Biochem. J. (1978) **175**, 1069–1077
Printed in Great Britain

α-Galactosidases II, III and IV from Seeds of *Trifolium repens*

PURIFICATION, PHYSICOCHEMICAL PROPERTIES AND MODE OF GALACTOMANNAN HYDROLYSIS *IN VITRO*

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(*Received* 24 *April* 1978)

Five α-galactosidases (α-D-galactoside galactohydrolase, EC 3.2.1.22) were identified by chromatography and by their different electrophoretic mobilities, in the germinated seeds of *Trifolium repens* (white clover). α-Galactosidases II, III and IV were purified to homogeneity, with increases in specific activity of approx. 4600-, 4900- and 2800-fold respectively. The enzymes were purified by a procedure that included $(NH_4)_2SO_4$ precipitation, hydroxyapatite, Sephadex G-75 and DEAE-cellulose chromatography, and preparative polyacrylamide-gel disc electrophoresis. The purified enzymes showed a single protein band, corresponding to the α-galactosidase activity, when examined by polyacrylamide-gel electrophoresis. The pH optimum was determined with *o*-nitrophenyl α-D-galactoside and the galactomannan of *T. repens* T_0 as substrate. All three enzymes are highly thermolabile. Hydrolysis of oligosaccharides and galactomannans was examined, including two galactomannans from the germinated seed of *T. repens* (T24 and T36). By sodium dodecyl sulphate/polyacrylamide-gel electrophoresis the mol.wts. of the multiple forms of enzyme were found to be identical (41000).

Galactomannans are found as a major polysaccharide storage reserve in the seeds of the Leguminosae (Anderson, 1949; Smith & Montgomery, 1959). The mature seeds of *Trifolium repens* (tribe Trifoliaceae) contain galactomannan (Andrews *et al.*, 1952; Courtois & LeDizet, 1963; Horvei & Wickström, 1964) as the major component of the endosperm, as well as significant quantities of sucrose, raffinose, stachyose and galactosylsucrose oligosaccharides.

Galactomannans from different species of plants have different proportions of D-galactose and D-mannose, but consist of a common basic 'backbone' of β1→4-linked D-mannopyranose residues to which the D-galactopyranose units are attached α1→6 (Smith & Montgomery, 1959). During the initial period of germination (Reid, 1971; Reid & Meier, 1972) the galactomannan is rapidly metabolized by α-galactosidase (EC 3.2.1.22) and β-mannanase (EC 3.2.1.25) (Hylin & Sawai, 1964; Emi *et al.*, 1972; Villarroya & Petek, 1976).

The occurrence of multiple forms of α-galactosidase was first reported in seeds of *Coffea arabica* by Petek & ToDong (1961) and in seeds of *Plantago ovata* (Courtois *et al.*, 1963). Dey & Pridham (1968) and Pridham & Dey (1974) later showed that extracts of *Vicia faba* seeds contained several forms of α-galactosidase differing in their molecular weights. The seeds from a number of other plant species have also been shown to exhibit multiple forms of this enzyme (Barham *et al.*, 1971; Balasubramaniam *et al.*, 1974; Dey & Dixon, 1974). The existence of several forms of α-galactosidase has also been observed in the fungi *Aspergillus niger* (Lee & Wacek, 1970), *Mortierella vinacea* (Suzuki *et al.*, 1970) and *Corticium rolfsii* K2 (Kaji & Yoshihara, 1972). Evidence for the occurrence of three forms of α-galactosidase in the leaves of *Cucurbita pepo* (Thomas & Webb, 1977) has been obtained.

In a previous paper (Williams *et al.*, 1977) it was shown that five forms of α-galactosidase could be identified by chromatography and polyacrylamide-gel disc electrophoresis in the germinated seeds of *Trifolium repens*. α-Galactosidase I was shown to be a monomeric protein of mol.wt. 41000–43000 and to exhibit two enzymic activities, namely α-D-galactoside galactohydrolase and galactosyltransferase. In the present paper we describe the purification and properties of three forms of α-galactosidase from the same seed.

Experimental and Results

Materials

The dry seeds of *Trifolium repens* (white clover; variety Trefle nain blanc mira) were obtained from Vilmorin, Paris, France. Hydroxyapatite was pre-

pared by a modification of the method of Tiselius *et al.* (1956) and Levin (1962). The sources of other materials have been given previously (Williams *et al.*, 1977).

Preparation of galactomannan from Trifolium repens

Dormant seeds (100g) were boiled in ethanol (95°C) for 10min. The seeds were then dried thoroughly before being macerated in benzene (50ml) and then washed on a Buchner funnel with approx. 400ml of benzene. The preparation was then washed with 200ml of chloroform, followed by 50–100ml of diethyl ether, to remove lipid contaminants. The preparation was left to dry overnight on filter paper at room temperature. To the dry powder 5 litres of water was added and the mixture stirred thoroughly for 24h at 4°C. After centrifugation for 30min at 13000*g*, the clear solution so obtained was filtered (Whatman filter paper no. 1) and precipitated by the addition of an equal volume of ethanol. The precipitate was collected by centrifugation at 13000*g* for 30min, redissolved in 1 litre of water and subsequently dialysed for 24h at 4°C against water. The resulting solution was centrifuged if turbid and reprecipitated with ethanol. The precipitate was collected as described above and dissolved with stirring in approx. 500ml of water before being dialysed (4°C) for 24h. The non-diffusible residue was centrifuged (20000*g*) for 30min and the clear solution so obtained re-precipitated with ethanol. This precipitate was collected by centrifugation (13000*g* for 30min), suspended in acetone and washed several times with acetone on a Buchner funnel. The precipitate was left to dry in a desiccator in the presence of $CaCl_2$ and conc. H_2SO_4. Once dry the galactomannan was ground to a fine powder with a pestle and mortar. The galactomannan extracted from dormant seeds was designated *T. repens* T_0. The same protocol was followed to extract the galactomannans from seeds of *T. repens* after 24h (*T. repens* T24) and 36h (*T. repens* T36) of germination.

Assay of enzyme activity

α-Galactosidase activity was assayed by incubating 25μl of suitably diluted enzyme with 50μl of *o*-nitrophenyl α-D-galactoside (25mM) and 25μl of buffer at 37°C for 4min. The buffer used was McIlvaine's (1921) at the required pH optimum for α-galactosidases II, III or IV. The enzymic reaction was stopped by addition of 3ml of 0.2M-Na_2CO_3 and the yellow colour so developed read spectrophotometrically at 400nm. Specific activity was expressed as μmol of galactose liberated/min per mg of protein at 37°C.

Hydrolysis of galactomannan, raffinose, planteose, stachyose and phenyl α-D-galactoside was determined by the Somogyi–Nelson technique (Somogyi, 1945; Nelson, 1944). The original method was used where the quantity of hexose liberated was greater than 50μg. Under conditions where the quantity of hexose liberated was 10–50μg the following micro-method was used. The enzymic reaction was stopped by the addition of 0.5ml of solution A (Somogyi, 1945) diluted to 50% (v/v). After incubation at 100°C in a boling-water bath for 20min the assay tubes were allowed to cool and 0.25ml of solution B (Nelson, 1944) was added, followed by 3ml of water. After 10min the stabilized blue coloration was read spectrophotometrically at 650nm against suitably prepared blanks. The assay medium for these incubations consisted of 25μl of enzyme (at pH4.2 and 37°C) with 50μl of substrate and 25μl of McIlvaine (1921) buffer. Protein was determined by the method of Zamenhof and Chargaff (Zamenhof, 1957) with bovine serum albumin as the protein standard.

Assay of galactosyltransferase activity

The incubation mixture for the transgalactosylation experiments contained 50μl of suitably diluted enzyme, 50μl of galactosyl donor, 50μl of acceptor sugar and 50μl of McIlvaine (1921) buffer, pH4.2. Galactosyl donors used were *o*-nitrophenyl α-D-galactoside or melibiose (Gal*p*α1→6Glc) at final concentrations of 10mM and 25–50mM respectively in the incubation medium. Acceptor-sugar concentrations used were 250mM (galactose, galactinol, mannose or glucose) and 5–50mM for mannobiose (Man*p*β1→4Man), cellobiose, maltose, sucrose, melibiose, raffinose, planteose or stachyose. Incubation was carried out at 37°C for 1–24h and the reaction stopped by heating at 100°C for 2min.

Transfer products were detected by t.l.c. on silica-gel-coated plastic sheets. The solvent system was propan-1-ol/ethanol/ethyl acetate/pyridine/water (7:3:3:2:3, by vol.). The solvent front was allowed to migrate 14cm from the baseline. Chromatograms were subsequently developed with a solution of β-naphthylamine (Petek, 1962) followed by treatment with 2% (v/v) H_2SO_4 in acetone.

Gel electrophoresis

Electrophoresis was carried out on 7.5% (w/v) polyacrylamide disc gels by the technique described by Maizel (1964). Experiments were carried out at 4°C with a current of 6mA/gel. The gels were fixed and stained with Coomassie Brilliant Blue (0.25%, w/v) in methanol/acetic acid/water (5:1:4, by vol.) for 2h, and subsequently destained at 40°C in a solution containing 25% (v/v) methanol and 7% (v/v) acetic acid in water.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Molecular-weight determinations were carried out

on 7.5% (w/v) polyacrylamide disc gels by the procedure of Weber & Osborn (1969).

Reference proteins used (with molecular weights in parentheses) were: phosphorylase *a* (94000); serum albumin monomer (68000); egg albumin (43000); pepsin (35000); trypsin (23000). Electrophoresis was carried out overnight (18h) at room temperature (20°C) with a current of 3mA/gel (5mm diam., 110mm long). The gels were stained with Coomassie Brilliant Blue for 5h and bleached as described above.

Purification

Dry seeds of *T. repens* (200g) were soaked in water for 16h and subsequently germinated in the dark at 20–22°C for 48h. The crude extract was prepared at 4°C by maceration of the seeds with 3% (w/v) NaCl solution containing 0.2ml of β-mercaptoethanol/litre, in a Waring commercial blender. All subsequent procedures were carried out at 4°C and all solutions contained β-mercaptoethanol (0.2ml/litre). The extract was left for 3h before being centrifuged for 30min at 13000*g* and the resulting supernatant dialysed overnight (16h) against 0.2M-citrate/sodium phosphate buffer, pH 3.5. The precipitate so formed was removed by centrifugation (30min at 13000*g*) and the supernatant precipitated by addition of $(NH_4)_2SO_4$ to 80% saturation. This precipitate was dissolved in 80ml of water, dialysed against water (24h) and the remaining precipitate removed by centrifugation. The resulting 120–130ml of supernatant was immediately applied to a column of hydroxyapatite (column 1: see below and Table 1).

Column chromatography

Column 1 (200mm×22mm) was of hydroxyapatite equilibrated with 5mM-potassium phosphate buffer, pH6.0. After elution of fraction 1, containing α-galactosidase I (Williams *et al.*, 1977), the second fraction containing α-galactosidase activity (fraction 2) was eluted with 100mM-potassium phosphate buffer. Fraction 3, containing α-galactosidases II, III and IV, was eluted, after washing with 100mM buffer, with 200mM-potassium phosphate buffer containing 200mM-NaCl. Fraction 3 represented approx. 65% of the total α-galactosidase activity eluted from this column (see Table 1). The column was eluted at a flow rate of 80–100ml/h and collected in 10ml portions, with an average pooled volume of 60ml. This fraction was concentrated against sucrose, by placing the enzyme in a dialysis bag in contact with the crystalline sucrose; no loss of activity was noted. The concentrated enzyme was subsequently dialysed for 1h against water before being applied (2–4ml) to a column (140cm×2.5cm) of Sephadex G-75 (column 2) equilibrated with 10mM-potassium phosphate buffer, pH6.0. The rate of elution of column 2 was 10ml/h and the eluate collected in 7ml portions. The average pooled volume of the most active fractions was 60ml. This fraction was concentrated against sucrose as described above and the concentrated enzyme applied, after dialysis against water (1h), to a column (140mm×15mm) of DEAE-cellulose (column 3). This column was equilibrated with 10mM-potassium phosphate buffer, pH7.0, and eluted in a stepwise manner with increasing concentrations of NaCl, namely 0, 10, 20, 50 and 100mM. α-Galactosidase II was eluted with buffer plus 20mM-NaCl. After limited washing with the latter buffer α-galactosidases III and IV were eluted with buffer plus 50mM-NaCl.

α-Galactosidase II represented 41% of the total activity recovered from this column (see Table 1). α-Galactosidase II (average pooled volume, 100ml) was subsequently concentrated by an Amicon ultrafiltration apparatus, fitted with a UM2 membrane, at a pressure of 152kPa (1.5atm). The enzyme so obtained showed a single protein band on examination by polyacrylamide-gel disc electrophoresis (Fig. 1).

α-Galactosidases III and IV represented (together) 59% of the total activity recovered from column 3, with an average pooled volume of active fractions of 70ml. This fraction was concentrated, as described previously, against sucrose and subjected to polyacrylamide-gradient-gel electrophoresis (Doly & Petek, 1977).

Preparative polyacrylamide-gel electrophoresis

The concentrated enzyme (1–2ml) was layered on the gel surface; 150–200μl portions were applied to each of the eight gels. Gels were of 6.5% polyacrylamide, and electrophoresis was carried out at 4°C with a constant voltage of 90V during 8.5h. After electrophoresis one of the gels was immediately sliced (Doly & Petek, 1977) and half of each slice (2mm) incubated with *o*-nitrophenyl α-D-galactoside, to locate exactly the positions of α-galactosidases III and IV (Fig. 1). Active slices were pooled to give eight active fractions. Each fraction was injected, by means of a syringe, into one of the eight chambers of the elution apparatus designed by Doly & Petek (1977). Elution was carried out at a constant 250V (1mA/chamber) for 16h. Each of the eight fractions (2.5ml) was subsequently dialysed for 6h against water.

α-Galactosidase III represented 35% of the activity recovered after elution (and dialysis), and α-galactosidase IV represented 65% (see Table 1). Both enzymes III and IV, when examined by polyacrylamide-gel disc electrophoresis, proved to be homogeneous protein preparations (Fig. 1).

Table 1. *Purification of α-galactosidases II, III and IV*

The substrate used was *o*-nitrophenyl α-D-galactoside. α-Galactosidases II, III and IV represented 65% of the total α-galactosidic activity, as measured with the above substrate, eluted from column 1. The Table has subsequently been corrected in steps 1, 2 and 3 to show only the activity corresponding to enzymes II, III and IV, their collective specific activity and percentage recovery. Calculation of the recovery and purification of each enzyme is based on the proportion of the total activity that each enzyme represented. Thus enzyme II represents 41% of the total activity eluted from column 3 (DEAE-cellulose) and α-galactosidases III and IV represent 35 and 65% respectively of the activity recovered after separation by preparative electrophoresis (21 and 38% overall).

Step	Volume (ml)	Total activity (μmol of galactose/min)	Total protein (mg)	Specific activity (units/mg of protein)	Recovery (%)	Purification (-fold)
(1) Crude extract	880	1401	9.797	0.143	100	
(2) After acid dialysis at pH3.5	880	1370	4.463	0.307	97.8	2.14
(3) After 80%-satd.-$(NH_4)_2SO_4$ precipitation and dialysis	110	1290	1.451	0.889	92.1	6.22
(4) Column 1 (hydroxyapatite)	58	687.4	60.03	11.45	49.1	80.0
(5) Column 2 (Sephadex G-75)	62	433.1	10.43	41.55	30.9	290.6
(6) Column 3 (DEAE-cellulose)						
α-Galactosidase II	100	73.13	0.271	268.97	12.7	4590
α-Galactosidases III and IV	70	104.9	1.125	93.2	12.7	883.1
(7) Preparative electrophoresis						
α-Galactosidase III	4	7.36	0.051	144.1	2.55	4885
α-Galactosidase IV	4	13.97	0.089	157.2	2.60	2863

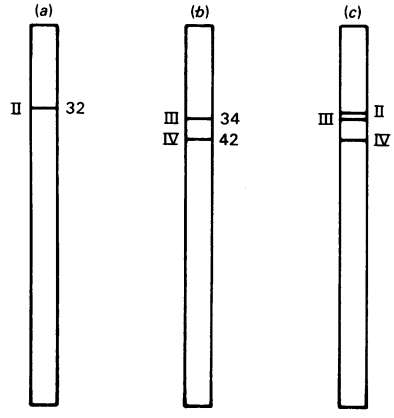

Fig. 1. *Diagram to show the different electrophoretic mobilities of α-galactosidases II, III and IV*

(*a*) α-Galactosidase II after step 6 (DEAE-cellulose: column 3). (*b*) α-Galactosidases III and IV before separation by preparative polyacrylamide-gel electrophoresis. (*c*) Mixture of α-galactosidases II, III and IV after purification to show the relative electrophoretic mobilities. Gels of 7.5% polyacrylamide (14cm×1.0cm) were used; migration was for 4h and 180V at 40°C. The distances of migration are given in cm from the anode.

General properties

The pH–activity profiles for α-galactosidases II, III and IV with *o*-nitrophenyl α-D-galactoside as substrate show a broad optimum between pH4.5 and 5.8, with a slight maximum at approx. pH5.5 (Fig. 2). This contrasts with the pH–activity profiles obtained with the galactomannan of *T. repens* T_0 (Fig. 3), which for this substrate show a more restricted optimum: α-galactosidase II, pH3.6–4.4; α-galactosidase III, pH4.2–4.8; α-galactosidase IV, pH4.2–4.6.

During purification of the three α-galactosidases the enzyme preparation was stable at 4°C. Preparations at the stage of $(NH_4)_2SO_4$ fractionation (step 5, Table 2) could be stored at −20°C without loss of enzymic activity. However, after this stage enzyme fractions could only be stored at −20°C if already concentrated in sucrose. All three enzymes proved to be unstable to freeze-drying.

Fig. 4 shows the inactivation of hydrolytic activity for α-galactosidase II as a function of temperature and time. Identical experiments were carried out with α-galactosidases III and IV. At 45°C the time for 50% inactivation was 29min for enzyme II, 7.5min for III and 8min for IV. The

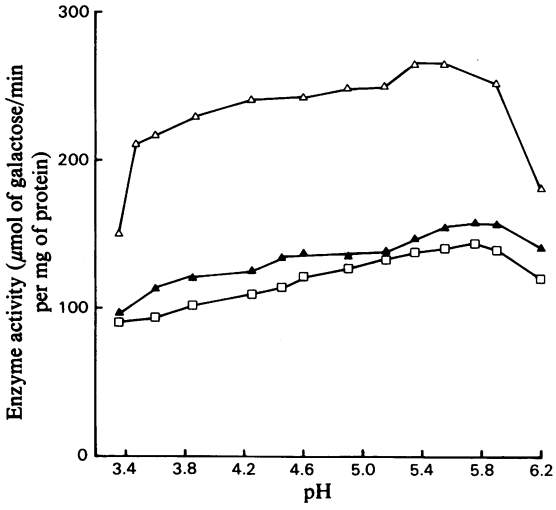

Fig. 2. *Effect of pH on the activities of α-galactosidases II, III and IV with o-nitrophenyl α-D-galactoside as substrate*
McIlvaine buffer with a pH range of 2.5–8 was used. The incubation mixture contained 25μl of enzyme, 50μl of substrate (25mM) and 25μl of buffer. The incubation time was 4min at 37°C. The reaction was stopped by the addition of 3ml of 0.2M-Na_2CO_3. The liberated aglycone was determined at 400nm. Symbols: △, α-Galactosidase II; □, α-galactosidase III; ▲, α-galactosidase IV.

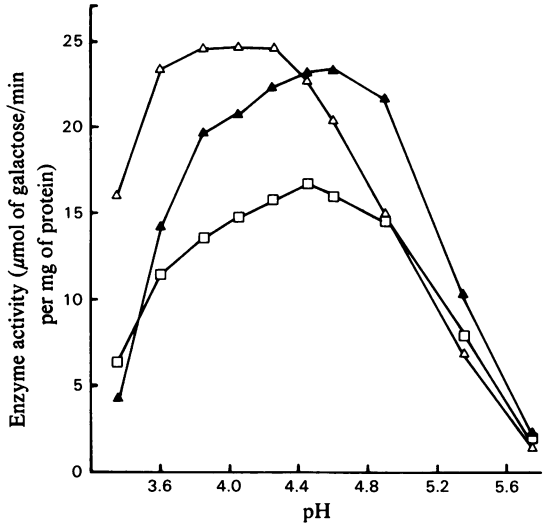

Fig. 3. *Effect of pH on the activities of α-galactosidases II, III and IV with the galactomannan of T. repens as substrate*
McIlvaine buffer with a pH range of 2.5–8 was used. The reaction mixture contained 25μl of enzyme, 50μl of substrate (1%, w/v) and 25μl of buffer. Incubation was for 20min at 37°C and the galactose liberated was estimated by a micro-dosage technique adapted from the Somogyi–Nelson method (see under 'Assay of enzyme activity'). Symbols: △, α-Galactosidase II; □, α-galactosidase III; ▲, α-galactosidase IV.

difference in stability at 37°C was notable, as compared with α-galactosidase I (Williams *et al.*, 1977); whereas after 40min of incubation enzyme II retains 85% of its initial activity, enzymes III and IV retain 22 and 53% respectively.

Molecular-weight determinations

For each of the enzymes II, III and IV, electrophoretically pure enzyme (7.5% gels) was examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. α-Galactosidases II, III and IV each gave a single protein band of mol.wt. 41000. When a mixture of α-galactosidases was examined by the same technique a single protein band of identical molecular weight (41000) was obtained; when α-galactosidase I was included with enzymes II, III and IV we obtained the same result. When examined by the method of Hendrick & Smith (1968) α-galactosidases II, III and IV showed a single protein band of mol.wt. 43000.

Inhibition or activation of hydrolytic activity

Under the conditions provided (Fig. 5), the liberation of galactose *in vitro* did not inhibit the hydrolysis, by enzymes II, III and IV, of the galactomannans examined: *T. repens*, *Medicago sativa* and *Ceratonia siliqua*.

Over the concentration range 10μm–10mM, Ca^{2+}, Mg^{2+}, K^+, Na^+ and EDTA had no apparent effect on the activities of enzymes II, III and IV. The substrate used was *o*-nitrophenyl α-D-galactoside under standard assay conditions.

Substrate specificity

The kinetics of hydrolysis of galactomannans with a range of galactose/mannose ratios were examined: *T. repens* T_0, 1:1.1; *M. sativa*, 1:1; *C. siliqua*, 1:4. The values of K_m and $V_{max.}$ were calculated as Lineweaver–Burk double-reciprocal plots and are shown in Table 2. No values of K_m or $V_{max.}$ were obtained for *T. repens* T24 owing to the limits of solubility and viscosity of this galactomannan.

The hydrolysis with time, of the various galactomannans, was also examined with α-galactosidases II (Fig. 5), III and IV. The concentrations of α-galactosidases II, III and IV used were respectively 53.3, 12.8 and 22.2μg of protein/ml. All three α-galactosidases were capable of the hydrolysis of almost all of the galactopyranose residues of the galactomannan, irrespective of the initial galactose/mannose ratios. The times of precipitation of the

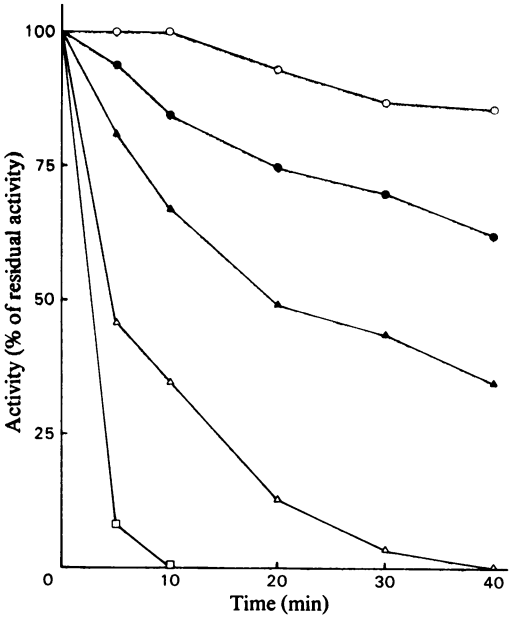

Fig. 4. *α-Galactosidase II: inactivation of hydrolytic activity as a function of temperature and time*

Suitably diluted samples of electrophoretically pure α-galactosidase II were incubated at 37°C (○), 40°C (●), 45°C (▲), 50°C (△) and 55°C (□). At the indicated times samples (25 μl) were withdrawn and residual hydrolytic activity was assayed with *o*-nitrophenyl α-D-galactoside as substrate under standard assay conditions; full details are given in the legend to Fig. 2. The ordinate represents percentage residual activity (initial specific activity was 269 μmol of galactose/min/per mg of protein).

mannans so produced varied, according to both the α-galactosidase and the galactomannan examined. With α-galactosidase II precipitation times were: *C. siliqua*, 3h; *T. repens* T_0, 4h; *M. sativa*, 5h;

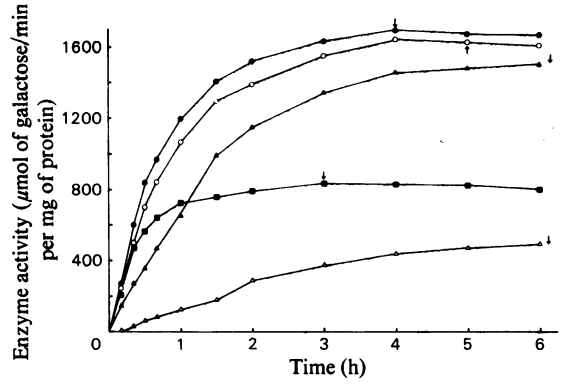

Fig. 5. *α-Galactosidase II: hydrolysis of various galactomannans as a function of time*

The incubation mixture consisted of 0.84ml of galactomannan (1%), 0.21ml of buffer, pH4.2 (McIlvaine), and 0.35ml of enzyme (53 μg/ml). At the indicated times (at 37°C) samples (100 μl) were withdrawn and the galactose liberated was measured by the Somogyi–Nelson technique (micro-method or standard assay) (see under 'Assay of enzyme activity'). Times at which precipitation of the different mannans occurred are indicated by arrows. Galactomannans: ●, *T. repens* T_0; ▲, *T. repens* T24; △, *T. repens* T36; ○, *M. sativa*; ■, *C. siliqua*. (Specific activity is 269 μmol of galactose/min per mg of protein, with 25mM-*o*-nitrophenyl α-D-galactoside as substrate.)

Table 2. *Specificity of α-galactosidases II, III and IV in McIlvaine buffer, pH4.2 or 4.4, at 37°C*

Hydrolysis of *o*-nitrophenyl α-D-galactoside and *p*-nitrophenyl α-D-galactoside was assayed by incubating 25 μl of enzyme, 50 μl of substrate and 25 μl of McIlvaine buffer (pH4.2 for enzyme II and pH4.4 for enzymes III and IV) at 37°C. After 4min the reaction was stopped by the addition of 3ml of 0.2M-Na_2CO_3. The liberated aglycone was determined at 400nm. For the other substrates the incubation medium was identical, whereas the incubation time was 15min (at 37°C). Galactose liberated was determined by a micro-dosage technique adapted from the Somogyi–Nelson method (see under 'Assay of enzyme activity'). Stock solutions of the galactomannans used as substrates were made up by percentage weight (w/v), since the initial degree of hydration and exact molecular weights are unknown. The results were plotted in Lineweaver–Burk double-reciprocal form.

Substrate	K_m (mM)			$V_{max.}$ (μmol of galactose liberated/min per mg of protein)		
	II	III	IV	II	III	IV
o-Nitrophenyl α-D-galactoside	7.0	2.7	11.1	435	196	476
p-Nitrophenyl α-D-galactoside	1.1	—	—	212	—	—
Phenyl α-D-galactoside	8.0	—	—	277	—	—
Raffinose	67	1000	120	48	100	29
Stachyose	83	—	—	8	—	—
		(g/100 ml)				
Galactomannan (*M. sativa*)	1	1	5	59	60	125
Galactomannan (*T. repens* T_0)	5	7	5	235	250	167
Galactomannan (*T. repens* T36)	1.7	—	1.7	8	—	25
Galactomannan (*C. siliqua*)	10	1.4	10	333	38	17

T. repens T24, 8h; *T. repens* T36, approx. 10h. With α-galactosidase III precipitation times of the mannans were approx. 24h for *C. siliqua*, approx. 48h for *M. sativa* and *T. repens* T_0 and approx. 60h for *T. repens* T24 and T36. With α-galactosidase IV all the mannans precipitated after approx. 24h of incubation, except *T. repens* T36, which precipitated after 36h. For α-galactosidases III and IV the longer precipitation times observed were only partly due to the lower enzyme concentrations used (see Table 2). Furthermore, the essentially similar hydrolysis profiles obtained with α-galactosidases II, III and IV differed in two respects. First, whereas for α-galactosidase II the galactomannan of *T. repens* was more rapidly hydrolysed than that of *M. sativa* (Fig. 5), for α-galactosidase III the opposite was true and for α-galactosidase IV the rates of hydrolysis were practically identical. Secondly, with α-galactosidase III the curves obtained for the hydrolysis of the galactomannans of *C. siliqua* and *T. repens* were almost identical.

The liberation of galactose by α-galactosidase II from raffinose, planteose and stachyose resembles the pattern of hydrolysis for these substrates shown by α-galactosidase I (Williams *et al.*, 1977). However, the proportions hydrolysed by α-galactosidase I are 16:2.5:1 (raffinose:planteose:stachyose), whereas for α-galactosidase II the respective proportions are 16:5.8:3.6. The rates of hydrolysis of planteose and stachyose by α-galactosidases III and IV were minimal compared with that of raffinose in each case.

Unlike α-galactosidase I (Williams *et al.*, 1977), enzymes II, III and IV did not give transfer products with either raffinose or melibiose. No transfer products could be detected by silica-gel t.l.c., when either melibiose (50mM) or *o*-nitrophenyl α-D-galactoside (10mM) were used as galactosyl donors with mannobiose, cellobiose or maltose as acceptors. Under conditions of high acceptor concentrations no significant transfer products were detected with monosaccharides or galactinol.

Discussion

The three forms of α-galactosidase, purified to homogeneity as described above, have identical molecular weights as estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (41000). α-Galactosidases II, III and IV thus appear to be distinct multiple forms of identical molecular weight, on the basis of their separation by column chromatography and their different mobilities on polyacrylamide gels (Fig. 1). α-Galactosidases II, III and IV (IUPAC–IUB Commission on Biochemical Nomenclature, 1978) would not appear to be artifacts resulting from the oxidation of a single form of the enzyme during the purification process (Payne *et al.*, 1972), since all three enzymes (and also α-galactosidase I) are present in the same proportions when purified in the presence or absence of β-mercaptoethanol. α-Galactosidase I has been previously shown to have a mol.wt. of 41000 (Williams *et al.*, 1977), which has now been confirmed to be identical with that of enzymes II, III and IV.

Unlike α-galactosidase I, no significant galactosyltransferase activity was observed for enzymes II, III and IV. No transfer products were noted when melibiose (galactosyl donor) was incubated, in the presence of enzyme, with a disaccharide such as maltose or mannobiose, at concentrations for which transfer products were observed with α-galactosidase I (Williams *et al.*, 1977). When monosaccharides were used at high concentration (galactosyl acceptor) only minimal quantities of a new core tentatively identified as a disaccharide were obtained.

α-Galactosidases II, III and IV show different kinetics of hydrolysis with the same galactomannan (Table 2) and with different galactomannans. We observe that, under identical assay conditions, the liberation of galactose with time from the galactomannans of *T. repens* and *M. sativa* differs significantly for enzymes II, III and IV. Thus for enzyme II the galactomannan of *T. repens* is more rapidly hydrolysed than that of *M. sativa*; for enzyme III the opposite is true and for enzyme IV the rates of hydrolysis are practically identical. It is also noteworthy that only for α-galactosidase III does the pattern of hydrolysis with time for the galactomannan of *C. siliqua* resemble that for *T. repens* T24.

Examination of the kinetic data (Table 2) shows that for the galactomannan of *T. repens* T_0, α-galactosidases II and IV have the same affinity (K_m, 5%), but that $V_{max.}$ is much larger for enzyme II, whereas for enzyme III both K_m (7%) and $V_{max.}$ are larger than for either enzymes II or IV. It is also noteworthy that enzyme IV has very similar K_m and $V_{max.}$ values for the galactomannans of *T. repens* and *M. sativa*, which suggests that the galactopyranose residues hydrolysed by α-galactosidase IV may be similar in both galactomannans. However, for enzymes II and III the values of K_m and $V_{max.}$ are very different for these two galactomannans. Consequently, it would seem that the specificity of hydrolysis of enzyme IV is different in this respect from that of enzymes II and III.

Despite these differences, all three enzymes are capable of the hydrolysis of virtually all of the galactopyranose residues in the galactomannan, irrespective of the initial galactose/mannose ratios. That is, hydrolysis results in the precipitation of the mannan so formed (Fig. 5), which contains only trace amounts of galactose. These results contrast with those obtained with α-galactosidase I (Williams

et al., 1977), with which, despite high affinity for the galactomannans of *T. repens* T_0 (0.04%), *C. siliqua* (0.1%) and *M. sativa* (0.27%), it was never possible to produce extensive hydrolysis or precipitation of mannan. It is also the first time in the literature that any α-galactosidase (Wallenfels & Malholtra, 1961; Petek, 1962; Petek *et al.*, 1969; Dey & Pridham, 1972; Dey & Wallenfels, 1974) has been shown to be capable of the elimination of the totality of the galactopyranose residues of a galactomannan to give mannan (J. Williams, unpublished work).

When examined under identical conditions of substrate and enzyme concentration the various galactomannans precipitate after different times of hydrolysis (Fig. 5). With enzyme II at a given enzyme concentration, precipitation times were: *C. siliqua*, 3h; *T. repens* T_0, 4h; *M. sativa*, 5h; *T. repens* T24, 8h; *T. repens* T36, approx. 10h. These differences would tend to confirm the differences in structure of the galactomannans of *T. repens* T_0 and *M. sativa* discussed above. Further evidence supporting this hypothesis has been obtained with the β-mannanases of *T. repens* (Villarroya *et al.*, 1978), which are able to hydrolyse the galactomannan of *T. repens* T_0 but not that of *M. sativa*, despite almost identical galactose/mannose ratios. For the galactomannans of *T. repens* T_0, T24 and T36, the differences in precipitation times reflect the very different patterns of hydrolysis for all three α-galactosidases. These galactomannans also have different solubilities and viscosities; that from *T. repens* T24 is less soluble (and more viscous) than that from *T. repens* T_0, whereas the viscosity of that from *T. repens* T36 decreases notably. These findings strongly indicate that the structure of the galactomannan of *T. repens* changes during germination, contrary to the observations of previous authors (Sömme, 1971; Reid & Meier, 1972; McCleary & Matheson, 1975).

Examination of the kinetic data as a whole (Table 2) poses certain difficulties, since the values of K_m for the galactomannans are expressed as percentages (w/v), compared with molar values for the other substrates. Nevertheless, comparison of the values of $V_{max.}$ for raffinose and for the galactomannan from *T. repens* T_0 shows that $V_{max.}$ is much greater for the latter. These results contrast with the results previously obtained for α-galactosidase I (Williams *et al.*, 1977), for which the value of $V_{max.}$ obtained for raffinose is six times that obtained for the galactomannan of *T. repens* T_0. It is also noteworthy that despite the relatively large value of $V_{max.}$ for enzyme III with raffinose, the value of K_m is extremely high (1 M).

The findings presented in the present paper are consistent with the hypothesis that α-galactosidases II, III and IV are primarily concerned with the breakdown of the galactomannan reserve polysaccharide in the germinating seed of *T. repens*. It seems unlikely that these α-galactosidases could be involved simultaneously in breakdown of galactomannan and oligosaccharides in the endosperm, since at the onset of germination galactomannan concentration in the endosperm greatly exceeds that of the galactosylsucrose oligosaccharides. It is possible, though difficult to envisage, owing to compartmentalization within the seed (Reid, 1971), that α-galactosidases II, III and IV could be responsible for galactomannan hydrolysis in the endosperm and oligosaccharide hydrolysis in the cotyledons.

Consistent with results obtained in other studies (Reid & Meier, 1972) we envisage that α-galactosidases II, III and V could be synthesized and secreted by the aluerone layer in the endosperm during germination, to bring about galactomannan mobilization in co-ordination with β-mannanase (Villarroya *et al.*, 1978).

This study was supported in part by a grant to J. W. from the C.N.R.S., sponsored by the University of London.

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