Glycosidases from the Culture Medium of *Physarum polycephalum*

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Eight exo-glycosidase activities were detected in the axenic culture medium of the myxomycete, *Physarum polycephalum*. The secretion of each enzyme examined followed the growth curve and continued during the stationary phase after the cessation of growth. Two or more forms of each enzyme were detected after electrophoretic separation. The β -N-acetyl-D-hexosaminidase activity was readily separated into its two electrophoretic forms, X and Y, which were purified 145- and 306-fold respectively. These β -N-acetyl-D-hexosaminidases had several similar characteristics. Evidence is presented that the major electrophoretic form of α -D-galactosidase is heterogeneous. The possible functions of extracellular glycosidases in the life-history of the organism are discussed in the light of their occurrence and properties.

Physarum polycephalum, a true slime mould or myxomycete, is a very suitable model system for biochemical studies of cellular interaction and differentiation (Dee, 1975). Its life-cycle may be said to begin with spore germination to uninucleate amoebae. Such amoebae of suitable mating types may fuse to form a multi-nucleate plasmodium, which, under unfavourable nutritional conditions, will ultimately differentiate to form either sclerotia or fruiting bodies.

Before differentiation, the usually immobile plasmodium may be provoked into migration across a solid substratum in search of nutrients. When the advancing margins of two plasmodia of the same strain meet they rapidly fuse and protoplasmic mixing occurs (Gray, 1945). Plasmodia of incompatible strains, however, may remain in contact for days without fusing (Gray, 1945; Carlile & Dee, 1967). Fusion is thought to be controlled by factors located at the plasmodial surface (Poulter & Dee, 1968; Ross & Cummings, 1970; Ling, 1972; Jeffrey & Rusch, 1975), and Ross & Shipley (1973) have suggested that enzymic alteration of the polysaccharide slime sheath that covers the plasmodial membrane may be a prerequisite for normal plasmodial fusion. Sublethal autolysis (Poste, 1971) involving glycosidases has also been invoked (Bosmann, 1972; Poste, 1972; Bosmann *et al.*, 1974) as a possible mechanism in the normal growth control of avian and mammalian cells, and similar enzymes may mediate the cessation of plasmodial growth and subsequent differentiation. It is therefore clearly important to establish whether *P. polycephalum* secretes glycosidases, and if so, to determine their sugar specificities and other properties.

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P. polycephalum normally feeds on bacteria, yeasts and fungi, but in the laboratory it can be cultivated conveniently in the form of microplasmodia in a semi-defined axenic medium (Carlile, 1971). From a similar medium, extracellular ribonucleases (Braun & Behrens, 1969) and proteases (Farr *et al.*, 1974) have been detected, but except for short communications on β -(1 $\rightarrow$ 3)-glucanases (Farr *et al.*, 1973, 1975) and β -N-acetyl-D-hexosaminidases (Kilpatrick & Stirling, 1975), there have been no reports of enzymes that might be expected to degrade complex carbohydrates. Here we describe some of the characteristics of extracellular glycosidases of *P. polycephalum* grown in this way, and report the purification and properties of α -D-galactosidase and β -N-acetyl-D-hexosaminidase in some detail.

Experimental

Materials

Cultures of *P. polycephalum* (strain i $\times$ A7029) and samples of spent growth medium were kindly given to us by Dr. M. Carlile, Imperial College, University of London. ^3H -labelled ceramide trihexoside was a gift from Dr. M. W. Ho of this Department. *NN'*-Diacetylchitobiose (chitobiose) was prepared by the method described by Stirling (1974). All 4-methylumbelliferyl glycoside substrates were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and *p*-nitrophenyl α -D-glucoside was also purchased from the same suppliers.

Other materials were purchased from the following commercial sources: Azocoll-100 mesh from Calbiochem., La Jolla, CA, U.S.A.; Blue Dextran, bovine serum albumin and horseradish peroxidase from Sigma Chemical Co., St. Louis, MO, U.S.A.;

chitin from Eastman Kodak Co., Rochester, NY, U.S.A.; cytochrome *c* from BDH Chemicals Ltd., Poole, Dorset, U.K.; hydrolysed starch from Connaught Medical Research Laboratories, Toronto, Canada; DEAE-cellulose (DE 22) from W. and R. Balston Ltd., Maidstone, Kent, U.K.; hydroxyapatite (Bio-Gel HT) from Bio-Rad Laboratories, Richmond, CA, U.S.A.; Sephadex from Pharmacia (Great Britain) Ltd., London W.5., U.K. General laboratory chemicals were obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K.

Methods

Stocks of *P. polycephalum* were maintained in the form of plasmodia growing on nutrient agar and as microplasmodia growing in liquid culture by the methods described by Carlile (1971). To obtain spent growth medium for investigation of glycosidases, flasks containing microplasmodia were left to shake (200 rev./min at 23°C) for 2–3 days after maximum growth had been attained. The microplasmodia were removed by centrifugation for 5 min at 1000g in an MSE High-Speed 18 refrigerated centrifuge at 4°C and the supernatant was stored at –20°C until required. Growth of *P. polycephalum* was assessed by measuring the increase in dry weight of microplasmodia. Samples (3 ml) were taken periodically from flasks containing growing microplasmodia. The microplasmodia were removed by centrifugation (500g for 5 min), washed once with distilled water at 4°C, then dried overnight at 100°C. Culture medium from these centrifuged samples was retained for the assay of enzyme activity. It is not known whether the onset of the stationary phase in these cultures is associated with the limitation of carbon, nitrogen or phosphorus.

Enzyme assays

β -N-Acetyl-D-glucosaminidase and β -N-acetyl-D-galactosaminidase were assayed by the fluorimetric method of Dance *et al.* (1969). *NN'*-Diacetylchitinase was assayed by the method of Stirling (1974). Assay of α -D-mannosidase, β -D-glucosidase and β -D-galactosidase were performed by incubating 1.0 ml of a suitable aqueous dilution of the sample with 1.0 ml of the appropriate 4-methylumbelliferyl glycoside (1.0 mM) in McIlvaine (1921) sodium citrate/sodium phosphate buffer, pH 4.5, for 15 min at 37°C. The reaction was stopped by the addition of 3.0 ml of glycine/NaOH buffer, pH 10.4. α -L-Fucosidase was assayed by the method of Robinson & Thorpe (1974). α -D-Galactosidase was usually assayed by incubating 10 μ l of the sample with 50 μ l of 4-methylumbelliferyl α -D-galactoside (0.2 or 5.0 mM) in McIlvaine sodium citrate/phosphate buffer, pH 3.5, or in 0.1 M-sodium phosphate buffer, pH 6.75, for 30 min at 37°C. The precise conditions used for each experiment are given in the Results section. The reaction was stopped

by the addition of 1.0 ml of glycine/NaOH buffer, pH 10.4. The 4-methylumbelliferone liberated in the course of these enzyme assays was measured in a Locarte fluorimeter (activating at 350 nm and measuring at 440 nm) using a standard quinine sulphate solution (4 mg per litre of 0.05 M-H₂SO₄).

α -D-Glucosidase was assayed by incubating 0.1 ml of a suitably diluted sample of enzyme with 0.5 ml of 1.0 mM-*p*-nitrophenyl α -D-glucoside in McIlvaine sodium citrate/phosphate buffer, pH 4.5, for 1 h at 37°C. The reaction was stopped by adding 3.0 ml of glycine/NaOH buffer, pH 10.4, and the *A*₄₀₀ measured on a Unicam SP. 600 spectrophotometer.

There were linear relationships between aglycone liberated and the time of incubation over the periods used in the fixed time assays. One unit of enzyme activity is the amount of enzyme that liberates 1 μ mol of 4-methylumbelliferone or *p*-nitrophenol per min under the conditions of assay.

Protease activity was assayed by a modification of the method of Farr *et al.* (1974). Azocoll (25 mg) was suspended in 5.0 ml of McIlvaine sodium citrate/phosphate buffer, pH 4.5, and to this 1.0 ml of suitably diluted enzyme sample was added. The reaction mixture was shaken at 37°C for 30 min and then filtered through Whatman no. 1 filter paper. The *A*₅₂₀ of the filtrate was measured against a blank prepared in the same way except that 1.0 ml of distilled water was added to the Azocoll suspension.

The activity of α -D-galactosidase towards human ceramide trihexoside was measured by the method of Ho (1973).

Protein was estimated by a modification of the method of Lowry *et al.* (1951) in which trisodium citrate was used in place of sodium tartrate/potassium tartrate. Bovine serum albumin was used as a standard.

Preparation of crude enzyme extract

Culture medium was centrifuged at 15000g for 30 min in an MSE High-Speed 18 centrifuge at 4°C. The supernatant was brought to 65% satn. with solid (NH₄)₂SO₄ and, after stirring at 4°C for 3 h, the precipitate was collected by centrifugation at 15000g for 45 min. The precipitate was then dissolved in a small volume of distilled water and used for investigation of the general properties of the glycosidases.

Partial purification of β -N-acetyl-D-hexosaminidase and α -D-galactosidase

Spent growth medium was centrifuged at 1000g for 15 min and the supernatant (500 ml) mixed with an equal volume of ethanol at 4°C to precipitate the slime present in the medium. The supernatant recovered after centrifugation of the suspension at 15000g for 40 min was dialysed against 3 $\times$ 12 litres of water over 19 h. The diffusate (1.9 litres) was adjusted to 90% satn. with (NH₄)₂SO₄, stirred for 3 h at 4°C

and the precipitate collected by centrifugation at 15000g for 40 min. Precipitate still remaining in the supernatant was recovered by filtration through Whatman no. 1 filter paper. The precipitate was dissolved in 80 ml of 5 mm-sodium phosphate buffer, pH 6.5, concentrated to about one-half this volume against polyethylene glycol 4000P (Shell Chemicals, London S.E.1., U.K.), and dialysed against distilled water (12 litres) at 4°C overnight. The sample (48 ml) was dialysed against 5 mm-sodium phosphate, pH 6.5, before being applied to a column (1.6 cm × 3.4 cm diam.) of hydroxyapatite equilibrated in the same buffer. The column was eluted with several column volumes of this buffer at a flow rate of 30 ml/h. Bound enzyme activity was removed by eluting with 200 mm-sodium phosphate buffer, pH 6.5, and finally with 1.0 M-NaCl in the same buffer. These fractions were pooled, dialysed against distilled water (8 litres) overnight, and concentrated to 35 ml against polyethylene glycol. After dialysis against 5 mm-sodium phosphate buffer, pH 7.0, the sample (33 ml) was applied to a column (4 cm × 1.2 cm diam.) of DEAE-cellulose equilibrated with this buffer. The column was eluted with the equilibration buffer at 30 ml/h until the breakthrough protein peak (50 ml) had been collected and then with a linear (0–0.4 M) NaCl gradient in 400 ml of the same buffer. The fractions containing peaks X and Y (which also contained α -D-galactosidase activity) were each concentrated to 5 ml against polyethylene glycol and applied separately to a column (33 cm × 2.6 cm diam.) of Sephadex G-150 equilibrated with 5 mm-sodium phosphate buffer, pH 7.0. Form Y was almost completely separated from α -D-galactosidase by gel filtration under these conditions.

Electrophoresis in starch gel was carried out as described by Smithies (1955) using 11% hydrolysed starch in 5.3 mm-sodium phosphate buffer, pH 7.0, with 40 mm-sodium phosphate, pH 6.85, in the buffer compartments. After electrophoresis at 40 mA (220–250 V) for 4 h, enzyme activity on the cut surfaces of the gel was detected by incubation with buffered solutions of the appropriate 4-methylumbelliferyl glycoside as described by Robinson *et al.* (1967).

The kinetic parameters K_m and V were determined by the method of Lineweaver & Burk (1934).

Results

The spent growth medium of *P. polycephalum* was assayed for eight glycosidase activities using synthetic substrates. All the assays gave positive results but there was more than a 100-fold range of activities between β -N-acetyl-D-glucosaminidase and α -L-fucosidase (Table 1). The activities of β -N-acetyl-D-glucosaminidase and β -N-acetyl-D-galactosaminidase co-purify, have identical electrophoretic mobilities, and are probably attributable to the same enzymes (Kilpatrick & Stirling, 1975). These enzymes will

subsequently be referred to as β -N-acetyl-D-hexosaminidases, although 4-methylumbelliferyl β -D-glucosaminide was used as the routine substrate.

Kinetics of accumulation of glycosidases in the medium during growth

During the exponential phase of growth all the extracellular glycosidases showed a steady increase in concentration compared with the amounts introduced with the inoculum at zero time. When the activities were expressed as a function of the mass of the microplasmidia, however, the enzymes appeared to behave in one of two ways (Figs. 1a and 1b). The relative activity (arbitrary units per mg dry wt. of microplasmidia) of β -N-acetyl-D-hexosaminidase and β -D-glucosidase rose slowly but steadily until day 4 or 5 of culture respectively. In contrast the relative activities of α -D-galactosidase, β -D-galactosidase and α -D-mannosidase declined slightly over this period, reaching a minimum after 4 days of culture. Transition from exponential growth to the stationary phase was accompanied by a marked increase in the relative activities of all the enzymes.

Dependence of activity of glycosidases on pH

The pH-activity curves exhibited a number of noteworthy features. Each enzyme displayed its optimum activity between pH 3.0 and 5.5. The curve for β -N-acetyl-D-hexosaminidase was broad with an optimum at 4.25. The profiles for α -D-glucosidase and β -D-glucosidase were symmetrical and of similar shape with optima at pH 4.5 and 5.0 respectively.

β -D-Galactosidase had a single peak of activity at pH 3.0, but α -D-galactosidase had both a major peak at pH 3.5 and a smaller one at pH 6.75 possibly indicating the presence of two enzymes. α -D-Manno-

Table 1. *Glycosidase activities in the spent culture medium of P. polycephalum*

Details of assays are given in the Materials and Methods section. Assays for all enzymes were performed with 4-methylumbelliferyl glycosides except for α -D-glucosidase where *p*-nitrophenyl α -D-glucopyranoside was used. To determine α -D-galactosidase activity 5 mm substrate, buffered at pH 3.5, was used.

Enzyme	Activity (units/litre of medium)
β -N-Acetyl-D-glucosaminidase	160
β -N-Acetyl-D-galactosaminidase	150
β -D-Glucosidase	80
β -D-Galactosidase	30
α -D-Galactosidase	14
α -D-Glucosidase	8
α -D-Mannosidase	6
α -L-Fucosidase	1

Fig. 1. Kinetics of accumulation of glycosidases in the growth medium of *P. polycephalum*

Enzyme accumulation is expressed as activity (arbitrary units) per mg dry wt. of *P. polycephalum*: (a) β -N-acetyl-D-hexosaminidase (Δ) and β -D-glucosidase ($\blacksquare$); (b) α -D-galactosidase ($\bullet$), β -D-galactosidase ($\blacktriangle$) and α -D-mannosidase ($\circ$). α -D-Galactosidase was assayed using 0.2mM substrate buffered at pH 3.5. Full details of enzyme assays and dry wt. ($\square$) determination are given in the Materials and Methods section.

sidase had an optimum at pH 3.5. A biphasic curve was obtained for α -L-fucosidase with a broad peak at pH 3.0 and a taller and sharper peak at pH 5 and it is possible that α -L-fucosidase is present in two forms with different pH optima.

Multiple electrophoretic forms of glycosidases

In an attempt to relate the biphasic pH-activity curves to different forms of the glycosidases, a crude enzyme preparation was subjected to starch-gel electrophoresis. All the enzymes for which we had fluorogenic substrates were found to exist in at least two electrophoretic forms, usually one migrating a short distance and the other a much greater distance from the origin towards the anode (Fig. 2). The intensity of the fluorescent bands were assessed subjectively but no difference was apparent in the relative activity of the α -D-galactosidase bands when buffered substrate (5mM) was applied at pH 3.5 or 6.75. It appears unlikely therefore that the two electrophoretic forms correspond directly to the putative enzyme forms with these pH optima. The slower-migrating minor form of α -D-galactosidase was unstable but loss of this component had no appreciable effect on the pH-activity curve for α -D-galactosidase. No clear relationship between the pH-activity curves of α -L-fucosidases and the electrophoretic forms was established. β -N-Acetyl-D-hexosaminidase activity was clearly separated by electrophoresis under these

Fig. 2. Starch-gel electrophoresis of extracellular glycosidases

A crude glycosidase preparation was subjected to electrophoresis in starch gel (pH 7.0) for 4 h at 40mA and enzyme activity detected by incubation with appropriate substrates, as described in the Materials and Methods section. (1) β -N-Acetyl-D-glucosaminidase, (2) β -N-acetyl-D-galactosaminidase, (3) β -D-glucosidase, (4) β -D-galactosidase, (5) α -D-galactosidase (buffered at pH 3.5), (6) α -D-galactosidase (buffered at pH 6.75), (7) α -D-mannosidase, and (8) α -L-fucosidase. ■, ■ and □ denote high, medium and low intensity of fluorescence, judged subjectively.

conditions into two forms which we have called X and Y (Kilpatrick & Stirling, 1975), the latter being the more acidic.

Separation and partial purification of β -N-acetyl-D-hexosaminidases

The two major forms of β -N-acetyl-D-hexosaminidase, X and Y, identified by their behaviour on electrophoresis, were separated and partially purified from spent culture medium. The purification procedure also resulted in partial purification of α -D-galactosidase and the results for both these glycosidases are given in Table 2. β -N-Acetyl-D-hexosaminidase forms X and Y were purified 145- and

306-fold respectively and α -D-galactosidase, which was separated from β -N-acetyl-D-hexosaminidase Y by the final step of gel filtration, was purified 240-fold.

Separation of β -N-acetyl-D-hexosaminidases X and Y during the purification procedure was achieved by chromatography on a column of DEAE-cellulose. Approx. 5% of the activity recovered from the column was eluted with the breakthrough peak, and then forms X and Y were eluted in a linear NaCl gradient. A similar separation to the one obtained in the purification procedure is shown in Fig. 3.

Electrophoresis of the partially purified β -N-acetyl-D-hexosaminidases in starch gel showed that forms X and Y migrated as single bands of activity,

Table 2. *Partial purification of β -N-acetyl-D-hexosaminidase and α -D-galactosidase from *P. polycephalum**

Total activities are expressed as μ mol of 4-methylumbelliferone released per min at 37°C, specific activities as μ mol of 4-methylumbelliferone released/min per mg of protein at 37°C. α -D-Galactosidase was assayed using 0.2mM substrate buffered at pH3.5. All other conditions are described in the Materials and Methods section.

Purification step	β -N-Acetyl-D-hexosaminidase			α -D-Galactosidase		
	Total activity	Specific activity	Purification (fold)	Total activity	Specific activity	Purification (fold)
Culture medium	81.2	0.050	1	7.4	0.005	1
Ethanol treatment	74.1	0.050	1	10.6	0.007	1.4
(NH ₄) ₂ SO ₄ precipitation	30.1	0.425	8.5	2.0	0.028	5.6
Hydroxyapatite	14.3	1.07	21.4	1.26	0.094	18.8
DEAE-cellulose						
Form X	5.7	4.72	94			
Form Y	4.2	1.21	24	0.8	0.229	45.8
Sephadex G-150						
Form X	3.3	7.26	145			
Form Y	3.7	15.29	306	0.43	1.2	240

Fig. 3. *Fractionation of β -N-acetyl-D-hexosaminidase on DEAE-cellulose*

Glycosidases were partially purified from the culture medium of *P. polycephalum* by (NH₄)₂SO₄ precipitation and chromatography on hydroxyapatite, then applied to a column of DEAE-cellulose equilibrated at pH 7.0. After washing the column with the same buffer, a linear NaCl gradient (----) was begun, reaching 0.4M in 180ml. Fractions were assayed for β -N-acetyl-D-hexosaminidase activity by using either 4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucoside (○) or NN'-diacetylchitobiose (▲) as substrate. For further details see the Materials and Methods section.

from X corresponding to the slow-moving and from Y to the faster-moving electrophoretic forms present in the crude medium.

Samples from each of the purification steps were also assayed for the acid protease described by Farr *et al.* (1974). Protease activity was present in the β -N-acetyl-D-hexosaminidase preparation until its elution from DEAE-cellulose when it was fully separated from β -N-acetyl-D-hexosaminidase X and partially separated from β -N-acetyl-D-hexosaminidase Y. Elution from Sephadex G-150 completely separated the remaining protease activity from β -N-acetyl-D-hexosaminidase Y.

Comparison of β -N-acetyl-D-hexosaminidases X and Y

β -N-Acetyl-D-hexosaminidases X and Y were both eluted from Sephadex G-150 as symmetrical peaks with the same elution volume, corresponding to a mol.wt. of about 10^5 , and have a similar K_m value of approx. $3 \times 10^{-5} M$ with 4-methylumbelliferyl β -N-acetyl-D-glucosaminide at pH 4.5.

β -N-Acetyl-D-hexosaminidases X and Y were both remarkably stable when incubated at pH 4.5 and $70^\circ C$, neither form showing any loss of activity after 20 min. Incubation at pH values above and below pH 4.5 led to rapid denaturation of both forms of the enzyme. The difference in the stability of the two forms at $70^\circ C$ was greatest at pH 6.0; form X lost 70% of its activity and form Y lost 12% after 10 min incubation under these conditions. The difference in the pH-activity curves for β -N-acetyl-D-hexosaminidases X and Y (Fig. 4) may in part reflect this difference in pH stability.

Fig. 4. pH-activity curves of purified β -N-acetyl-D-hexosaminidases X and Y

The dependence of activity of form X (▲) and form Y (△) on pH was examined using the sodium citrate/phosphate buffers for the pH range 2.5–5.5 and the sodium phosphate buffers for the pH range 5.5–8.0. Each point represents the mean of four determinations.

The activity of hexosaminidases X and Y towards the disaccharide *NN'*-diacetylchitobiose was assayed in eluate fractions from a DEAE-cellulose column (Fig. 3). The profile of activities towards the 4-methylumbelliferyl glycoside and *NN'*-diacetylchitobiose were closely similar, suggesting that both β -N-acetyl-D-hexosaminidases X and Y hydrolyse both the synthetic substrates and the disaccharide, but the ratio of chitobiase to β -N-acetyl-D-hexosaminidase activities was higher in form Y than in form X. Chitobiase activity was still associated with both X and Y peaks after the final (Sephadex G-150) step in the purification scheme, but the chitobiase activity of form X (relative to β -N-acetyl-D-hexosaminidase activity) was now about three times that of form Y. It is likely that chitobiase activity also exists in a molecular form independent of β -N-acetyl-D-hexosaminidase and can be separated from form Y by gel filtration but we have made no attempt to identify this other form. In this respect, *P. polycephalum* chitobiase may be similar to that of human liver (Stirling, 1974).

Further properties of α -D-galactosidase

Dependence of the reaction rate of α -D-galactosidase on substrate concentration was measured at pH 3.5, 5.5 and 6.75 and similar K_m values of $6 \times 10^{-5} M$ were obtained. At pH 6.75 the double-reciprocal plot was linear but at pH 3.5 and 5.5 (Fig. 5) there was marked substrate inhibition. At pH 3.5 activity at 4.2 mM was 24% less than the activity calculated from extrapolation of the Lineweaver–

Fig. 5. Dependence of α -D-galactosidase reaction rate on substrate concentration

α -D-Galactosidase was assayed as described in the Materials and Methods section with the substrate buffered at pH 3.5 (■), pH 5.5 (○), or pH 6.75 (●). Values were plotted by the double-reciprocal method of Lineweaver & Burk (1934).

Fig. 6. Variation in pH-activity profile of α -D-galactosidase with substrate concentration

α -D-Galactosidase was assayed as described in the Materials and Methods section with a final substrate concentration of either 4.55 mM (○) or 0.18 mM (●).

Fig. 7. α -D-Galactosidase fractionation on DEAE-cellulose

Glycosidases partially purified from the culture medium of *P. polycephalum* by $(\text{NH}_4)_2\text{SO}_4$ precipitation and hydroxyapatite chromatography were next applied to a column of DEAE-cellulose equilibrated with 5 mM-sodium phosphate, pH 7.0, as described in the Materials and Methods section. The applied α -D-galactosidase activity remained bound to the column when washed with starting buffer, but was eluted with a linear NaCl gradient (----). Fractions were assayed for α -D-galactosidase using 0.2 mM substrate buffered at either pH 3.5 (○) or pH 6.75 (●).

Burk plot, and at pH 5.5 and the same substrate concentration it was 50% less.

Re-examination of the pH-activity curves of α -D-galactosidase at substrate concentrations of 0.18 and 4.55 mM showed that the profiles were strongly dependent on the substrate concentration (Fig. 6). It is possible that this behaviour might be due to the existence of multiple forms of the enzyme with overlapping pH-activity curves and differing in their susceptibility to substrate inhibition.

Some evidence for the existence of such multiple forms of the enzyme was obtained by examining the behaviour of α -D-galactosidase on a column of

DEAE-cellulose (Fig. 7). Two main peaks of activity were evident when the fractions were assayed at pH 3.5, although these were incompletely resolved. Activity detected at pH 6.75 was eluted as a single peak between them but whether this can be ascribed to the activity at pH 6.75 of the overlapping main peaks or to a distinct form of the enzyme is at present uncertain. The multiple forms of α -D-galactosidase appear to be the same size judging from their elution profile from Sephadex G-150. A single peak of α -D-galactosidase activity with a mol.wt. of approx. 60000 was obtained when a sample of crude extract was run on a calibrated Sephadex G-150 column (Andrews, 1964) and the eluate assayed at both pH 3.5 and 6.75.

The α -D-galactosidases have only weak activity towards the neutral glycolipid, ceramide trihexoside. The assay using human heart ceramide trihexoside (Ho, 1973) was performed at pH 4.0 and at 6.75 on a preparation of α -D-galactosidases obtained from the crude medium by $(\text{NH}_4)_2\text{SO}_4$ fractionation and gel filtration on Sephadex G-150, representing a 12-fold purification of the activity towards the fluorogenic substrate. Ceramide trihexosidase activity after 24 h incubation at 37°C was 2 munits/ml (as defined by Ho, 1973) at pH 4.0 and 0.24 munit/ml at pH 6.75. This corresponds to 0.03 and 0.01% respectively of the activity towards 4-methylumbelliferyl α -D-galactoside.

Discussion

Carbohydrates occur widely if not universally at the cell periphery, and their involvement in a number of cell-cell interaction phenomena has been suggested (Winzler, 1970; Cook & Stoddart, 1973). The appearance in the culture medium of enzymes that might degrade or modify the cell periphery of *P. polycephalum* could be an indication of their involvement in such processes.

In common with glycosidases from many sources the extracellular glycosidases of *P. polycephalum* have maximum activities at acid pH values. The pH-activity curves of α -D-galactosidase and α -L-fucosidase had two optima, which possibly indicates the existence of two forms of both these enzymes. We were not able to relate these putative enzyme forms with different pH optima to the isoenzymic forms of these enzymes revealed by electrophoresis. Each of the enzymes for which we had fluorogenic substrates was resolved into at least two forms by electrophoresis but the functions and origins of these multiple forms are not known, nor is the pattern of their expression during different phases of the life cycle.

β -N-Acetyl-D-hexosaminidases X and Y bear a striking resemblance to the A and B forms of the same enzyme from human tissues. The latter enzymes can also be separated by electrophoresis or ion-exchange

chromatography and have similar molecular weight, pH optimum and K_m values (Robinson, 1974). In contrast the more acidic form (Y) from *P. polycephalum* is the more stable of the two, whereas the more acidic human hexosaminidase (A) is less stable than form B. Both forms (X and Y) from *P. polycephalum* have β -N-acetyl-D-galactosaminidase as well as β -N-acetyl-D-glucosaminidase activities (Kilpatrick & Stirling, 1975). Co-purification of these hexosaminidase activities is of general occurrence (Findlay & Levvy, 1960; Woollen *et al.*, 1961; Muramatsu, 1968; Mega *et al.*, 1970; Li & Li, 1970; Every & Ashworth, 1973), although usually the β -N-acetyl-D-glucosaminidase is hydrolysed at a greater rate than the C4 epimer.

Evidence has been presented that the broad acidic band of α -D-galactosidase activity obtained after starch-gel electrophoresis may consist of two or more isoenzymic forms that have been partially separated on DEAE-cellulose. The acid forms of α -D-galactosidase are subject to substrate inhibition at concentrations above 1.0 mM when assayed at pH 3.5 and 5.5. This property is shared by the α -D-galactosidases of *Vicia faba* and the sweet almond (Dey & Pridham, 1972), and may indicate that the enzyme functions as a transferase at high substrate concentrations.

α -D-Galactosidase might act in concert with β -D-galactosidase to modify the extracellular slime, which is known to be a sulphated galactan with predominantly β -glycosidic links (McCormick *et al.*, 1970; Farr *et al.*, 1972). It has been suggested by Ross & Shipley (1973) that enzymic modification of the slime sheath might be a prerequisite for plasmodial fusion in *P. polycephalum* but it is difficult to envisage how the observed specificity of fusion could be based on such an enzyme-substrate system. Sublethal autolysis (Poste, 1971) of membrane-bound glycoproteins catalysed by these enzymes may hold greater possibilities for specific mechanisms.

The spent culture medium of *P. polycephalum* contains several β -(1 $\rightarrow$ 3)-glucanases (Farr *et al.*, 1973, 1975) and chitinase (G. A. Cook & J. L. Stirling, unpublished results) in addition to the exoglycosidases here described. This mixture of lytic enzymes could fulfil a number of physiological roles but when considering these it should be remembered that the activities of the enzymes towards synthetic substrates might not truly represent their hydrolytic potential for naturally occurring substrates. β -N-Acetyl-D-hexosaminidases X and Y both have appreciable activity towards NN'-diacetylchitobiose, a disaccharide likely to arise from the degradation of chitin, but α -D-galactosidase had very low activity towards the naturally occurring galactoside, ceramide trihexoside.

The kinetics of accumulation of various glycosidases in the culture medium were closely similar, increasing more or less in parallel with the mass of

microplasmodia during the exponential phase of growth and showing an increased rate of accumulation as the culture entered stationary phase. Prolonged incubation of microplasmodia under these conditions leads to differentiation into spherules, and it is possible that extracellular glycosidases are involved in this process. Huttermann (1973) has noted an increase in the activity of β -N-acetyl-D-glucosaminidase and β -glucosidase during the formation of spherules in a non-nutrient salts medium. Developmentally related secretion of glycosidases has also been described in *Dictyostelium discoideum* (Every & Ashworth, 1973) and *Polysphondylium pallidum* (O'Day, 1973), in both organisms the enhanced glycosidase secretion accompanying the transition from growth to differentiation.

Alternatively, the secretion of glycosidases may be part of a mechanism to locate fresh supplies of food. Release of monosaccharides from a distant potential food source by extracellular glycosidases could initiate a positive chemotactic response in plasmodia leading them to a source of nutrients. Plasmodia of *P. polycephalum* are known to respond by positive chemotaxis to solutions of certain sugars (Carlile, 1970).

Another possible function of extracellular glycosidases and other lytic enzymes bathing the plasmodium may be to confer non-specific immunity to the organism during early stages of differentiation before formation of the resistant wall of the spherules.

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