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PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR ACID PHOSPHATASE OF *TETRAHYMENA PYRIFORMIS*

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An extracellular acid phosphatase secreted into the medium during growth of *Tetrahymena pyriformis* strain W was purified about 900-fold by $(NH_4)_2SO_4$ precipitation, gel filtration and ion exchange chromatography. The purified acid phosphatase was homogeneous as judged by polyacrylamide gel electrophoresis and was found to be a glycoprotein. Its carbohydrate content was about 10% of the total protein content. The native enzyme has a molecular weight of 120000 as determined by gel filtration and 61000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The acid phosphatase thus appears to consist of two subunits of equal size. The amino acid analysis revealed a relatively high content of aspartic acid, glutamic acid and leucine. The purified acid phosphatase from *Tetrahymena* had a rather broad substrate specificity; it hydrolyzed organic phosphates, nucleotide phosphates and hexose phosphates, but had no diesterase activity. The K_m values determined with *p*-nitrophenyl phosphate, adenosine 5′-phosphate and glucose 6-phosphate were $3.1 \cdot 10^{-4}$ M, $3.9 \cdot 10^{-4}$ M and $1.6 \cdot 10^{-3}$ M, respectively. The optima pH for hydrolysis of three substrates were similar (pH 4.6). Hg^{2+} and Fe^{3+} at 5 mM were inhibitory for the purified acid phosphatase, and fluoride, L-(+)-tartaric acid and molybdate also inhibited its activity at low concentrations. The enzyme was competitively inhibited by NaF ($K_i = 5.6 \cdot 10^{-4}$ M) and by L-(+)-tartaric acid ($K_i = 8.5 \cdot 10^{-5}$ M), while it was inhibited noncompetitively by molybdate ($K_i = 5.0 \cdot 10^{-6}$ M). The extracellular acid phosphatase purified from *Tetrahymena* was indistinguishable from the intracellular enzyme in optimum pH, K_m, thermal stability and inhibition by NaF.

Introduction

Extracellular enzymes have been commonly found in bacteria [1] and phytoflagellate [2]. Large amounts of lysosomal acid hydrolyses were also found in the cultured medium of lower eukaryotic cells, *Tetrahymena* [3] and *Dictyostelium* [4]. We have previously reported that the extracellular protease released by *Tetrahymena* was almost identical to the cellular enzyme [5]. However, whether the presence of extracellular protease was due to true secretion or due to egestion by a different route has not as yet been clarified. Hasilik and Neufeld [6] suggested that the structure of carbohydrate moiety, especially mannose 6-phosphate residue, may be important in processing of lysosomal enzymes biosynthesis and in secretion of lysosomal enzymes. Like most mammalian lysosomal enzymes, acid phosphatases of bacteria [7,8] and *Rhodotorula glutinis* [9] have also been demonstrated to be glycoproteins. Therefore, it seems to be of interest whether *Tetrahymena* lysosomal enzymes contain the carbohydrate moiety with such a recognition marker. In our previous paper it was

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not proved whether the highly purified extracellular protease of *Tetrahymena* contained the carbohydrate moiety [10]. In this paper we have extended the previous work on extracellular protease to acid phosphatase by comparing properties of intra- and extracellular enzymes in *Tetrahymena.*

Materials and Methods

Materials. The chemicals used were of the highest purity available and all substrates and inhibitors used were purchased from Sigma Chemical Co. Sephacryl S-300, CM-Sephadex and DEAE-Sephadex were obtained from Pharmacia Fine Chemicals, Inc. The marker proteins for the determination of molecular weight were obtained from Seikagaku Kogyo Co., Japan.

Cell growth. Tetrahymena pyriformis strain W was grown with shaking at 28°C in an enriched 2% proteose/peptone medium as previously described [11].

Enzyme assays. Acid phosphatase activity was routinely assayed by incubating for 10 min at 37°C with *p*-nitrophenyl phosphate as substrate. The assay mixture (1.0 ml total volume) contained 0.5 ml of 15 mM *p*-nitrophenyl phosphate in acetate buffer, 0.2 ml of 0.25 M acetate buffer, pH 4.6, and 0.2 ml homogenate which contained 20–30 μg total protein or 0.2 ml cultured medium. The reaction was initiated by the addition of substrate and was terminated by adding 2 ml of 0.1 M NaOH. The released *p*-nitrophenol was measured at 410 nm in a Hitachi Model 139 Spectrophotometer by the method of Bessey et al. [12]. The rate of reaction was linear with respect to time and enzyme concentration under these conditions.

Enzyme activity toward a number of other phosphate esters was determined by the liberation of inorganic phosphate. Assay mixtures (1.0 ml total volume) contained 0.5 ml substrate solution in buffer, 0.2 ml of 0.25 M acetate buffer, pH 4.6, and 0.2 ml enzyme solution. After incubation for 10–15 min the reaction was stopped by the addition of 1.0 ml of 10% trichloroacetic acid. The liberated inorganic phosphate was determined by the method of Chen et al. [13] as modified by Ames and Dubin [14].

Enzyme units are expressed as μmol *p*-nitrophenol or inorganic phosphate liberated per min.

Protein was determined by the method of Lowry et al. [15] with crystalline bovine serum albumin as standard.

Purification procedure. After removal of cells by centrifugation at $1500 \times g$ for 5 min, solid ammonium sulfate to 35% saturation was added to the medium (40 l) and the solution was stirred on the magnetic stirrer for 60 min. After subsequent centrifugation at $12000 \times g$ for 30 min, the resulting supernatant fraction was collected, to which was added solid ammonium sulfate to 55% saturation and after gentle stirring the suspension was centrifuged as before. The precipitated protein was then redissolved in 40 ml of 0.1 M Tris-HCl buffer (pH 7.2). The solution was applied onto a column of Sephacryl S-300 (4.0×90 cm) equilibrated with 0.1 M Tris-HCl buffer (pH 7.2), containing 0.3 M NaCl and the column was eluted with the same buffer. The eluted pool was concentrated by precipitation with 65% saturated ammonium sulfate. The redissolved precipitate was chromatographed on a CM-Sephadex column (3.0×20 cm) equilibrated with 0.01 M acetate buffer, pH 5.5. Non-adsorbed protein fractions with enzyme activity were pooled and concentrated by ultrafiltration using an Amicon YM-10 membrane filter. This concentrated sample was then chromatographed twice on a DEAE-Sephadex column (2.0×30 cm) by linear salt gradient elution (0–0.2 M NaCl, 600 ml total gradient).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of the purified enzyme was carried out with 5% gels as described by Laemmli [16]. Gels were stained for protein with 0.2% Coomassie blue in 50% methanol/acetic acid [10]. Gels were stained for enzyme activity with sodium α-naphthyl phosphate (1 mg/ml) and Farst Garnet GBC (1 mg/ml) in 50 mM sodium acetate buffer, pH 5.0. After incubation for 30 min at 37°C, the gels were rinsed with distilled water and covered with 7% acetic acid. The detection of carbohydrate was performed by the periodic acid-Schiff reaction [17]. Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate (SDS) was carried out in 7.5% gels. The samples were pretreated at 100°C for 2–3 min in 1% SDS; 25 μg was applied for purity check and 10 μg used for molecular weight determination. Logarithmic plots of relative migration vs. molecular weight were

obtained with the following markers: RNA polymerase β'-subunit (M_r 180 000), β (M_r 140 000), χ (M_r 100 000), α (M_r 42 000) and z (M_r 39 000).

Determination of molecular weight. The molecular weight of the purified enzyme was estimated using gel filtration through a Sephacryl S-300 column (2 × 90 cm) equilibrated with 0.1 M Tris-HCl buffer (pH 7.2), containing 0.3 M NaCl [18]. Calibration for molecular weight was followed using the standard proteins (M_r); glucose oxidase (190 000), aldolase (158 000), serum albumin (68 000) and amylase (43 000).

Amino acid analyses. Samples of purified acid phosphatase were dialyzed exhaustively against distilled water and lyophilized. Amino acid analysis was performed using a standard procedure [19]. The samples were hydrolyzed in 6 M HCl in sealed evacuated tubes for 24 h, 48 h or 72 h at 110°C. Tryptophan was determined on duplicate samples in the amino acid analyzer after hydrolysis with *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole as described by Liu and Chang [20]. Half-cystine was determined on duplicate samples by performic acid oxidation described by Moore [21] followed by hydrolysis in 6 M HCl for 24 h.

Carbohydrate determination. Total carbohydrate was determined by the phenol/sulfuric acid method as modified by Barenett and Tawab [22]. A calibration using mannose as standard was carried out. The carbohydrate content of the enzyme was given as a ratio of carbohydrate to protein.

Results

Changes in the acid phosphatase activity during growth. It is known that *Tetrahymena pyriformis* strain HMS releases several lysosomal acid hydrolyzing enzymes into the cultured medium during growth [23]. We have previously found that *Tetrahymena* released a large amount of protease into the medium during growth [5]. To examine whether *T. pyriformis* strain W also releases acid phosphatase into the medium in a growth-dependent fashion, activities of extra- and intracellular acid phosphatase were measured at various stages of growth. As shown in Fig. 1, the specific activity of intracellular acid phosphatase underwent a small increase in the early logarithmic phase, while the enzyme activity in the medium was found to rise considerably as the age of the culture increased.

Purification of extracellular acid phosphatase. Allen et al. [24] have studied the electrophoretic profile on starch gel of the acid phosphatase activity in *Tetrahymena* cell extracts, describing that acid phosphatase of *Tetrahymena* was present in various heterogeneous enzyme forms. In order to examine whether acid phosphatase is released in heterogeneous forms into the cultured medium, we attempted to purify extracellular acid phosphatase of *Tetrahymena*. The acid phosphatase activity was assayed by using *p*-nitrophenyl phosphate as substrate at each purification step. Six steps were involved with a final purification of about 820-fold and with at least 26% recovery (Table I).

TABLE I

PURIFICATION PROCEDURE OF EXTRACELLULAR ACID PHOSPHATASE OF *TETRAHYMENA*

Acid phosphatase activity was assayed with *p*-nitrophenyl phosphate as substrate.

Fraction	Total protein (mg)	Spec. act. (U/mg protein)	Total act. (U)	Recovery (%)
Medium	6313	1.1	7016	100
35–45% $(NH_4)_2SO_4$ precipitate	1788	4.3	7606	108
Sephacryl S-300	234	23.9	5586	79.6
CM-Sephadex	25	150.2	3755	53.5
First DEAE-Sephadex	5	609.3	3046	43.3
Second DEAE-Sephadex	2	898.2	1796	25.6

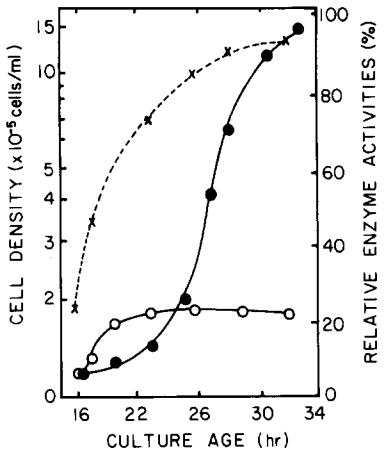

Fig. 1. Changes in acid phosphatase activity of *Tetrahymena* during growth. *Tetrahymena* cells were grown from a single inoculum for the time indicated in 500 ml Erlenmeyer flasks, each with 200 ml of cultured medium, at an initial density of 5000 cells/ml. At the time indicated, cells were collected and washed once with saline. The acid phosphatase activity toward *p*-nitrophenyl phosphate was determined. ●———●, extracellular acid phosphatase; ○———○, intracellular acid phosphatase; ×------×, cell density.

When the pooled fraction from gel filtration was applied on the CM-Sephadex column chromatography, two fractions have acid phosphatase activities were obtained; one peak with 43% recovery of total activity in the void and the other with about 10% total activity eluted with 0.3 M NaCl. The former fraction was further purified twice by the DEAE-Sephadex column chromatography. The first chromatographic pattern of the DEAE-Sephadex column is shown in Fig. 2. A single peak with acid phosphatase activity was eluted with 0.12 M NaCl and was subjected to the second chromatography on DEAE-Sephadex column, by which a single protein fraction with activity was obtained by elution with the same ion strength.

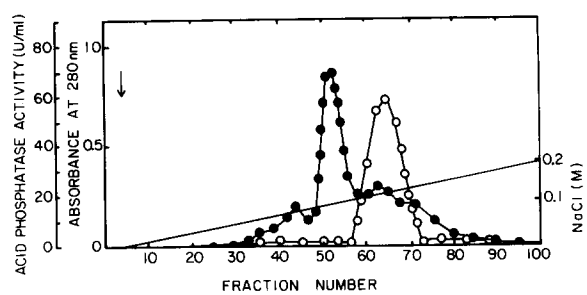

Fig. 2. First chromatography on DEAE-Sephadex of extracellular acid phosphatase from *Tetrahymena*. Chromatography was performed on 2.0×30 cm column of DEAE-Sephadex equilibrated with 0.01 M Tris-HCl buffer, pH 7.2. After sample application, the column was washed with the same buffer and activity was eluted with linear NaCl gradient (———) in 0.01 M Tris-HCl buffer, pH 7.2, with 600 ml total gradient. A flow rate of 40 ml/h was maintained and 5 ml fractions were collected. Protein elution profile (●———●) monitored at 280 nm. Activity toward *p*-nitrophenyl phosphate (○———○).

Physical and chemical properties. The final enzyme preparation gave a single symmetrical peak in molecular exclusion chromatography on Sephacryl S-300 column. A single protein band was shown on polyacrylamide gel electrophoresis at pH 8.3 (Fig. 3, lane 2) and on the gel in the presence of 0.1% SDS (Fig. 3, lane 4). This band was found to have acid phosphatase activity (Fig. 3, lane 3) and carbohydrate (Fig. 3, lane 1), indicating that the purified acid phosphatase was a glycoprotein. This purified enzyme contained 9.8 ± 1.3% by weight of carbohydrate.

The molecular weight of the purified acid phosphatase of *Tetrahymena* was determined by gel filtration on Sephacryl S-300 and by polyacrylamide gel electrophoresis in the presence of 0.1% SDS. When the purified acid phosphatase was

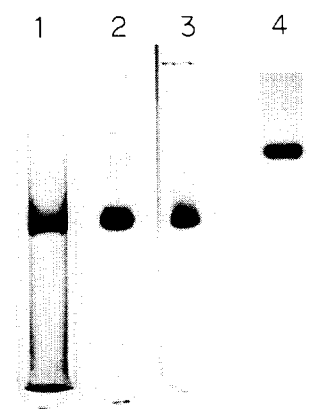

Fig. 3. Polyacrylamide gel electrophoresis of the purified extracellular acid phosphatase of *Tetrahymena*. Electrophoresis of the purified acid phosphatase (30 μg protein/gel) on 5% acrylamide gels carried out at pH 8.3. Lane 1: gel stained for carbohydrate by the Schiff reaction. Lane 2: gel stained for protein with Coomassie blue. Lane 3: gel stained for activity with α-naphthyl phosphate and Farst Garnet GBC. Lane 4: 7.5% acrylamide gel electrophoresis in the presence of 0.1% SDS.

TABLE II

AMINO ACID COMPOSITION OF THE EXTRACELLULAR ACID PHOSPHATASE FROM *TETRAHYMENA PYRIFORMIS*

Values are the averages of 24, 48 and 72 h hydrolysis samples. The number of residues was calculated assuming a subunit molecular weight of 61 000 for *Tetrahymena* acid phosphatase.

Amino acids	g/100 g of protein	No. of residues per molecule
Lysine	4.8	21
Histidine	3.4	14
Arginine	5.1	20
Aspartic acid	15.1	74
Threonine	5.1	29
Serine	3.7	24
Glutamic acid	13.4	60
Proline	3.8	22
Glycine	2.0	17
Alanine	3.8	29
Half-cystine	1.4	8
Valine	5.1	28
Methionine	1.5	7
Isoleucine	3.6	19
Leucine	9.4	47
Tyrosine	9.5	41
Phenylalanine	7.7	31
Tryptophan	1.4	5

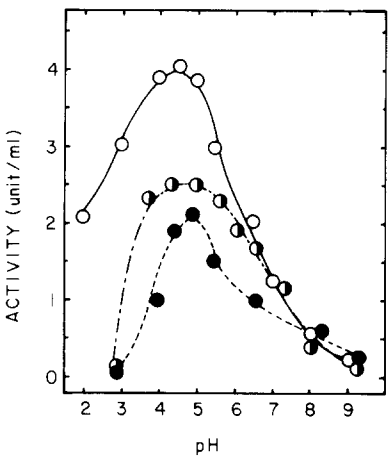

Fig. 4. The effect of pH on the activity of the acid phosphatase purified from the cultured medium of *Tetrahymena*. The buffers used were 50 mM; sodium citrate for pH 2 and pH 3; sodium acetate for pH 4 and pH 5; imidazole-HCl buffer for pH 6 and pH 7; Tris-HCl for pH 8 and pH 9. ○———○, hydrolysis of *p*-nitrophenyl phosphate; ①-·-①, hydrolysis of adenosine 5′-phosphate; ●------●, hydrolysis of glucose 6-phosphate.

subjected to a 7.5% polyacrylamide gel electrophoresis in 0.1% SDS the migration relative to the marker proteins corresponded to a subunit molecular weight of 61 000 ± 2000. The elution position of the native enzyme corresponded to a molecular weight of 120 000 ± 11 000 as compared to proteins of known molecular weight. These results showed that the acid phosphatase of *Tetrahymena* consists of two equal-sized subunits.

The amino acid composition is shown in Table II. The high content of aspartic acid, glutamic acid and leucine residues in this protein is noteworthy.

Optimal pH. We tested optimum pH of the purified extracellular acid phosphatase of *Tetrahymena* with using three different substrates, *p*-nitrophenyl phosphate, adenosine 5′-phosphate and glucose 6-phosphate in the pH range from 2 to 10. As shown in Fig. 4, the enzyme has the optimum pH in the almost same range (pH 4.6) toward all three substrates.

Substrate specificity. It has been known that nonspecific acid phosphatases are widely distributed which hydrolyzed a variety of phosphate esters including nucleotides and sugar phosphates [25]. The purified extracellular acid phosphatase of *Tetrahymena* was assayed for activity against a variety of phosphate esters. Table III represents the activities of the enzyme toward various substrates. The relative activities of the acid phosphatase toward different substrates were expressed as percentage based on the activity for *p*-nitrophenyl phosphate. The most active hydrolysis was directed toward *p*-nitrophenyl phosphate and α-naphthyl phosphate, and other physiologically important phosphates, ribonucleotide phosphates and carbohydrate phosphate were hydrolyzed at significant rates. Pyrophosphates, ADP and ATP were degraded to much lesser extents. On the other hand, there were no activities toward phosphodiesters, cyclic 3′,5′-AMP, cyclic 3′,5′-GMP and bis(*p*-nitrophenyl) phosphate. The effects of substrate concentration on the velocity of hydrolysis of three substrates, *p*-nitrophenyl phosphate, adenosine 5′-phosphate and glucose 6-phosphate were determined under conditions of constant pH, and the K_m values for these substrates are shown in Table III. This implies that *Tetrahymena* acid phosphatase's affinity for its substrates was in the

TABLE III

SUBSTRATE SPECIFICITIES OF *TETRAHYMENA* ACID PHOSPHATASE

Activities are expressed as percent for that with *p*-nitrophenyl phosphate. Each phosphate compound was tested at pH 4.6 in 0.2 ml of the reaction mixture at 0.01 M concentration. The reaction mixture was incubated for 15 min at 37°C.

Substrates	Relative rate of hydrolysis (%)	K_m (M)	V_{max} (μmol/min per mg protein)
p-Nitrophenyl phosphate	100	$3.1 \cdot 10^{-4}$	608.4
α-Naphthyl phosphate	98.2	—	—
Phenolphthalein phosphate	47.7	—	—
Fructose 1,6-diphosphate	67.1	—	—
Glucose 6-phosphate	35.2	$1.6 \cdot 10^{-3}$	196.4
Adenosine 5′-phosphate	76.3	$3.9 \cdot 10^{-4}$	323.4
Adenosine diphosphate	16.5	—	—
Adenosine triphosphate	0.4	—	—
Flavin mononucleotide	58.4	—	—
Cyclic 3′,5′-AMP	0	—	—
Cyclic 3′,5′-GMP	0	—	—
Bis (*p*-nitrophenyl)phosphate	0	—	—

order of glucose 6-phosphate < adenosine 5′-phosphate < *p*-nitrophenyl phosphate.

Effect of inhibitors. A number of compounds was tested as inhibitors of the *Tetrahymena* acid phosphatase activity toward three substrates (Table IV). The effect of *p*-chloromercuribenzoate upon the acid phosphatase of *Tetrahymena* crude extract has been reported by Allen et al. [24], and the involvement of a sulfhydryl group in enzyme activity was suggested [26]. All the cations indicated in Table IV brought about varying degrees of inhibition on the purified acid phosphatase, but other tested cations, Ca^{2+}, Mn^{2+}, Mg^{2+}, and Zn^{2+} had no effect. The strong inhibition by Hg^{2+} and the slight inhibitory effect by *p*-chloromercuribenzoate suggests that the presence of a sulfhydryl group would be required for the enzyme activity. However, cysteine and EDTA were without any effect on the acid phosphatase of *Tetrahymena*. These results would not prove

TABLE IV

EFFECTS OF VARIOUS INHIBITORS ON THE PURIFIED EXTRACELLULAR ACID PHOSPHATASE FROM *TETRAHYMENA*

Assay medium contained 50 mM acetate buffer, pH 4.6, 5 mM substrates, enzyme (4 μg) and inhibitors. The enzyme and inhibitors were preincubated at 37°C for 5 min before addition of the substrates to initiate the reaction. The incubation was performed at 37°C for 20 min. *p*-NPP; *p*-nitrophenyl phosphate, AMP; adenosine 5′-phosphate, G-6-*P*; glucose 6-phosphate.

Inhibitors	Final concentration (M)	Relative activity (%)		
		p-NPP	AMP	G-6-*P*
None	—	100	100	100
Hg^{2+}	$1 \cdot 10^{-3}$	3.8	13.4	33.5
Fe^{3+}	$5 \cdot 10^{-3}$	4.3	2.1	4.2
La^{3+}	$1 \cdot 10^{-3}$	47.6	41.4	44.1
NaF	$2 \cdot 10^{-4}$	0	0	0
Molybdate	$5 \cdot 10^{-6}$	0	0	0
L-(+)-Tartaric acid	$1 \cdot 10^{-3}$	0	0	0
D-(−)-Tartaric acid	$5 \cdot 10^{-3}$	99.0	98.0	98.9
p-Chloromercuribenzoate	$5 \cdot 10^{-4}$	88.1	80.0	82.7

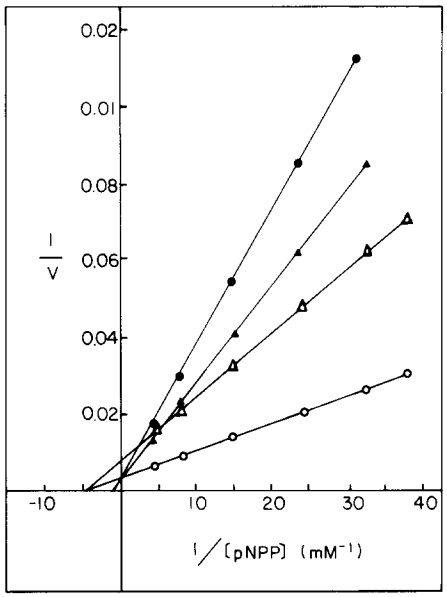

Fig. 5. Inhibition of *p*-nitrophenyl phosphate hydrolysis by the purified acid phosphatase of *Tetrahymena*. Rates of hydrolysis of *p*-nitrophenyl phosphate were measured at six different substrates concentrations at pH 4.6 in 50 mM acetate buffer with and without the addition of inhibitors. ○——○, no inhibitor; ●——●, 0.6 mM NaF; △——△, 15 μM molybdate; ▲——▲, 0.75 mM L-(+)-tartaric acid.

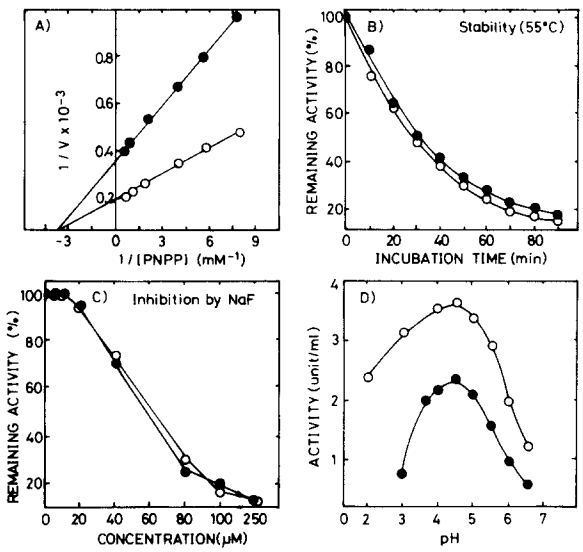

Fig. 6. Comparison of K_m, thermal stability at 55°C, inhibition by NaF and optimum pH of intra- and extracellular acid phosphatases from *Tetrahymena*. (A) Rate versus substrate concentration for intra- and extracellular acid phosphatases. The reaction mixture contained in a total volume of 1.0 ml; each purified acid phosphatase preparation (3.0 μg protein for intra- or 6.0 μg for extracellular enzyme), and different concentrations of *p*-nitrophenyl phosphate, pH 4.6. Initial velocity is expressed as μmol *p*-nitrophenol liberated/min per ml. ●——●, intracellular; ○——○, extracellular acid phosphatase. (B) Thermal denaturation of the acid phosphatase activity. Both enzyme solutions were heated at 55°C in 50 mM acetate buffer, pH 4.6, for the times indicated. Heating was terminated by transferring the sample into an ice bath, and the remaining activity was assayed at 37°C with *p*-nitrophenyl phosphate as substrate. ●——●, intracellular; ○——○, extracellular acid phosphatase. (C) Effect of NaF on the intra- and extracellular acid phosphatase acitivities. Enzymes were preincubated at 37°C for 10 min with varying concentrations of NaF before the addition of *p*-nitrophenyl phosphate as substrate. ●——●, intracellular; ○——○, extracellular acid phosphatase. (D) pH activity curve for purified intra- and extracellular acid phosphatases. Intracellular (2 μg protein) and extracellular (3.5 μg protein) enzymes were assayed with *p*-nitrophenyl phosphate as substrate at various pH values. The buffers used were same as described in Fig. 4. ●——●, intracellular; ○——○, extracellular acid phosphatase.

whether sulfhydryl group is essential for the maximal activity of the *Tetrahymena* acid phosphatase.

This enzyme was inhibited to a considerable degree by fluoride, L-(+)-tartaric acid and molybdate at a low concentration. As shown by the intersection on the $1/V$ axis of a plot of $1/V$ against $1/S$ both with and without the inhibitors present (Fig. 5), fluoride and L-(+)-tartaric acid act as competitive inhibitors while molybdate exerts a noncompetitive inhibition on the hydrolysis of *p*-nitrophenyl phosphate. The dissociation constant (K_i) for three inhibitors, fluoride, L-(+)-tartaric acid and molybdate were $5.6 \cdot 10^{-4}$ M, $8.5 \cdot 10^{-5}$ M and $5.0 \cdot 10^{-6}$ M, respectively. The dissociation constant for the enzyme-molybdate complex was two order of magnitude lower than that of the enzyme-fluoride complex.

Comparison of extra- and intracellular acid phosphatases. We have previously reported that the protease purified from cultured medium of *Tetrahymena* was very similar or identical to the intracellular enzyme [5]. On the other hand, Patni and Aaronson [27] have described that intra- and extracellular acid phosphatases purified from photoheterotrophic *Ochromonas danica* differed in heat inactivation, substrate specificity and inhibition by various inhibitors. This has prompted us to compare acid phosphatases from two sources (intracellular and extracellular) in *Tetrahymena*. Be-

cause of the limited quantity due to the low activity (Fig. 1) of the purified intracellular enzyme from *Tetrahymena,* the properties of critical importance, i.e. the kinetics, heat stability, inhibition by NaF and optimum pH were examined for the purified extracellular and intracellular acid phosphatases.

The K_m for the extra- and intracellular enzymes was almost identical $3.2 \cdot 10^{-4}$ M with *p*-nitrophenyl phosphate as substrate (Fig. 6A). Fig. 6B shows that heating the enzyme solution for 90 min at 55°C caused complete loss of both extra- and intracellular acid phosphatases and their half-life was 30 min at this temperature.

Fluoride was found to be potent inhibitor extracellular acid phosphatase of *Tetrahymena.* When the various concentrations of NaF were incubated at 37°C for 10 min before addition of *p*-nitrophenyl phosphate as substrate, the equally inhibitory action was observed for both enzymes (Fig. 6C). Fifty percent inactivation of both enzymes was caused with approx. 50 μM NaF.

Both extra- and intracellular acid phosphatases had a similar pH optimum for *p*-nitrophenyl phosphate as substrate with an optimum at 4.6 (Fig. 6D). At higher pH both acid phosphatase activities fell very sharply.

These results suggest that both extra- and intracellular acid phosphatases of *Tetrahymena* were identical or, if not, very similar enzymes.

Discussion

The purified *Tetrahymena* acid phosphatase closely resembles those of animal, bacteria and plant in molecular weight (110000–120000) and pH optimum (4.5–5.0). The substrate specificity of the *Tetrahymena* acid phosphatase differs somewhat from prostatic acid phosphatase which has been most extensively studied among the acid phosphatases of animal origin [28]. The prostatic enzyme is a true phosphomonoesterase in that it did not hydrolyze terminal pyrophosphate bonds such as ADP or ATP. On the other hand, nonspecific acid phosphatases, which hydrolyze terminal pyrophosphate bonds at the same rate for *p*-nitrophenyl phosphate, have also been found in the induced enzymes of *Euglena* [29], bacteria [30.31] and plants [32,33]. We think that *Tetrahymena* acid phosphatase would be classified as a nonspecific acid phosphatase, since the substrate specificity of this enzyme was rather broad for nucleotide phosphates and hexose phosphates and it is able to hydrolyze pyrophosphates even at a slow rate, 16% for ADP and 0.4% for ATP. The low hydrolyzing activity for pyrophosphates was also observed in both intra- and extracellular acid phosphatases of *Ochromonas danica* [27]. *Tetrahymena* acid phosphatase was more active with adenosine 5′-phosphate than with glucose 6-phosphate, while lupine enzyme has higher activity toward glucose 6-phosphate (68%) than toward adenosine 5′-phosphate (9%) [33]. Glucose 6-phosphate was hydrolyzed by *Tetrahymena* enzyme with a relative acitivity of 35% but is not hydrolyzed by prostatic acid phosphatase [28].

Fluoride and L-(+)-tartaric acid competitively inhibited the hydrolytic activity of the *Tetrahymena* acid phosphatase toward *p*-nitrophenyl phosphate, while these were noncompetitive inhibitors for adenosine 5′-phosphate as substrate. Thus this behaviour of inhibitors was similar to those of human prostatic [28], *Neurospora* [34] and *Paramecium* acid phosphatase [35], but different from that for tobacco and seeds enzymes [32,33].

Acid phosphatases have been purified to homogeneity from various sources and identified to be glycoproteins [7–9]. All of these enzymes contain nearly 50% or more mannose by weight, except that from *S. pombe,* which may also have high proportion of associated galactose [8]. *Tetrahymena* acid phosphatase purified from medium in the present study was also demonstrated to be a glycoprotein (10% by weight of carbohydrate content), while being less content of sugars than that of fungal enzymes. Like the extracellular acid phosphatase of *Rhodotorula glutinis* [9], the extracellular acid phosphatase of *Tetrahymena* was assumed to consist of two identical 61-kDa subunits, each associated with carbohydrate. In mammals most lysosomal enzymes are glycoproteins containing large amount of mannose, and Hasilik and Neufeld [6] suggested that the structure of the carbohydrate moiety may be important in transferring these enzymes into lysosomal vesicles. Acid phosphatase was found to localize principally in mitochondria/lysosome fraction of *Tetrahymena* cells. Unlike the distinct difference of

intra- and extracellular acid phosphatases of *Ochromonas danica* [27], the extracellular acid phosphatase of *Tetrahymena* was indistinguishable from the intracellular enzyme in K_m, thermal stability, inhibition by NaF and optimum pH. The carbohydrate moiety of *Tetrahymena* extracellular enzyme was resistant to endoglucosaminidase H, which cleaves carbohydrate chains of high mannose type. To clarify the mechanism of release into the medium of acid phosphatase by *Tetrahymena*, more extensive work including carbohydrate structure of both enzymes remains to be done.

Disclosure of the physiological function of extracellular acid phosphatase secreted by *Tetrahymena* is of great importance for better understanding of the cell biology of this organism but it remains unknown. It has been thought that the fungal acid phosphatase would provide inorganic phosphates necessary for cell growth by degrading of phosphate esters present in the cultured medium [36]. An earlier paper reported that the increase in acid phosphatase activity observed when *Euglena* is deprived of phosphate would be due to synthesis de novo of an induced enzyme which is different from the constitutional acid phosphatase [29]. Consequently one would expect that extracellular nonspecific acid phosphatase of *Tetrahymena* may play a role in providing nutritional inorganic phosphate for cell growth.

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