Purification and Properties of an α -D-Galactoside Galactohydrolase from the Seeds of *Trifolium repens* (White Clover)

By JANICE WILLIAMS, HENRI VILLARROYA and FAHRETTIN PETEK
*Laboratoire de Chimie Biologique, Equipe de Recherche Associée au CNRS no. 99,
Faculté des Sciences Pharmaceutiques et Biologiques, 4 Avenue de l'Observatoire,
75270 Paris Cedex 06, France*

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Five α -D-galactosidases (α -D-galactoside galactohydrolase; EC 3.2.1.22) have been identified by chromatography and polyacrylamide-disc-gel electrophoresis in the germinated seeds of *Trifolium repens* (white clover). α -Galactosidase I has been purified to homogeneity with an approx. 2000-fold increase in specific activity. The enzyme was purified by a procedure which included precipitation by dialysis against citrate/phosphate buffer, pH 3.5; $(\text{NH}_4)_2\text{SO}_4$ precipitation; hydroxyapatite, DEAE-cellulose and ECTEOLA-cellulose column chromatography. Each stage of purification was controlled by polyacrylamide-disc-gel electrophoresis; the purified enzyme showed a single protein band that corresponded to the α -D-galactosidic activity. The pH optimum was found to be between pH 3.8 and 4.2; the enzyme is highly thermostable. Hydrolysis of oligosaccharides and galactomannans has been examined, and it has been found that α -galactosidase I exhibits two enzymic activities, namely α -D-galactoside galactohydrolase and galactosyltransferase. By the polyacrylamide-gel-electrophoresis method of Hendrick & Smith (1968), and by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the mol.wt. has been estimated to be 43000 and 41000 respectively. These results indicate that α -galactosidase I is a monomeric protein and that both enzymic activities associated with the enzyme reside on the same polypeptide chain.

α -Galactosidases have been found to be widely distributed in Nature (Wallenfels & Malhotra, 1961; Petek, 1962) but it is only comparatively recently that they have been purified to apparent homogeneity (Petek *et al.*, 1969; Dey & Pridham, 1969; Suzuki *et al.*, 1970; Beutler & Kuhl, 1972; Dey & Wallenfels, 1974). Consequently relatively little is known about their kinetics, the situation being further complicated by the lack of readily available, naturally occurring oligosaccharides and polysaccharides as substrates. Moreover, as techniques of purification and identification have improved, the existence of more than one α -galactosidase has been shown in the coffee bean (Petek & To Dong, 1961), *Vicia faba* (broad bean) (Dey & Pridham, 1969), coconut (Balasubramaniam *et al.*, 1974) and *Medicago sativa* (alfalfa) (F. Petek, H. Villarroya & E. Bar, unpublished work). The α -galactosidase from germinated seeds of *Vicia sativa* (Petek *et al.*, 1969) has been shown to hydrolyse raffinose, planteose, stachyose and galactinol but not melibiose or galactomannans. *Lens esculenta* contains an α -galactosidase in the dormant seed which hydrolyses raffinose, melibiose to a lesser extent, but not galactomannan (Dey & Wallenfels, 1974).

α -Galactosidases have also been shown to be

capable of the transfer of a galactosyl moiety to certain acceptor sugars under given conditions (Anagnostopoulos *et al.*, 1954, 1955; Courtois & Petek, 1957; Li & Shetlar, 1964; Dey & Pridham, 1972). The α -galactosidase from *Vicia sativa* (Petek *et al.*, 1969) mentioned above, exhibits a galactosyltransferase activity with three D-aldohehexoses and also raffinose, cellobiose and sucrose. An α -galactosidase from dormant seeds of sweet almond (Dey, 1969) has been shown to give transfer products with monosaccharides and disaccharides.

Experimental and Results

Materials

The dry seeds of *Trifolium repens* (white clover; variety Treffe nain blanc mira) were obtained from Vilmorin, Paris, France. DEAE-cellulose was from Whatman Biochemicals Ltd., Maidstone, Kent, U.K., and ECTEOLA-cellulose from Bio-Rad, Richmond, CA, U.S.A. Hydroxyapatite was prepared by a modified method of Tiselius *et al.* (1956) and Levin (1962). Both DEAE-cellulose and ECTEOLA-cellulose were precycled before use and stored at 4°C in 10mM-Tris/HCl, pH 8.0.

o-Nitrophenyl α -D-galactoside, *o*-nitrophenyl α -D-glucoside, *p*-nitrophenyl α -D-galactoside and *p*-nitrophenyl α -D-mannoside were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Raffinose and galactose were recrystallized in this laboratory. Mannitol was obtained from Prolabo, Rhone-Poulenc, Paris, France. Galactinol (Petek *et al.*, 1966), methyl β -D-galactoside (Helferich, 1944) and the galactomannans of *T. repens* and *M. sativa* (Courtois *et al.*, 1958) were prepared in this laboratory. Melibiose and cellobiose were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., trehalose and sucrose from Merck, Darmstadt, Germany. Stachyose (Tanret, 1902), planteose (Herissey, 1957) and the galactomannan of *Ceratoniasiliqua* (locust bean) (Courtois *et al.*, 1958) were gifts from M. Le Dizet; mannobiose was a gift from Mme. Coulombel (Taylor & Whelan, 1962). Maltose and lactose were from the Bertrand collection of this laboratory (G. Bertrand, unpublished work). Nycoline 6000 (Carbowax) was obtained from Lyon Schmidt, Noisy-le-Sec, France. T.l.c. ready plastic sheets F 1500 silica-gel, were from Schleicher und Schüll, Dassel, Germany. Coomassie Brilliant Blue was from Sigma. Bovine albumin was from Koch-Light, Colnbrook, Bucks., U.K.; egg albumin from Calbiochem, Los Angeles, CA, U.S.A.; α -phosphorylase and pepsin from Worthington Biochemical Corp., Freehold 2, NJ, U.S.A.; trypsin from Armour Laboratories, Armour and Co., Ltd., London E.C.1, U.K.

Assay of enzyme activity

α -Galactosidase activity was assayed by incubating 50 μ l of suitably diluted enzyme with 50 μ l of 20mM-*o*-nitrophenyl α -D-galactoside and 25 μ l of buffer at 37°C for 4 min. The buffer used was that of McIlvaine (1921), which gave a final pH of 4.0 in a total assay volume of 125 μ l. The enzymic reaction was stopped by addition of 3 ml of 0.2M-sodium carbonate, and the yellow colour so developed was read spectrophotometrically at 400nm by using a Beckman DB-GT instrument with a digital display. Specific activity was expressed as μ mol of galactose liberated/min per mg of protein at 37°C. Raffinose, phenyl α -D-galactoside, planteose, stachyose and galactomannan hydrolysis were determined by the Somogyi-Nelson micro-dosage technique (Somogyi, 1945; Nelson, 1944). The assay medium for these dosages consisted of 50 μ l of suitably diluted enzyme incubated (at pH4.0 and 37°C) with 100 μ l of substrate and 50 μ l of McIlvaine buffer. The blue coloration was read spectrophotometrically at 650nm against suitably prepared blanks. Protein was determined by the Zamenhof method (Zamenhof, 1957) with bovine albumin as the protein standard.

Assay of galactosyltransferase activity

For the transgalactosylation experiments the incubation mixture contained 50 μ l of suitably diluted enzyme, 50 μ l of galactosyl donor, 50 μ l of acceptor sugar and 50 μ l of McIlvaine buffer, pH 4.0. Galactosyl donors used were *o*-nitrophenyl α -D-galactoside or melibiose, at final concentrations of 5–10mM and 10–200mM respectively in the incubation mixture. Acceptor-sugar concentrations used were 400mM for the monosaccharides and 5–50mM for the disaccharides raffinose, planteose and stachyose (see Tables 3 and 4 below). Incubation was carried out for 1 h at 37°C, and the reaction stopped by heating at 100°C for 2 min.

The 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 either 2% (v/v) sulphuric acid in acetone or by a solution of β -naphthylamine followed by treatment with 2% (v/v) sulphuric acid in acetone.

Gel electrophoresis

Electrophoresis was performed on 7.5% (w/v) polyacrylamide disc gels by the technique of Maizel (1964). Experiments were carried out at 4°C with a current of 6mA per 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 afterwards bleached 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 using the procedure of Weber & Osborn (1969).

Reference proteins used (with molecular weights in parentheses) were: α -phosphorylase (94000); serum albumin monomer (68000); egg albumin (43000); pepsin (35000); and trypsin (23000). A current of 3mA per gel (5mm diam., 110mm long) was used. Electrophoresis was performed overnight (18h) at room temperature (20°C). 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 were germinated in the dark for 48h. The crude extract was prepared at 4°C by maceration with 0.9% (w/v) NaCl solution containing 100 μ l of

Table 1. *Purification of α -galactosidase I*

The substrate was *o*-nitrophenyl α -D-galactoside. α -Galactosidase I represented 25% 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 α -galactosidase I activity and its corresponding specific activity and percentage recovery.

Fraction	Volume (ml)	Total activity (μ mol of galactose/min)	Total protein (mg)	Specific activity (corrected units/mg of protein)	Recovery (%)	Purification (fold)
(1) Crude extract	890	396.8	9235	0.043	100	
(2) After acid dialysis at pH 3.5	870	394.6	4287	0.092	99.4	2.14
(3) After 80%-satd. $-(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysis	110	370	1100	0.386	93.2	7.8
(4) Column 1 (hydroxyapatite)	410	210.3	58.63	3.58	52.9	83.5
(5) Column 2 (hydroxyapatite)	175	104.3	11.03	9.46	26.3	220
(6) Column 3 (DEAE-cellulose)	110	59.04	3.23	18.25	14.9	425
(7) Column 4 (ECTEOA-cellulose)	34	45.93	0.466	98.58	11.6	2295

mercaptoethanol/litre in a Waring commercial blender. All subsequent procedures were also carried out at 4°C and all solutions contained 100 μ l of mercaptoethanol/litre. The extract was centrifuged for 30 min at 4°C and at 13000g, and the resulting supernatant dialysed overnight against 0.2M-citrate/sodium-phosphate buffer, pH 3.5. The resulting precipitate was removed by centrifugation for 30 min at 4°C and at 13000g, and the clear supernatant precipitated by $(\text{NH}_4)_2\text{SO}_4$ saturation to 80% (w/v). The precipitate was dissolved in 80 ml of water, dialysed for 48 h against water, and the remaining undissolved precipitate removed by centrifugation. The resulting 120–130 ml of clear supernatant was immediately applied, without further concentration, to a column of hydroxyapatite (Column 1: see below and Table 1).

Column chromatography

All buffers contained 100 μ l of mercaptoethanol/litre, and elution was always by stepwise increase of salt concentration.

Column 1 (200 mm $\times$ 22 mm) contained hydroxyapatite at pH 6.0, equilibrated with 5 mM-potassium phosphate buffer. After washing with 10 mM buffer, the first fraction (Fraction 1) containing α -galactosidic activity was eluted with 50 mM buffer; this fraction, α -galactosidase I, represented approx. 25% of the total α -galactosidase activity eluted from this column (see Table 1). The remaining α -D-galactosidase activity was eluted with 100 mM buffer containing 500 mM-KCl. The column was eluted at a flow rate of 80–100 ml/h, and the average pool volume of Fraction 1 was 250 ml. α -Galactosidase I was concentrated in Carbowax overnight by placing the enzyme in a dialysis bag in contact with the Carbowax powder; no loss of enzymic activity was noted. The concentrated enzyme was subsequently dialysed against water for 3–4 h.

This enzyme was then applied to a second hydroxyapatite column (column 2; 150 mm $\times$ 22 mm) also equilibrated with 5 mM buffer, pH 6.0. After adequate washing with 10 mM buffer, α -galactosidase I was eluted with 50 mM buffer (fraction 2) and collected in 10 ml fractions with an average total pooled volume of 150–200 ml. The flow rate of the column was as for Column 1.

Active fraction 2 was concentrated in Carbowax as described above, and the dialysed enzyme (4–6 ml) applied to a column (100 mm $\times$ 15 mm) of DEAE-cellulose (Column 3) equilibrated with 10 mM-potassium phosphate buffer, pH 6.2. This column was eluted in stepwise manner with increasing concentrations of NaCl namely 0, 10, 50, 100 and 150 mM. α -Galactosidase I was eluted with buffer plus 150 mM-NaCl and collected in 5 ml fractions, giving an average pooled volume of 80–100 ml. Fraction 3 so obtained was concentrated in Carbowax, dialysed as before and the resulting 3–5 ml applied to a column (Column 4; 50 mm $\times$ 15 mm) of ECTEOA-cellulose (Cellex-E Biorad) equilibrated with 10 mM-potassium phosphate, pH 8.1. Elution was the same as for column 3, with NaCl. α -Galactosidase I was eluted with 100 mM-NaCl (fraction 4) and subsequently concentrated by using an Amicon ultrafiltration apparatus fitted with a UM2 membrane at a pressure of $\sim$ 152 kPa (1.5 atm). The enzyme so obtained showed a single protein band on polyacrylamide-disc-gel electrophoresis (Plate 1).

General properties

The pH-activity profile for pure α -galactosidase I shows a maximum at pH 3.8–4.2 (Fig. 1). The enzyme, whether in a pure or impure state, was unstable to freezing (at -20°C) and thawing and also to freeze-drying. During its purification the enzyme preparation was stable at 4°C. Glycerol was added as a protective agent to the homogeneous enzyme

Fig. 1. Effect of pH on the activity of α -galactosidase I. McIlvaine buffer with a pH range of 2.5–8 was used. The incubation mixture contained 50 μ l of suitably diluted enzyme, 100 μ l of either phenyl α -D-galactoside (50 mM) or raffinose (100 mM) used as substrate, and 50 μ l of buffer. The incubation time was 15 min at 37°C, and the galactose liberated was determined by the Somogyi–Nelson micro-dosage method (see the Experimental and Results section). $\circ$, Phenyl α -D-galactoside; $\bullet$, raffinose.

preparation to a concentration of 10% (v/v), since it was found that it allows storage for at least 2 months at 4°C. Fig. 2 shows that the enzyme was rapidly inactivated by heating.

Molecular-weight determinations

An initial value for molecular weight of approx. 40000 was indicated by column chromatography with Sephadex G-75. Subsequently the electrophoretically pure enzyme was examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; a single protein band was obtained of mol.wt. 41000. When examined by the electrophoretic method of Hendrick & Smith (1968), the enzyme again showed a single protein band of mol.wt. 43000.

Inhibitors and activators

Possible inhibition of the enzyme's hydrolytic activity by ribose, arabinose, glucose, fructose,

Fig. 2. Inactivation of hydrolytic activity as a function of temperature and time

Suitably diluted samples of electrophoretically pure α -galactosidase I (containing only 0.1 ml of mercaptoethanol/litre) were incubated at 37° ($\circ$), 45° ($\bullet$), 50° ($\blacktriangle$) and 55°C ($\triangle$). At the indicated times samples (50 μ l) were withdrawn, and residual hydrolytic activity assayed with *o*-nitrophenyl α -D-galactoside as substrate; full details of this assay are described in Table 2 and in the Experimental and Results section. The ordinate represents residual activity (μ mol of galactose liberated/min per mg of protein).

mannose, xylose and sucrose was examined under standard assay conditions with *o*-nitrophenyl α -D-galactoside. Even at a final concentration of 0.1 M, no inhibition was apparent. With 8 mM-galactose, 15% inhibition was observed when *o*-nitrophenyl α -D-galactoside was at a concentration of 4 mM in the assay medium. Further, no inhibition was observed with *myo*-inositol. Inhibition (12% and 25% respectively) of the enzyme by Fe^{2+} and Cu^{2+} occurred at concentrations of 100 μ M with 4 mM-*o*-nitrophenyl α -D-galactoside used as substrate in the incubation medium. Ca^{2+} , Mg^{2+} , K^{+} , Na^{+} and EDTA showed no apparent effect on α -galactosidase I activity over the concentration range 10 μ M–10 mM under the same assay conditions.

Substrate specificity

The liberation of galactose by α -galactosidase I from raffinose, planteose and stachyose is shown in

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis (7.5%) of α -galactosidase I fractions at successive stages of purification
(a) Fraction 1, column 1 (hydroxyapatite); (b) fraction 2, column 2 (hydroxyapatite); (c) fraction 3, column 3 (DEAE-cellulose); (d) fraction 4, column 4 (ECTEOLA-cellulose).

Fig. 3. The significant difference in rates of galactose liberation from these substrates demonstrates that not simply the chain length but also the arrangement of glucose and fructose in the molecule is significant.

In addition to the hydrolysis of raffinose to give free galactose, the enzyme (under the conditions discussed below) produced two new cores by transfer of galactose from raffinose to raffinose, although at 40mM-raffinose this transfer is slight. In striking contrast, planteose and stachyose did not react as acceptors and showed no formation of transfer products under identical conditions. Hydrolysis of galactinol was minimal. The kinetics of hydrolysis of three galactomannans with a range of galactose/mannose ratios were examined; *T. repens* (1:1.3), *M. sativa* (1:1) and *C. siliqua* (1:4). The values for K_m and V_{max} were calculated as Lineweaver-Burk plots and are shown in Table 2. As the initial extent of hydration and the exact molecular weights of the three galactomannans are unknown, stock solutions were made up on the basis of percentage weight/volume.

Galactosyltransferase activity

A summary of the experimental results and conditions is given in Tables 3 and 4. No transfer products could be detected by silica-gel t.l.c. with monosaccharides, galactinol, mannitol, methyl β -D-galactoside, planteose or stachyose. The Tables show further that the enzyme transfers galactose preferentially to the three disaccharides linked 1 $\rightarrow$ 4, namely mannobiose, cellobiose and maltose. When 10mM-*o*-nitrophenyl α -D-galactoside was used as a donor (Table 3), new transfer products were observed with either mannobiose, cellobiose or maltose as acceptor at concentrations from 5 to 50mM. Under the same

conditions, the formation of a trisaccharide by transfer to sucrose, lactose or trehalose was not observed below 50mM acceptor. We also observed (Table 4) that melibiose (Gal α 1 $\rightarrow$ 6Glc), at a concentration of 10mM, was capable of acting as a galactosyl donor; α -galactosidase I transferred to cellobiose, maltose or mannobiose at concentrations of 5mM

Fig. 3. Different rates of hydrolysis for planteose, raffinose and stachyose to demonstrate the importance of the position and number of sugar residues in these substrates

Portions (50 μ l) of suitably diluted enzyme were incubated with 100 μ l of 80mM substrate and 50 μ l of McIlvaine buffer, pH 4.0, for the indicated times. Galactose liberated was determined by using the Somogyi-Nelson micro-dosage method (see the Experimental and Results section). $\circ$, Raffinose; $\bullet$, planteose; $\blacktriangle$, stachyose.

Table 2. Specificity of α -galactosidase I of *T. repens* in McIlvaine buffer, pH 4.0, at 37°C

Hydrolysis of the substrates *o*-nitrophenyl α -D-galactoside and *p*-nitrophenyl α -D-galactoside by α -galactosidase I was assayed by incubating 50 μ l of suitably diluted enzyme, 50 μ l of substrate and 25 μ l of buffer. After 4 min the reaction was stopped by adding 3 ml of 0.2M-sodium carbonate. The liberated aglycone was determined at 400 nm. For the other substrates the incubation mixture was 50 μ l of suitably diluted enzyme, 100 μ l of substrate and 50 μ l of buffer. The incubation time was 15 min. Galactose liberated by hydrolysis was determined by the Somogyi-Nelson micro-dosage method (see the Experimental and Results section). Stock solutions of the galactomannans used as substrates were made up by percentage weight (w/v), since the initial degree of hydration and the exact molecular weights are unknown. The data was plotted in Lineweaver-Burk double-reciprocal form.

Substrate	K_m		V_{max} (μ mol of galactose liberated/min per mg of protein)
	(mol)	(g/100ml)	
<i>o</i> -Nitrophenyl α -D-galactoside	6.6×10^{-3}		181.8
<i>p</i> -Nitrophenyl α -D-galactoside	4.7×10^{-3}		161.29
Phenyl α -D-galactoside	1.6×10^{-2}		45.4
Raffinose	4.34×10^{-2}		7.69
Stachyose	6.25×10^{-2}		2.08
Galactomannan (<i>Medicago sativa</i>)		0.27	5.56
Galactomannan (<i>Ceratonia siliqua</i>)		0.10	2.22
Galactomannan (<i>Trifolium repens</i>)		0.04	1.30

Table 3. *Transgalactosylation products formed by α -galactosidase I when *o*-nitrophenyl α -D-galactoside was used as galactosyl donor*

o-Nitrophenyl α -D-galactoside was used as donor at a final concentration of 5–10 mM in the assay medium. Acceptor concentrations used were 400 mM for the monosaccharides and 5–50 mM for the disaccharides, raffinose, planteose and stachyose. The relative amounts of products shown above were determined at 50 mM acceptor. After 30, 60 and 120 min incubation at pH 4.0 and at 37°C, suitable samples were removed from the incubation mixture and chromatographed on silica-gel plates. Transfer products were identified by using marker spots of authentic samples. Other assay conditions, the procedure and the solvent system used were as described in the Experimental and Results section.

Acceptor	Relative amounts of products	Examination, by silica-gel t.l.c. after 30, 60 and 120 min of incubation
Mannobiose	+++++	Trisaccharide
Cellobiose	+++++	Tri- and tetra-saccharide
Maltose	+++++	Trisaccharide
Sucrose	+++	Trisaccharide
Lactose	++	Trisaccharide
Melibiose	++	Three new cores
Trehalose	+	Trisaccharide
Raffinose	+	Stachyose, verbascose
Planteose	—	None
Stachyose	—	None
Galactose	—	None
Galactinol	—	None
β -Methylgalactose	—	None
Mannose	—	None
Mannitol	—	None
Fructose	—	None
Glucose	—	None
Ribose	—	None
Xylose	—	None

galactosyl acceptor. After 1 h of incubation, one transfer could be detected, whereas with 10 mM-melibiose alone (as galactosyl donor and acceptor) no new cores were produced. With higher concentrations of melibiose alone and 2–3 h incubation, up to three new cores were formed (Table 4). It appears that, whereas melibiose is a ready galactosyl donor to mannobiose, cellobiose or maltose, only at 200 mM-melibiose, when alone, will it serve readily as acceptor as well as donor. However, when incubated in the presence of 10 mM-*o*-nitrophenyl α -D-galactoside (galactosyl donor) and melibiose at concentrations from 50 to 200 mM, α -galactosidase I readily catalysed galactosyl transfer to melibiose with formation of three new cores after 1 h.

Discussion

α -Galactosidases hydrolyse α 1 $\rightarrow$ 6 galactosyl linkages in both polysaccharides and in substrates of much

Table 4. *Transgalactosylation products formed by α -galactosidase I when melibiose was used as galactosyl donor*

The incubation was carried out at pH 4.0, 37°C for 1 h in experiments when mannobiose, cellobiose or maltose were used as galactosyl acceptors, and for 2–3 h when melibiose was used alone as galactosyl donor and acceptor. Values in parentheses are the concentrations (mmol/litre) of the acceptors/donors in the assay. Other assay conditions and the procedure were as described in Table 3 and in the Experimental and Results section.

Galactosyl donor	Galactosyl acceptor	Products observed by silica-gel t.l.c.
Melibiose (10)	Mannobiose (5)	Trisaccharide
(10)	Cellobiose (5)	Two new cores
(10)	Maltose (5)	Trisaccharide
Melibiose (as acceptor and donor) (10)		None
(50)	Trisaccharide	
(200)		Three new cores

lower molecular weight. α -Galactosidase I is capable of hydrolysing galactomannans of different mannose/galactose ratios (e.g. *M. sativa*, 1.0; *T. repens*, 1.1; *C. siliqua*, 4.0). However, its affinity for the galactomannan of *T. repens* is significantly higher than its affinity for the galactomannan of either *M. sativa* or *C. siliqua* (Table 2). This suggests, first, that the conformations in solution of galactomannans of *T. repens* and *M. sativa* are certainly different, despite almost identical mannose/galactose ratios, and, secondly, that an increase in the ratio mannose/galactose does not in this case result in an increased accessibility of the molecule to hydrolytic attack.

The interpretation of the kinetic data as a whole is more difficult, as suggested by the presentation of results in Table 2. Whereas the galactomannans of *M. sativa* and *C. siliqua* are soluble to the extent of 0.5% (w/v) in water, that of *T. repens* is only soluble to the extent of 0.2% (w/v), and the viscosity of the latter solution (at 0.2%, w/v) is much greater than that of *M. sativa* or *C. siliqua* at 0.5% (w/v). The K_m values for the galactomannans are expressed as percentages and not in mol/litre as for the other substrates. It is in any case difficult to compare directly these highly viscous polysaccharides in which the galactose moiety is linked singly to an unbranched chain of Man β 1 $\rightarrow$ 4Man (Courtois & Le Dizet, 1963; Horvei & Wickström, 1964; Andrews *et al.*, 1952) with the other substrates examined.

As noted above, the different rates of hydrolysis of raffinose and planteose, under the same conditions, indicate the importance of the arrangement of sugar residues in these otherwise identical trisaccharides.

When we compare the conditions of hydrolysis of raffinose and stachyose, we note that the affinity of α -galactosidase I for the tetrasaccharide is only slightly smaller than for raffinose, whereas the value of $V_{\max}$ is almost four times larger for raffinose than for stachyose. α -Galactosidase I shares with certain other plant α -galactosidases a hydrolytic activity accompanied by a galactosyltransferase activity (Anagnostopoulos *et al.*, 1954; Petek, 1962; Dey, 1969; Suzuki *et al.*, 1970). Of interest in this respect is a 'Galactinol-sucrose 6-galactosyltransferase' (Lehle & Tanner, 1973) which has been reported in a highly purified extract of ripe seeds of *Vicia faba*. The extract exhibits three features: an α -galactosyltransferase activity, catalysis of an exchange reaction between sucrose and raffinose, and a hydrolytic activity. By contrast, the α -galactosidase I of *T. repens* has not been observed to use galactinol as donor.

We have noted that α -galactosidase I is capable of galactosyl transfer from certain donor molecules (notably melibiose) to specific acceptors. The best of these galactosyl acceptors are mannobiose and cellobiose, in terms of both the quantities of new cores produced and the minimum concentrations of donor and acceptor possible (see Tables 3 and 4). Unlike previously studied α -galactosidases which have capacity as a galactosyltransferase, α -galactosidase I from *T. repens* does not catalyse galactosyl transfer to galactose, glucose or mannose. α -Galactosidase I catalyses transfer preferentially to disaccharides linked $\beta 1 \rightarrow 4$ (mannobiose, cellobiose) whereas transfer to trehalose, where the linkage is $\alpha 1 \rightarrow 1$, is minimal. However, the presence of galactose in the disaccharide lactose (galactosyl acceptor), which also has a $\beta 1 \rightarrow 4$ linkage, decreased greatly the quantity of trisaccharide produced by transfer. Raffinose and melibiose are hydrolysed by α -galactosidase I (see Table 2) but are very poor acceptors relative to the disaccharides mentioned above. Whereas previous authors have used high concentrations of acceptors: 1M (Petek, 1962), 1M (Dey, 1969), 500mM (Suzuki *et al.*, 1970), 25mM (Anagnostopoulos *et al.*, 1955), we have observed transfer products by using acceptor concentrations as low as 5mM.

The present results indicate that α -galactosidase I is a monomeric protein of mol.wt. 41 000–43 000. Since the enzyme was homogeneous by electrophoresis it seems that both enzymic activities, α -D-galactoside galactohydrolase and galactosyltransferase, reside on the same polypeptide chain. α -Galactosidase I is the first of five α -galactosidases that we have been able to distinguish in the germinated seeds of *T. repens*. The existence of several forms of α -galactosidase in the dormant and germinated seeds of *T. repens* and *M. sativa* and the results discussed above suggests that these enzymes may have a more complex metabolic role than simple hydrolases.

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References

- Anagnostopoulos, C., Courtois, J. E. & Petek, F. (1954) *Bull. Soc. Chim. Biol.* **36**, 1115–1123
Anagnostopoulos, C., Courtois, J. E. & Petek, F. (1955) *Arch. Sci. Biol.* **39**, 631–640
Andrews, P., Hough, L., & Jones, J. K. N. (1952) *J. Am. Chem. Soc.* **74**, 4029–4032
Balasubramaniam, K., Dey, P. M. & Pridham, J. B. (1974) *Biochem. Soc. Trans.* **2**, 1128–1130
Beutler, E. & Kuhl, W. (1972) *J. Biol. Chem.* **247**, 7195–7200
Courtois, J. E. & Le Dizet, P. (1963) *Bull. Soc. Chim. Biol.* **45**, 731–741
Courtois, J. E. & Petek, F. (1957) *Bull. Soc. Chim. Biol.* **39**, 715–723
Courtois, J. E., Anagnostopoulos, C. & Petek, F. (1958) *Bull. Soc. Chim. Biol.* **40**, 1277–1285
Dey, P. M. (1969) *Biochim. Biophys. Acta* **191**, 644–652
Dey, P. M. & Pridham, J. B. (1969) *Biochem. J.* **113**, 49–55
Dey, P. M. & Pridham, J. B. (1972) *Adv. Enzymol.* **36**, 91–130
Dey, P. M. & Wallenfels, K. (1974) *Eur. J. Biochem.* **50**, 107–112
Helferich, B. (1944) *Ber. Dtsch. Chem. Ges.* **77**, 194–201
Hendrick, J. L. & Smith, A. (1968) *Arch. Biochem. Biophys.* **126**, 155–164
Herissey, H. (1957) *Bull. Soc. Chim. Biol.* **39**, 1553–1555
Horvei, K. F. & Wickström, A. (1964) *Acta Chem. Scand.* **18**, 833–837
Lehle, L. & Tanner, W. (1973) *Eur. J. Biochem.* **38**, 103–110
Levin, O. (1962) *Methods Enzymol.* **5**, 27–32
Li, Y.-T. & Shetlar, M. R. (1964) *Arch. Biochem. Biophys.* **108**, 523–530
McIlvaine, T. C. (1921) *J. Biol. Chem.* **49**, 183–188
Maizel, J. V., Jr. (1964) *Ann. N. Y. Acad. Sci.* **121**, 382–390
Nelson, N. (1944) *J. Biol. Chem.* **153**, 375–380
Petek, F. (1962) Thèse de Doctorat en Pharmacie, Université de Paris
Petek, F. & To Dong (1961) *Enzymologia* **23**, 133–142
Petek, F., Villarroja, E. & Courtois, J. E. (1966) *C. R. Hebd. Séances Acad. Sci. Ser. D* **263**, 195–198
Petek, F., Villarroja, E. & Courtois, J. E. (1969) *Eur. J. Biochem.* **8**, 395–402
Somogyi, M. (1945) *J. Biol. Chem.* **160**, 61–68
Suzuki, H., Li, S.-C. & Li, Y.-T. (1970) *J. Biol. Chem.* **245**, 781–786
Tanret, C. (1902) *Bull. Soc. Chim. Fr.* **27**, 947–952
Taylor, P. M. & Whelan, W. J. (1962) *Chem. Ind. (London)* **44–45**
Tiselius, A., Hjerten, S. & Levin, O. (1956) *Arch. Biochem. Biophys.* **65**, 132–155
Wallenfels, K. & Malhotra, O. P. (1961) *Adv. Carbohydr. Chem.* **16**, 239–298
Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
Zamenhof, S. (1957) *Methods Enzymol.* **3**, 696–704