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α- AND β-GALACTOSIDASE ACTIVITIES IN PROTEIN BODIES AND CELL WALLS OF LENTIL SEED COTYLEDONS

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Key Word Index—*Lens culinaris*; Leguminoseae; lentil; seeds; cell wall; protein bodies; galactosidases.

Abstract—Cotyledons of mature *Lens culinaris* seeds contain two forms of both α- and β-galactosidase which can be separated by ion exchange chromatography. These forms are present in cotyledon cell walls and protein bodies except β-galactosidase II, which is undetectable in the cell walls of these organs. All the enzymatic forms were active in an acid pH range but each behaved differently with different substrates, both natural and synthetic, and in the action of different effectors on the activity. α-Galactosidase I and II were able to release free sugars from several putative substrate oligosaccharides and all the forms of α- and β-galactosidase were seen to release galactose from lentil storage glycoproteins.

INTRODUCTION

High activities of several glycosidases, including α-D-galactosidase (EC 3.2.1.22) and β-D-galactosidase (EC 3.2.1.23) have been reported in nature. α-Galactosidase has been found to be widely distributed in plant species and the enzyme is involved in the primary attack on galactosyl oligosaccharides [1, 2]. Multiple forms of this enzyme have been described and these may reflect differences in their distribution between tissues. β-Galactosidases have received little attention in plant species [3, 4], particularly in seeds, and besides their ability to split β-galactosidic linkages, their physiological functions remain unknown.

In the present work α- and β-galactosidases were partially purified from the cotyledons of *Lens culinaris* seeds in order to investigate their basic properties and distribution in cell walls and protein bodies.

RESULTS

Separation of α- and β-galactosidase

Preliminary studies were carried out in crude cotyledon extracts. α- and β-Galactosidase were predominantly recovered from the pellets of 60% $(NH_4)_2SO_4$ fractions of crude extracts. Following dialysis the concentrated fractions were chromatographed on DEAE-cellulose (Fig. 1A). Part of the α- and β-galactosidase activity (Form I), was eluted immediately from the bed with 10 mM Na-phosphate buffer, pH 6.8, and the remainder (Form II), was eluted by a NaCl gradient (0–0.5 M), prepared in the elution buffer. α-Galactosidase II eluted at 0.11 M and β-galactosidase II at 0.17 M salt. After dialysis concentrates of form I of the enzymes were chromatographed on CM-cellulose. They were separated with McIlvaine buffer (25 mM citrate 50 mM phosphate [5]), pH 5.5, using a NaCl gradient (0–0.5 M). β-Galactosidase I eluted at 0.18 M and α-galactosidase I at 0.26 M NaCl (Fig. 1B).

α- and β-Galactosidase in cell walls

In the preparations of cell walls, the utmost care must be taken to free the walls from adhering cytoplasm or plasmalemma. To exclude any possible contamination, the crude material was washed at least 20 times. Since no protein could be detected in the supernatants of the last four washings, cell walls prepared by the procedure described in the Experimental were considered free from cytoplasm or plasmalemma. Moreover, inspection of these walls under the microscope failed to show any contamination; spectrometric determination at 260 nm showed the absence of nucleic acids. Cell wall proteins were extracted with 3 M NaCl. Three M LiCl or 0.5 M KCl, as used by some workers [6, 7], did not increase the extraction of enzyme activity.

DEAE-cellulose chromatography of cell wall protein extracts also revealed the presence of two forms of α-galactosidase but only one form of β-galactosidase was detected; this could be due to the inactivation of form II during the isolation or elution procedure. Form I of both enzymes was separated by cation-exchange chromatography (Fig. 2). Table 1 shows the degree of purification achieved.

α- and β-Galactosidase in protein bodies

The stability of protein bodies in nonaqueous media [8] permitted the isolation of intact protein bodies by centrifugal sedimentation in glycerol. The percentage of rupture was less than 10%. Prior to isolation of enzymes, protein bodies prepared in glycerol were observed in the electron microscope and this observation confirmed that the preparation was homogeneous and free of wall contamination. This result was in agreement with that reported by Yatsu and Jacks [9] and Plant and Moore [10].

Protein body extracts exhibited α- and β-galactosidase activity. Anion-exchange chromatography also revealed the presence of two forms of each enzyme which were

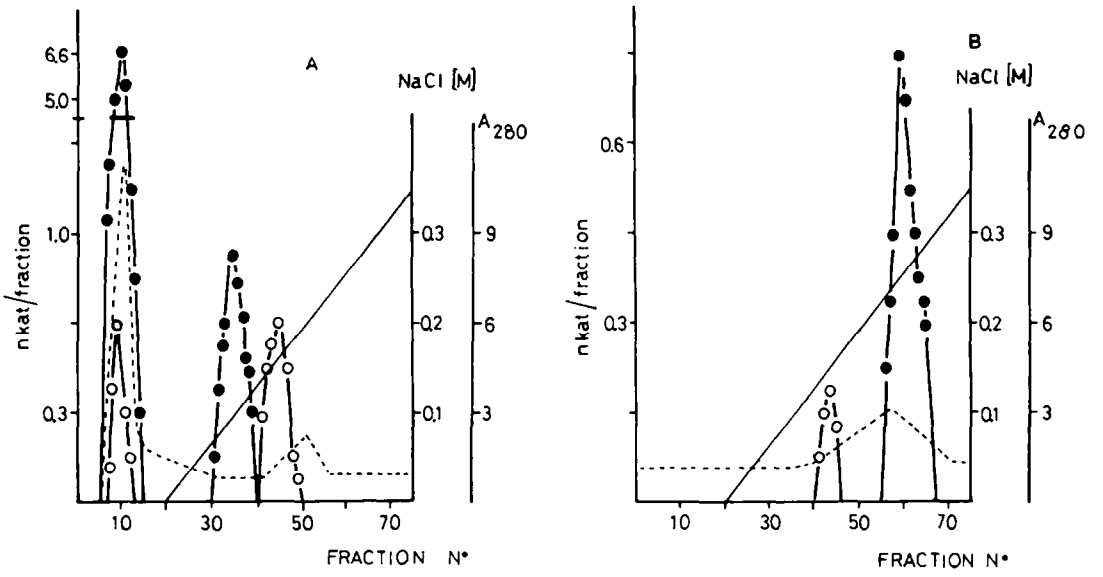

Fig. 1. Ion exchange chromatography of galactosidases from crude extracts: (—●—) α-galactosidase; (—○—) β-galactosidase; (——) NaCl gradient; (- - - -) absorption at 280 nm. A: DEAE-cellulose chromatography, B: CM-cellulose.

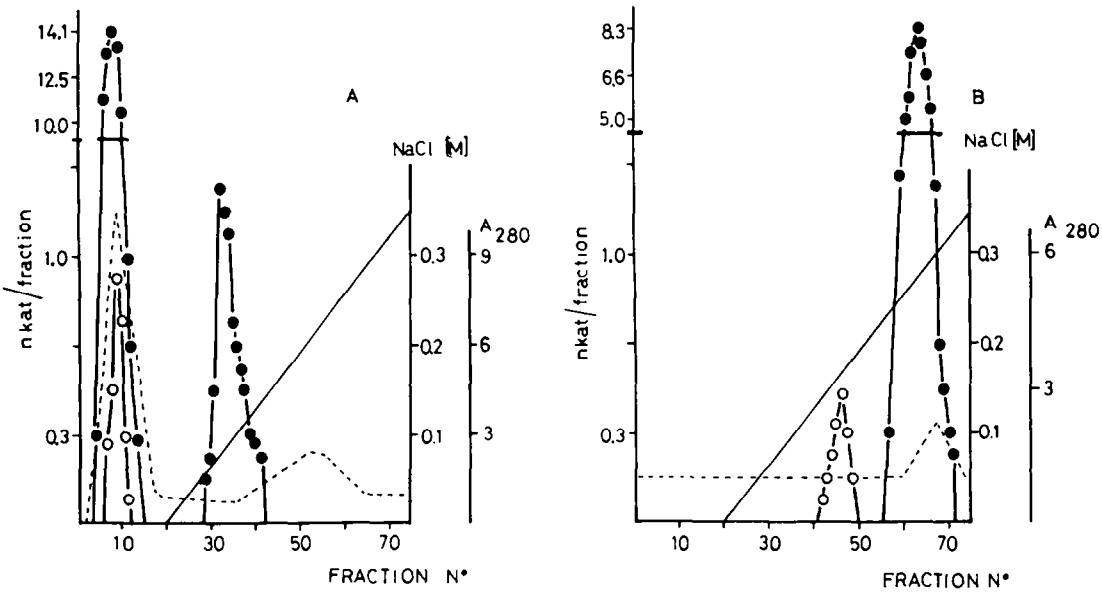

Fig. 2. DEAE-cellulose (A) and CM-cellulose (B) chromatography of galactosidases from cotyledon cell walls: (—●—) α-galactosidase; (—○—) β-galactosidase; (——) NaCl gradient (- - - -) absorption at 280 nm.

separated by a combination of DEAE-and CM-cellulose chromatography (Fig. 3). Table 2 shows the degree of purification achieved. Cell wall and protein body enzymatic preparations were free of α- and β-glucosidase, α- and β-mannosidase, β-*N*-acetyl hexosaminidase, α-xylosidase and β-arabinosidase activities.

Properties of enzymes

When cell wall extracts were subjected to polyacrylamide gel electrophoresis, only one form of β-galactosidase could be detected whose relative mobility was 0.03. Nevertheless, electrophoresis was able to separate two forms of both α- and β-galactosidase from protein body extracts and two forms of α-galactosidase from cell wall extracts. The relative mobility (R_m) of galactosidases from anode (−) to cathode (+) was similar in each compartment: the R_m of α-galactosidases I and II was 0.07 and 0.32, respectively, and the R_m of forms I and II of β-galactosidase was 0.03 and 0.25.

Maximum activity for cell wall α-galactosidase I and II was seen at pH 6; pH values above neutrality resulted in a

Table 1. Partial purification of galactosidases from lentil cotyledon cell walls

	Activity (nKat)	Protein (mg)	Specific activity (nKat/mg)	Recovery (%)
α-Galactosidase isolation				
Supernatant	159	235	0.67	100
$(NH_4)_2SO_4$	138	126	1.09	87
Dialysis				
DEAE-cellulose				
Eluate I	115.5	64	1.8	73
Eluate II	12.3	0.6	23.5	7.7
CM-cellulose				
Eluate I	62	1.1	56.36	39
β-Galactosidase isolation				
Supernatant	7	235	0.029	100
$(NH_4)_2S_4$	5.2	126	0.041	74
Dialysis				
DEAE-cellulose				
Eluate I	3.3	53	0.061	47
CM-cellulose				
Eluate I	1.55	0.5	3.1	22.5

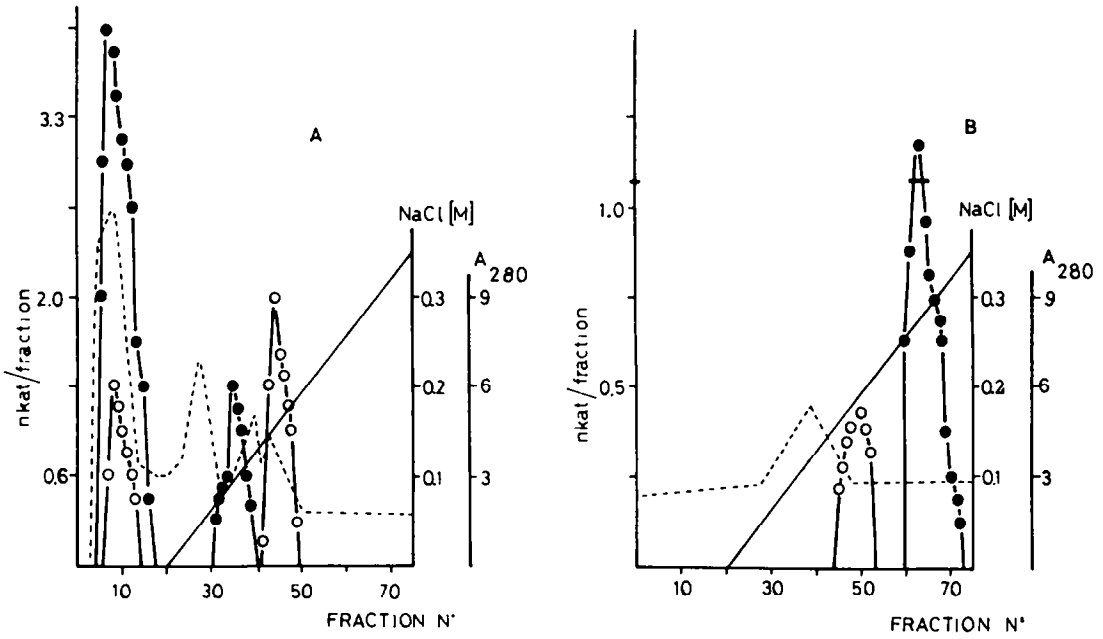

Fig. 3. DEAE- (A) and CM-cellulose (B) chromatography of galactosidases from protein bodies: (—●—) α-galactosidase; (—○—) β-galactosidase (——) NaCl gradient; (- - - -) absorption at 280 nm.

reduction in activity of both α-galactosidases. The pH optima of protein body α-galactosidases were 5.5 (Form I) and 6.5 (Form II). β-Galactosidases from cell walls and protein bodies showed a substantial loss of activity at low or high pH and they were more sensitive to pH changes than the α-galactosidase. pH optima were 4.5 (β-galactosidase from cell walls and protein bodies) and 5.0 (β-galactosidase II from protein bodies). These pH values are similar to the values reported in the literature for galactosidases isolated from leguminous seeds [10, 11].

All enzymes showed Michaelis–Menten kinetics. Table 3 shows the K_m and V_{max} values of all the enzyme forms in the different compartments. The results revealed the high affinity for *p*-nitrophenylglycoside as substrate.

Table 4 shows the action of certain compounds on enzymatic activity. D-Galactose inhibited all the enzyme forms; this is logical because it is the final reaction product and competes with the substrate for the active sites of the

Table 2. Partial purification of galactosidases from lentil protein bodies

	Activity (nKat)	Protein (mg)	Specific activity (nKat/mg)	Recovery (%)
α-Galactosidase isolation				
Crude extract	89	565	0.16	100
$(NH_4)_2SO_4$	61	96	0.63	68
Dialysis				
DEAE-cellulose				
Eluate I	22	23.6	0.93	24
Eluate II	5.3	1.9	6.3	5.9
CM-cellulose				
Eluate I	5.9	0.9	6.5	6.6
β-Galactosidase isolation				
Crude extract	11	565	0.019	100
$(NH_4)_2SO_4$	8.2	96	0.085	79
Dialysis				
DEAE-cellulose				
Eluate I	1.6	14.7	0.11	16
Eluate II	2	1.3	1.53	19
CM-cellulose				
Eluate I	0.54	0.6	0.9	5

Table 3. Kinetics of glycosidases

		K_m (mM)	V_{max} (nKat)
Cell wall			
α-Galactosidase	I	0.26	0.66
	II	0.31	0.75
β-Galactosidase	I	0.56	0.24
Protein bodies			
α-Galactosidase	I	0.29	0.07
	II	0.27	0.82
β-Galactosidase	I	0.13	0.05
	II	0.25	0.03

enzymes. γ-D-Galactonolactone, a specific inhibitor of β-galactosidases in animals and microorganisms, also inhibited the enzyme forms present in the different compartments. Myo-inositol selectively inhibited α-galactosidase. This result is similar to findings reported by other authors [12–14].

α-Galactosidases I and II were able to release galactose residues from raffinose and stachyose, though release was greater in the presence of the latter. The enzymes also released galactose from legumin and vicilin obtained from dry lentil cotyledons according to the procedure of Basha and Beevers [15]. Legumin and vicilin are glycosylated and, as neutral sugars, contain: rhamnose (5%), arabinose (8%), mannose (22%), galactose (10%) and glucose (55%) for legumin and mannose (25%), galactose (8%) and glucose (67%) for vicilin [16]. Our results suggest that at least some of the galactose residues are terminal in the carbohydrate chains and that they may be bound at α- and

Table 4. Effect of metal ions and sugars on the activity of α- and β-galactosidase*

Addition	Concentration† (mM)	Cell wall α-I	Cell wall α-II	Cell wall β-I	Protein bodies α-I	Protein bodies α-II	Protein bodies β-I	Protein bodies β-II
Control	—	100	100	100	100	100	100	100
$CaCl_2$	4	72	80	100	82	80	107	125
$BaCl_2$	4	77	70	100	84	89	85	100
$MnCl_2$	4	73	67	102	98	100	105	141
NaCl	4	82	79	94	83	87	98	100
$ZnSO_4$	4	65	72	104	81	85	103	141
$MgSO_4$	4	66	84	108	91	90	85	116
D-Galactose	8	32	31	40	32	31	33	40
Myo-inositol	140	15	14	100	38	39	100	100
γ-Galactonolactone	5	100	100	60	100	100	47	53

*Results are expressed as relative activity (%).

†Final concentration.

β-positions since the enzymes are specific for this kind of linkage.

β-Galactosidase I from cell walls was unable to release galactose from legumin; this could be interpreted in two different ways: either the substrate was inaccessible to enzymatic action, or the number of units present in the enzyme preparations was insufficient to hydrolyse this glycoprotein. α- and β-Galactosidase from cotyledon cell walls released galactose from dry cell walls previously treated with organic solvents to eliminate associated enzymatic activity.

All results on substrate specificity are summarized in Table 5.

DISCUSSION

The results presented show that in *L. culinaris* cotyledons the enzymes α- and β-galactosidase can be resolved into two forms by ion-exchange chromatography. The identification of these forms does not exclude the possibility that other forms of galactosidases may be present in cotyledon cells. The enzymes are present in protein bodies and in the cell wall. However, in view of the very similar properties of the enzyme fractions isolated from both subcellular compartments and the substrate specificity shown by the enzymes (α-galactosidase from cell wall and protein bodies was able to hydrolyse raffinose and stachyose, both cytoplasm or vacuolar soluble oligosaccharides [17–19]), the distribution data should be treated with caution. However, the fact that the enzymes are compartmentalized suggest that their function is probably different: in the cell wall, during water uptake by the seed, the α- and β-galactosidase enzymes would modify the wall to permit the expansion of cotyledon cells, and in the protein bodies they would release glycosidic units of reserve glycoproteins to later facilitate proteolytic attack. Such a notion is supported by the fact that in lentils proteolytic activity increases from the second day of germination onwards [20], and glycosidases reach their peak activity levels between 24 and 48 hr [21]. A possible direct involvement of carbohydrate in the metabolic stabilization of glycoproteins is highly likely [22, 23]. In addition, the actual mechanism by which carbohydrates could protect these biologically important molecules against proteolytic degradation is not known. Studies in our laboratory have shown that the *in vitro* action of caseinase isolated from protein bodies on reserve proteins underwent a 10% increase if the glycoprotein was previously incubated in the presence of an extract with only galactosidase activity.

Table 5. Substrate specificity of α- and β-galactosidase*

	Leg	Vic	Raf	Sta	CW
Cell wall					
α-I	3.4	0.9	29.6	40.1	2.9
α-II	2.2	0.7	16.6	23.9	1.8
β-I	—	0.5	—	—	1.2
Protein bodies					
α-I	0.7	0.4	12.2	16.6	—
α-II	0.6	0.3	3.5	5.0	—
β-I	0.1	0.1	—	—	—
β-II	0.2	0.2	—	—	—

*Results are expressed as nmol of galactose released per min per mg of protein. Leg: legumin; Vic: vicilin; Raf: raffinose; Sta: stachyose; CW: dry cell walls.

EXPERIMENTAL

Cell wall preparation. Cotyledons (30 g) of mature seeds of *L. culinaris* Medik. were homogenized in a Potter homogenizer with McIlvaine buffer, pH 5.5 (1/4 w/v). After stirring for 1 hr at 4°, the slurry was centrifuged at 20000 *g* for 20 min. The supernatant was considered 'crude extract' and the pellet was further used for isolation of cell walls. The pellet thus obtained was resuspended in McIlvaine buffer, pH 5.5, rehomogenized and sonicated for 10 min. The pellet was successively washed in the following steps, each followed by centrifugation at 1000 *g*: three times with McIlvaine buffer pH 5.5; three times with H_2O; once with 1% (v/v) Triton X-100; once with H_2O; once with 1% Triton X-100 and 10–12 times with H_2O. After each washing the protein content in the supernatant was determined. Commercial α-amylase from porcine pancreas was used for removing starch contamination according to the procedure of ref. [24].

Isolation of wall-bound proteins. Wall material was resuspended in McIlvaine buffer pH 5.5 and 3 M NaCl for 24 hr at 4° and centrifuged at 12500 *g* for 10 min. The supernatant was collected and used as the source of galactosidases.

Protein body isolation. Dry seeds (30 g), were dehulled and cotyledons ground to a meal. Protein bodies were isolated according to the procedure of ref. [9].

Isolation of protein body enzymes. Protein bodies were extracted by continuous stirring for 6 hr in McIlvaine buffer, pH 5.5, containing 0.5 M NaCl + 5 mM 2-mercaptoethanol. After centrifugation at 12000 *g* for 1 hr, the supernatant was collected for further purification of galactosidases.

General procedures. Enzymatic analysis and partial purification of galactosidases were performed according to the procedures previously described [21]. An additional step of purification by chromatography on CM-cellulose was introduced. Protein was determined by *A* at 280 nm and by the method of ref. [25], with BSA as std. Gel electrophoresis was performed according to the Maurer procedure [26]. One set of the gels was stained for protein using Coomassie Blue R [26]. The others were assayed for enzyme activity.

Kinetic studies. K_m values were determined from double reciprocal plots using at least 9 concns of *p*-nitrophenyl-α- or β-galactopyranoside. Enzymes were incubated under std conditions for 15 min after which time reaction rates were linear.

Effect of inhibitors. All assays were performed under std conditions with 3 mM substrate. Effectors were preincubated with enzymes for 15 min before substrate was added.

Effect of pH. The pH dependence of enzyme activity was determined by incubation of the isolated galactosidases with 3 mM *p*-nitrophenylgalactosides for 15 min in McIlvaine buffer, pH range 3–8.

Activity on potential substrates. A qualitative assessment of enzyme activity was performed against the following: legumin and vicilin (satd); stachyose and raffinose (Sigma), 15 mM, and dry cell walls (satd). Substrate soln (500 μl) was incubated with 500 μl of enzyme (24 hr at 30° in the case of reserve proteins, 1 hr with oligosaccharides and 2 hr with cell walls). Galactose released was detected by the galactose-dehydrogenase procedure [27]. In all cases N_3Na (0.02% w/v) was added to the incubation medium in order to avoid bacterial contamination (N_3Na did not affect galactosidase activity *in vitro*).

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