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Alkaline Phosphatase of *Blastocladiella emersonii*: Partial Purification and Characterization

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Alkaline phosphomonoesterase (EC 3.1.3.1) activity from *Blastocladiella emersonii*, while displaying typically broad substrate specificity for phosphorylated organic compounds, exhibited nearly complete substrate preference for *N*-acetylglucosamine-6-phosphate over *N*-acetylglucosamine-1-phosphate. Enzyme in zoospore extracts was purified 43-fold by differential centrifugation followed by gel filtration (Sephadex G-200) and then by ion-exchange chromatography (diethylaminoethyl-cellulose). The partially purified enzyme displayed an apparent molecular weight (Sephadex G-200) of ~170,000. The activity of partially purified enzyme exhibited a pH optimum of pH 8.5, did not require a metal divalent cation, but was inhibitable by ethylenediaminetetraacetic acid. During the life cycle of the organism, the specific activity of the phosphatase decreased slightly during germination and early exponential growth but then increased about 4.5-fold during sporulation. *B. emersonii* alkaline phosphatase does not appear to be a repressible enzyme.

This paper reports some characteristics of an alkaline phosphomonoesterase (EC 3.1.3.1) activity present in extracts of the aquatic phycomycete, *Blastocladiella emersonii*. This enzyme activity became of interest to us because of the following information. (i) The enzyme activity was initially observed (C. P. Selitrennikoff and D. R. Sonneborn, Biochim. Biophys. Acta, in press) during the development of a forward-direction assay for the third pathway-specific enzyme of hexosamine biosynthesis, acetylaminodeoxyglucose phosphomutase (EC 2.7.5.2). Under the conditions of assay, zoospore extracts were capable of dephosphorylating the substrate for the above enzyme activity (i.e., *N*-acetylglucosamine-6-phosphate [GlcNAc-6-P]) but not the product (i.e., *N*-acetylglucosamine-1-phosphate [GlcNAc-1-P]). (ii) The hexosamine biosynthetic pathway appears to be feedback inhibited in the zoospore by virtue of end-product (uridine-5'-diphospho-*N*-acetylglucosamine [UDPGlcNAc]) inhibition of the first pathway-specific enzyme activity (9, 13). Accompanying the de novo formation of the cell wall during zoospore germination, chitin is abruptly synthesized and inhibition of the hexosamine biosynthetic pathway is abruptly relieved (13; Selitrennikoff and Sonneborn, Dev. Biol., in press). (iii) In *B. emersonii* extracts, *N*-acetylglucosamine (GlcNAc) can stimulate the incorporation of radioactivity from uridine 5'-diphosphate (UDP)-[^{14}C]GlcNAc into chitin. Moreover, in the presence of unlabeled UDPGlcNAc, *B. emersonii* chitin synthetase can catalyze the incorporation of [^{14}C]GlcNAc into chitin and this incorporation cannot be accounted for by an exchange reaction between GlcNAc and UDPGlcNAc (2).

The presence of GlcNAc-6-P dephosphorylating activity in zoospore extracts provided a possible route whereby the production of GlcNAc for the chitin synthetase reaction could be regulated in concert with the regulation of the hexosamine biosynthetic pathway. As such, it was deemed of value to characterize this enzyme activity.

MATERIALS AND METHODS

Chemicals. Diethylaminoethyl (DEAE)-cellulose (exchange capacity, 0.95 meq/g), bovine serum albumin (BSA), deoxyribonucleic acid (DNA, type 1, calf thymus), β-galactosidase, aldolase, and various organophosphate compounds were purchased from Sigma Chemical Co. Yeast ribonucleic acid (RNA) was obtained from Schwarz Bio Research, Inc., whereas apoferritin and α-chymotrypsinogen were from Calbiochem and Schwarz/Mann, respectively. Sephadex G-200 was from Pharmacia Fine Chemicals, Inc. GlcNAc-1-P, GlcNAc-6-P, and [1-^{14}C]-GlcNAc-6-P were synthesized and characterized by methods described elsewhere (Selitrennikoff and Sonneborn, Biochim. Biophys. Acta, in press). All other chemicals were of reagent grade, and double-distilled water was used.

Procedures: (i) growth of *B. emersonii*. Zoospores were grown, harvested, and lyophilized as described previously (13). For the experiments examining enzyme activity throughout the life cycle (see Fig. 8),

zoospore inocula were harvested by flooding first-generation cultures grown on peptone-yeast extract-glucose (PYG) agar with distilled water (after 12 h of incubation at 27°C or 24 h of incubation at 20°C). The harvests were filtered through Whatman 541 paper and washed once by centrifugation (200 × *g*; 10 min); both procedures were performed sterilely and in the cold (4°C). The washed inocula were added to 10 liters of filter-sterilized liquid medium in a New Brunswick Microfirm Fermentor at a final cell density of 0.7×10^5/ml. The liquid medium was a modification of the previously reported defined growth medium (14) containing twice the original concentration of the amino acid mixture, 0.3 mg of glucose per ml, 0.4 mg of glutamic acid per ml, 0.04 μg of thiamine per ml, 10^{-2} M NaCl, 10^{-4} M Na_3PO_4, 5×10^{-3} M NH_4NO_3, 10^{-3} M $MgCl_2$, trace metals (per liter: 0.6 mg of $FeSO_4$, 0.2 mg of $MnSO_4$, 0.2 mg of $ZnSO_4$, 0.1 mg of $CuSO_4$), 3.5×10^{-4} M tris(hydroxymethyl)aminomethane (Tris)-maleate buffer (pH 6.8), and a mixture of KOH and KCl to bring the final pH to pH 6.8 and the final K^+ concentration to 10^{-2} M. The fermentor was maintained at 27°C with the propeller rotating at 400 to 500 rpm. Cultures were aerated with an O_2-air mixture (0.5:2.8) at 9 to 10.5 liters/min and were in the exponential phase of growth (dry weight doubling time, approximately 1.4 h) from no later than 2 h of incubation until the time of induced sporulation. To induce sporulation, growth cultures were filtered and washed over Whatman No. 1 paper, transferred to the same volume of sporulation solution (10^{-3} M $CaCl_2$ in 10^{-3} M Tris-maleate buffer, pH 6.8) in the fermentor, and stirred and aerated as above. At various times during the life cycle, samples were removed, washed, and lyophilized.

(ii) Preparation of crude extracts. Extracts of zoospores derived from PYG-agar cultures were prepared by suspending lyophilized cells in ice-cold 0.05 M Tris-hydrochloride (pH 8.5) and passing the suspension through Pasteur pipettes several times. Extracts of cell types (including zoospores) obtained from liquid cultures were prepared by suspending lyophilized cells (~5 mg) in 1 ml of 0.05 M Tris-hydrochloride (pH 8.5) and disrupting them by using a Sonifier Cell Disruptor (50 W, three 10-s bursts with 1-min intervals to allow for cooling). The extracts were centrifuged for 10 min at 20,000 × *g* (4°C), and the supernatant portions were retained.

Enzyme assays: (i) alkaline phosphatase. Enzyme activity was measured by the release of orthophosphate from certain phosphorylated substrates (routinely, GlcNAc-6-P); orthophosphate liberated was estimated by the method described by Ames (1), using phosphate buffer as standard. Reaction mixtures routinely contained 7.4 mM GlcNAc-6-P and 175 μl of 0.05 M Tris-hydrochloride (pH 8.5) and were initiated by addition of cell extracts or column fractions derived from zoospore extracts (final volume, 200 μl). Reactions were terminated by boiling for 1 min. The change in absorbance at 820 nm of incubated reaction mixtures containing active extracts was subtracted from that of incubation mixtures lacking substrate. For purified fractions, the subtraction was unnecessary. For certain experiments, blanks lacking cell extract were included. Units of enzyme activity are defined as nanomoles of phosphate liberated per minute at 25°C.

(ii) Ribonuclease activity. Enzyme activity of partially purified zoospore phosphatase was measured by using slight modifications of the procedures described by Ishikawa et al. (5). Reaction mixtures contained 200 μg of RNA and 0.9 μg of partially purified zoospore enzyme protein in 200 μl of 0.05 M Tris-hydrochloride (pH 8.5). Reactions were terminated by the addition of 100 μl of 15% perchloric acid. After incubation in ice for 10 min, the mixtures were clarified by centrifugation, a 200-μl sample was diluted 10-fold, and the optical density at 260 nm was determined.

(iii) Deoxyribonuclease activity. Enzyme activity was measured by a modification of the method of Kunitz (7). A 10-mg amount of DNA was suspended in 30 ml of Tris-hydrochloride (pH 8.5) and left overnight at 4°C. A 2.5-ml sample of the DNA solution was added to 0.5 ml (18.5 μg of protein) of partially purified zoospore enzyme, and the increase in optical density of 260 nm was compared to that of a blank (no enzyme added).

(iv) Protein concentration. The method of Lowry et al. (8) was used with BSA as a standard. Specific enzyme activity is defined as units per milligram of protein.

Column chromatography: (i) Sephadex G-200. Samples were applied to a column (40 by 2 cm) previously equilibrated with 0.05 M Tris-hydrochloride (pH 8.5) and developed with the same buffer. One-milliliter fractions were collected.

(ii) DEAE-cellulose. DEAE-cellulose was washed by the method of Peterson and Sober (11). A column (20 by 1.5 cm) was prepared and equilibrated with 5 liters of 0.05 M Tris-hydrochloride (pH 8.5). Samples were applied to the column, and the column was washed with 50 ml of buffer and developed with a 0 to 0.2 M linear KCl gradient (in buffer) with 2-ml fractions collected.

RESULTS

Phosphatase activity of zoospore extracts. To document the presence of the enzyme activity, low-speed (10,000 × *g*) supernatants of crude extracts from agar culture-derived zoospores (see Materials and Methods) were incubated with [1-^{14}C]GlcNAc-6-P. At each time point, two samples were taken: one sample was assayed for inorganic phosphate released from GlcNAc-6-P and the other sample was separated by descending chromatography using 1 M ammonium acetate (pH 7.0)-ethanol (3:7, vol/vol) as solvent. Autoradiograms of the chromatographs revealed only two radioactive spots, one of the radioactive substrate and the other at the position of authentic GlcNAc. The latter spot was not observed in autoradiograms of zero-time mixtures or if boiled extracts were used, but when present, the corresponding area on the chromatograph gave a positive Morgan-

Elson test (12) for *N*-acetylhexosamine. Such areas were cut out, and the radioactivity was determined. The nanomolar amounts of free orthophosphate and of (radioactive) GlcNAc produced from GlcNAc-6-P in the reaction mixtures were indistinguishable at each time point and the appearance of either product was linear with time for at least 30 min (Fig. 1).

Partial purification of zoospore phosphatase activity. Crude extracts from agar culture-derived zoospores were subjected to differential centrifugation, and each fraction was assayed for phosphatase activity using GlcNAc-6-P as substrate. The data presented in Table 1 demonstrate that the vast majority of the activity was recovered in the high-speed supernatant. When such supernatants were fractionated by Sephadex G-200 column chromatography, one major peak and one minor peak of phosphatase activity were observed and the former peak was separated from a major peak of total protein (Fig. 2). When the fractions from the major phosphatase peak were pooled and chromatographed on DEAE-cellulose (elution with a linear gradient of KCl), a major and a minor peak of phosphatase activity were observed, again with the major peak separated from significant amounts of the remaining bulk protein (Fig. 3). The fractions from this major peak were pooled, dialyzed against buffer to remove KCl, and retained for further characterization. Table 2 presents a summary of the results obtained through the successive steps in the enzyme purification procedure. The approximately 43-fold-purified enzyme preparation was completely destroyed by freeze-thaw or lyophilization, but was completely stable for at least 10

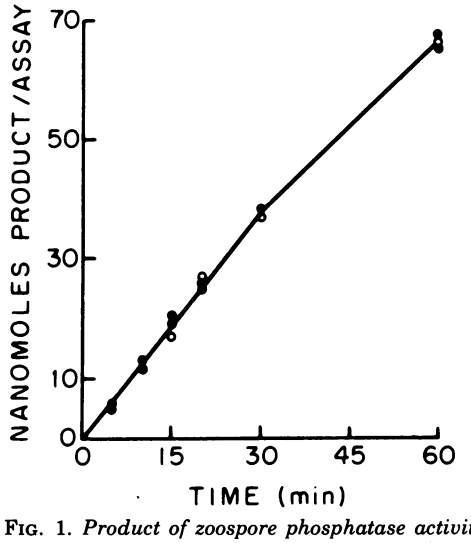

FIG. 1. *Product of zoospore phosphatase activity. Zoospore extract (10,000 × g supernatant; 310 μg of protein) was incubated with 7.2 mM [1-^{14}C]GlcNAc-6-P (2.8 × 10^{-2} μCi/μmol) in a final volume of 3.2 ml. At the indicated times 200-μl samples were removed for phosphate determinations (●) and 50-μl samples were removed for determination of GlcNAc production (○). The latter samples were boiled, quantitatively spotted on Whatman No. 1 paper, and separated by descending chromatography. Material comigrating with authentic GlcNAc was cut out and the radioactivity was determined (liquid scintillation counting).*

TABLE 1. *Distribution of zoospore phosphatase activity in centrifugal fractions*[a]

Fraction	Total units	Activity	Percent of crude lysate
Crude lysate	750	68	100
10,000 × *g* supernatant	724	75.4	96.5
10,000 × *g* pellet	25	6	3.3
100,000 × *g* supernatant	720	85	96
100,000 × *g* pellet	19	12	2.5

[a] Lyophilized zoospores (20 mg) were disrupted in 3 ml of 0.05 M Tris-hydrochloride (pH 8.5) and centrifuged for 20 min at 10,000 × *g*. The resulting pellet was resuspended in 2 ml of buffer and recentrifuged as above. The supernatants were pooled and centrifuged at 100,000 × *g* for 60 min (4°C). Phosphatase activity and protein concentration were determined as described in Materials and Methods.

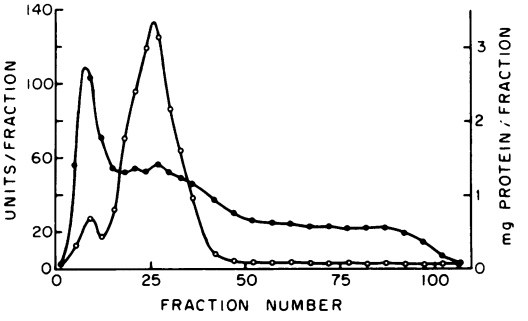

FIG. 2. *Fractionation of zoospore high-speed supernatant by Sephadex G-200 chromatography. Lyophilized zoospores (350 mg) were disrupted in 4 ml of 0.05 M Tris-hydrochloride (pH 8.5) and processed exactly as in Table 1 with the exception that the low-speed pellet was washed twice by resuspension in 1 ml of buffer. The 100,000 × g supernatant (5 ml) was carefully removed, applied to a Sephadex G-200 column, and chromatographed exactly as described in the text. From the indicated fractions, 50-μl samples were assayed for phosphatase activity (○) and for protein concentration (●) as described in the text.*

weeks at 4°C. (Samples were frozen and thawed or lyophilized or dried on a Virtis Biodryer immediately or after the addition of 0.5 M sucrose, 0.1 M sucrose, 1 mg of BSA per ml, 1 mg of ovalbumin per ml, 5 mM $MgCl_4$, and 5 mM $CaCl_2$. Less than 5% recovery of enzyme activity resulted from any of these treatments.)

Partial characterization of 43-fold-purified zoospore phosphatase activity. As with crude enzyme activity (see Fig. 1), the production of orthophosphate from GlcNAc-6-P by the partially purified phosphatase was linear for 25 min at 25°C and linear with respect to protein concentration (0.37 to 3.7 μg of protein per assay).

pH optimum. The data presented in Fig. 4 demonstrate that the partially purified enzyme activity had a pH optimum of pH 8.5 with a broad shoulder extending to the highest pH tested—pH 9.75. Identical results were obtained using 10,000 × *g* supernatants derived from zoospores separately lysed in 0.05 M Tris buffer adjusted to the different pH values.

Substrate specificity. The apparent K_m with respect to GlcNAc-6-P was graphically determined to be approximately 5×10^{-3} M (Fig. 5), the same as that for crude enzyme preparations (10,000 × *g* supernatants). The K_m and V_{max} were identical when 6 mM GlcNAc was included in reaction mixtures (data not presented).

The relative rates of hydrolysis of various

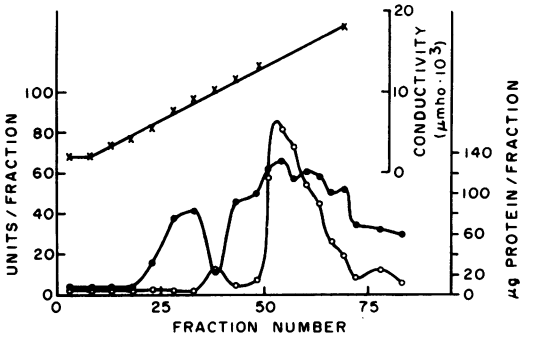

FIG. 3. *DEAE-cellulose chromatography. Peak fractions from Fig. 2 containing ≥40 units of enzyme activity were pooled (16 ml) and chromatographed on DEAE-cellulose exactly as described in the text. The indicated fractions were assayed for phosphatase activity* (○) *and protein concentration* (●) *using 100-μl and 200-μl samples, respectively. Conductivity was determined using a YSI conductivity bridge* (×).

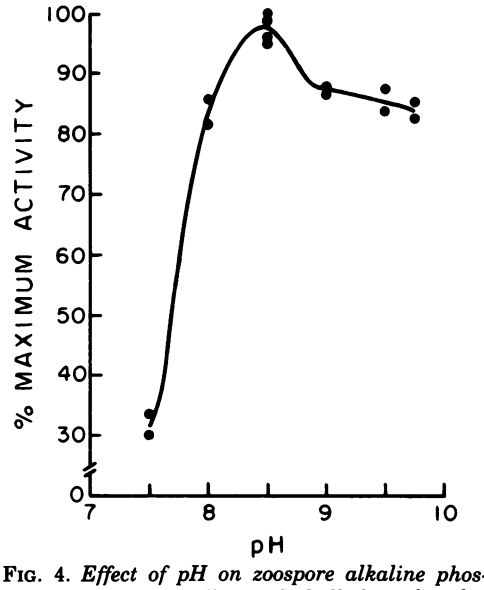

FIG. 4. *Effect of pH on zoospore alkaline phosphatase activity. Partially purified alkaline phosphatase (0.3 μg of protein) and 7.4 mM GlcNAc-6-P were incubated for 30 min in 0.05 M Tris-hydrochloride adjusted to the indicated pH. After incubation, 200-μl samples were assayed for phosphate released as described in the text. The points represent individual determinations.*

TABLE 2. *Partial purification of zoospore phosphatase activity*

Fraction	Total units	Protein (mg)	Sp act	Yield (%)	Purification
Crude lysate	9,311	126	73.9		1
High-speed supernatant	9,545	96.5	98.9	102	1.3
Sephadex G-200[a] chromatography	5,151	18.1	284.6	55	3.8
DEAE-cellulose[b] chromatography			3,172	41	42.9

[a] Represents assays on the pooled fractions containing ≥40 units of phosphatase activity in Fig. 2.

[b] Represents assays on the pooled fractions containing ≥40 units of phosphatase activity in Fig. 3. The pooled fractions were dialyzed twice against 100 volumes of 0.05 M Tris-hydrochloride (pH 8.5) prior to assay.

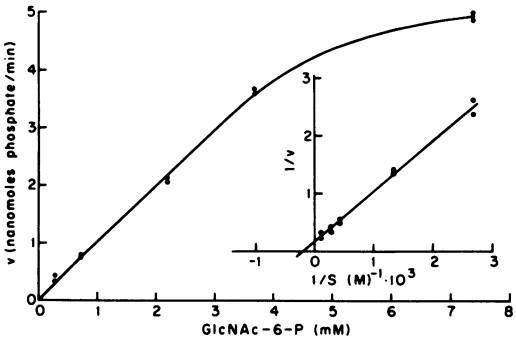

FIG. 5. *Effect of varying GlcNAc-6-P concentration on zoospore alkaline phosphatase activity. Partially purified zoospore alkaline phosphatase (0.37 μg of protein) was incubated with the indicated concentrations of GlcNAc-6-P. After 0, 5, and 10 min of incubation, 200-μl samples were assayed for phosphate released as described in the text, and the velocity (nanomoles of phosphate per minute per assay) was calculated. Points represent individual determinations.*

phosphorylated organic compounds by the partially purified enzyme activity is documented in Table 3. Clearly, the substrate specificity of the zoospore phosphatase is broad; however, phosphodiesterase, ribonuclease, deoxyribonuclease, or GlcNAc-1-P phosphatase activities were not detected.

Ion requirements. Partially purified enzyme activity was neither enhanced nor inhibited (90 to 104% of control activity) by the addition of up to 10 mM NaCl, KCl, $CaCl_2$, $MgCl_2$, or $MnCl_2$. However, $ZnSO_4$ and NaF could mildly inhibit enzyme activity and, at 1 mM ethylenediaminetetraacetic acid (EDTA), enzyme activity was markedly inhibited (Table 4). Dialysis of the 43-fold-purified enzyme activity against distilled water led to a partial loss of enzyme activity which was not restored by the addition of up to 5 mM $MgCl_2$. The enzyme activity of the dialyzed preparation remained further inhibitable by 1 mM EDTA (Table 4).

Heat stability. The effects of incubation of partially purified phosphatase activity at various temperatures is shown in Fig. 6. Further incubation at 30°C for up to 4 h resulted in no apparent decrease in enzyme activity.

Apparent molecular weight. Chromatography of the partially purified enzyme preparation on Sephadex G-200 revealed a single homogenous peak of activity which corresponded to an apparent molecular weight of about 170,000 (Fig. 7).

Alkaline phosphatase activity during the life cycle of *B. emersonii*. The specific activity of alkaline phosphatase was monitored during the life cycle utilizing 10,000 × *g* supernatants of cell lysates and GlcNAc-6-P as substrate (Fig. 8). The specific enzyme activity of agar culture-derived zoospores decreased to about 70% of the initial value during the first 5.5 h of incubation in defined liquid growth medium (exponential growth was achieved by no later than 2 h of incubation; the specific activity remained constant at this level during three additional hours of exponential growth; data not shown). In contrast, during sporulation, specific activity abruptly increased by more than 4.5-fold and was highest in the zoospores released into the sporulation solution (last time point, Fig. 8). Neither the rate nor extent of specific activity increase during sporulation was affected by the inclusion of up to 10^{-3} M inorganic phosphate in the sporulation solution (data not shown).

DISCUSSION

Zoospores of *B. emersonii* were found to contain alkaline phosphomonoesterase activity capable of metabolizing GlcNAc-6-P to GlcNAc and inorganic phosphate. The zoospore phos-

TABLE 3. *Substrate specificity of partially purified zoospore alkaline phosphatase activity*[a]

Substrate	Relative rate of hydrolysis
GlcNAc-6-P	100
2-Deoxyglucose-6-phosphate	104
Galactose-6-phosphate	95
Fructose-6-phosphate	52
Glucosamine-6-phosphate	100
Fructose-1,6-diphosphate	75
GlcNAc-1-P	<5
p-Nitrophenyl-phosphate	190
Bis-*p*-nitrophenyl-phosphate	<3
UDPGlcNAc	<5
DNA	<1
RNA	<5
Uridine-5'-monophosphate	210
Adenine-5'-monophosphate	310
Inosine-5'-monophosphate	300
Phosphoenolpyruvate	213
cAMP	<8
cUMP	<17
Phosvitin	<8
Phosphoserine	160
Phosphothreonine	190

[a] The indicated compounds were incubated with 0.9 μg of protein of partially purified phosphatase activity and the rate of hydrolysis relative to that of GlcNAc-6-P was determined. The concentrations of all compounds except DNA (50 μg per assay), RNA (200 μg per assay), and phosvitin (25 μg per assay) were 5 mM. cAMP, Cyclic adenosine 3',5'-monophosphate; cUMP, cyclic uridine 3',5'-monophosphate.

TABLE 4. *Effect of various compounds on partially purified zoospore alkaline phosphatase activity*

Additions to reaction mixture[a]	Sp act	Percent of control
None	3,172	
$ZnSO_4$, 0.5 mM	2,410	76
$ZnSO_4$, 1 mM	2,380	75
NaF, 5 mM	2,410	76
NaF, 10 mM	2,060	65
EDTA, 0.1 mM	2,695	85
EDTA, 1.0 mM	950	30
Dialysis against water[b]	2,030	64
$MgCl_2$, 5 mM[c]	2,125	67
EDTA, 1 mM[d]	985	31

[a] The indicated compounds were added to reaction mixtures containing 7 mM GlcNAc-6-P and 0.9 μg of partially purified enzyme preparation. After 15 min of incubation at 25°C, reaction mixtures were assayed for phosphate released as described. Each number represents the average of at least two determinations.

[b] A 500-μl sample of the partially purified enzyme preparation was dialyzed against 4 liters (twice) of ice-cold distilled water. The protein concentration was redetermined and 0.9 μg was assayed for activity as above and compared to the activity of an undialyzed control.

[c] $MgCl_2$ (5 mM) was added to a sample of the preparation described in footnote *b* and assayed for phosphatase activity as described in footnote *a*.

[d] Same as in footnote *c* except 1 mM EDTA was added instead of 5 mM $MgCl_2$.

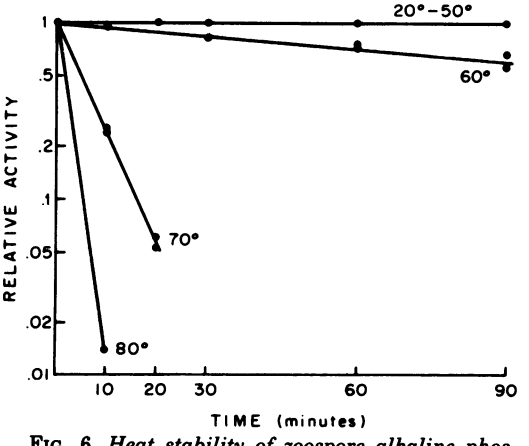

FIG. 6. *Heat stability of zoospore alkaline phosphatase activity. Samples (500 μl; 18.5 μg of protein) of the partially purified phosphatase were incubated at the indicated temperatures for selected times (x-axis) after which 25-μl samples were removed and incubated at 25°C for 15 min in 175 μl of 0.05 M Tris-hydrochloride (pH 8.5) containing 7.4 mM GlcNAc-6-P. Each reaction mixture was then assayed for the amount of phosphate released as described in the text. Each point represents an individual determination.*

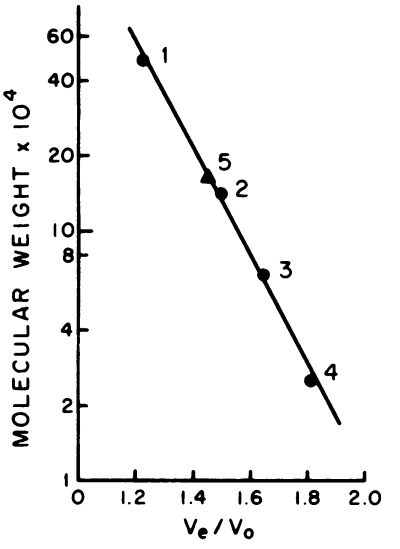

FIG. 7. *Determination of molecular weight by Sephadex G-200 column chromatography. Partially purified zoospore alkaline phosphatase (37 μg of protein) was applied to a Sephadex G-200 column (40 by 2 cm) and eluted with 0.05 M Tris-hydrochloride (pH 8.5). A 200-μl sample of each 1-ml fraction was assayed for phosphatase activity as described in Materials and Methods. The position of the peak (5) was compared to those for the following standards: (1) apoferritin, 460,000 to 490,000 daltons; (2) aldolase, 140,000 daltons; (3) BSA, 67,000 daltons; (4) α-chymotrypsinogen, 25,000 daltons. Positions of standards were determined by absorbance at 230 nm.*

phatase exhibits several properties typical for "nonspecific" alkaline phosphatase activity: pH profile, broad substrate phosphomonoesterase activity, "solubility" after differential centrifugation, and estimated molecular weight of approximately 170,000, similar to the 150,000 to 185,000 molecular weight range reported for the enzyme from several other fungi (3, 6). However, special note should be made of three properties not commonly reported for other alkaline phosphatase activities. First, the nearly complete specificity of the enzyme activity for GlcNAc-6-P over GlcNAc-1-P deserves comment. These two compounds have not previously been tested as substrates with other alkaline phosphatases, as far as we are aware, but some sugar -6- and -1- phosphates have been tested (especially glucose) and do not exhibit nearly the extent of difference as is reported here. The potential interest in this difference is that the two compounds are, respectively, the substrate and product of the third enzyme in the hexosamine biosynthetic pathway (see below). The second property is that, in contrast to alkaline phosphatase activity studied in sev-

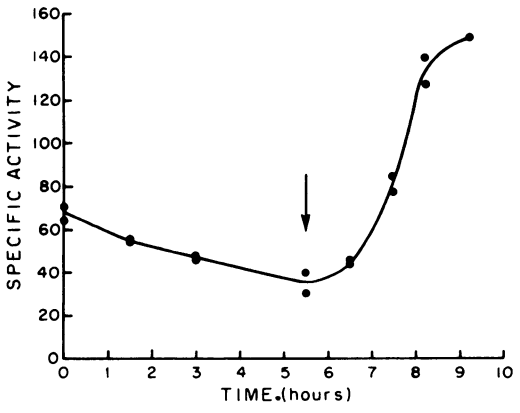

FIG. 8. *Specific activity of alkaline phosphatase during the life cycle of B. emersonii. Zoospores (0.7 × 10⁹) were inoculated into 10 liters of defined liquid growth medium (see text). After 5.5 h of growth (↓), cells were harvested by filtration, the medium was replaced with sporulation solution, and the cells were incubated for an additional 3.75 h. At the indicated times, samples were harvested, washed, and lyophilized. Crude cell extracts (10,000 × g supernatants) were assayed for phosphatase activity and protein concentration as described in the text. Each point represents an individual determination. For the last time point, the released zoospores were separated from the empty sporangial "ghosts" by filtration and centrifugation.*

eral other organisms, zoospore alkaline phosphatase activity does not appear to require a divalent metal ion for maximal activity. However, the observation that EDTA inhibits enzyme activity may mean that a metal ion-containing moiety associated with the phosphatase (from which the metal ion is only partially removed at best by dialysis against distilled water) is required. The third property is that the *B. emersonii* alkaline phosphatase does not appear to be a repressible enzyme whose in vivo level is sensitive to the inorganic phosphate concentration in the medium. Repressibility has been documented with several lower eukaryotic organisms (4, 10, 15), but the levels of *B. emersonii* enzyme activity through the life cycle were not affected by altering the phosphate level from 0.1 to 1.0 mM in the growth medium (data not shown) or by the inclusion of up to 1 mM phosphate in the sporulation solution. (However, a twofold difference in specific activity was observed between zoospores derived from PYG-agar cultures and from defined medium liquid cultures [Fig. 8] and this difference was reproducible. The cause of this difference remains unknown.) These data suggest that the changes in alkaline phosphatase activity through the life cycle, and particularly during sporulation (Fig. 8), may indeed be developmentally regulated.

In the zoospore, both the hexosamine biosynthetic pathway and chitin synthetase appear to be post-translationally regulated (see above; 13; Selitrennikoff and Sonneborn, Dev. Biol., in press). During germination, the hexosamine pathway is abruptly activated to provide substrates for the chitin synthetase reaction. The latter reaction appears to require both UDPGlcNAc, the end product of hexosamine synthesis, and GlcNAc as substrates for maximal activity (2). GlcNAc may be produced from one or both of two possible routes: one utilizing UDPGlcNAc as substrate and producing UDP and GlcNAc, the other utilizing zoospore alkaline phosphatase activity and GlcNAc-6-P as substrate. The former enzyme activity, if present, is presumably associated with easily sedimentable particles, as is chitin synthetase activity (see Selitrennikoff and Sonneborn, Dev. Biol., in press, for discussion), whereas the latter enzyme activity resides in the "soluble" fraction after differential centrifugation (Table 1), as do all four enzyme activities of the hexosamine pathway (Selitrennikoff and Sonneborn, Dev. Biol., in press). The route of GlcNAc production via the zoospore phosphatase should be regulated by the concentration of UDPGlcNAc and by the ratio of UDPGlcNAc to UDP plus uridine 5'-triphosphate concentrations since GlcNAc-6-P is an intermediate in the hexosamine biosynthetic pathway and the entire pathway appears to be regulated by the feedback of the above metabolites on the first pathway-specific enzyme activity (13; Selitrennikoff and Sonneborn, Dev. Biol., in press). More discriminating tests of the involvement of alkaline phosphatase during germination as well as during sporulation await the selection of appropriate temperature-conditional mutants.

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