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α-D-MANNOSIDASE AND α-D-GALACTOSIDASE FROM PROTEIN BODIES OF *LUPINUS ANGUSTIFOLIUS* COTYLEDONS

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Key Word Index—*Lupinus angustifolius*; Leguminosae; blue lupin; seeds; protein bodies; α-D-mannosidase; α-D-galactosidase; purification; properties.

Abstract—Two forms of *p*-nitrophenyl α-D-mannosidase and *p*-nitrophenyl α-D-galactosidase were purified from the protein bodies of mature *Lupinus angustifolius* seeds. A MW of 300000 was calculated for both α-mannosidase A and B with $K_m = 1.92$ and 2.70 mM and activation energies of 10.9 and 10.8 kcal/mol, respectively. α-Galactosidase I and II had MWs of 70800 and 17000 with $K_m = 0.282$ and 0.556 mM and activation energies 17.7 and 11.5 kcal/mol, respectively. The enzymes had acid pH optima and were inhibited by various metal ions, carbohydrates and glycoproteins. They were able to release free sugar from several putative natural substrate oligosaccharides and the *Lupinus* storage glycoprotein, α-conglutin.

INTRODUCTION

High activities of several glycosidases including α-D-mannosidase (EC 3.2.1.24) and α-D-galactosidase (EC 3.2.1.22) have been recorded from the mature dry seeds of many plant species. During imbibition and germination there is often reported to be an increase in activity [1], correlated with elevated metabolic activity in the developing seedling. The enzymes are believed to be involved in a variety of processes, most importantly the hydrolysis of oligosaccharides such as raffinose and stachyose during the early germinative period, and of cell wall storage polysaccharides such as mannan and galactomannan at a later period [2]. The enzymes may also have a synthetic function as transglycosylation reactions have been reported for most glycosidases [3]. They have been implicated in the removal of toxic accumulants [4] and their ability to hydrolyse phenolic glycosides could provide a means of control of the levels of plant growth substances.

Glycosidases have been reported from the cell walls, vacuoles, lysosomes, protein bodies and soluble extracts of various plant organs [5–7]. In the cotyledons they are largely but not exclusively located in the principal storage protein site, in the protein body [7]. Multiple forms of some of the enzymes, particularly α-galactosidase [8] have frequently been reported and in seeds these may in part reflect differences in their distribution between the tissues [9]. However, little is known of their subcellular distribution, since most isolations have been performed from whole seed meal (e.g. [10]) using conditions likely to remove bound activity from cell walls as well as extracting cytosolic and protein body associated activities [5].

In this paper, we report on some of the glycosidases found in purified protein bodies from the cotyledons of *Lupinus angustifolius*. Two forms of α-mannosidase and α-galactosidase were detected and some of their basic properties investigated. A role in storage glycoprotein metabolism is tentatively suggested.

RESULTS AND DISCUSSION

Glycosidase purification

α-D-Galactosidase and α-D-mannosidase were recovered predominantly in the respective pellets of 53 and 70% ammonium sulphate fractions of the crude protein body extract. Upon acidification and subsequent dialysis, considerable precipitation of protein occurred, giving a substantial degree of purification (Table 1). The precipitated proteins were predominantly storage globulins which exhibit limited solubility at low pH and after reduction of salt concentration by dialysis [11].

When the α-D-mannosidase dialysate was concentrated and applied to an ion exchange column, part of the activity (peak A) was eluted immediately from the bed with Tris–MES buffer (pH 5.5) whilst the remainder (peak B) which remained bound to the resin was displaced by elution with buffered 0.5M NaCl (Fig. 1A). Concentrated dialysates of peaks A and B, separately applied to a Sephadex G-200 column gave very similar elution profiles (Fig. 1B) indicating similar MW, which was determined to be 300000 by reference to the measured elution volumes of proteins of known MW. A purification factor of 3200 was achieved for α-mannosidase A, but only 390 for α-mannosidase B.

When the α-galactosidase dialysate was concentrated and applied to a Sephadex G-200 column a single peak of activity was eluted. This was concentrated again and applied to a Sephadex G-150 column from which two poorly resolved peaks of activity were eluted (Fig. 1C). When these peaks (galactosidase I and II) were concentrated and separately applied to Sephadex G-75 columns, the two components were clearly resolved with elution volumes

Table 1. Purification of glycosidases from protein bodies of *L. angustifolius* cotyledons

	Enzyme activity (μmol/min)	Protein (mg)	Specific activity (nmol/min/mg protein)	Purification*
α-Galactosidase isolation				
Crude protein body extract	74.4	5251	14.2	1.0
0–53% $(NH_4)_2SO_4$ ppt	22.5	725	31.1	2.2
Acid supernatant	19.9	400	49.0	3.5
Dialysed acid supernatant	12.0	84	142.3	10.0
Sephadex G-200 eluate	11.4	38	300.1	21.2
Sephadex G-150 eluate I	2.6	3.6	721	51
II	2.5	10.5	240	17
Sephadex G-75 eluate I	2.4	1.48	1600	113
II	1.5	5.23	285	20
α-Mannosidase isolation				
Crude protein body extract	25.7	5251	4.9	1.0
53–70% $(NH_4)_2SO_4$ ppt	10.4	1005	10.4	2.1
Acid supernatant	9.8	629	15.6	3.2
Dialysed acid supernatant	14.2	36	399.5	81.7
CM52 eluate A	9.7	8.0	1215	249
B	1.7	9.3	180	37
Sephadex G-200 eluate A	7.3	0.46	15940	3262
B	1.3	0.66	1933	395

*Purification is expressed relative to the crude extract.

giving MW values of 70800 and 17000 respectively (Fig. 1D).

Two forms of α-D-galactosidase have been demonstrated from the seeds of several plant species [8] and in some cases these appear to represent monomeric and tetrameric forms of the enzyme [10, 12]. The MW ratio of the two galactosidases is also consistent with form I being a tetramer of form II.

A frequent problem in the isolation of α-mannosidase and α-galactosidase is the co-purification of the other glycosidases [1]. We were unable to detect hydrolysis of *p*-nitrophenyl linked α-L-fucose, *N*-acetyl-β-D-glucosamine, α- or β-mannose by either galactosidase I or II. The galactosidase was partially active against *p*-nitrophenyl β-D-galactose, but lack of reproducibility of this result makes its significance uncertain. α-Mannosidase was totally specific to *p*-nitrophenyl α-D-mannose when tested over the same range of substrates.

*p*H *optima and stability*

Pre-incubation for 2 hr at pH values above neutrality resulted in substantial reduction in activity of both α-galactosidases. α-Galactosidase I was also labile at low pH. The α-mannosidases also showed some loss of activity, particularly at high pH, but were generally far less sensitive to pH changes than the α-galactosidases. pH optima, determined from short incubations to avoid substantial enzyme inactivation, were at pH 6.5 (α-galactosidase I); pH 5.5 (α-galactosidase II); a broad optimum at pH 4.0 (α-mannosidase A); a sharp peak at pH 4.6 (α-mannosidase B).

Activation energy and K_m

Arrhenius plots of log initial rate against reciprocal of temperature showed linear relationships and activation energies of 10.9 kcal/mol (α-mannosidase A), 10.8 kcal/mol (α-mannosidase B), 17.9 kcal/mol (α-galactosidase I) and 11.5 kcal/mol (α-galactosidase II) were calculated. These values are similar to those reported for other seed α-galactosidase and α-mannosidase activities [8, 13].

α-Mannosidase A and B demonstrated simple Michaelis–Menten kinetics, K_m values being 1.92 and 2.70 mM respectively when the assays were performed in the presence of 1 mM Zn^{2+} to ensure maximal activity. Both α-galactosidase I and II showed deviations from simple kinetics at high substrate concentrations. Plots of initial rate against log substrate concentration were bell-shaped curves characteristic of substrate inhibition, in which the rate of reaction tends to zero as substrate concentration increases. It is of interest to note that Dey and Pridham [14] have also reported substrate inhibition in a galactosidase isolated from *Vicia faba*. Values of K_m could be calculated from the linear portion of the double reciprocal plots (Fig. 2) at low substrate concentration and were found to be 0.282 mM for I and 0.556 mM for galactosidase II.

Inhibition studies

Table 2 reports the effects of metal ions, sugars and glycoproteins on the activity of α-mannosidase A and α-galactosidase II. Both enzymes were inhibited to some extent by Ag^+, Hg^{2+} or Cu^{2+} and are similar in this respect to the majority of α-galactosidases and α-mannosidases [1, 8]. Prolonged incubation with

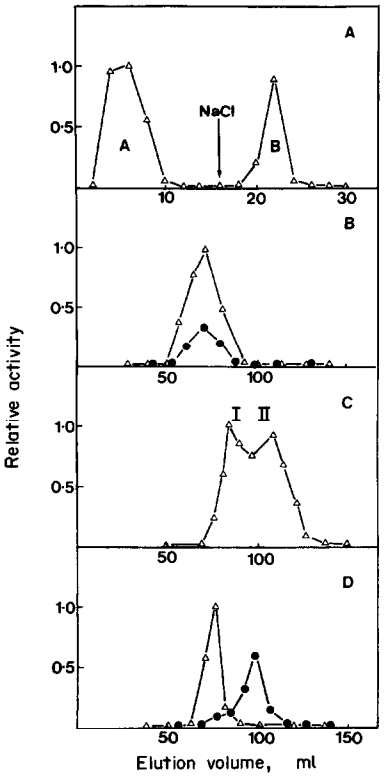

Fig. 1. Column chromatography of α-mannosidase and α-galactosidase from *Lupinus* protein bodies: (A) ion exchange chromatography on CM52 on mannosidase acid supernatant. Peak A was eluted by pH 5.5 buffer, peak B by buffered NaCl; (B) elution profiles of mannosidase A (△) and mannosidase B (●) after separate application to Sephadex G-200 columns; (C) Sephadex G-150 chromatography of galactosidase acid supernatant; (D) elution profiles of galactosidase I (△) and galactosidase II (●) after separate application to Sephadex G-75 columns.

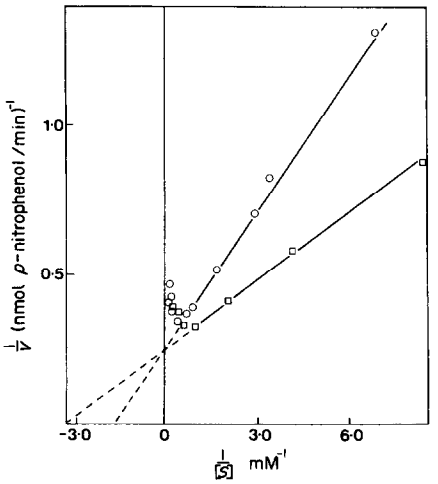

Fig. 2. Effects of substrate concentration on α-galactosidase activity: double reciprocal plot for α-galactosidase I (□) and α-galactosidase II (○).

EDTA inhibited mannosidase and activity was restored by the addition of Zn^{2+}. Many seed mannosidases require the formation of a protein–Zn^{2+} complex for maximal activity, the ion binding to a specific site on the enzyme. EDTA is thought to cause inhibition largely by competing for bound Zn^{2+} in the complex [1]. The slight inhibitory effect of Co^{2+} and Cu^{2+} may also be attributable to competition for bound Zn^{2+} resulting in an inactive metalloenzyme complex [1].

Mannose and galactose were found to inhibit the α-mannosidase and α-galactosidase respectively (Table 2). Double reciprocal plots at a range of mannose and galactose concentrations showed that the inhibition of mannosidase A by D(+)-mannose was competitive, with $K_i = 15.0$ mM. D(+)-Galactose apparently caused mixed inhibition of α-galactosidase II, suggesting both competitive and uncompetitive binding of galactose to the enzyme.

It is possible that the substrate inhibition by *p*-nitrophenyl galactose may also have a competitive element due to the galactose moiety, as suggested by Dey and Pridham [18] for *Vicia faba* α-galactosidase. Unlike the uncompetitive component however, a competitive element would be undetectable on double reciprocal plots [15].

Haemagglutinin tests

Some legume α-galactosidases have been reported to have lectin activity [10, 16]. However, α-galactosidase I/II and α-mannosidase A/B were unable to cause agglutination of trypsinized rabbit erythrocytes at concentrations comparable or in excess of the concentrations of Con A required for this effect.

Natural substrates

Qualitative determinations of the activity of mannosidase A and galactosidase II on some saccharides and glycoproteins showed that the galactosidase could release galactose residues from α-D-melibiose, methyl α-D-galactose, raffinose and stachyose and also from ovomucoid, but not from ovalbumin nor from the lupin seed storage glycoproteins α-, β- or γ-conglutin. The mannosidase was incapable of releasing mannose from ovalbumin, ovomucoid, yeast mannan, β- or γ-conglutin, but did hydrolyse methyl α-D-mannose and release mannose from α-conglutin.

α-Conglutin contains 0.71% of its anhydrous protein weight as mannose, along with smaller amounts of galactose and glucosamine [17]. Our data suggest that at least some of this is terminal to the carbohydrate chains. These mannose residues may be responsible for the inhibition of *p*-nitrophenyl mannosidase by α-conglutin reported in Table 2. The inability of α-mannosidase A to release mannose from β-conglutin and the less pronounced inhibition of the enzyme by this glycoprotein suggests that despite its six-fold greater mannose content [17], these residues are not as readily accessible to the enzyme as are those of α-conglutin.

We have demonstrated that multiple forms of α-mannosidase and α-galactosidase occur within the protein body. Their presence within the organelle containing the major seed storage protein reserve might also allow the glycosidases to play a role in the

Table 2. Effects of metal ions, sugars and glycoproteins on the activity of mannosidase A and galactosidase II

Addition	Concentration (mM)	% Inhibition	
		α-Mannosidase A	α-Galactosidase II
Control	0	0	0
$AgNO_3$	10	97	100
$HgCl_2$	10	45	100
$CuSO_4$	10	10	0
$Co(NO_3)_2$	10	5	0
EDTA	1	11	—
EDTA + $ZnSO_4$	0.2	0	—
Galactose	10	—	76
Mannose	10	29	—
Methyl α-D-mannose	10	9	—
Raffinose	10	6	—
α-Conglutin	saturation	29	—
β-Conglutin	saturation	11	—

modification or degradation of glycoprotein oligosaccharides during germination. Analysis of the products of [^{14}C] glucosamine-labelled polypeptide breakdown during the germination of *Pisum* seeds, suggests that proteolysis precedes oligosaccharide degradation [18], whereas the present work shows that sugar release can take place from the intact glycoprotein. Further work is required to understand the significance of glycosidase action to the overall lytic function of protein bodies during germination.

EXPERIMENTAL

Protein body isolation. Dry seeds of *L. angustifolius* (cv New Zealand Bitter Blue) were hulled, and the cotyledons ground to a meal. Protein bodies were isolated from the meal by the non-aq. glycerol method, essentially as in [19]. 95% of the sol. protein of the preparations exhibited solubility characteristics typical of the storage globulin and was resolved by cellulose acetate electrophoresis as α-, β- and γ-conglutin. Light microscopy confirmed that the preparation was composed of protein bodies, and calcofluor straining showed that little cell wall contamination was present.

Extract preparation. All isolation procedures were performed at 4°. Protein bodies (30 g) were extracted by continuous stirring for 2 hr in 1 l. 50 mM Na–KPi buffer (pH 7.0), containing 0.3 M NaCl, 5 mM 2-mercaptoethanol and 0.06% (w/v) Polyclar AT. After centrifugation (12000 *g* for 1 hr) the supernatant was made 53% satd with $(NH_4)_2SO_4$ and the ppt collected for use in further purification of the galactosidases. Remaining soln was brought to 70% satn and the ppt used as a source of mannosidases.

Acid precipitation. $(NH_4)_2SO_4$ ppts were suspended in 50 mM Tris–MES (pH 7.0) and then made pH 5.0 with 1 M HCl. After standing for 5 min, the ppt was removed by centrifugation (2000 *g* for 15 min) and the supernatant readjusted to neutrality with 1 M NaOH. Supernatants were decanted off, dialysed against H_2O and concd against PEG and finally suspended in 5 ml 50 mM Tris–MES buffer (pH 5.5) containing 1 mM $ZnSO_4$ (for the 53–70% cut which was enriched in mannosidase activity) or 50 mM Na citrate (pH 7.2) (for the 53% cut which was enriched in galactosidase activity).

Isolation of glycosidases. Mannosidase-enriched acid supernatants (5.0 ml) were applied to a Whatman CM52 cation exchange column (8.0 × 1.5 cm) equilibrated with 50 mM Tris–MES (pH 5.5). Peak A was eluted immediately, after which buffered 0.5 M NaCl was applied and peak B recovered. Eluates were dialysed against H_2O, concd against PEG and applied separately to a column of Sephadex G-200–120. Galactosidase-enriched acid supernatants were concd against PEG and applied to a Sephadex G-200–120 column. The single galactosidase peak was concd and applied to a Sephadex G-150–40 column, eluting as two peaks (I and II) which were applied separately to a Sephadex G-75–40 column.

Estimations of the glycosidase MW were based on the elution vols from Sephadex G-200–120 (mannosidase) or G-75–40 (galactosidase) using haemoglobin, myoglobin, cytochrome *c* and ferritin as standards. Gel columns were 40 × 2.6 cm and equilibrated with 50 mM Na citrate (pH 7.2) throughout. Buffers used in mannosidase gel separations contained 1 mM $ZnSO_4$.

Enzyme assays. α-D-Galactosidase and α-D-mannosidase were assayed by following the release of *p*-nitrophenol from *p*-nitrophenyl α-D-galactose and *p*-nitrophenyl α-D-mannose respectively. Assays containing 50 μl enzyme and 0.5 ml 2 mM substrate in 200 mM NaOAc buffer (pH 5.6) were incubated at 30° for 10 min (mannosidase) or 15 min (galactosidase). Mannosidase assays were performed in the presence of 1 mM $ZnSO_4$. Reactions were terminated with 0.5 ml 1 M Na_2CO_3, the A_{410} measured, and values expressed as *p*-nitrophenol.

Kinetic studies. K_m values were determined from double reciprocal plots using at least eight concns of *p*-nitrophenyl α-D-mannose or *p*-nitrophenyl α-D-galactose. Enzymes were incubated under standard conditions for 10 min (mannosidase) or 15 min (galactosidase) during which reaction rates were linear with respect to time. The inhibition of mannosidase A and galactosidase II by mannose and galactose was examined by double reciprocal plots using five mannose concns and three galactose concns.

Effects of inhibitors. All assays were performed under standard conditions with 2 mM substrate. $AgNO_3$, $HgCl_2$, $CuSO_4$ and $Co(NO_3)_2$ (final concn 10 mM) were pre-in-

cubated with enzyme for 20 min before substrate was added. EDTA (1 mM) was pre-incubated with enzyme for 24 hr and enzyme activity assayed with or without $ZnSO_4$ (0.2 mM). The effects of galactose, mannose, methyl α-D-mannose, raffinose (10 mM), α- and β-conglutin (satn) were determined directly, without pre-incubation.

Effect of temp. The purified enzyme prepns were all stable for at least 20 min in the temp. range 4–50°. Activation energies were determined from Arrhenius plots which were linear over at least four temps in this range.

Effects of pH. The pH dependence of enzyme activity was determined by incubation of the isolated glycosidases with 2.0 mM *p*-nitrophenyl glycosides for 10 min in 200 mM NaOAc buffer at 30°. Stability at different pH was determined by pre-incubation for 2 hr with 200 mM NaOAc (pH 3.4, 4.5, 5.6), 200 mM Na–KPi (pH 7.5) or 200 mM Tris–HCl (pH 8.0, 9.0) at 30°. Assays were then performed at pH 5.6 using 2.0 mM substrates at 30°.

Activity on potential substrates. A qualitative assessment of enzyme activity was performed against the following: α-, β- and γ-conglutins (satn); ovalbumin and ovomucoid (2% w/v); Me α-D-mannose, Me α-D-galactose, α-D-melibiose, raffinose (0.5% w/v); stachyose and yeast mannan (0.7% w/v). 300 μl substrate soln was incubated with 50 μl enzyme for 20 hr at 30° and sugar release detected by TLC using the procedures of Hansen [20].

General methods. The *Lupinus* storage proteins, α-, β- and γ-conglutin were extracted from dry seeds according to the method of ref. [21]. Protein was routinely estimated by the Hartree modification of the Lowry method [22] using bovine serum albumin as standard. Haemagglutinin activity was assayed using trypsinized rabbit erythrocytes according to ref. [10].

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