosidase reported to date.

MATERIALS AND METHODS

Plant Material. Seeds of *Cucurbita pepo* L. var *melopepo* F. *torticolis* Bailey (Early Prolific Straight-Neck squash from W.A. Burpee Co., Warminster, PA) were germinated in perlite and grown in a controlled environment cabinet as previously described (19). Sugars and all other fine chemicals were purchased from Sigma Chemical Co. Galactinol was extracted from *C. pepo* tissue and isolated chromatographically pure in our laboratory (20). All other chemicals used were of the highest analytical grade commercially available.

Enzyme Extraction and Purification. Mature leaf blades from plants 3 to 5 weeks old were used as source material. The extraction and all purification steps were carried out at 4°C. Approximately 100 g fresh weight of whole leaf tissue were homogenized in a Waring Blendor for 2 min in 250 ml 100 mM Na-phosphate (pH 7.0) containing 20 mM BME[3]. The homogenate was filtered through four layers of cheesecloth and centrifuged at 40,000*g* for 30 min. The pellet was discarded. The supernatant (crude fraction) was brought to 30% $(NH_4)_2SO_4$ saturation by addition of saturated $(NH_4)_2SO_4$ (pH 7.0). The resulting precipitate was removed by centrifugation at 10,000*g* for 10 min and the supernatant brought to 60% $(NH_4)_2SO_4$ saturation by additional saturated $(NH_4)_2SO_4$ (pH 7.0). The precipitate was collected by centrifugation at 10,000*g* for 10 min and resuspended in 100 mM Na-phosphate (pH 7.0) containing 20 mM BME. The solution was desalted by passage through a 2.2- × 30-cm column containing Sephadex G-25 which had been previously equilibrated with 100 mM Na-phosphate (pH 7.0) containing 20 mM BME. The desalted fraction was applied to a 2.2- × 15-cm DEAE column (Sephadex A-50) which had been previously equilibrated with 100 mM Na-phosphate (pH 7.0) con-

[1] Supported by an Operating Grant No. A 2827 (to J. A. W.) from the Natural Sciences and Engineering Research Council of Canada.

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[3] Abbreviations: BME, 2-mercaptoethanol; PNPG, *p*-nitrophenyl-α-D-galactopyranoside; TEA, triethanolamine-HCl-NaOH buffer; PAGE, polyacrylamide gel electrophoresis; PCMB, *p*-chloromercuribenzoate.

taining 20 mM BME. The enzyme was eluted with a 200-ml linear salt gradient from 0 to 0.5 M NaCl at a flow rate of 0.25 ml min^{-1}. Fractions of 4.5 ml were collected and assayed for alkaline α-galactosidase activity. The active fractions were pooled and concentrated by ultrafiltration under N_2 using an Amicon PM 10 membrane in 50 mM Na-phosphate (pH 7.0) containing 20 mM BME. The concentrated fraction was run onto a 2.2- × 85-cm column containing Sephadex G-100 previously equilibrated with 50 mM Na-phosphate (pH 7.0) containing 20 mM BME and passed through at a flow rate of 0.24 ml min^{-1}. Fractions of 5.0 ml were collected and assayed for alkaline α-galactosidase activity. The active fractions were pooled and applied to a 2.2- × 15-cm hydroxyapatite column (type 1, Sigma Chemical Co.) previously equilibrated with 50 mM Na-phosphate (pH 7.0) containing 5 mM BME. The enzyme was eluted with a 300-ml linear 50 to 250 mM Na-phosphate gradient at a flow rate of 0.24 ml min^{-1}. Fractions of 5 ml were collected and assayed for alkaline α-galactosidase activity. The active fractions were pooled and concentrated by ultrafiltration, as above, into McIlvaine buffer (pH 7.5). The McIlvaine buffer used at this stage, and in other procedures described in this report, was prepared by mixing appropriate volumes of 100 mM citric acid and 200 mM Na_2-phosphate to obtain the required pH (10). The concentrated enzyme fraction was either used directly as the enzyme source and is referred to as the purified enzyme, or it was appropriately diluted with McIlvaine buffer (pH 7.5) before use.

Enzyme Assays. Alkaline α-galactosidase activity was assayed during the purification steps by preincubating 100 μl of the enzyme source in 400 μl of McIlvaine buffer (pH 7.5) at 30°C for 10 min. The reaction was started by adding 100 μl 30 mM PNPG in aqueous solution and was terminated after 15 min by adding 2.4 ml 5% (w/v) Na_2CO_3. The *A* was read at 400 nm and, for quantifying enzyme activity, the amount of *p*-nitrophenol released was calculated using a molar extinction coefficient, determined under these assay conditions, of 1.83×10^4 M^{-1} cm^{-1}. One unit of enzyme activity (U) was defined as one μmol of PNPG hydrolyzed min^{-1}. Total protein was estimated by the method of Bradford (1) using BSA (Cohn, fraction V) as the standard. For determination of enzyme properties, some minor changes were made in the assay procedures and these are indicated in the results section.

Rates of raffinose and stachyose hydrolysis were calculated by enzymically determining the amounts of galactose released using an aliquot of a suspension of β-galactose dehydrogenase in 3.2 M $(NH_4)_2SO_4$ containing 1 mM EDTA at approximately 25 units ml^{-1} (Sigma Chemical Co.). The hydrolysis reaction was terminated by adding 3.0 ml freshly prepared 100 mM Tris-HCl (pH 8.7) containing 4 mM GSH and 0.5 mM NAD^+. The *A* at 340 nm was recorded and was followed by the addition of 3 μl containing approximately 0.075 units of β-galactose dehydrogenase. After 1 h of incubation at 30°C, the *A* at 340 nm was again recorded and the amount of free galactose calculated from the difference in optical density. Appropriate controls were run containing no galactosides.

Gel Electrophoresis. PAGE was performed in duplicate at between 2 and 4°C using 7.5% (w/v) medium pore gels (pH 8.9) at 4 mamp/gel as described by Maurer (9). One of the gels was stained for protein using Coomassie Blue R (9). The other was assayed for enzyme activity. It was frozen on dry ice immediately after electrophoresis, and 1-mm serial slices of the entire gel were cut with a mechanical gel slicer. Each slice was incubated with 400 μl of McIlvaine buffer (pH 7.5) containing 5 mM PNPG for 1 h at 30°C. The reaction was terminated by adding 2.6 ml of 5% (w/v) Na_2CO_3 and the *A* read at 400 nm to determine the *p*-nitrophenol released.

Thin-Layer Chromatography. For a visual assessment of the hydrolysis of a number of sugars, 10-μl aliquots of the assay volume were spotted onto TLC plates (20 × 20 cm) precoated with 250-μm Silica Gel-60 F (Baker TLC plates). The plates were developed for 10 cm twice in the same direction in ethyl acetate:glacial acetic acid:methanol:water (13:3:5:2, v/v). There was a 5-min drying time between the two developments. The developed plates were sprayed with a solution containing 3 g phenol and 5 ml concentrated H_2SO_4/100 ml anhydrous ethanol and heated at 110°C for 10 to 15 min.

RESULTS

Purification of L_{IV}. A typical purification sequence for L_{IV} is shown in Table I. Less than 5% of the total activity of the crude fraction was found in the pellet obtained after centrifugation of the tissue homogenate. Activity in the crude fraction precipitated at 30% $(NH_4)_2SO_4$ saturation and that remaining in the supernatant after precipitation at 60% $(NH_4)_2SO_4$ saturation amounted in total to less than 10% of the activity precipitated at 60% saturation. The overall recovery however was low and, as previously noted (6), a large and irreversible loss of activity occurred in the presence of high $(NH_4)_2SO_4$ concentrations. Desalting the 30 to 60% $(NH_4)_2SO_4$ fraction with Sephadex-G-25 produced no further change in activity. L_{IV} activity emerged between fractions 36 to 44 from the DEAE column at approximately 0.4 to 0.45 M NaCl; between fractions 28 to 34 from the Sephadex G-100 column and at an elution concentration beginning at approximately 190 mM phosphate from the hydroxyapatite column. A PAGE comparison of the active fractions collected from the G-100 and hydroxyapatite columns revealed that the latter step had removed most of the contaminating protein (Fig. 1). The stained gel of the purified enzyme fraction showed one sharp protein band accompanied by two other diffuse bands. When the gel slices were incubated with PNPG the sharp band seen in both the G-100 and hydroxyapatite gels was the only region possessing L_{IV} activity. Activity of the concentrated enzyme fraction from the hydroxyapatite column was stable for at least 1 week in McIlvaine buffer (pH 7.5) when stored at 2°C.

Effect of pH. PNPG hydrolysis was determined by incubating 100 μl of the purified enzyme in the assay medium containing 400 μl of either a range of McIlvaine buffers from pH 3.0 to 8.0 or with 100 mM TEA from pH 7.5 to 8.5. The reaction was started by adding 100 μl of 30 mM PNPG in aqueous solution and terminated after 20 min by adding 2.4 ml of 5% (w/v) Na_2CO_3. The *A* at 400 nm were used directly for a relative comparison of hydrolysis rates (Fig. 2). pH optima for raffinose and stachyose hydrolysis were found by preincubating 50 μl of the purified enzyme at 30°C with 400 μl of one of the above buffer solutions for 10 min. The reaction was started by adding 50 μl of 100 mM raffinose or stachyose in aqueous solution. The reaction was terminated after 1 h, and the galactose released was determined with β-galactose dehydrogenase. Stachyose and PNPG hydrolysis were optimal at pH 7.5 (Fig. 2). Raffinose hydrolysis (not shown in Fig. 2) was extremely slow by comparison and under these conditions appeared optimal between pH 6.5 and 7.0. None of the above three substrates were detectably hydrolyzed below pH 6.0 and neither stachyose nor raffinose were hydrolyzed by the purified enzyme in 100 mM Tris-HCl (pH 8.7), which were the conditions under which the galactose dehydrogenase assays were performed. There was a significant decrease in stachyose hydrolytic activity in TEA compared with McIlvaine buffer at a similar pH but this did not occur for PNPG hydrolysis.

Substrate Specificity and Kinetic Properties. The hydrolysis of melibiose, raffinose, stachyose, and galactinol at pH 7.5 was visually assessed by TLC. Twenty-five μl of the purified enzyme was incubated with 25 μl of 10 mM aqueous solutions of each of the sugars at 30°C. Aliquots of 10 μl were taken at 15, 30, 45, and 60 min and spotted onto the thin-layer plates. After spraying the developed plates with the phenol reagent, the results clearly showed a rapid hydrolysis of stachyose but a much slower rate for

Table I. *Typical Purification Sequence for Alkaline α-Galactosidase (L_{IV}) Isolated from 100 Grams Fresh Weight of Mature Leaves*

Procedure	Volume	Total Activity	Total Protein	Specific Activity	Yield	Purification
	ml	*munits*	*mg*	*munits/mg*	*%*	*-fold*
Crude extract	450	29,508	1,575	18.7	100	1.0
A.S. (30–60%) desalted	30	11,431	1,257	9.1	38.7	0.5
DEAE-Sephadex A-50	35	4,705	133	35.4	15.9	1.9
Sephadex G-100	14.5	3,974	8	497.0	13.4	26.6
Hydroxyapatite	9.4	1,452	0.47	3,089.0	4.9	165

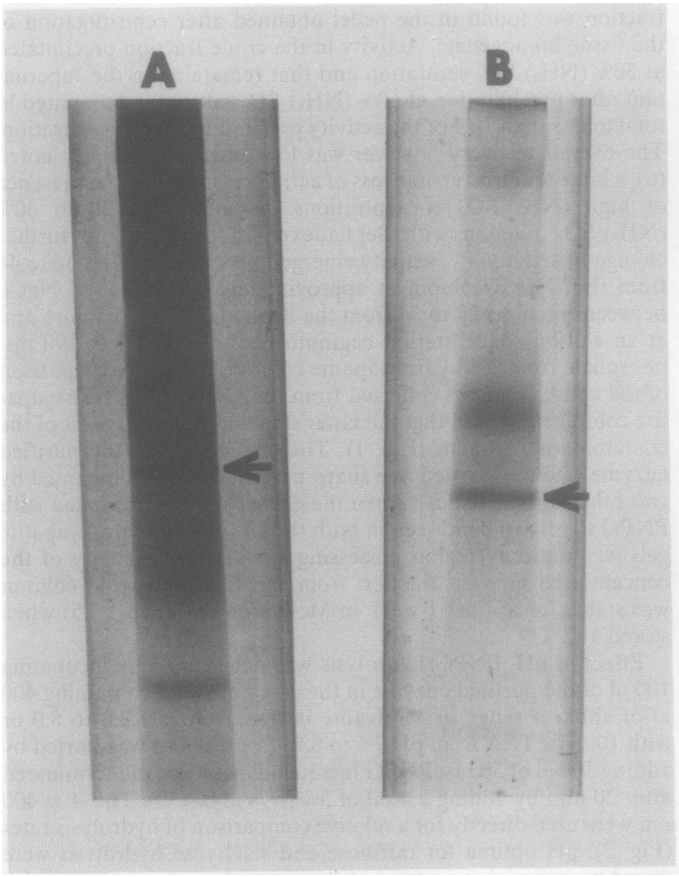

FIG. 1. Polyacrylamide gels stained with Coomassie Blue R of fractions containing alkaline α-galactosidase (L_{IV}) activity. A, Sample of pooled fraction from Sephadex G-100 column; B, sample of pooled fraction from the hydroxyapatite column. Arrows indicate the band corresponding to L_{IV} activity as determined in unstained duplicate gels.

raffinose, while hydrolysis of melibiose and galactinol were barely detectable.

To determine whether a number of other glycoside derivatives of *p*-nitrophenol could be hydrolyzed, 100 μl of 0.9% (w/v) aqueous solutions of the derivatives were added to 100 μl of purified enzyme and 100 μl of McIlvaine buffer (pH 7.5) at 30°C. The reactions were terminated after 30 min by adding 2.5 ml of 5% (w/v) Na_2CO_3, and the *p*-nitrophenol released was determined at 400 nm. No hydrolysis of any of these glycoside derivatives were detected (Table II).

K_m and V_{max} values were determined by standard Lineweaver-Burk plots for *o*-, *m*-, and *p*-nitrophenyl-α-D-galactoside, raffinose, and stachyose (Table II). For the aryl galactosides, 50 μl of 0.5 to 20 mM substrate in aqueous solution were incubated at 30°C with 200 μl of appropriately diluted enzyme in McIlvaine buffer (pH 7.5). The reaction was terminated after 20 min by adding 2.5 ml 5% (w/v) Na_2CO_3, and the amount of nitrophenol released was measured at 400 nm. Molar extinction coefficients, determined under these assay conditions, used to quantify the results were 4.74×10^3 M^{-1} cm^{-1} for the *ortho* derivative, and 1.83×10^4 M^{-1} cm^{-1} for both the *meta* and *para* derivatives. K_m and V_{max} values for raffinose and stachyose were determined by incubating 100 μl of appropriately diluted enzyme in McIlvaine buffer (pH 7.5) with 100 μl of the same buffer containing from 10 to 50 mM raffinose or 5 to 40 mM stachyose at 30°C. After 1 h, the reaction was terminated, and the galactose released was determined with β-galactose dehydrogenase. Melibiose and galactinol were hydrolyzed extremely slowly under these conditions, and K_m and V_{max} values for these two substrates were not determined.

Effect of Other Sugars and Cyclitols. The effect of a number of naturally occurring sugars on the hydrolysis of PNPG was determined by incubating 100 μl of appropriately diluted enzyme with 400 μl of McIlvaine buffer (pH 7.5) containing 7.5 mM PNPG and a range of concentrations (0–25 mM) of the compound under study at 30°C. The reaction was terminated after 20 min by adding 2.5 ml 5% (w/v) Na_2CO_3, and the *p*-nitrophenol released was determined at 400 nm. Galactose, stachyose, raffinose, melibiose, and galactinol all showed inhibitory activity, and standard Dixon plots of inhibitor concentration against reciprocal of reaction velocity were made for these sugars. From the graphs, it was calculated that in the above assay system 6.4 mM galactose, 15.6 mM stachyose, 70 mM melibiose, 70 mM galactinol, and 244 mM raffinose reduced the reaction velocity by 50%. The following sugars and derivatives did not show significant inhibition at the 0.5 level of confidence in the above assays: D-xylose, L-arabinose, L-fucose, D-glucose, D-mannose, sucrose, *myo*-inositol, 1-*O*-methyl-α-D-galactopyranoside or the corresponding β-form, and *N*-acetyl-D-galactosamine.

To observe the effects of metal ions on L_{IV}, the purified enzyme was initially transferred from McIlvaine buffer into either 10 mM TEA (pH 7.5) or 10 mM Hepes-NaOH (pH 7.5) by ultrafiltration under N_2 using an Amicon PM 10 membrane. For each study, 100 μl of enzyme in the appropriate buffer was added to 200 μl of the same buffer (10 mM, pH 7.5) containing 15 mM PNPG and 4.5 mM of the metal ion under study. The reaction was incubated at 30°C and terminated after 20 min by adding 2.4 ml 5% (w/v) Na_2CO_3, and the *p*-nitrophenol released was determined at 400 nm. A similar assay was used to study the effect of 100 mM PCMB in place of the metal ion. The results are shown in Table III where activities are expressed relative to controls in the respective buffers. No metal ion promotion was observed, but a range of inhibitions was found for a number of divalent ions. L_{IV} was completely inhibited in both buffers by small concentrations of PCMB. In other assays, the inhibitory effect of Mg^{2+} and Ca^{2+} ions was found to increase gradually as the concentrations of these ions were raised from 0.1 to 3.0 mM final concentration.

Transfer of L_{IV} from McIlvaine buffer (pH 7.5) to TEA (pH 7.5) or Hepes-NaOH (pH 7.5) consistently caused a 30 to 40% loss of PNPG hydrolysis activity. It was subsequently discovered that this loss could be almost completely restored by adding 2 mM EDTA to the assays and that significant restoration could also be obtained by adding a similar concentration of citrate. Additions of similar concentrations of either pyruvate or malate also partially

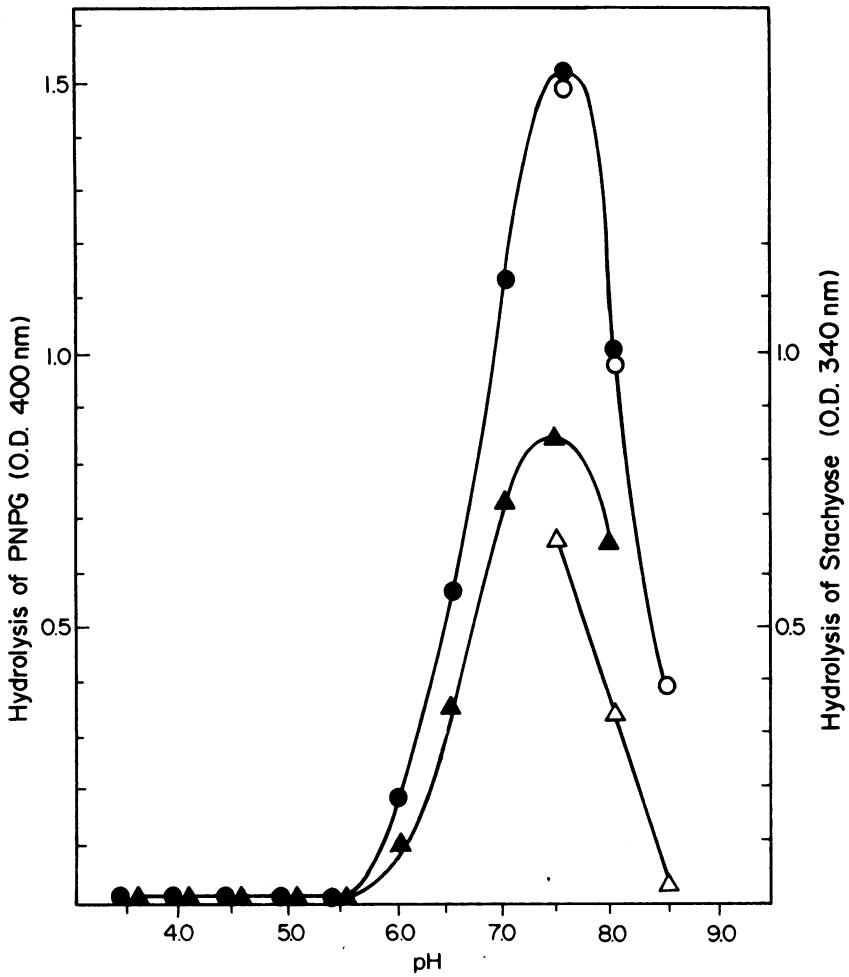

FIG. 2. pH activity curve for the hydrolysis of PNPG (○, ●) and stachyose (△, ▲) by alkaline α-galactosidase (L_{IV}). (○, △), Determinations in triethanolanine hydrochloride-NaOH containing diluted McIlvaine buffer; (●, ▲), determination in McIlvaine buffer.

Table II. *Kinetics and Specificity of Alkaline α-Galactosidase (L_{IV})*

Substrate	K_m	V_{max}[a]
	mM	μmol galactose/ min·mg protein
p-Nitrophenyl-α-D-galactoside	1.40	3.5
o-Nitrophenyl-α-D-galactoside	1.00	5.25
m-Nitrophenyl-α-D-galactoside	2.00	0.33
Raffinose	36.40	0.14
Stachyose	4.50	1.58
Melibiose		+[b]
Galactinol		+[b]
p-Nitrophenyl-α-D-mannoside	No hydrolysis	
p-Nitrophenyl-α-D-fucoside	No hydrolysis	
p-Nitrophenyl-α-D-glucoside	No hydrolysis	
p-Nitrophenyl-β-D-galactoside	No hydrolysis	
p-Nitrophenyl-β-D-glucoside	No hydrolysis	
o-Nitrophenyl-β-D-galactoside	No hydrolysis	

[a] V_{max} values were determined in McIlvaine buffer (pH 7.5), 30°C.
[b] Very slow hydrolysis; K_m and V_{max} not determined.

Table III. *Effect of Metal Ions and PCMB on the Hydrolysis of PNPG by Alkaline α-Galactosidase (L_{IV}) after Transfer by Ultrafiltration into TEA and Hepes-NaOH Buffers (pH 7.5)*

Metal Ion[a]	Activity	
	TEA	Hepes
	% of control	
Na^+, K^+	100	100
Mn^{2+}	62	77
Ca^{2+}	58	35
Mg^{2+}	43	72
MoO_4^{2-}	ND[b]	42
Co^{2+}, Fe^{3+}	9	0
Ni^{2+}, Zn^{2+}	6	0
Hg^{2+}, Cu^{2+}, Ag^{2+}	0	0
100 μM PCMB	0	0

[a] All metal ions at 3 mM final assay concentration.
[b] Not determined.

restored activity (Table IV, for TEA). The addition of succinate at 2 mM had no effect. Stachyose hydrolysis showed an even more phenomenal change in the presence of these compounds (Table IV). The McIlvaine buffer used for the control for comparing activities in either TEA or Hepes-NaOH may have reflected a loss of citrate rather than a direct inhibition by the buffer reagents. This possibility was investigated by pooling fractions containing L_{IV} obtained directly from the hydroxyapatite column. The pooled fractions were assayed for PNPG hydrolysis by preincubating 25 μl of the pooled enzyme fraction with 200 μl McIlvaine buffer (pH 7.5) for 10 min at 30°C. The reaction was started by adding 75 μl of 30 mM PNPG in aqueous solution and terminated after 20

Table IV. *Effect of Adding Organic Acids on the Hydrolysis of PNPG and Stachyose by Alkaline α-Galactosidase (L_{IV}) after Transfer to TEA by Ultrafiltration*

Addition	Relative Enzyme Activity	
	PNPG hydrolysis[a]	Stachyose hydrolysis[b]
Control, McIlvaine[c]	100	ND[f]
Control, TEA[d]	68.1	100
+ 3 mM Citrate[e]	85.1	460
+ 3 mM Pyruvate[e]	79.7	260
+ 3 mM Malate[e]	79.7	260
+ 3 mM Succinate[e]	68.1	100
± 3 mM EDTA[e]	96.0	460

[a] Enzyme activity expressed as a percentage relative to activity in McIlvaine buffer (pH 7.5). Assay system composed of 100 μl enzyme in appropriate buffer added to 200 μl of the same buffer containing 15 mM PNPG with or without 3 mM acid anion or EDTA.

[b] Enzyme activity expressed as a percentage relative to activity in control TEA (pH 7.5).

[c] Control, activity in McIlvaine buffer pH 7.5, no additions.

[d] Control, activity of L_{IV} after transfer from McIlvaine buffer to TEA (pH 7.5) by ultrafiltration, no additions.

[e] Additions to L_{IV} in TEA (pH 7.5).

[f] Not determined.

Table V. *Effect of Adding EDTA and Organic Acids on Alkaline α-Galactosidase (L_{IV}) Activity as Determined with PNPG or Stachyose as Substrates after L_{IV} Had Been Dialyzed for 72 Hours against Various Buffers at pH 7.5*

Activator	Activator Concn.	Recovery of Enzyme Activity[a]			
		PNPG			Stachyose
		TEA	Hepes	Na-Phosphate	Na-Phosphate
	mM			%	
None		9	0	41	52
EDTA	0.25	86	86	96	223
	10	86	86	111	270
Citrate	0.25	59	52	88	209
	10	67	81	111	234
Pyruvate	1.5	29	24	47	
	10	57	63	72	223
Malate	1.5	14	5	41	61
	10	23	40	51	94
Succinate	10	14	24	41	52

[a] Recovery values are expressed as a percentage of the activity present before dialysis which was determined with PNPG or stachyose as substrates in McIlvaine buffer (pH 7.5).

min by adding 2.4 ml of 5% (w/v) Na_2CO_3, and the *p*-nitrophenol released was determined at 400 nm. The pooled fractions were then divided into four equal aliquots (of about 4 ml), each of which were dialyzed for 72 h at 4°C against 2 L of either McIlvaine buffer (pH 7.5), 10 mM Na-phosphate (pH 7.5), 10 mM TEA (pH 7.5), or 10 mM Hepes-NaOH (pH 7.5). After dialysis, L_{IV} activity in each buffer was determined by preincubating a 25-μl aliquot of each buffer with 200 μl of the same buffer for 10 min at 30°C. The reaction was started and terminated as in the immediately preceding assay. The effects of EDTA and the organic acid anions were determined by adding them to the buffer solution used in the preincubation step giving final assay concentrations of from 0.25 to 10.0 mM. The results are shown in Table V where for brevity only the most significant data are tabulated. The 72-h dialysis of L_{IV} against McIlvaine buffer caused an approximately 8% loss of PNPG hydrolytic activity (this is not shown in Table V). The results confirmed the considerable loss in activity following dialysis against either Na-phosphate or TEA while dialysis against Hepes-NaOH completely eliminated L_{IV} activity. EDTA and citrate were by far the most efficient reagents to restore activity, while pyruvate and malate were relatively poor promoters and succinate extremely marginal.

The effect of prolonged dialysis of L_{IV} on stachyose hydrolysis was investigated using the enzyme fraction dialyzed against 10 mM Na-phosphate (pH 7.5). In these experiments, the assay protocol, including the addition of restoring agents, remained the same as in the preceding assays with the exceptions that the reaction was started by substituting 75 μl of 33.3 mM stachyose in 10 mM Na-phosphate (pH 7.5) for PNPG, and it was allowed to run at 30°C for 1 h. The galactose released was determined with β-galactose dehydrogenase (Table V). Again, the effects of prolonged dialysis and the addition of activators on L_{IV} hydrolysis of stachyose resembled the results from the PNPG experiments. However, there was one very significant difference. The increase in activity of L_{IV} in the presence of either EDTA, citrate, or pyruvate greatly exceeded the activity of L_{IV} in McIlvaine buffer (pH 7.5) before dialysis.

DISCUSSION

A fourth molecular form of α-galactosidase, designated L_{IV}, from mature leaves of *C. pepo* has been purified 165-fold. PAGE analysis of the purified form indicated some minimal protein contamination, but the nature of the contaminants and whether they contained any other glycosidase activity was not determined. L_{IV} has the unusual feature of possessing an alkaline pH optimum, hence our introduction of the term alkaline α-galactosidase. All α-galactosidases previously isolated from higher plants have displayed acidic pH optima (3). It was also distinguished by having a high specificity for stachyose, which significantly is a major sugar translocated in *C. pepo*. The ability of L_{IV} to hydrolyze stachyose 10 times faster than raffinose is also unusual since the rate of hydrolysis of an homologous series of α-D-galactosides by α-galactosidases from higher plants usually diminishes with increasing chain length (3, 11, 14, 21, 22). Furthermore, high PNPG concentrations caused no inhibition, contrasting with the other forms of α-galactosidase isolated from *C. pepo* tissues (16) and with most other forms of α-galactosidase reported (3).

There was an appreciable and irreversible loss of L_{IV} activity during the $(NH_4)_2SO_4$ fractionation. Nevertheless, we continued to include this step during our study as the procedure enabled us to reduce the volume of the extract quickly, and a sufficient amount of L_{IV} activity was still retained for subsequent purification. We have not explored an alternative fractionation step, but in future work designed to optimize yield this will clearly be necessary. Recently, an α-galactosidase was purified from the golden-brown alga *Poterioochromonas malhamensis* (2) which possessed several similar properties to L_{IV}. It was optimally active at pH 7.0 and was also strongly denatured during fractionation with ammonium sulfate. It was also very unstable in an acidic medium and was inhibited by galactose, PCMB, and $HgCl_2$ but not by high concentrations of the substrate PNPG. EDTA, on the other hand, was inhibitory. This α-galactosidase, while capable of rapidly hydrolyzing the synthetic PNPG, was highly specific for its physiological substrate isofloridoside (*O*-α-D-galactopyranosyl(1→1)-glycerol), and failed to hydrolyze galactinol, melibiose, raffinose, or stachyose.

Both K_m and V_{max} values for L_{IV} were affected when the position of the nitro substituent in phenyl-α-D-galactoside was changed. Similar results have been obtained with α-galactosidases from several sources (3, 4, 15, 21, 22). L_{IV} activity was shown to depend upon a specific configuration of the hydroxyl groups at least at carbon atoms 1, 2, and 4 of the glycosyl moiety of the aryl derivatives which has been commonly observed for α-galactosid-

ases (3). The absence of any inhibitory effect of *N*-acetyl-D-galactosamine on L_{IV} activity resembles that reported for α-galactosidase obtained from *M. vinacea* (15) and reaffirms other results (12) that show a very strict requirement for a hydroxyl group at C-2 of the glycosyl moiety. Unlike α-galactosidase from *V. faba* (3), however, substitution at C-6 (e.g. D-fucose) prevented L_{IV} activity. The inhibition of L_{IV} by galactose is a typical property of α-galactosidases (3). *myo*-Inositol has been reported to be a specific inhibitor of α-galactosidases (13), but this is not invariable since it has no effect either on L_{IV} as reported here or L_{III} from *C. pepo*, (unpublished result) or α-galactosidase I from *T. repens* (21). No inhibition of L_{IV} activity was observed in the presence of 1-*O*-methyl-α-D-galactoside (3, 15).

L_{IV} activity was highly inhibited by Hg^{2+}, Cu^{2+}, and Ag^{2+} and PCMB, as are several other α-galactosidases (3, 4, 7, 11, 16) and suggests the involvement of sulfhydryl groups within the active site region. The divalent cations Mg^{2+}, Ca^{2+}, and Mn^{2+} were also significantly inhibitory, a feature that has not been previously encountered for α-galactosidases. No metal ion activation was observed. Some of the metals investigated here have been reported to activate weakly α-galactosidases from plant tissues (7, 11), but most forms of the enzyme have been reported to be indifferent to metal ion activation.

There was strong stimulation of L_{IV} by the chelating agent EDTA and the organic anions citrate, pyruvate, and malate, and the presence or absence of these compounds in various buffers appeared to be responsible to a large extent for the apparent buffer effects on L_{IV} catalysis. The enzyme appeared most sensitive to EDTA and citrate. For example, after the prolonged dialysis of L_{IV} against Na-phosphate buffer (pH 7.5), the recovery of L_{IV} hydrolysis of PNPG in Na-phosphate buffer containing 10 mM citrate (a citrate concentration which approximated that in the initial McIlvaine buffer [pH 7.5]) exceeded the control in McIlvaine buffer (pH 7.5) by more than 10%, while the recovery of L_{IV} activity in TEA and Hepes-NaOH containing 10 mM citrate was only 67 and 81%, respectively. These results were remarkable when one considers that L_{IV} activity after prolonged dialysis against these last two buffers was barely detectable in the absence of citrate. On the other hand, the failure of citrate to completely restore L_{IV} activity in either TEA or Hepes-NaOH suggested that these buffer reagents might also be slightly inhibitory.

The extent of loss of L_{IV} activity in TEA and Hepes-NaOH after prolonged dialysis was initially surprising since L_{IV} had retained 60 to 70% of its residual activity after transfer by ultrafiltration into these buffers. However, L_{IV} transferred into TEA or Hepes-NaOH by ultrafiltration had previously been in McIlvaine buffer and therefore in contact with citrate, whereas L_{IV} dialyzed against TEA or Hepes-NaOH had been taken directly from the hydroxyapatite column, thus avoiding all prior contact with citrate. It was therefore possible that traces of citrate from the McIlvaine buffer remained associated with the enzyme after the relatively rapid process of ultrafiltration. Experiments recently completed with citrate revealed that concentrations as low as 16 μM citrate were sufficient to cause 40 and 48% recovery of activity of L_{IV} which had previously been dialyzed for 72 h against Hepes-NaOH and TEA, respectively. These results indicate a very high affinity of the enzyme for citrate which may not be readily removed from the enzyme during short periods of ultrafiltration or dialysis. Some recent exploratory experiments in which L_{IV} obtained directly from the hydroxyapatite column, had been dialyzed for 72 h against either TEA (pH 7.5) or Na-phosphate (pH 7.5), then incubated for 30 min at 4°C in the presence of 15 mM citrate, then redialyzed against 2 L of the same buffer for a further 24 h, showed that the stimulation of enzyme activity caused by citrate was not completely eliminated by the second dialysis. These preliminary results indicated that L_{IV} activity present after the incubation in citrate and second dialysis were about 1.4 and 3 times higher than controls in Na-phosphate and TEA buffers, respectively. L_{IV} therefore appears to be highly sensitive to citrate.

The greatly increased stachyose hydrolysis activity of 10 mM Na-phosphate-dialyzed-L_{IV} in the presence of EDTA, citrate, or pyruvate, compared to controls in McIlvaine buffer (pH 7.5), suggested that the relatively high phosphate concentration (200 mM) present in the McIlvaine buffer may have inhibited L_{IV} hydrolysis of stachyose. But this was not the case for PNPG. The inhibition would have been much reduced following dialysis of the enzyme for a prolonged time against 10 mM phosphate, thereby permitting an enhancement of the stimulatory effects of EDTA and citrate. This observation cautions against generalizing about the optimal conditions for L_{IV} catalysis. The optimal conditions for one substrate may not necessarily apply to all other utilizable substrates.

Presumably, the stimulatory effects of EDTA and citrate are due to chelation, a possibility that may be extended to the other stimulatory organic acid anions. The question arises as to whether we have exposed a true regulatory control of L_{IV} that exists *in vivo* or whether it is an artifact arising from the experimental conditions. The results resemble the recent findings concerning hexokinase assays (18, 23). It had been generally accepted that EDTA, citrate, malate, and several other compounds were effective activators of hexokinases, but it has now been shown that these compounds are doing no more than chelating Al^{+3} ions, which are inhibitory to the enzyme, and which had been artificially introduced into the assay systems as contaminants in commercial sources of the substrate ATP (18, 23). The source and purity of commercially supplied chemicals clearly require very careful screening, particularly when used with metal ion-sensitive enzymes. We have demonstrated the sensitivity of L_{IV} to several metals, and it is therefore very tempting to draw a parallel here with the problems inherent in the hexokinase assay systems. Further work is in progress to clarify this problem.

The discovery of an alkaline α-galactosidase, L_{IV}, raises the possibility that the distribution of this enzyme might be responsible for the different patterns of utilization of the galactosyl-sucrose oligosaccharides by mature and immature leaves. The high specificity of L_{IV} for stachyose, the major sugar translocated in *C. pepo*, and the fact that L_{IV} was found to undergo a 6-fold decrease in specific activity over the developmental period during which immature, importing leaves become net exporting organs (6) support this hypothesis.

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