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Glycosidases induced in *Aspergillus tamarii*

Mycelial α-D-galactosidases

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Two α-D-galactosidases (α-D-galactoside galactohydrolase, EC 3.2.1.22) produced by *Aspergillus tamarii* were purified from the mycelial extract by a procedure including chromatography on hydroxyapatite, DEAE-cellulose and ECTEOLA-cellulose. Each of these enzymes showed a single protein band corresponding to the α-D-galactosidase activity when examined by polyacrylamide-gel electrophoresis. They catalysed the hydrolysis of *o*-nitrophenyl α-D-galactoside, melibiose, raffinose and stachyose, but did not attack the galactomannans. Their M_r values were respectively 265000 ± 5000 and 254000 ± 5000 by the method of Hedrick & Smith [(1968) *Arch. Biochem. Biophys.* **126**, 155–164]. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate in each case showed a single protein band, with M_r 88000 and 77500 respectively. The purified enzymes contained carbohydrate, consisting of *N*-acetylglucosamine, mannose, glucose and galactose in the estimated molar proportions of 1:9:5:8 in α-galactosidase I.

Investigations of fungal hydrolases, especially in *Aspergillus niger*, have been predominantly concerned with extracellular glycoenzymes: α- and β-D-glucosidases [(EC 3.2.1.3 and EC 3.2.1.6) (Rudick & Elbein, 1973, 1974, 1975)], α- and β-D-galactosidases [(EC 3.2.1.22 and EC 3.2.1.23) (Bahl & Agrawal, 1969; Adya & Elbein, 1977; Widmer & Leuba, 1979)], glucoamylases [(EC 3.2.1.3) (Lineback *et al.*, 1969; Pazur *et al.*, 1971)] and β-D-mannosidase [(EC 3.2.1.25) (Elbein *et al.*, 1977; Bouquelet *et al.*, 1978)]. Two intracellular glycoenzymes have been studied in this same organism: α-glucosidase (Rudick *et al.*, 1979) and acid phosphatase [(EC 3.1.3.2) (Shimada *et al.*, 1977)]. Nevertheless the relationship between intra- and extra-cellular forms and the role of their carbohydrate moieties remain to be elucidated.

Several hypotheses have been suggested for the biological function of carbohydrate moieties in glycoproteins. Eylar (1966) has suggested that they could play a role in the transport of glycoproteins into the extracellular environment. The use of tunicamycin, a specific inhibitor of protein glycosylation through the lipid-linked oligosaccharide pathway, seems to contradict this hypothesis. Thus the inhibition of glycosylation by tunicamycin has been shown to have little or no effect on secretion of ovalbumin by the oviduct (Keller & Swank, 1978), of interferon by leucocytes in culture (Mizrahi *et al.*, 1978), of transferrin by rat hepatocytes, or of apoprotein B chains of very-low-density lipoprotein synthesized by chick hepatocytes (Struck *et al.*, 1978). Nonetheless Kuo & Lampen (1974) have shown that tunicamycin can stop the synthesis of glycoproteins such as invertase (β-D-fructofuranosidase) and acid phosphatase in *Saccharomyces* strain 1016.

The resistance of glycoproteins to proteolytic attack suggests a possible protective effect of the carbohydrate moiety, as demonstrated by Loh & Gainer (1978), who showed that glycosylation of a prohormone appeared to be important for its protection against non-specific proteolysis.

The results obtained by Pazur *et al.* (1970) on *A. niger* glucoamylase I, showing its stability on storage at low temperatures, the partial loss of activity by periodate oxidation of its carbohydrate residues and the total inactivation by reduction with $NaBH_4$, also suggest that the carbohydrate stabilizes the three-dimensional structure of this enzyme.

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We undertook the comparative study of mycelial and secreted glycosidases, of glycoprotein nature, induced in *Aspergillus tamarii* in order to elucidate the role of their carbohydrate moieties. Two mycelial α-D-galactosidases, namely α-galactosidase I and α-galactosidase II, were obtained with raffinose as carbon source; replacement of raffinose by galactomannan in the culture medium led to the formation of α-D-galactosidase III and a β-D-mannanase, which were secreted into the medium. In the present paper, we describe the purification and some properties of the mycelial α-D-galactosidases induced by raffinose.

Experimental and results

Materials

O-Nitrophenyl α-D-galactoside was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Melibiose was purchased from BDH Chemicals (Poole, Dorset, U.K.). Raffinose and stachyose were recrystallized in this laboratory. The galactomannans of *Trifolium repens* (white clover), *Medicago sativa* [lucerne (alfalfa)] and *Ceratonia siliqua* (locust bean) were prepared by the procedure described by Courtois *et al.* (1958). *Cyamopsis* sp. (guar) galactomannan was prepared from guar gum (Sigma). Hydroxyapatite was prepared by a modification (Levin, 1962) of the method of Tiselius *et al.* (1956). DEAE-cellulose DE-52 was from Whatman Biochemicals (Maidstone, Kent, U.K.), and ECTEOLA-cellulose from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Both were precycled before use and stored at 4°C in 20mM-potassium phosphate buffer, pH 6.5. Concanavalin A–Ultrogel was provided by the Institut Biologique Français (Villeneuve La Garenne, France). Coomassie Brilliant Blue R-250 and G-250 were from Sigma. Bovine serum albumin was from Koch–Light Laboratories (Colnbrook, Bucks., U.K.), and egg albumin from Calbiochem (Los Angeles, CA, U.S..). Phosphorylase *a* and pepsin were from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.), and trypsin was from Armour Laboratories (London E.C.1, U.K.). Test-Combination Glucose was from Boehringer (Mannheim, Germany). Phenylmethanesulphonyl fluoride was from Feinbiochemica, Heidelberg, Germany.

Micro-organisms

Aspergillus tamarii (IP 1017-10) was provided by the Service de Mycologie de l'Institut Pasteur (Paris, France). Raulin medium used for growth contained (g/l): L(+)-tartaric acid (2.6), NH_4NO_3 (2.6), $(NH_4)_2HPO_4$ (0.4), $Mg(HCO_3)_2$ (0.27), $(NH_4)_2SO_4$ (0.17), $ZnSO_4$ (0.04), $FeSO_4$ (0.04); to which was added raffinose (5g/l). The solution (1500ml) was then distributed in Roux bottles (125ml) and autoclaved for 20min at 120°C. Each bottle was inoculated with 1ml of aqueous suspension of conidia. The fungus was cultivated at 29°C during 64h.

Assay of enzyme activity

α-D-Galactosidase activity was assayed by incubating 50μl of enzyme solution with 50μl of 20mM-*o*-nitrophenyl α-D-galactoside and 25μl of McIlvaine (1921) buffer (0.2M-Na_2HPO_4/0.1M-citric acid, pH 4.2). The reaction was stopped by addition of 3ml of 0.2M-Na_2CO_3. The coloration developed by *o*-nitrophenol liberation was read at 420nm.

The activity was expressed as μmol of galactose liberated/min per mg of protein at 37°C. Standard kinetic assay conditions for α-D-galactosidase activity were those under which initial velocity was proportional to enzyme concentration.

The glucose liberated by the hydrolysis of melibiose was determined by the glucose oxidase colorimetric method (Werner *et al.*, 1970), with the Test-Combination Glucose reagent (Boehringer).

The liberation of reducing sugars during the hydrolysis of raffinose, stachyose and various galactomannans was determined by the Somogyi–Nelson (Nelson, 1944; Somogyi, 1945) microdosage technique (Williams *et al.*, 1978).

Protein was determined by the Coomassie Brilliant Blue G-250 technique, with bovine serum albumin as the standard (Sedmak & Grossberg, 1977).

Gel electrophoresis

Electrophoresis was usually performed on 7.5% (w/v) polyacrylamide disc gels (100mm × 5mm) (Maizel, 1964), with a current of 6mA/gel. The gels were fixed and stained with Coomassie Brilliant Blue R-250 (0.2%, w/v) in methanol/acetic acid/water (5:1:4, by vol.) for 2h and afterwards destained in methanol/acetic acid/water (3:1:10, by vol.). The technique described by Kapitany & Zebrowski (1973) was used for Schiff staining of the glycoprotein band.

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis

M_r determinations were performed on 7.5%-polyacrylamide disc gels by the procedure of Weber & Osborn (1969). Reference proteins used were: phosphorylase *a* monomer (M_r 94000), bovine albumin monomer (68000), egg albumin (43000), pepsin (35000) and trypsin (23300).

Gas–liquid chromatography

The molar ratios of neutral monosaccharides and hexosamine were determined after methanolysis with methanolic 0.5M-HCl at 80°C during 20h

followed by trifluoroacetylation (Zanetta *et al.*, 1972). A Varian Aerograph type 2100 instrument fitted with a flame ionization detector was used. The glass column (300cm × 0.3cm) was packed with Chromosorb HP WAW (100–120 mesh). The N_2 flow rate was 15ml/min and column temperature increased from 110 to 200°C with a temperature gradient of 2°C/min.

Preparation of crude extract

All operations during the purification procedure were performed at 0–5°C. All steps of extraction and purification were done in the presence of 100 μM-phenylmethanesulphonyl fluoride to protect against any proteolysis.

The collected mycelia were washed with 150mM-NaCl and compressed with filter paper, then suspended in 20mM-potassium phosphate buffer, pH5.3. The suspension was macerated (in an Ultra-Turrax type 18-10 homogenizer) and then sonicated eight times for 1min each, with 3min intervals between each operation (Branson Sonifier model B-15). The extract was centrifuged at 13000*g* for 30min, the supernatant was collected, and the pellet, resuspended in the same buffer, was sonicated eight times as above. The supernatants obtained from both steps constituted the crude extract. A sample of this crude extract was submitted to 7.5%-polyacrylamide-gel electrophoresis, and was stained with Coomassie Blue or tested for galactosidase activity. At this step, two bands migrating at different positions have shown activity.

Purification

Column I. The crude extract was applied to a column (200mm × 22mm) of hydroxyapatite equilibrated with 20mM-potassium phosphate buffer, pH5.3. After successive washings with the same buffer but with increasing phosphate concentrations (50mM, 100mM and 200mM), α-galactosidase I was eluted with 300mM buffer containing 1M-KCl (F1), and α-galactosidase II with 600mM buffer containing 1M-KCl (F2). At this stage the second fraction, F2, was still contaminated with α-galactosidase I.

Column IIa. After dialysis, the first fraction (F1) was applied to a column (130mm × 22mm) of DEAE-cellulose equilibrated with 20mM-potassium phosphate buffer, pH6.5. By stepwise elution with KCl (0, 50mM, 100mM and 200mM), α-galactosidase I was eluted with 20mM-potassium phosphate buffer, pH6.5, containing 200mM-KCl (F1a).

Column IIIa. The dialysed eluate (F1a) was then applied to a second hydroxyapatite column (100mm × 22mm) equilibrated with 20mM-potassium phosphate buffer, pH6.2. After washing with successive potassium phosphate buffers (50mM, 100mM, 200mM and 300mM), α-galactosidase I was eluted with 450mM buffer containing 1M-KCl.

Column IVa. Final purification was achieved with an ECTEOLA-cellulose column (60mm × 15mm) under the same conditions as for column IIa. The enzyme was eluted with 20mM-potassium phosphate buffer, pH6.5, containing 200mM-KCl. A single protein band was observed by polyacrylamide-disc-gel electrophoresis.

A similar procedure was followed for α-galactosidase II purification.

Column IIb. The fraction containing α-galactosidase II (F2) was applied after dialysis to a DEAE-cellulose column (100mm × 22mm) equilibrated as above (column IIa). The contaminating α-galactosidase I activity was eliminated by washing with 20mM-potassium phosphate buffer, pH6.5, containing 200mM-KCl; α-galactosidase II was eluted with the same buffer containing 400mM-KCl (F2b).

Column IIIb. After dialysis, this fraction (F2b) was applied to an hydroxyapatite column (80mm × 22mm) equilibrated with 20mM-potassium phosphate buffer, pH6.2, and, after washing as described above (column IIIa), α-galactosidase II was eluted with 300mM buffer containing 1M-KCl.

Column IVb. As a final step, an ECTEOLA-cellulose column (60mm × 15mm) was equilibrated as described above (column IIa). The enzyme was eluted with 20mM-potassium phosphate buffer, pH6.5, containing 400mM-KCl. It showed a single protein band on polyacrylamide-disc-gel electrophoresis.

A flow rate of 75ml/h for hydroxyapatite and 35ml/h for ion-exchanger columns was used. In all cases, 8ml fractions were collected.

Table 1 summarizes the purification procedure beginning with 35g of mycelia, and Fig. 1 shows α-galactosidases I and II at successive stages of purification.

Determination of M_r

The electrophoretically pure enzymes were examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. A single band was obtained with each enzyme, corresponding to M_r 88000 for α-galactosidase I and M_r 77500 for α-galactosidase II. By the electrophoretic method of Hedrick & Smith (1968), M_r values of 265000 ± 5000 and 254000 ± 5000 were obtained respectively for these enzymes. The difference in M_r between the monomers of enzymes I and II could be attributed to variable carbohydrate content.

Table 1. *Purification of mycelial α-D-galactosidases I and II*

The substrate used as *o*-nitrophenyl α-D-galactoside. As α-D-galactosidase II activity from column I was still contaminated with α-galactosidase I, the Table has not been corrected in the following steps to show their corresponding specific activities.

	Volume (ml)	Total activity (μmol of galactose/min)	Total protein (mg)	Specific activity (units/mg of protein)	Recovery (%)	Purification (fold)
Crude extract	360	1254.9	105.9	11.8	100	1
Column I (hydroxyapatite)						
Fraction 1	184	455.1	9.5	47.5	36.2	4
Fraction 2	89	271.8	2.0	135.8	21.6	11.4
α-Galactosidase I						
Column IIa (DEAE-cellulose)	72	339.4	2.4	141.1	27.0	11.9
Column IIIa (hydroxyapatite)	53	281.5	0.2	1021.5	22.4	86.2
Column IVa (ECTEOLA-cellulose)	22	224.9	0.1	1597.7	17.9	135.0
α-Galactosidase II						
Column IIb (DEAE-cellulose)	43	72.0	0.2	401.8	5.7	33.9
Column IIIb (hydroxyapatite)	24	40.9	0.04	1111.7	3.2	94
Column IVb (ECTEOLA-cellulose)	10	26.3	0.02	1642.1	2.1	138.7

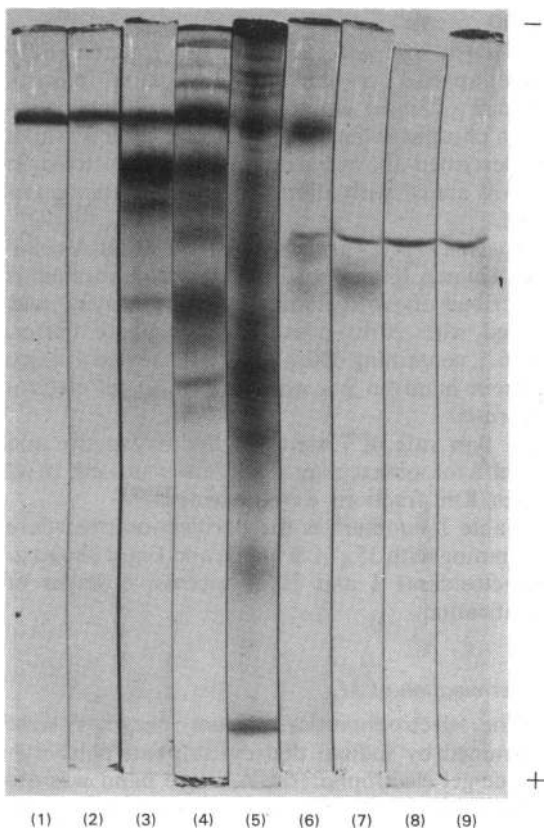

Fig. 1. *Polyacrylamide (7.5%)-gel electrophoresis of mycelial α-D-galactosidase I and II fractions at successive stages of purification*

α-Galactosidase I fractions: (1) column IVa (ECTEOLA-cellulose); (2) column IIIa (hydroxyapatite); (3) column IIa (DEAE-cellulose); (4) fraction 1, column I (hydroxyapatite); (5) crude extract. α-Galactosidase II fractions: (6) fraction 2, column I (hydroxyapatite); (7) column IIb (DEAE-cellulose); (8) column IIIb (hydroxyapatite); (9) column IVb (ECTEOLA-cellulose).

Glycoprotein nature of α-galactosidases

Both enzymes were detected by periodate/fuchsin coloration on polyacrylamide-disc-gel electrophoretograms. They were also retained by concanavalin A–Ultrogel, α-galactosidase II being retained to a lesser extent. The enzymic activities were recovered after addition of 0.2M-α-methyl glucoside to the lectin, with stirring.

The results obtained by g.l.c. gave *N*-acetylglucosamine, mannose, glucose and galactose in the molar proportions 1:9:5:8 for α-galactosidase I. These monosaccharides and hexosamine were also obtained with α-galactosidase II, but there was insufficient material to determine their molar proportions.

Optimum pH

Both enzymes showed the same pH–activity profile for the hydrolysis of *o*-nitrophenyl α-D-galactoside, with a maximum at pH 4.2–4.3 (see Fig. 2). The enzymes were stable over the pH range 3.5–6.5 after 1 h preincubation at 37°C.

Stability and conservation

α-Galactosidase I remained stable at 4°C for 1 month, but α-galactosidase II did not remain stable for 10 days. Both enzymes were unstable to freezing (at −20°C) and thawing as well as to freeze-drying, and were also rapidly inactivated by heat treatment. α-Galactosidase I was more resistant at 45 and 50°C (see Fig. 3).

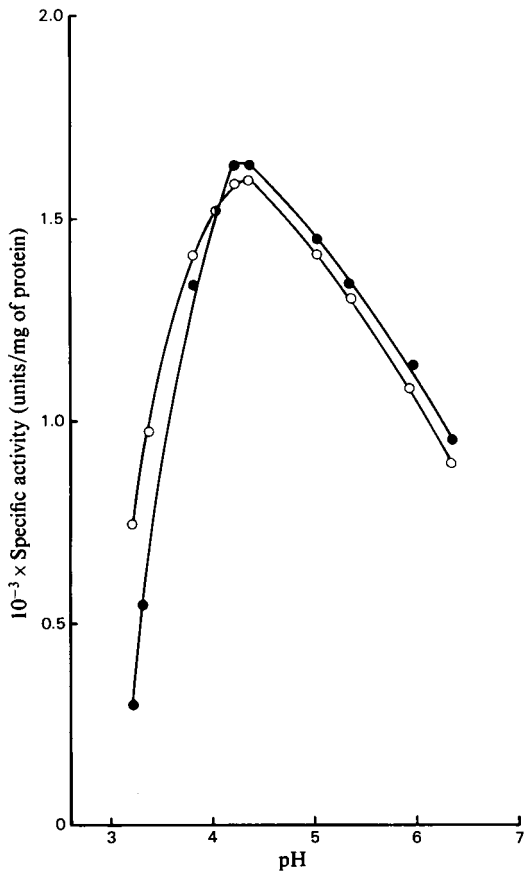

Fig. 2. *Effect of pH on the activity of α-galactosidases I and II*

McIlvaine buffer with a pH range of 3.2–6.6 was used. Assays contained 50 μl of enzyme solution, 25 μl of buffer and 50 μl of 20 mM-*o*-nitrophenyl α-D-galactoside. The incubation time was 5 min at 37°C. The ordinate represents the specific activities of both enzymes: ○, α-galactosidase I; ●, α-galactosidase II.

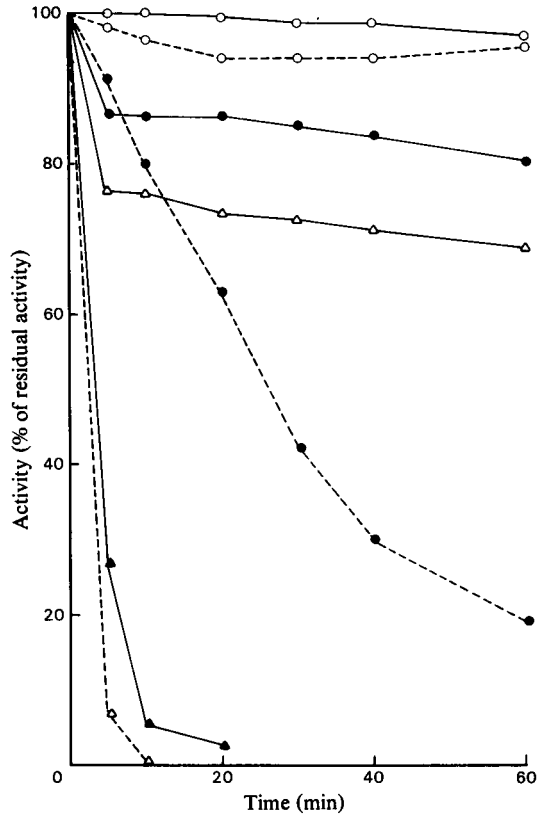

Fig. 3. *α-Galactosidases I and II: inactivation of hydrolytic activity as a function of temperature and time*

Samples of electrophoretically pure α-galactosidases I and II of approximately the same protein concentration (0.9 μg/ml) were incubated at 37°C (○), 45°C (●), 50°C (△) and 55°C (▲). At the indicated times 50 μl samples were withdrawn and residual activity was assayed with *o*-nitrophenyl α-D-galactoside as substrate under standard assay conditions. The ordinate represents percentage residual activity: ——, α-galactosidase I; – – – –, α-galactosidase II.

Effect of cations

Na^+, K^+, Ca^{2+} and Mg^{2+} showed no apparent effect on the enzyme activity over the concentration range 10 μM–10 mM. Under the same conditions, they were inactivated by Ag^+ and Cu^{2+}, α-galactosidase II being also inhibited by Zn^{2+}, Co^{2+} and Cd^{2+}. EDTA over the concentration range 10 μM–10 mM had no effect on the enzyme activity.

Substrate specificity

The effect of varying the substrate concentration on the reaction rate was studied with *o*-nitrophenyl α-D-galactoside, melibiose, raffinose and stachyose. The apparent Michaelis constant (K_m) and maximum velocity ($V_{max.}$) for each substrate were calculated by Lineweaver–Burk plots and are summarized in Table 2.

α-Galactosidase I showed a greater affinity, with a higher value of $V_{max.}$ for each substrate. The hydrolysis of *o*-nitrophenyl α-D-galactoside was inhibited at high substrate concentration (beginning at 6.6 mM for α-galactosidase I and at 2.8 mM for α-galactosidase II), whereas an activation at high substrate concentration (above 25 mM) was observed with melibiose as substrate. With raffinose, the simple Michaelis law seemed to be followed.

The uncompetitive inhibition of hydrolytic activity by glucose and mannose must be noted for each enzyme. Both enzymes were non-competitively inhibited by galactose.

Table 2. *Specificity of α-galactosidases I and II in McIlvaine buffer, pH 4.2, at 37°C*
Hydrolysis of *o*-nitrophenyl α-D-galactoside was assayed by incubating 50 μl of enzyme, 50 μl of substrate and 25 μl of McIlvaine buffer, pH 4.2, at 37°C. After 5 min the reaction was stopped by the addition of 3 ml of 0.2 M-Na_2CO_3. The liberated enzyme was determined at 420 nm. For melibiose, the incubation medium was identical, but the incubation time was 10 min at 37°C. Glucose liberated was determined by the glucose oxidase technique (see under 'Assay of enzyme activity' in the text). For other substrates the incubation medium consisted of 100 μl of enzyme, 100 μl of substrate and 50 μl of McIlvaine buffer, pH 4.2. The incubation time was 10 min at 37°C. Galactose liberated was determined by a micro-dosage technique adapted from the Somogyi–Nelson method (see under 'Assay of enzyme activity' in the text). The results were plotted in Lineweaver–Burk double-reciprocal form.

	K_m (mM)		$V_{max.}$ (μmol of galactose liberated/min per mg of protein)	
	I	II	I	II
o-Nitrophenyl α-D-galactoside	2.3	3.8	2272.7	1818.2
Melibiose	3.7	3.8	312.6	142.8
Raffinose	27.7	71.4	667.0	555.5
Stachyose	35.5	72.0	392.1	227.3

Neither enzyme showed any activity towards the galactomannans of *C. siliqua*, *T. repens* or *M. sativa*.

Discussion

We have demonstrated that, in the presence of raffinose, two α-D-galactosidases are produced by *A. tamarii*. The lack of any α-D-galactosidase activity when a glycerol-containing medium was used showed that these enzymes are inducible. A constitutive β-D-fructosidase activity was always present in the growth medium and in the cells, with an increased activity in raffinose-containing cultures. This suggests that melibiose, which is produced by the action of extracellular β-D-fructosidase on raffinose, could also play an important role in the induction mechanism. The high and very similar affinities of the two α-D-galactosidases I and II for melibiose are in general agreement with this assumption.

Both enzymes show similar pH–activity profiles, the same type of inhibition by glucose and mannose and non-competitive inhibition by galactose. Furthermore, both were inactive on various galactomannans. These enzymes could, however, be differentiated by their elution profiles on hydroxyapatite and ion-exchange columns, α-galactosidase II also showing a greater mobility than α-galactosidase I on polyacrylamide-gel electrophoresis.

They are both large-M_r enzymes. This feature is highly similar to that of an intracellular acid phosphatase isolated from *A. niger* by Shimada *et al.* (1977), which has been shown to be a glycoprotein of M_r 310000 with subunit of M_r 89000. However, the carbohydrate moiety of this enzyme, consisting of *N*-acetylglucosamine, mannose and galactose in the molar proportions 1:8:1.8, is different from that of α-galactosidase I, where the *N*-acetylglucosamine/mannose/glucose/galactose proportions are estimated to be 1:9:5:8. In this respect, and with respect to M_r, the intracellular α-glucosidase of *A. niger* (Rudick *et al.*, 1979), a monomeric glycoprotein (M_r 95000) containing glucosamine, mannose and glucose, is also quite different from the α-galactosidase I. But the glycoprotein nature of the α-galactosidases I and II of *A. tamarii* shows that glycosylation does not necessarily lead to secretion.

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