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Glycosidases of Ehrlich Ascites Tumor Cells and Ascitic Fluid—Purification and Substrate Specificity of α-*N*-Acetylgalactosaminidase and α-Galactosidase: Comparison with Coffee Bean α-Galactosidase[1]

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Ehrlich ascites tumor cells and ascitic fluid were assayed for glycosidase activity. α-Galactosidase and β-galactosidase, α- and β-mannosidase, α-*N*-acetylgalactosaminidase, and β-*N*-acetylglucosaminidase activities were detected using *p*-nitrophenyl glycosides as substrates. α-Galactosidase and α-*N*-acetylgalactosaminidase were isolated from Ehrlich ascites tumor cells on ε-aminocaproylgalactosylamine–Sepharose. α-Galactosidase was purified 160,000-fold and was free of other glycosidase activities. α-*N*-Acetylgalactosaminidase was also purified 160,000-fold but exhibited a weak α-galactosidase activity which appears to be inherent in this enzyme. Substrate specificity of the α-galactosidase was investigated with 12 substrates and compared with that of the corresponding coffee bean enzyme. The pH optimum of the Ehrlich cell α-galactosidase centered near 4.5, irrespective of substrate, whereas the pH optimum of the coffee bean enzyme for PNP-α-Gal was 6.0, which is 1.5 pH units higher than that for other substrates of the coffee bean enzyme. The reverse was found for α-*N*-acetylgalactosaminidase: the pH optimum for the hydrolysis of PNP-α-GalNAc was 3.6, lower than the pH 4.5 required for the hydrolysis of GalNAcα1,3Gal. Coffee bean α-galactosidase showed a relatively broad substrate specificity, suggesting that it is suited for cleaving many kinds of terminal α-galactosyl linkages. On the other hand, the substrate specificity of Ehrlich α-galactosidase appears to be quite narrow. This enzyme was highly active toward the terminal α-galactosyl linkages of Ehrlich glycoproteins and laminin, both of which possess Galα1, 3Galβ1,4GlcNAcβ-trisaccharide sequences. The α-*N*-acetylgalactosaminidase was found to be active toward the blood group type A disaccharide, and trisaccharide, and glycoproteins with type A-active carbohydrate chains.

Glycoconjugates with α-galactosyl termini have been found on the surface of many tumor cells (1–6). In Ehrlich ascites tumor cells (7), the presence of a high density of α-galactosyl end groups was reported and a family of glycoproteins with this terminal sugar group was isolated on a *Griffonia simplicifolia* I–Sepharose affinity column, which binds terminal linked α-galactosyl groups (8). Recently, an α-(1,3)-galactosyltransferase was also isolated from Ehrlich ascites tumor cells (9) and its substrate specificity was extensively investigated (10). It was suggested that most carbohydrate chains with α-galactopyranosyl end groups present in Ehrlich cells have the structure Galα1,3Galβ1,4G1NAcβ–. However, the accumulation of such carbohydrate chains may depend on a balance between biosynthesis and catabolism. This, in turn, depends on factors including the localization of the degradative enzymes and the substrate glycoproteins, and the specificity of the glycosidases, (e.g., α-galactosidase) toward the carbohydrate chains.

In this study, we surveyed Ehrlich cells for the presence of glycosidases and purified an α-galactosidase from these cells and compared its substrate specificity with that of coffee bean α-galactosidase. The substrate specificity of an α-*N*-acetylgalactosaminidase, isolated together with the α-galactosidase by affinity chromatography on ε-aminocaproylgalactosylamine–Sepharose, was also examined.

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MATERIALS AND METHODS

Bovine serum albumin (BSA),[4] was a product of Miles Laboratories (Naperville, IL). Coffee bean α-galactosidase (EC 3.2.1.22) and galactose dehydrogenase (EC 1.1.1.48) were purchased from Boehringer-Mannheim (Indianapolis, IN). Coomassie protein assay reagent was obtained from Pierce (Rockford, IL).

Saccharides and glycoproteins. Methyl α-D-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide, and raffinose were obtained from the Sigma Chemical Co. (St. Louis, MO). Galactose, melibiose, and *N*-acetyl-D-galactosamine were purchased from Pfanstiehl Laboratories (Waukegan, IL). *p*-Nitrophenyl-α-D-galactopyranoside, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-α-D-mannopyranoside, *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitrophenyl-*N*-acetyl-α-D-galactosaminide, *p*-nitrophenyl-*N*-acetyl-α-D-glucosaminide, and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide were from Koch-Light Laboratory, Colnbrook, England. Ethyl 4-*O*-α-D-galactopyranosyl-α-D-galactopyranoside was obtained from Pierce, and stachyose from Aldrich Chemicals (Milwaukee, WI). Galα1,3Gal, Galα1,6Gal, GalNAcα1,3Gal, and GalNAcα1,6Gal were synthesized by Nike R. Plessas of this laboratory. Galα1,3Galβ1,4GlcNAcβ-*O*-$(CH_2)_8CO_2CH_3$ was a gift of Dr. Murray Ratcliffe, Chembiomed, Edmonton, Ontario. Bovine submaxillary mucin and hog gastric mucin from Pierce, were desialylated in 0.1 N H_2SO_4 at 80°C for 1 h and the desialylated glycoproteins were lyophilized after dialysis against water. Ehrlich cell surface glycoproteins and laminin were purified on a *Griffonia simplicifolia* I–Sepharose column by the methods of Eckhardt and Goldstein (7) and Shibata *et al.* (11). Galactomannan from *Cassia alata* was prepared in this laboratory.

Protein concentration. Proteins were estimated using the Coomassie protein assay reagent (Pierce) using BSA as standard.

Preparation of Ehrlich ascites fluid and cells. Ehrlich ascites tumor cells were passaged by interperitoneal injection of female Swiss CD-1 white mice and harvested 7–10 days after transfer. The ascites cells and fluid were filtered through cheesecloth into a threefold volume of ice-cold saline (0.15 M). The solution was centrifuged at 900 rpm, and separated into supernatant and cell fractions. The supernatant was used as an ascites fluid fraction for the measurements of glycosidase activities and protein. The cells were washed repeatedly in the above saline solution followed by two washes with a homogenizing buffer (15 mM Tris–HCl, pH 7.5, 15 mM NaCl, and 1 mM $MgCl_2$) and centrifugation at 2000*g* for 3 min. Ehrlich cells were suspended in the homogenizing buffer and kept for 20 min to swell the cells. The swollen cells were homogenized in a glass Dounce apparatus fitted with a tight pestle. The homogenate was used as an ascites cell fraction for the measurements of glycosidase activities and protein.

Measurements of glycosidase activities. Enzyme assays were carried out at 37°C in a reaction mixture of 0.4 ml containing 50 mM citrate buffer and 0.1 mg/ml BSA, when *p*-nitrophenyl glycosides were used as substrate. The reaction was initiated by addition of enzyme solution to the otherwise complete reaction mixture and terminated by addition of 1 ml of 0.5 M sodium carbonate solution. The extent of hydrolysis was determined by measuring liberated *p*-nitrophenol at 400 nm. When the homogenate from Ehrlich ascites cells was used, the reaction was terminated by the addition of 0.2 ml of 10% trichloroacetic acid. The solution was centrifuged for removal of precipitated protein and an aliquot of the supernatant solution was added to 1 ml of 0.5 M sodium carbonate solution.

The hydrolysis of oligosaccharide substrates with α-galactosidase was measured by using galactose dehydrogenase (12). The enzyme reaction carried out in a reaction mixture of 0.2 or 0.4 ml containing 50 mM citrate buffer and 0.1 mg/ml BSA was terminated by addition of 0.2 ml of 1 N HCl followed by the addition of 0.2 ml of 1 N NaOH and 0.6 or 0.8 ml of 1 M Tris–HCl buffer, pH 8.6. After the addition of galactose dehydrogenase (4 μl, 5 mg/ml) and NAD (20 μl of 6 mg/ml solution) the solution was kept at room temperature for 1 h, and the fluorescence at 465 nm was measured by excitation at 340 nm.

α-*N*-Acetylgalactosaminidase activity toward oligosaccharides and glycoproteins, except for *p*-nitrophenyl *N*-acetyl-α-D-galactosaminide and GalNAcα1,3Gal, was measured by the Morgan–Elson reaction (13). The enzyme reaction was terminated by addition of 0.3 ml of 0.8 M borate buffer, pH 9.1, and the free *N*-acetyl-D-galactosamine was determined. When GalNAcα1,3Gal was used as substrate, liberated galactose was determined by the galactose dehydrogenase procedure, as described above.

Most glycosidase reactions for evaluating initial velocity were terminated when the reaction products formed were less than 10% of the substrate concentrations. However, when laminin or Ehrlich cell glycoprotein was hydrolyzed by Ehrlich cell or coffee bean α-galactosidase, the initial hydrolysis rates were obtained when product formation reached between 15 and 25% of the substrates, because these substrates were employed at very low concentrations (0.0095–0.019 mM).

Estimation of the terminal sugar in glycoproteins and galactomannans. The amount of terminal galactosyl residues in Ehrlich cell surface glycoproteins, laminin and galactomannan, was determined by galactose dehydrogenase as described above after exhaustive hydrolysis of the glycoproteins by coffee bean α-galactosidase.

The amount of galactosamine residues in asialo-bovine submaxillary mucin and asialo-hog gastric mucin was determined by the Elson–Morgan reaction (14) after hydrolysis of these glycoproteins with 6 N HCl at 100°C for 3 h. The values were used as the tentative amount of terminal *N*-acetyl-D-galactosamine in these proteins.

Purification of α-galactosidase. The homogenate prepared as described above was made 70% saturated with respect to $(NH_4)_2SO_4$ and the precipitate was collected by centrifugation. The α-galactosidase activity in the precipitate was extracted three times with cold 50 mM citrate buffer, pH 4.5. After removing the sediment by centrifugation, the extract was added directly to an affinity column of ε-aminocaproyl-galactosylamine–Sepharose (0.5 × 7 cm), previously equilibrated with 50 mM citrate buffer, pH 4.5. The column was washed with the same buffer, until A_{280} was reduced below 0.005. Subsequently, the column was washed with 0.5 M NaCl containing 50 mM citrate buffer, pH 4.5, followed by 50 mM *N*-acetyl-D-galactosamine in 50 mM citrate buffer, pH 4.5. α-Galactosidase activity was eluted with 200 mM galactose in citrate buffer, pH 4.5. The enzyme fractions were combined, BSA was added to a concentration of 0.5 mg/ml, and the solution was dialyzed against 50 mM citrate buffer, pH 4.5, and stored at −20°C.

Purification of α-N-acetylgalactosaminidase. The α-*N*-acetylgalactosaminidase activity, present in the ammonium sulfate precipitate of the homogenate, was extracted twice with cold 50 mM citrate buffer, pH 4.0. Protein in the extract was precipitated with $(NH_4)_2SO_4$ and the precipitate was dialyzed against 50 mM citrate buffer, pH 4.5. After centrifuging the sediment, the dialyzate was added to the ε-aminocaproylgalactosylamine–Sepharose affinity column (0.5 × 7 cm), previously equilibrated with 50 mM citrate buffer, pH 4.5. The column was washed as described for α-galactosidase. α-*N*-Acetylgalactosaminidase together with a weak α-galactosidase activity was eluted by 50 mM *N*-acetyl-D-galactosamine. Most of the α-galactosidase activity was eluted with 200 mM galactose. Fractions with α-*N*-acetylgalactosaminidase activity were combined and BSA was added to a concentration of 0.5 mg/ml. After dialysis against 50 mM citrate buffer, pH 4.5, the α-*N*-acetylgalactosaminidase was stored at −20°C.

RESULTS

Glycosidase activities in Ehrlich ascites fluid and cells. Table I presents a survey of glycosidase activities

[4] Abbreviations used: BSA, bovine serum albumin; PNP, *p*-nitrophenyl.

TABLE I

Glycosidase Activities in Ehrlich Ascites Fluid and Cells

Substrate	Activity (μmol/h) Fluid	Cell	pH
PNP-α-Man	4.3	1.5	4.2
PNP-β-Man	0.75	0.69	4.4
PNP-α-Gal	0.83	3.8	4.5
PNP-β-Gal	3.5	2.6	3.8
PNP-α-GalNAc	0.77	2.6	3.6
PNP-β-GlcNAc	98.5	198	4.0

Note. Each substrate at 5 mM was hydrolyzed at the indicated pH, which was optimum for the hydrolysis of each substrate by the cell extract. Total activity was expressed per 10 ml ascitic fluid. Protein present in the fluid and cells was 145 and 84 mg/10 ml ascitic fluid, respectively.

in Ehrlich ascites fluid and cells. α-Mannosidase, β-galactosidase, β-*N*-acetylglucosaminidase, and α-galactosidase activities might be expected to be involved in the processing of carbohydrate chains with terminal α-galactosyl units in Ehrlich ascites. Each activity in the fluid was found to be similar to the activity in the cells. Among these activities, β-*N*-acetylglucosaminidase was highest in both the fluid and the cells.

Purification of α-galactosidase and α-N-acetylgalactosaminidase from Ehrlich cells. Figure 1 shows the purification by affinity chromatography of α-galactosidase and α-*N*-acetylgalactosaminidase on ε-aminocaproylgalactosylamine–Sepharose. By eluting stepwise with 50 mM *N*-acetyl-D-galactosamine and 200 mM galactose, it was possible to separate α-galactosidase from α-*N*-acetylgalactosaminidase activity (Fig. 1a). On the other hand, when α-galactosidase was extracted from an ammonium sulfate precipitate of the homogenate and the extract was added directly to the affinity column (Fig. 1b), only α-galactosidase was recovered by elution from the affinity column.

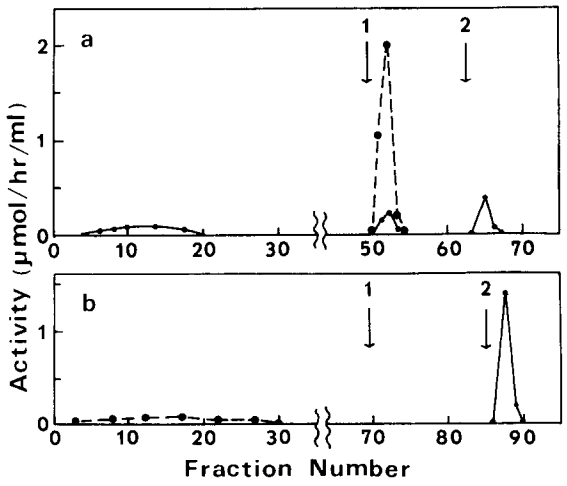

FIG. 1. Affinity chromatography of α-*N*-acetylgalactosaminidase and α-galactosidase. (a) Sample solution after dialysis was applied onto an ε-aminocaproylgalactosylamine–Sepharose column (0.5 × 7 cm). (b) Sample solution after extraction with 50 mM citrate buffer was applied on the column. α-Galactosidase activity was assayed with 10 mM PNP-α-Gal in 50 mM citrate buffer, pH 4.5, in the presence of 0.1 mg/ml BSA at 37°C, solid line; and α-*N*-acetylgalactosaminidase was assayed with 5 mM PNP-α-GalNAc in 50 mM citrate buffer, pH 3.6, in the presence of 0.1 mg/ml BSA at 37°C, broken line. Fraction volume was 200 drops before elution with 50 mM *N*-acetyl-D-galactosamine and 80 drops after elution with *N*-acetyl-D-galactosamine. The vertical arrow 1, indicates elution with 50 mM *N*-acetyl-D-galactosamine; arrow 2, with 200 mM D-galactose.

TABLE II

Purification of α-Galactosidase and α-*N*-Acetylgalactosaminidase

Step	Protein (mg)	Total activity (μmol/h)	Specific activity (μmol/h/mg)
		α-Galactosidase	
Homogenate	3200	23.0	0.007
Extract at pH 4.5	151	20.0	0.133
Affinity chromatography (Fig. 1b)	0.0036	4.22	1160
		α-*N*-Acetylgalactosaminidase	
Homogenate	1430	39.5	0.028
After dialysis	111	15.6	0.14
Affinity chromatography (Fig. 1a)	0.0016	7.3	4600

Note. α-Galactosidase activity was assayed at 37°C with 10 mM PNP-α-Gal at pH 4.5 and α-*N*-acetylgalactosaminidase was assayed with 5 mM PNP-α-GalNAc at pH 3.6.

Table II presents the purification scheme for both enzymes. α-Galactosidase (Fig. 1b) was purified over 160,000-fold with a yield of 18%, and α-*N*-acetylgalactosaminidase (Fig. 1a) also 160,000-fold with a yield of 18%. The α-*N*-acetylgalactosaminidase preparation displayed a weak α-galactosidase activity whereas all other glycosidase activities were absent from both preparations. Although highly purified, neither the α-galactosidase nor the α-*N*-acetylgalactosaminidase were homogeneous on polyacrylamide gel electrophoresis at pH 4.3.

Comparison of pH-optima of Ehrlich cell α-galactosidase and coffee bean α-galactosidase. Figure 2 shows the pH-activity profile for the Ehrlich tumor cell and coffee bean α-galactosidases using 5 mM stachyose and 1 mM PNP-α-galactopyranoside as substrates. For both enzymes, the optimum pH for the hydrolysis of stachyose was lower and broader than for PNP-α-galactopyranoside. The difference was especially large for the coffee bean enzyme. Previously, Courtois *et al.* (15) reported 3.6–4.2 as the optimum pH of this enzyme using oligosaccharides as substrates, whereas Petek and Dong (16) determined 5.3 and 6.0 for the pH optima of the isozymes, using phenyl α-galactopyranoside as substrate. It

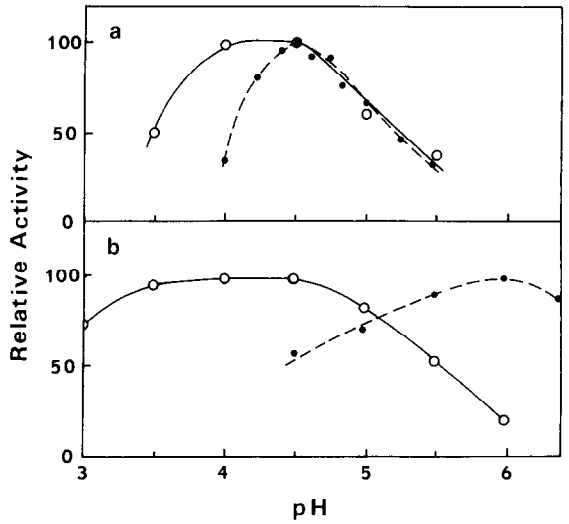

FIG. 2. pH-Activity profiles of two α-galactosidases. (a) Ehrlich α-galactosidase, and (b) coffee bean α-galactosidase. Solid line with open circles, 5 mM stachyose as substrate; broken line with closed circles, 1 mM PNP-α-Gal as substrate. Enzyme assays were carried out in 50 mM citrate buffer at 37°C, in the presence of 0.1 mg/ml BSA for the Ehrlich cell enzyme and, in the absence of BSA, for coffee bean enzyme. The Ehrlich cell enzyme purified by affinity chromatography was used for these experiments.

is apparent that these differences are due to the nature of the substrates employed.

When we compared the hydrolysis rates of seven substrates (six oligosaccharides and laminin) for coffee bean α-galactosidase at pH 4.5 and 6.0, the hydrolysis rates for all the substrates were greater at pH 4.5 than at pH 6.0. The lower pH is recommended when assaying for the coffee bean enzyme. We also determined hydrolysis rates of the Ehrlich cell α-galactosidase for oligosaccharides and glycoproteins at pH 4.5.

In the case of α-*N*-acetylgalactosaminidase, the optimum pH for the hydrolysis of PNP α-*N*-acetylgalactosaminide was lower than that for GalNAcα1,3Gal (Fig. 3). This enzyme preparation also exhibited a weak α-galactosidase activity. The optimum pH for the hydrolysis of PNP α-galactopyranoside was 4.5, which was comparable to that for GalNAcα1,3Gal. This pH-activity profile for the α-galactosidase activity present in the *N*-acetylgalactosaminidase preparation was similar to that for α-galactosidase. It appears that a single enzyme is responsible for both α-galactosidase activity and α-*N*-acetylgalactosaminidase activity, and that this enzyme is distinct from the α-galactosidase whose purification from ascites cells was described in the preceding section. The evidence for the dual activity of this enzyme is presented below.

Substrate specificity of Ehrlich cell and coffee bean α-galactosidases. Kinetic parameters for Ehrlich cell and coffee bean α-galactosidase toward 12 substrates were determined by Lineweaver–Burk plots, and are tabulated in Table III. Figure 4 shows a typical example of such a plot for the hydrolysis of melibiose by Ehrlich cell α-galactosidase. The K_m values for the Ehrlich cell enzyme were very high in comparison with those for the coffee bean enzyme. With increase in molecular size, proceeding from melibiose to stachyose, the change in the values of the parameters was different for the two enzymes.

The V_{max} of the coffee bean α-galactosidase for the hydrolysis of raffinose was approximately 3 times those for melibiose and stachyose, and the K_m gradually increased with the molecular size of the substrate, whereas for the Ehrlich enzyme, the change in V_{max} was not significant, and the K_m values for raffinose and stachyose were about 10 times that for melibiose. As a result, raffinose is a good substrate for the coffee bean enzyme, but a poor one for the Ehrlich enzyme. Under the condition K_m S, $v = V_{max}/K_m \cdot S$. The comparison V_{max}/K_m has the same meaning as the comparison with the specificity constant k_{cat}/K_m. The better substrate has the higher V_{max}/K_m. Some substrate concentrations might not be very low to satisfy the condition K_m S. However, the value for the ratio of velocity/substrate concentration is always lower than the true V_{max}/K_m. The estimated V_{max}/K_m ratios for laminin and the cell surface glycoprotein from the Ehrlich cells were high for both enzymes, showing these to be good substrates. The trisaccharide sequence Galα1,3Galβ1,4GlcNAc which occurs on both the Ehrlich cell surface and laminin was the best substrate for Ehrlich α-galactosidase.

Substrate specificity of α-N-acetylgalactosaminidase. The substrate specificity of α-*N*-acetylgalactosaminidase is shown in Table IV. The enzyme was active toward aryl-glycosides, the blood group type A disaccharide and trisaccharide and glycoproteins with A-active carbohydrate chains. The K_m for the trisaccharide (GalNAcα1,3[α1,2Fuc]Gal) was lower than that for the

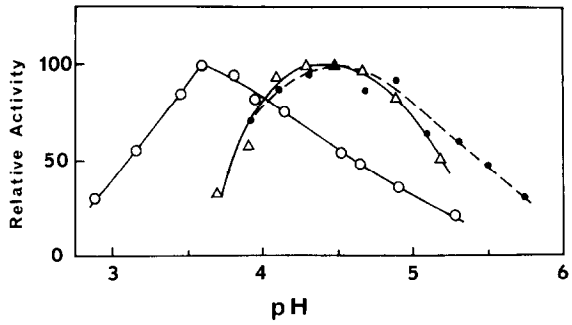

FIG. 3. pH-Activity profile of α-*N*-acetylgalactosaminidase. Enzyme assays were carried out in 50 mM citrate buffer at 37°C in the presence of 0.1 mg/ml BSA. Solid line with open circles, 5 mM PNP-α-GalNAc; solid line with triangles, 5 mM PNP-α-Gal; broken line with closed circles, 2 mM GalNAcα1,3Gal. α-*N*-Acetylgalactosaminidase, purified by affinity column chromatography, was used for these experiments.

TABLE III

Substrate Specificity of α-Galactosidase from Ehrlich Cells and Coffee Beans

Substrate	Ehrlich cell α-galactosidase			Coffee bean α-galactosidase		
	V_{max} (μmol/min/mg)	K_m (mM)	V_{max}/K_m	V_{max} (μmol/min/mg)	K_m (mM)	V_{max}/K_m
PNP-α-Gal	25.8	3.4	7.59	28.4	0.12	236.4
Me-α-Gal	0.62	16.4	0.037	3.2	4.3	0.75
Melibiose	0.88	3.1	0.28	5.7	0.86	6.7
Raffinose	0.70	29	0.023	17.3	1.66	10.4
Stachyose	1.26	35	0.035	4.8	4.3	1.1
Galα1,3Gal	0.88	10.0	0.088	0.83	0.052	15.9
Galα1,4Gal-α-Et	0.48	5.5	0.086	2.7	1.67	1.61
Galα1,6Gal			0.055[a]	0.80	0.13	5.0
Galactomannan[b]			0.008[a]	1.22	5.1	0.24
Ehrlich cell glycoprotein[b]			0.73[a]			3.8[a]
Laminin[b]			1.58[a]			10.4[a]
Galα1,3Galβ1,4GlcNAcβ-O-$(CH_2)_8CO_2CH_3$	1.04	0.54	1.92	1.92	0.052	36.9

Note. The hydrolysis rates of all substrates for both enzymes were measured at pH 4.5, 37°C.

[a] Estimated values obtained from velocity/substrate concentration at 0.1, 0.34, 0.019, and 0.0095 mM for Galα1,6Gal, galactomannan, Ehrlich cell glycoprotein, and laminin, respectively.

[b] Substrate concentration was represented as galactose concentration released after complete hydrolysis by coffee bean α-galactosidase.

blood group type A disaccharide (Galα1,3GalNAc). However, the estimated V_{max}/K_m ratios for glycoproteins were not as high as found in the case of the α-galactosidase (Table III). Even if the substrate concentrations, 0.48 mM and 0.83 mM were five times K_m for both glycoproteins, the true V_{max}/K_m ratios were calculated to be of the same order with the V_{max}/K_m ratios of other oligosaccharides.

The enzyme showed a weak α-galactosidase activity but its K_m was much higher than that of the Ehrlich α-galactosidase (Table III). Therefore we examined by a mixed substrate experiment (17) whether both substrates were hydrolyzed at a single catalytic site. If two substrates are hydrolyzed at a single catalytic site, the hydrolysis rate, v, can be given as $v = (V_a \cdot A + V_b \cdot B)/(1 + A + B)$, where A and B are the substrate concentration/K_m for the substrates a and b, respectively and V_a and V_b are the velocity maxima for a and b. The K_m and V_{max} values for each substrate were determined separately. When two substrates are hydrolyzed at two independent sites of one enzyme or by two enzymes, the rate of hydrolysis is the sum of the two

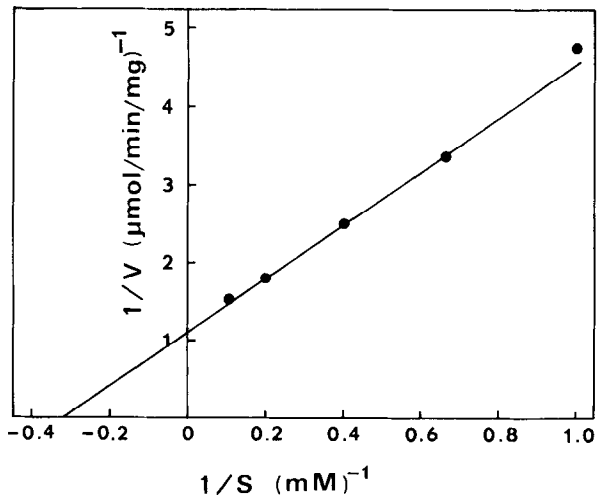

FIG. 4. A Lineweaver–Burk plot for the hydrolysis of melibiose by Ehrlich cell α-galactosidase.

TABLE IV

Substrate Specificity of α-*N*-Acetylgalactosaminidase

Substrate	V_{max} (μmol/min/mg)	K_m (mM)	V_{max}/K_m
PNP-α-GalNAc (pH 3.6)	173	5.5	29
PNP-α-GalNAc (pH 4.5)	105	4.8	22
PNP-α-Gal	13.0	14.1	0.92
GalNAcα1,3Gal	8.3	5.5	1.5
GalNAcα1,3(Fucα1,2)Gal	4.2	2.0	2.1
GalNAcα1,6Gal	2.8	2.4	1.1
AsialoBSM[a]			0.27[b]
AsialoHGM[a]			0.056[b]

Note. The hydrolysis rates were measured at pH 4.5, unless otherwise noted.

[a] BSM, bovine submaxillary mucin; HGM, hog gastric mucin.

[b] Estimated values obtained from velocity/substrate concentrations at 0.48 and 0.83 mM for asialoBSM and asialoHGM, respectively. Substrate concentrations were represented by galactosamine concentrations after acid hydrolysis, assuming that all galactosamine was released from the nonreducing terminal α-*N*-acetylgalactosamine.

TABLE V

Mixed Substrate Experiment for α-Galactosidase and α-*N*-Acetylgalactosaminidase Activities in α-*N*-Acetylgalactosaminidase Preparations

Substrate	*p*-Nitrophenol released (μmol/min/mg)		
		Calculated[a]	
	Observed	For single enzyme	For two enzymes
5 mM PNP-α-Gal (a)	3.4 ± 0.2		
5 mM PNP-α-GalNAc (b)	53.5 ± 0.2		
a + b	49.1 ± 0.7	47.7	56.9

Note. All the enzymatic reactions were conducted at pH 4.5 and 37°C.

[a] Calculated on the assumption of a single catalytic site, using the equation: $v = (V_a \cdot A + V_b \cdot B)/(1 + A + B)$, where V_a and V_b are the velocity maxima, and A and B are the substrate concentration/K_m for a and b.

values calculated independently for each substrate. Table V shows that the observed value was in accord with that calculated on the presumption of a single site.

DISCUSSION

Since Harpaz *et al.* (18) first reported the use of ε-aminocaproylgalactosylamine–Sepharose for the isolation of coffee bean α-galactosidase, this affinity chromatographic matrix has been used for the purification of α-galactosidases from many sources. However, in some cases α-*N*-acetylgalactosaminidase is also absorbed on the gel as found for the Ehrlich cell extract. The separation of α-galactosidase and α-*N*-acetylgalactosaminidase may be attained by ion exchange (19) or hydroxyapatite column chromatography (20, 21). In the present study, α-*N*-acetylgalactosaminidase was eliminated from α-galactosidase activity by a simple change of the eluting solution.

Ehrlich tumor cell α-*N*-acetylgalactosaminidase exhibited a weak α-galactosidase activity and it appeared by mixed substrate experiment that the α-galactosidase activity was catalyzed at the same site as α-*N*-acetylgalactosaminidase activity. α-Galactosidase activity has been observed in many α-*N*-acetylgalactosaminidase preparations (22–25). In the present study, Ehrlich cell α-*N*-acetylgalactosaminidase was also absorbed on this affinity gel. Human placenta α-galactosidase was reported to have α-*N*-acetylgalactosaminidase activity and this α-galactosidase was also purified using ε-aminocaproylgalactosylamine–Sepharose (19).

A family of membrane glycoproteins from Ehrlich cells has been shown to terminate in the Galα1, 3Galβ1,4GlcNAcβ– trisaccharide sequence based on studies with exoglycosidases and on the specificity of the α-(1,3)-galactosyltransferase isolated from the cell. The same structural feature has also been found in laminin (26). Both glycoproteins were good substrates for Ehrlich tumor cell α-galactosidase and coffee bean α-galactosidase. The K_m value for Galα1,3Gal was very low for the coffee bean enzyme but relatively high for the Ehrlich tumor cell enzyme. When the hydrolysis rates for raffinose were compared, it was found to be a good substrate for the coffee bean enzyme, as found for many plant α-galactosidases (27), but a poorer substrate than some oligosaccharides for the Ehrlich tumor cell enzyme. In many α-galactosidases, the increase in molecular size when proceeding from melibiose to raffinose resulted in an increase in the rate of hydrolysis. The same effect was observed for the action of Ehrlich cell α-galactosidase with an increase in the chain length from Galα1,3Gal to the carbohydrate chains of the glycoproteins. The substrate specificity of Ehrlich cell α-galactosidase appeared to be relatively narrow, whereas coffee bean enzyme has a broad substrate specificity.

Johnson *et al.* (28) reported 10 lysosomal hydrolase activities in eight organs of males and females from six inbred mouse strains. They showed that β-*N*-acetylglucosaminidase activity was high in brain, kidney, and spleen. Although their assay was different from ours, the β-*N*-acetylglucosaminidase activity in Ehrlich ascites appears to be comparable to the activities in these tissues. On the other hand, α-galactosidase activity in Ehrlich ascites fluid and cells was very low compared with β-*N*-acetylglucosaminidase activity, but its activity appeared to be of the same order as the activity in spleen and kidney. Kidney was also reported to contain glycoconjugates with terminal α-galactosyl groups (29). It is very interesting that Ehrlich ascites fluid and kidney have relatively higher levels of α-galactosidase activities than other mouse tissues. Further, the V_{max}/K_m value of Ehrlich α-galactosidase toward the Ehrlich membrane glycoprotein indicated that glycopeptides are good substrates for this enzyme. These observations might be consistent with the accumulation of α-galactosyl-terminated glycoprotein in Ehrlich cells, if the localization of α-galactosidase and the substrate glycoprotein in Ehrlich cells are different.

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