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The Purification and Properties of Extracellular Glycosidases of the Cellular Slime Mould *Dictyostelium discoideum*

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The purification of β-*N*-acetylhexosaminidase, α-glucosidase, α-mannosidase and β-glucosidase from the spent growth medium of *Dictyostelium discoideum* strain Ax-2 myxamoebae is described. β-*N*-Acetylhexosaminidase and α-glucosidase were obtained in high yield and as homogeneous preparations whereas the α-mannosidase preparation consisted of two electrophoretically distinct isoenzymes. The physical, chemical and kinetic properties of these enzymes are described.

Loomis (1969) reported that the enzyme β-*N*-acetylglucosaminidase was synthesized during the early stages of the cell differentiation of the cellular slime mould *Dictyostelium discoideum*. He showed that the synthesis of this enzyme, like that of a number of others (Sussman & Sussman, 1969), was aberrant in some morphologically deranged mutants and was dependent on prior RNA synthesis and concomitant protein synthesis, and consequently he described the synthesis of this enzyme as being part of the 'developmental programme'. Wiener & Ashworth (1970) found that this enzyme could be synthesized during axenic growth of the *D. discoideum* strain Ax-2 isolated by Watts & Ashworth (1970) although this strain behaved like that studied by Loomis (1969) after growth on bacteria (Quance & Ashworth, 1972). Ashworth & Quance (1972) also showed that this enzyme could be excreted as true exponential growth ceased and they suggested that it was the excretion, rather than the synthesis, of this enzyme that was of developmental significance. Similarly, it has been claimed that α-mannosidase (Loomis, 1970) and β-glucosidase (Coston & Loomis, 1969) are developmentally regulated whereas Ashworth & Quance (1972) have shown that both these enzymes can also be found extracellularly after growth of strain Ax-2 axenically. It is thus clearly of interest to determine the physiological role of these enzymes during the development of *D. discoideum*. However, glycosidases of this type are also noteworthy in their own right as putative reagents for the analysis of glycoprotein and glycolipid structures. A number of procedures for purifying to homogeneity β-glucosidase (Got & Marnay, 1968; Blakely & Mackenzie, 1969), β-*N*-acetylhexosaminidase (Mega *et al.*, 1970; Li & Li, 1970; Wadstrom & Hisatsune, 1970), α-mannosidase (Paus & Christensen, 1972), and α-glucosidase (Bruni *et al.*, 1969; Jeffrey *et al.*, 1970) are available.

We describe the preparation, in high yield, of highly purified samples of β-*N*-acetylhexosaminidase, α-mannosidase, β-glucosidase and α-glucosidase, from the spent growth medium of *D. discoideum* strain Ax-2.

Materials and Methods

Materials

p-Nitrophenyl glycosides, glyconolactones, 4-methylumbelliferyl glycosides, naphthol AS-B1 2-acetamido-2-deoxy-β-D-glucopyranoside and 6-bromo-2-naphthyl glycosides were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; Sephadex G-100 and G-200 were obtained from Pharmacia, Uppsala, Sweden; Whatman DEAE-cellulose was obtained from W. and R. Balston Ltd., Maidstone, Kent, U.K. Hydroxyapatite (Bio-Cel HT) was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Concanavalin A was obtained from Miles Laboratories, Elkhart, Ind., U.S.A. Coomassie Brilliant Blue was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Partially hydrolysed starch was obtained from Connaught Medical Laboratories, Toronto, Ont., Canada. All other materials were of the highest purity available from BDH Chemicals Ltd., Poole, Dorset, U.K.

Methods

Organism and culture conditions. D. discoideum strain Ax-2 was grown in 700ml portions of axenic medium as described by Watts & Ashworth (1970). Cells in stationary phase were removed from the growth medium by centrifugation (500*g*, 15min) when in the stationary phase of growth and the supernatant (spent growth medium) was stored at 4°C with a few drops of toluene to inhibit microbial growth.

Enzyme assays. The glycosidase activities were assayed in 0.02M-acetate buffer, pH5.0, as described by Loomis (1969) for β-*N*-acetylglucosaminidase, Loomis (1970) for α-mannosidase and Coston & Loomis (1969) for β-glucosidase, during the purifica-

tion procedures, but in 0.02M-sodium citrate–phosphate buffers at their optimum pH values when examining their properties. The enzyme preparations were incubated at 35°C for 5–60min in buffer containing 10mM-*p*-nitrophenyl glycoside and the reaction was stopped by addition of Na_2CO_3 solution to a final concentration of 0.5M. The liberated *p*-nitrophenol was determined by E_{410} measurements. One unit of enzyme activity (E.U.) liberates 1μmol of *p*-nitrophenol/min of incubation under the above conditions. Protein was determined either by E_{280} measurements (Warburg & Christian, 1941) or by the colorimetric method of Lowry *et al.* (1951) with dry bovine serum albumin as standard. During the fractionation procedures it was often convenient to use a qualitative spot test to assay the various enzymes. Drops of the test sample, buffer and substrate were placed on a 6mm×6mm piece of Whatman no. 1 filter paper and incubated in a Petri dish floating on a water bath at 35°C for 5–100min. The *p*-nitrophenol liberated was detected by the dropwise addition of 1M-Na_2CO_3 until the final pH was alkaline.

Enzyme purification. Unless otherwise stated all operations were carried out at 0–4°C.

DEAE-cellulose batch procedure. DE-1 (floc grade) DEAE-cellulose was equilibrated with 5mM-sodium phosphate buffer, pH6.5, and 600ml (approx. 200g) of this cellulose-powder suspension was then added to 10 litres of pooled spent growth media, and the suspension was stirred for 1–2h until all glycosidase activity had been adsorbed. The cellulose powder was collected by filtration on a Buchner funnel and washed with 1 litre of 5mM-sodium phosphate buffer, pH6.5, containing 0.1M-NaCl at room temperature. The glycosidases were removed from the cellulose pad by elution with 1 litre of 5mM-sodium phosphate buffer, pH6.5, containing 1.0M-NaCl.

$(NH_4)_2SO_4$ fractionation. The eluate from the previous step was adjusted to pH4.0 with acetic acid, and solid $(NH_4)_2SO_4$ was added to 65% saturation. The fine precipitate that formed after 1–2h of stirring was collected by centrifugation at 10000*g* for 15min and redissolved in 16ml of 5mM-sodium phosphate buffer, pH6.5. The supernatant was discarded.

Gel filtration. The redissolved $(NH_4)_2SO_4$ precipitate was applied to the top of a column (50cm×7cm) of Sephadex G-100 and eluted with 5mM-sodium phosphate buffer, pH6.5, at a flow rate of approx. 90ml·h^{-1}. Fractions (10ml) were collected, assayed for glycosidase activities and suitable fractions were pooled and concentrated to a volume of 9ml in an Amicon membrane ultrafiltration cell with a Diaflo PM-30 membrane and a pressure of 483kPa (70lbf·in^{-2}). All the glycosidase activities were eluted together at, and just after, the hold-up volume (630ml), but this step served to remove many low-molecular-weight impurities which otherwise affected the separation achieved at the next step.

The glycosidase mixture (9ml) was applied to the top of a column (100cm×4cm) of Sephadex G-200 and eluted with 5mM-sodium phosphate buffer, pH6.5, at a flow rate of approx. 9ml·h^{-1}. Fractions (10ml) were collected, assayed for enzyme activity and the protein concentration estimated by E_{280} measurements. A typical elution pattern is shown in Fig. 1. The glycosidases were eluted in two reasonably well-separated groups, which were separately pooled (Fig. 1) and concentrated to 9ml in 5mM-sodium phosphate buffer, pH6.5, as described above.

It is important, at this stage, to separate the β-glucosidase and β-*N*-acetylglucosaminidase activities as completely as possible (by, for example, rechromatographing fractions 41–46, Fig. 1), since they do not separate on hydroxyapatite columns.

Hydroxyapatite chromatography. The two resulting enzyme solutions were applied to separate columns (5cm×3.3cm) of hydroxyapatite equilibrated with 5mM-sodium phosphate buffer, pH6.5. The columns were eluted in a stepwise fashion with sodium phosphate buffers, pH6.5, of increasing molarity and at a flow rate of approx. 10ml·h^{-1}. Fractions (10ml) were collected and assayed for glycosidase activities and protein content (E_{280}). Typical elution patterns are shown in Figs. 2 and 3.

Molecular-weight estimations. The approximate molecular weights of the purified glycosidases were estimated by Sephadex G-200 gel filtration (Determann, 1967). The column (36cm×2.5cm) of Sephadex G-200 was equilibrated with 50mM-Tris–HCl buffer, pH7.0, and calibrated by Dr. D. Malchow by using proteins of known molecular weight (cytochrome *c*, 13000; ovalbumin, 49000; bovine serum albumin, 67000; glycerol phosphate dehydrogenase, 135000; rabbit immunoglobulin G, 156000). The glycosidases and Blue Dextran were dissolved in 50mM-Tris–HCl buffer, pH7.0, and eluted from the column at a flow rate of 36ml·h^{-1}. The elution volumes were calculated from the mid-points of the resulting peaks of enzymic activity. Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis was performed as described below. To estimate the molecular weight of the single polypeptide units of the glycosidases the following proteins were used to calibrate the gels: bovine serum albumin (67000), heavy chain of immunoglobulin G (50000), aldolase (40000), light chain of immunoglobulin G (23500) and cytochrome *c* (13000) (Shapiro *et al.*, 1967). These proteins were dissociated by being boiled for 10min in a solution containing 1% sodium dodecyl sulphate, 1% 2-mercaptoethanol and 0.01M-sodium phosphate buffer, pH7.0. The resulting solution was dialysed against 0.01M-sodium phosphate buffer, pH7.0, containing 0.1% sodium dodecyl sulphate and 0.1% 2-mercaptoethanol. Sucrose (final concn. 10%) and Bromophenol Blue were added and the mixture was subjected to electrophoresis.

Electrophoresis. Polyacrylamide-gel electrophoresis was carried out as described by Coston & Loomis (1969) in 5% (w/v) polyacrylamide gels in 0.05M-sodium phosphate buffer, pH7.2, or as described by Davis (1964) in 7% (w/v) polyacrylamide gels in 0.2M-Tris–HCl buffer, pH8.9, or as described by Shapiro *et al.* (1967) in 10% (w/v) polyacrylamide gels containing 0.2M-Tris–HCl buffer, pH8.9, and 0.1% sodium dodecyl sulphate. In all cases polymerization was effected by using a final concentration of 0.025% *NNN'N'*-tetramethylethylenediamine and 0.07% ammonium persulphate. Sucrose was added to a final concentration of 10% to all samples which were then subject to electrophoresis at 0.5mA/gel for 20min and then run at 5–6mA/5% gel for 2–3h with 0.1M-sodium phosphate running buffer, pH7.2, and at 2–4mA/7% gel for 1–2h with 0.025M-Tris–glycine running buffer, pH8.3. Sodium dodecyl sulphate–polyacrylamide gels were run at 2–4mA/gel for 1–2h in a 0.05M-Tris–glycine running buffer, pH8.3, which contained 0.1% sodium dodecyl sulphate.

The gels were removed and stained with Coomassie Blue (Weber & Osborn, 1968) or assayed for enzyme activity in the intact gels or in gels sliced into 2mm fractions. The whole gels were assayed for enzyme activity by first preincubating the gels for 10min in 0.2M-sodium acetate buffer, pH5.0, and then incubating in the appropriate *p*-nitrophenyl glycoside substrate at pH5.0 and recording the liberation of *p*-nitrophenol continuously along the length of the gel at 405nm by using a Joyce–Loebl automatic recording microdensitometer.

Starch-gel electrophoresis was carried out by the methods of Smithies (1955) and Robinson *et al.* (1967) by using starch dissolved in 5mM-sodium phosphate or 5mM-citrate–phosphate buffers of pH5.0, 6.5 or 7.0; 40mM-sodium phosphate or 40mM-citrate–phosphate buffers of pH5.0, 6.5 or 7.0 were used in the electrode compartments and the enzyme samples were subjected to electrophoresis at 240V and 20–40mA for 3h. The activity of the glycosidases was detected on the gel surface with the appropriate 4-methylumbelliferyl glycoside fluorigenic substrate.

Concanavalin A–glycosidase reaction. The precipitation of glycosidases with concanavalin A was examined by the methods of Ouchterlony (1958) by using 1% agar in 0.075M-sodium phosphate buffer, pH7.2, containing 0.43M-NaCl. The purified glycosidases were dissolved at 5mg/ml in the above buffer and concanavalin A was 10mg/ml in 0.075M-sodium phosphate buffer, pH7.2, containing 1M-NaCl. Precipitation bands were examined with a light-source against a dark background. Glycosidase activity was detected on the agar surface by histochemical enzyme-staining techniques (Hayashi, 1965; Raunio, 1968) with naphthol AS-B1 2-acetamido-2-deoxy-β-D-glucopyranoside and 6-bromo-2-naphthyl glycosides as substrates or by using fluorigenic substrates in the same way as described for starch gels by Robinson *et al.* (1967). These experiments were done in collaboration with, and at the suggestion of, Dr. O. Wilhelms.

Results

Enzyme purification

Details of a typical purification are summarized in Tables 1 and 2. All the glycosidase activities are

Table 1. *Purification scheme for β-N-acetylglucosaminidase, β-N-acetylgalactosaminidase and α-glucosidase*

Total activities are expressed as μmol of *p*-nitrophenol released from the appropriate substrate/min at 35°C. Specific activities are expressed as μmol of *p*-nitrophenol released/min per mg of protein at 35°C. For full experimental details see the Materials and Methods section. The specific-activity ratio is specific activity of β-*N*-acetylglucosaminidase/specific activity of β-*N*-acetylgalactosaminidase.

	β-*N*-Acetylglucosaminidase activity		β-*N*-Acetylgalactosaminidase activity			α-Glucosidase activity	
Purification step	Total	Specific	Total	Specific	Specific-activity ratio	Total	Specific
1. Spent growth medium	8000	0.1	1350	0.017	5.9	31	0.00039
2. Batch DEAE-cellulose	8000	2.2	—	—	—	28	0.0075
3. $(NH_4)_2SO_4$ precipitation	5700	9.1	1120	1.8	5.1	22	0.035
4. Sephadex G-100	5600	21	980	3.7	5.7	22	0.083
5. Sephadex G-200	4800	78	800	13	6.0	21	0.34
6. Hydroxyapatite	2000	500	360	90	5.6	11	2.0
Final products							
Protein (mg)	4.0		4.0			5.5	
Yield (%)	25		27			36	

adsorbed on DEAE-cellulose (step 2), precipitated by 65% saturation with $(NH_4)_2SO_4$ (step 3) and largely excluded by Sephadex G-100 (step 4). Chromatography on Sephadex G-200 (step 5, Fig. 1) separated the activities into two groups. Fractions 33–41 (A) containing β-glucosidase, β-galactosidase and α-mannosidase activities were pooled, concentrated and dialysed and the proteins then chromatographed on a hydroxyapatite column (Fig. 2). Fractions 47–55 (B) containing predominantly β-*N*-acetylglucosaminidase, β-*N*-acetylgalactosaminidase and α-glucosidase activities were similarly pooled, concentrated, dialysed and chromatographed on a hydroxyapatite column (Fig. 3). In Figs. 2 and 3 the protein eluted

Table 2. *Purification scheme for α-mannosidase, β-glucosidase and β-galactosidase*

Total activities are expressed as μmol of *p*-nitrophenol released from the appropriate substrate/min at 35°C. Specific activities are expressed as μmol released/min per mg of protein at 35°C. For full experimental details see the Materials and Methods section. The specific-activity ratio is specific activity of β-glucosidase/specific activity of β-galactosidase.

	α-Mannosidase activity		β-Glucosidase activity		β-Galactosidase activity		
Purification step	Total	Specific	Total	Specific	Total	Specific	Specific-activity ratio
1. Spent growth medium	410	0.005	18	0.00023	23	0.00029	0.8
2. Batch DEAE-cellulose	360	0.097	18	0.005	14	0.004	1.3
3. $(NH_4)_2SO_4$ precipitation	250	0.4	14	0.022	7	0.011	2.0
4. Sephadex G-100	220	0.83	12	0.047	6	0.023	2.0
5. Sephadex G-200	83	0.85	10	0.1	1	0.009	11
6. Hydroxyapatite	53	4.4	4	1.6	0.2	0.08	20
Final products							
Protein (mg)	12		2.5		2.5		
Yield (%)	13		22		1		

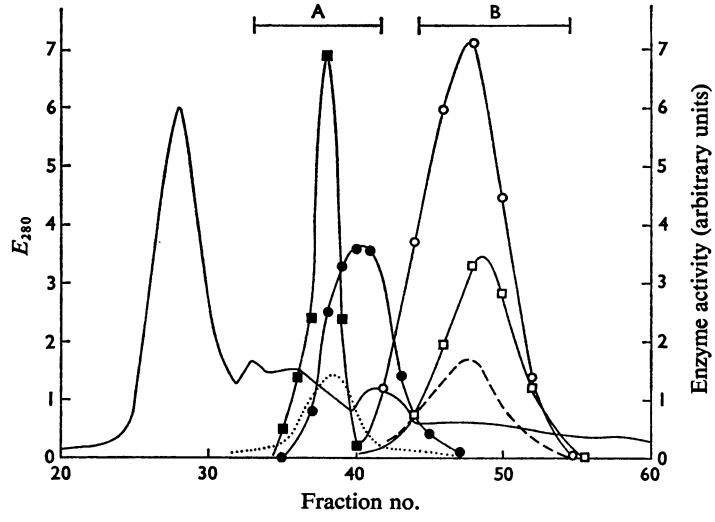

Fig. 1. *Elution diagram of glycosidases from a Sephadex G-200 column*

For details see the Materials and Methods section. One arbitrary unit of activity is equivalent to 10E.U./ml for β-*N*-acetylglucosaminidase (○) and β-*N*-acetylgalactosaminidase (---), 0.5E.U./ml for α-mannosidase (■), 0.1E.U./ml for α-glucosidase (□), 0.05E.U./ml for β-glucosidase (●) and 0.01E.U./ml for β-galactosidase (·····). E_{280} measurements were used as a measure for protein (——).

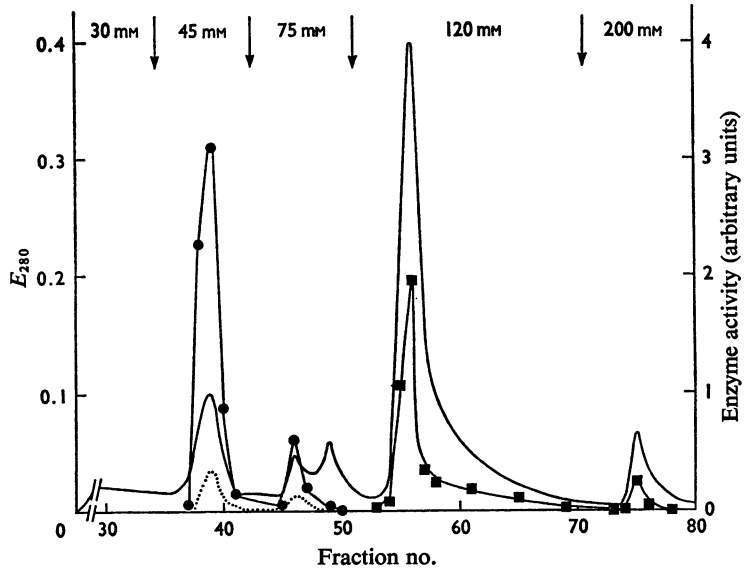

Fig. 2. *Chromatography of glycosidases on hydroxyapatite*

The partially purified group A glycosidases from the Sephadex G-200 column (Fig. 1) in 9ml of 5mM-sodium phosphate buffer, pH6.5, were applied to a hydroxyapatite column, washed into the column with 5mM-sodium phosphate buffer, pH6.5, followed by 19mM-sodium phosphate buffer, pH6.5, and finally, the column was eluted stepwise with sodium phosphate buffers, pH6.5, of increasing molarity as indicated by ↓↓. For full experimental details see the Materials and Methods section. One arbitrary unit of activity is equivalent to 1E.U./ml for α-mannosidase (■), 0.05E.U./ml for β-glucosidase (●) and 0.01E.U./ml for β-galactosidase (·····). Protein (——) was determined from E_{280} measurements.

at lower and higher molarity phosphate buffers not shown here contained insignificant amounts of enzyme activity. In both hydroxyapatite columns each activity tended to give more than one peak in the elution pattern. The number and relative area under these peaks changed markedly when different sodium phosphate buffer concentrations were used for elution. The concentrations used in the experiments shown in Figs. 2 and 3 gave the best separation of the various activities, but the appearance of, for example, two peaks of β-*N*-acetylglucosaminidase activity (Fig. 3) did not indicate two species of enzyme since preparations from either peak gave, on rechromatography on a similar column, more than one peak of activity. The fractions with the highest specific activity were pooled (fractions 110–140 in the case of β-*N*-acetylglucosaminidase), concentrated and salts were removed by ultrafiltration before freeze-drying. The fractions with lower specific activities were pooled, rechromatographed on Sephadex G-200 and hydroxyapatite columns reproducing fractionations similar to those shown in Figs. 1, 2 and 3. In Tables 1 and 2 'step 6' records the values obtained for the highest-specific-activity peaks from Figs. 2 and 3. The β-galactosidase activity proved unstable and we have not attempted to characterize this enzyme further.

Characterization of the enzymes

Homogeneity. The β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activities appear to be associated with the same protein species since the ratio of the two activities remained constant during purification (Table 1). Other evidence justifying this conclusion is discussed below, and here we refer to this protein as β-*N*-acetylhexosaminidase. We have assessed the homogeneity of our enzyme preparations by (1) disc-gel electrophoresis at pH7.2 and pH8.9 and (2) sedimentation-velocity analysis in an MSE analytical ultracentrifuge. β-*N*-Acetylhexosaminidase and α-glucosidase show one protein-staining band coincident with the enzymic activity after electrophoresis at either pH value, and both preparations appear greater than 90% homogeneous in the ultracentrifuge. The α-mannosidase preparation showed two protein-staining bands after electrophoresis at either pH value and both bands possessed

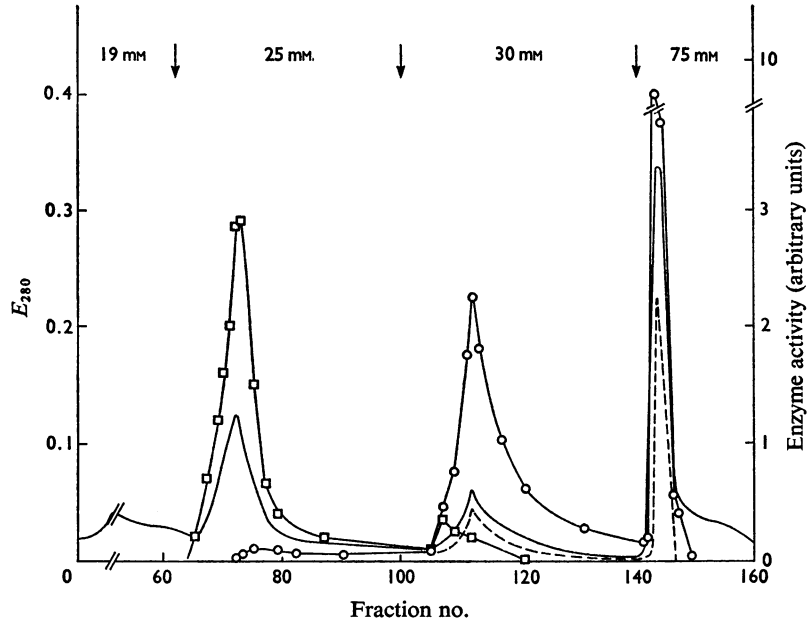

Fig. 3. *Chromatography of glycosidases on hydroxyapatite*

The partially purified group B glycosidases from the Sephadex G-200 column (Fig. 1) in 9ml of 5mM-sodium phosphate buffer, pH6.5, were applied to a hydroxyapatite column, washed into the column with the same buffer, and then the column was eluted stepwise with sodium phosphate buffers, pH6.5, of increasing molarity as indicated by ↓↓. For full experimental details see the Materials and Methods section. One arbitrary unit is equivalent to 10E.U./ml for β-*N*-acetylglucosaminidase (○) and β-*N*-acetylgalactosaminidase (---) activities, and 0.05E.U./ml for α-glucosidase activity (□). Protein (——) was determined from E_{280} measurements.

α-mannosidase activity. The β-glucosidase preparation showed several protein-staining bands after electrophoresis at either pH value, one of these bands had β-glucosidase activity and another β-*N*-acetylhexosaminidase activity. Both the α-mannosidase and β-glucosidase preparations appear heterogeneous in the ultracentrifuge.

pH optima and stability. The effect of alterations of pH value on the activity and stability of the purified glycosidases was studied by using sodium phosphate–citrate buffers of pH2.4–8 and a glycine–HCl buffer, pH2.1. Fig. 4 shows that the pH optima of all the glycosidases lie in the acid region; sodium acetate buffers (0.02M; pH3.6–6.4) gave results identical with those shown in Fig. 4.

Studies on the effect of pH on the stability of these enzymes were carried out by incubating the purified enzymes in 0.02–0.05M-sodium phosphate–citrate buffers of the appropriate pH values for 2h at 35°C and then diluting the samples 25–50-fold in 0.02M buffers of the pH optimum for the enzyme concerned and assaying enzyme activity. The results of such studies are shown in Fig. 5. β-*N*-Acetylhexosaminidase was completely stabilized at low pH values by the addition of bovine serum albumin (final concn. 1.0mg/ml) or by addition of the axenic growth medium (Watts & Ashworth, 1970). The presence of *p*-nitrophenyl β-*N*-acetylglucosaminide also stabilizes the purified enzyme to acid inactivation. The other purified glycosidases were stable over pH ranges as follows: α-mannosidase, pH4.5–7.2; α-glucosidase, pH2.8–8.9; and β-glucosidase, pH3.0–6.7. The α-mannosidase activity was also stabilized at low pH values by the addition of protein solutions to the purified preparations.

Temperature stability. All the purified enzymes were stable for long periods at 4°C when dissolved in water or in dilute buffers at or near their pH optima. Such solutions could be frozen and thawed and freeze-dried without appreciable loss of activity. The kinetics of thermal inactivation of the purified enzymes were studied over the range 40–70°C. The enzyme preparations were dissolved in 30mM-sodium phosphate–citrate buffer, pH6.4, and incubated at the appropriate temperature for up to 2h. Inactivation followed first-order kinetics, and a linear plot of log (relative

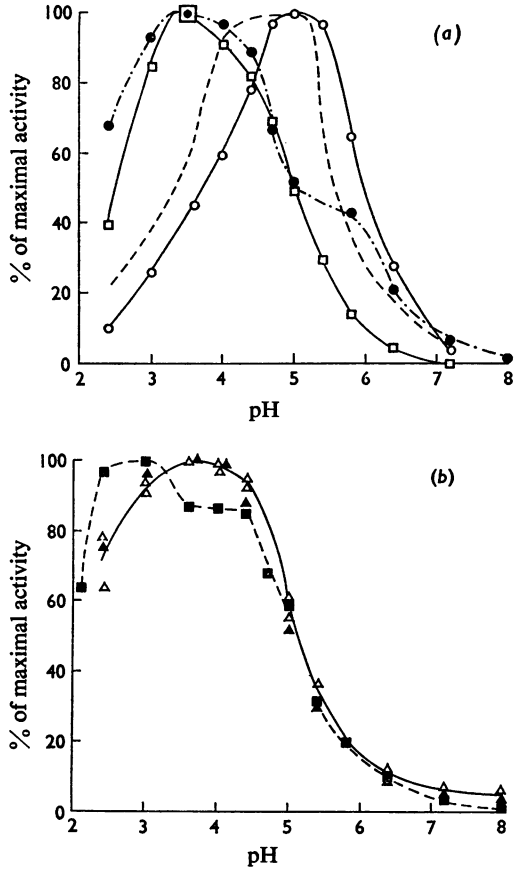

Fig. 4. *Effect of pH on glycosidase activity*

The enzymes were incubated with 10mM-*p*-nitrophenyl glycosides for 10–30min in 50mM buffers of different pH values at 35°C before assaying for the released *p*-nitrophenol. For full details see the Materials and Methods section. (*a*) pH curves of purified enzymes: β-*N*-acetylglucosaminidase (○); β-*N*-acetylgalactosaminidase (---); α-glucosidase (□); and β-glucosidase (●). (*b*) pH curves of α-mannosidase preparations: soluble extract from sonicated stationary-phase cells or culminating fruiting-body cells (△); spent growth medium from stationary-phase cells (▲); purified α-mannosidase (■).

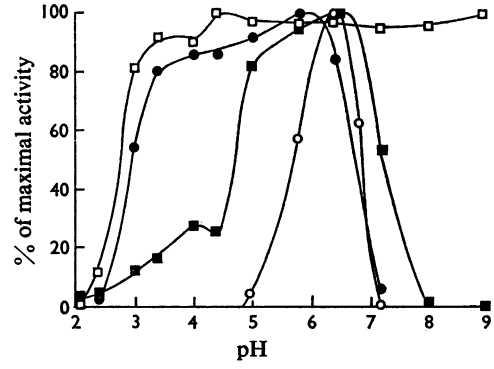

Fig. 5. *Effect of pH on stability of glycosidase activities*

Purified glycosidases were tested for stability at various pH values over a 2h period at 35°C; β-*N*-acetylglucosaminidase (○), α-mannosidase (■), α-glucosidase (□) and β-glucosidase (●). For full experimental details see the Materials and Methods section.

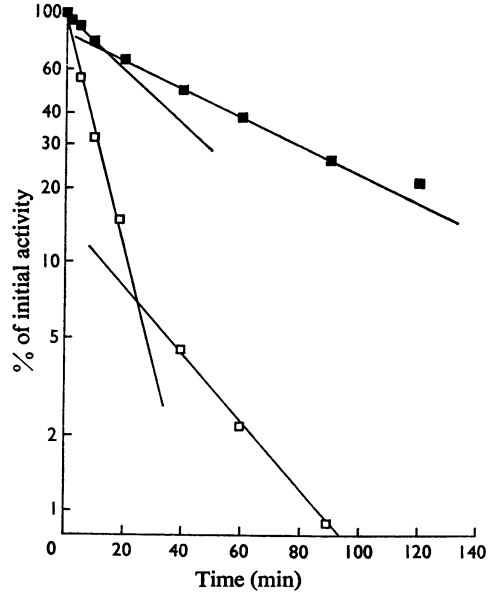

Fig. 6. *Effect of temperature on stability of purified α-mannosidase activity at 65°C (■) and 70°C (□)*

For full experimental details see the Materials and Methods section.

activity) against time of incubation was obtained for all the glucosidases. *N*-Acetylglucosaminidase and *N*-acetylgalactosaminidase activities had identical half-lives of 37min at 50°C. The half-lives of β-glucosidase and α-glucosidase were 12min at 50°C and 30min at 60°C respectively. The inactivation kinetics of the α-mannosidase activity (Fig. 6) suggested the presence of two enzymically active species with half-lives of 27min and 53min at 65°C and 6.5min and 22min at 70°C.

Kinetic properties. The effect of variations in the substrate concentration on the velocity of the reaction catalysed by the most highly purified enzyme preparations was studied by using concentrations of the appropriate *p*-nitrophenyl glycoside (0.34–10mM) in 0.02M buffers of the optimum pH at 35°C. In all cases, plots of (initial velocity)$^{-1}$ against (substrate concentration)$^{-1}$ gave straight lines, and Table 3 records the values of the Michaelis constants (K_m) and maximal velocities ($V_{max.}$) deduced therefrom. The effects of potential lactone inhibitors of the glycosidases were studied by the method of Dixon (1953). D(+)-Mannonic acid γ-lactone did not inhibit α-mannosidase at concentrations below 50mM. The other lactone inhibitors listed in Table 3 acted competitively with the *p*-nitrophenyl glycoside substrates.

Substrate specificity. The most highly purified samples of β-*N*-acetylhexosaminidase, α-mannosidase, α-glucosidase and β-glucosidase were incubated with the *p*-nitrophenyl derivatives of β-*N*-acetylglucosamine, β-*N*-acetylgalactosamine, α-glucose, β-glucose and α-mannose. The β-*N*-acetylhexosaminidase preparation hydrolysed all *p*-nitrophenyl glycosides other than those derived from the amino sugars at less than 0.02% of the rate observed with *p*-nitrophenyl β-*N*-acetylglucosaminide. The α-mannosidase preparation hydrolysed all other *p*-nitrophenyl glycosides other than the α-D-mannose derivative at less than 1% of the rate seen with *p*-nitrophenyl α-D-mannoside. The α-glucosidase preparation hydrolysed *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide at 2.5% of the rate observed with *p*-nitrophenyl α-D-glucoside and all other *p*-nitrophenyl derivatives at less than 0.5% of this rate. It is difficult to separate the β-glucosidase and β-*N*-acetylhexosaminidase activities and thus most of our β-glucosidase preparations hydrolyse the *p*-nitrophenyl derivatives of the amino sugars and β-D-glucoside but other derivatives are hydrolysed at less than 5% of the rate seen with *p*-nitrophenyl β-D-glucoside. In addition the 4-methylumbelliferyl derivatives of *N*-acetyl-β-D-glucosamine, α-D-mannose and β-D-glucose were hydrolysed by the appropriate enzyme preparation as were the 6-bromo-2-naphthyl derivatives of α-D-mannose, α-D-glucose and β-D-glucose. The β-*N*-acetylhexosaminidase preparations also hydrolysed naphthol AS-B1 2-acetamido-2-deoxy-β-D-glucopyranoside. None of our enzyme preparations hydrolysed the *p*-nitrophenyl derivatives of α-D-galactose, β-D-glucuronic acid, β-D-mannose, β-D-fucose, β-L-fucose, β-D-xylose, α-D-xylose or α-L-fucose.

Size. The molecular-weight values and polypeptide subunit molecular weights derived either from Sephadex G-200 chromatography or sodium dodecyl sulphate–polyacrylamide-gel electrophoresis are summarized in Table 4. On sodium dodecyl sulphate–polyacrylamide gels α-glucosidase gave only one band of protein but *N*-acetylhexosaminidase gave one strong peak (peak I) and one weak peak (peak II) of protein. Since the α-mannosidase and β-glucosidase preparations were obviously heterogeneous they were not analysed by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis.

Preliminary experiments on the behaviour of *N*-acetylglucosaminidase and α-glucosidase in the ultracentrifuge have suggested that the molecular weights of the native enzymes correspond to the molecular weights of the polypeptide chains and that the estimates for the molecular weights derived from the Sephadex G-200 column are therefore misleading. There is evidence (see below) that these proteins are glycoproteins, and Andrews (1965) has reported that glycoproteins often behave anomalously on Sephadex columns.

Reaction with concanavalin A. Concanavalin A produced strong precipitation bands on Ouchterlony plates with the four purified glycosidase samples and each precipitated enzyme reacted with its correspond-

Table 3. *Kinetic properties of purified glycosidases*

The most highly purified preparation of hexosaminidase was used for both β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase measurements. The other glycosidases were also the most highly purified preparations. For full experimental details see the Materials and Methods section.

Enzyme	K_m (mM)	$V_{max.}$ (μmol/min per mg of protein)	Inhibitor	K_i (μM)
β-*N*-Acetylglucosaminidase	1.5	600	2-Acetamido-2-deoxy-D-gluconolactone	2.0
β-*N*-Acetylgalactosaminidase	1.5	112	—	—
α-Glucosidase	1.0	3.6	D-(+)-Gluconic acid δ-lactone	10000
β-Glucosidase	2.0	4.4	D-(+)-Gluconic acid δ-lactone	0.4
α-Mannosidase	2.3	8.3	—	—

Table 4. *Apparent molecular weights of purified glycosidases*

The most highly purified glycosidase preparations were used. The molecular weight of the active enzyme was determined by Sephadex G-200 chromatography. The molecular weight of the polypeptide subunits was determined by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. For full experimental details see the Materials and Methods section.

Enzyme	Molecular weight of active enzyme	Molecular weight of polypeptide subunits
β-*N*-Acetylhexosaminidase	168000	Peak I 65000, peak II 51000
α-Glucosidase	150000	84000
β-Glucosidase	225000	—
α-Mannosidase	280000	—

ing histochemical and fluorigenic substrates. Thus these glycosidases must have a carbohydrate component containing α-mannose residues and possibly glucose and *N*-acetylglucosamine residues.

Starch-gel electrophoresis. Purified hexosaminidase, β-glucosidase and α-glucosidase gave only one band of enzyme activity in starch gels, whereas purified α-mannosidase gave two bands of enzyme activity; one band with strong enzyme activity moving only slightly to the anode at pH5 (α-mannosidase B) and one band with weak enzyme activity moving several centimetres to the anode at pH5 (α-mannosidase A). In crude α-mannosidase preparations from the spent growth medium or cell extracts from myxamoebae and culminating fruiting bodies, only one band of enzyme activity appeared corresponding to α-mannosidase A. α-Mannosidase A showed slightly stronger reaction at pH4 than at pH2.5, whereas the converse was observed for α-mannosidase B. α-Mannosidase B probably accounts for the peak in activity at pH2.4 (Fig. 4*b*) observed with the purified α-mannosidase sample. When a sample of spent growth medium was frozen and thawed several times and incubated at 22°C for several days there was no conversion of α-mannosidase A into α-mannosidase B.

Discussion

The spent growth medium of *D. discoideum* strain Ax-2 provides a very convenient starting material for the preparation of a number of glycosidase activities. Besides the glycosidases listed in Tables 1 and 2, low β-D-fucosidase activity could be detected in myxamoebae and in spent growth medium. α-D-Galactosidase, β-D-mannosidase, α-L-fucosidase, β-L-fucosidase, β-D-glucuronidase, β-D-xylosidase or α-D-xylosidase activities could not be detected with *o*-nitrophenyl glycoside substrates. Several other sources rich in glycosidase activities have been reported (Snaith *et al.*, 1970; Muramatsu & Egami, 1967; Bahl & Agrawal, 1969), for which methods of separating some of the activities have been worked out.

Hexosaminidase preparations of purity comparable with ours have been reported from *Aspergillus oryzae* (Mega *et al.*, 1970), jackbean meal (Li & Li, 1970) and *Staphylococcus aureus* (Wadstrom & Hisatsune, 1970), but the turnover number of our enzyme for *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide substrate is the highest yet reported. It appears that the *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities reside in the same enzyme molecule because the ratio of the two activities remained relatively constant throughout the purification procedure and the final product was homogeneous on polyacrylamide-gel electrophoresis and ultracentrifugation. Also, the thermal- and pH-inactivation properties of these two activities were identical. It has also been found (D. Every & J. M. Ashworth, unpublished work) that the enzyme molecules responsible for these two activities have identical antigenic properties; both activities in crude extracts and in the most highly purified preparations are coprecipitated by specific anti-*N*-acetylhexosaminidase antibodies in an identical fashion in quantitative immunoprecipitation experiments. The lower pH optimum for the *N*-acetylgalactosaminidase activity (Fig. 4*a*) has been observed for other purified hexosaminidases (Li & Li, 1970; Mega *et al.*, 1970) and does not necessarily mean that there are distinct enzymes specific for each substrate. Many other reports have been published suggesting the identity of these enzymes (Findlay & Levvy, 1960; Woollen *et al.*, 1961; Bahl & Agrawal, 1969; Robinson & Stirling, 1968; Muramatsu, 1968; Sandhoff & Wässle, 1971), although an enzyme from calf brain which is specific for β-*N*-acetylgalactosaminide residues has been reported (Frohwein & Gatt, 1966).

It was also found that the β-galactosidase and β-glucosidase activities from *D. discoideum* were difficult to separate. However, it appeared from the changing ratios of the two activities during the purification procedure (Table 2) and from the slight

separation of the activities on Sephadex G-200 chromatography (Fig. 1) that two distinct enzymes were present. Homogeneous preparations of β-hexosidase have been purified from snail (Got & Marnay, 1968) and yeast (Blakely & Mackenzie, 1969) that hydrolyse both β-galactoside and β-glucoside. On the other hand, enzymes have been purified which hydrolyse specifically β-galactoside (Biermann & Glantz, 1968; Craven *et al.*, 1965) or β-glucoside (Jermyn, 1955; Agrawal & Bahl, 1968).

Our purified α-mannosidase preparations appear to consist of two isoenzymes with different electrophoretic mobilities, pH optima (Fig. 4*b*) and thermal stabilities (Fig. 6). Only one form of α-mannosidase occurs in crude preparations whether from spent growth medium or from cell extracts prepared from the myxamoeba or the fruiting-body stage of the life cycle, and it appears to resemble that isoenzyme in the purified preparation with the more alkaline pH optimum and faster electrophoretic mobility (α-mannosidase A). α-Mannosidase, like the other glycosidases, reacts with concanavalin A and thus must contain mannose and possibly glucose and *N*-acetylglucosaminide residues and thus be a glycoprotein. It was therefore thought possible that the second isoenzyme with the more acid pH optimum (α-mannosidase B) was derived from the other by extracellular modification of the carbohydrate portion of the molecule catalysed by one of the extracellular glycosidases (perhaps by α-mannosidase itself). Robinson & Stirling (1968) reported the conversion of one form of human spleen *N*-acetylglucosaminidase into another form by the removal of sialic acid residues from the enzyme molecule by neuraminidase, and even spontaneous conversion of one form into the other without neuraminidase. However, no conversion of slime-mould α-mannosidase could be detected in crude extracts even though they were incubated for several days at 22°C. It must therefore be concluded that the purification procedure is responsible for the partial conversion of α-mannosidase from the A into the B form, perhaps by the removal of a component which stabilizes the A form of the enzyme. Jermyn (1955) reported that during the purification of an extracellular β-glucosidase from *Stachybotrys atra* a non-covalently bound carbohydrate component was removed which altered the stability of the enzyme.

The substrate-specificity studies show that these enzymes are reasonably specific for particular *p*-nitrophenyl glycosides. Although the β-glucosidase preparation has a high hexosaminidase activity, it can be seen from Fig. 1 that the two activities can be separated by Sephadex G-200 chromatography. Thus β-glucosidase is specific for *p*-nitrophenyl β-glucoside. It is unlikely, however, that such compounds represent the natural substrates of these enzymes. In principle the physiological substrate(s) could be of bacterial or slime-mould origin. Braun *et al.* (1972) have reported that at pH3 our purified hexosaminidase degrades purified murein from *Escherichia coli* W7 by cleavage of both the *N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramic acid bond and the *N*-acetylmuramyl-β-1,4-*N*-acetylglucosamine bond. The enzyme was also found to lyse *Aerobacter aerogenes* and *Micrococcus lysodeikticus* at pH3 (D. Every & J. M. Ashworth, unpublished work). However, the slime-mould hexosaminidases do not appear to be able to degrade the *O*-antigen specific chain of bacterial lipopolysaccharide which contains similar β-1,4-glycosidic bonds to those found in murein (Malchow *et al.*, 1969; Nigam *et al.*, 1970), nor can our purified hexosaminidase alone or in combination with other extracellular slime-mould glycosidases release *N*-acetylglucosamine from purified slime-mould cell-surface components that contain *N*-acetylglucosamine residues (Wilhelm, 1972). This would suggest that the physiological substrate for our purified hexosaminidase is bacterial murein. However, Loomis (1969) and Quance & Ashworth (1972) have reported that the specific activity of hexosaminidase is higher during differentiation (i.e. in the absence of bacteria) than during the growth phase, which is difficult to reconcile with an exclusively digestive role for this enzyme.

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