[Jap. J. Parasit., Vol. 21, No, 3, 159-167, 1972]

Biochemical Characterization of Acid Phosphatase in *Trichomonas vaginalis*

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(Received for publication: May 29, 1972)

Introduction

Physiological studies on *Trichomonas vaginalis*, particularly on the nature of malate dehydrogenase and acid phosphatase in the organism in relation to the localization of these enzymes have been made for the past several years in our laboratory. As regards acid phosphatase in *T. vaginalis* and *T. tenax*, one of the present authors (Ohashi 1971, 1972) performed a cytochemical analysis on localization of the enzyme by light and electron microscopy.

In the present study, biochemical property of acid phosphatase in *T. vaginalis* was investigated in several aspects using cultured form of the organism as the source of the enzyme.

Materials and Methods

(1) Reagents

All of the reagents used in the present experiments were special or analytical grade. Adenosine tri-phosphate (ATP) and α-glycerophosphate were purchased from Sigma Chem. Co. Ltd. Glucose-6-phosphate, glucose-1-phosphate, and fructose-1, 6-diphosphate were the products of Daiichi Chem. Co. Ltd. β-glycerophosphate was obtained from Merck Co. Ltd.

(2) Enzyme solution

A strain of *T. vaginalis* which has been retained in our laboratory by serial transfer using Asami medium (Asami 1952) was employed throughout the experiments. The organisms were grown for 48 hours in Asami medium without agar, and collected by centrifugation at 1,200 Xg for 15 minutes in cold. Then, the organisms were washed three times with 0.1 M acetate buffer pH 7.6 containing sodium chloride in 0.8% by centrifugation. The organisms were finally suspended in 5.0 ml of 0.1 M acetate buffer pH 4.8 containing sodium chloride in 0.8%. Depending on the puropose of the experiment, the enzyme solution was prepared in various ways as follows.: (1) In the experiment to ascertain optimal pH, and effect of some inorganic ions and well-known inhibitory substances other than fluoride and L-tartrate on the enzyme activity, the organisms collected in the way described above were disrupted by sonic vibration. After the organisms were disrupted almost completely, this material was centrifuged in cold at 1,800 Xg for 20 minutes. The supernatant was employed as the enzyme solution for the assay of the activity of acid phosphatase in this series of experiments. (2) In the cases of investigation of the inhibitory effects of sodium fluoride and L-tartrate, and substrate specificity of the enzyme, the initial material was prepared by centrifugation of the disrupted cells with Potter-Elvehjem glass homogenizer in cold at 900 Xg for 20 minutes. Then, the supernatant was centrifuged at 15,000 Xg 20 minutes in cold. Both the supernatant and the sediment which was re-suspended in the same quantity of ice chilled 0.1 M acetate buffer pH 4.8 containing sodium chloride in 0.8% as that of the supernatant were used as the cytoplasm fraction and the particle fraction respectively. (3) In the case in which distribution of acid

phosphatase was examined, the organisms collected in the method described above were disrupted by homogenation with Potter-Elvehjem glass homogenizer. Then, the resulting homogenate was fractionated by centrifugation in cold as follows. : (a) 1,500-Xg, 15 minutes sediment (Sed. 1), 11,000 Xg, 60 minutes sediment (Sed. 2), 24,000 Xg, 60 minutes sediment (Sed. 3), and final supernatant (Sup.) (b) Both the cytoplasm fraction and the particle fraction which were prepared in the procedure above described were also used for the investigation of distribution of the enzyme.

(3) Assay methods

(a) determination of inorganic phosphate (Pi)

Determination of Pi released by acid phosphatase reaction was made according to the method of Fiske and Subbarow (1925).

(b) optimal pH of acid phosphatase

The assay mixture to determine optimal pH of acid phosphatase was composed of 0.1 ml of the enzyme solution prepared in the procedure described above and 1.0 ml of 0.1 M acetate buffer containing β-glycerphosphate in 20 mM. PH of these assay mixtures varied from 2.0 to 7.0 After preincubation of the substrate solution at 37°C for 10 minutes, the enzyme solution was added and the mixtures were incubated at 37°C for 60 minutes. Then, 1.0 ml of the supernatant from each test tube was separated by centrifugation after the reaction was terminated by addition of 2.4 ml of 10% trichloroacetic acid. The supernatant thus recovered was used for the determination of Pi released.

(c) substrate specificity

In this series of experiments, several sorts of substances were used under the identical conditions to investigate substrate specificity of acid phosphatase in *T. vaginalis*. The substances employed in this experiment were as follows: α-glycerosphate, β-glycerophosphate, ATP, glucose-6-phosphate, glucose-1-phosphate, and fructose-1, 6-diphosphate. The assay mixtures were consisted of 0.1 ml of the enzyme solution and 1.0 ml of 0.1 M acetate buffer pH 4.8 containing each substrate in 50 mM. The assay mixtures were incubated at 37°C for 60 minutes after initiation of the reaction by addition of the enzyme solution. Termination of the reaction and determination of Pi released were made in the same way as in the investigation of optimal pH.

(d) effect of several substances on the enzyme activity

The control assay mixture was composed of 0.1 ml of the enzyme solution and 1.0 ml of 0.1 M acetate buffer pH 4.8 containing α- or β-glycerophosphate in 8 mM and 40 mM. Effect of the substance added to the assay

Figure 1. Optimal pH of acid phosphatase in *T. vaginalis*

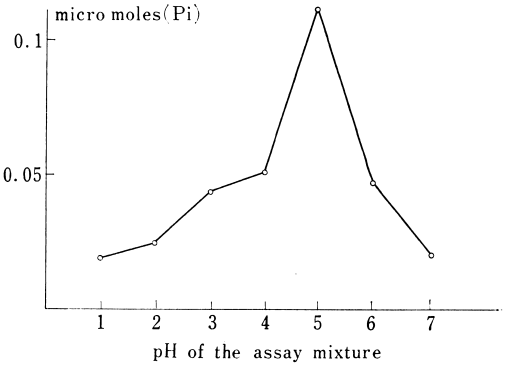

Table 1 Substrate specificity of acid phosphatase in *T. vaginalis*

Substrate	Relative rate of the activity	
	Cytoplasm fr.	Particle fr.
α-glycerophosphate	1.000*	1.235
glucose-1-phosphate	4.374	4.476
glucose-6-phosphate	1.944	1.048
fructose-1, 6-diphosphate	3.209	3.139
ATP	0.302	0.246
β-glycerophosphate	1.367	1.979

* The concentration of Pi released in this case was designated as 1.000.
In table 2-A, B, C, likewise, this figure means the concentration of Pi released in the control assay system of the series of experiment.

mixture was evaluated by the concentration of Pi released during 60 minutes incubation.
(e) localization of the enzyme.

The assay mixture was composed of 0.1 ml of the enzyme solution obtained as described above and 1.0 ml of 0.1 M acetate buffer pH 4.8 containing β-glycerophosphate in 20 mM. The activity of acid phosphatase in each fraction was represented by the concentration of Pi released during the incubation period per mg protein of the mixture.

Results

(1) optimal pH

As shown in Figure 1, the most prominent activity of acid phosphatase was recognized when pH of the assay mixture was adjusted to 5.0.

(2) substrate specificity.

The results obtained in the experiment were indicated in Table 1. When glucose-1-phosphate was employed as the substrate, Pi was released most markedly, almost four times as much as that in α-glycerophosphate both in the cytoplasm and the particle fraction. In addition, fructose-1, 6-diphosphate was also hydrolyzed to considerably high extent, about three times as much as α-glycerophosp-

Table 2-A Effect of several inorganicions on the acitvity of acid phosphatase in *T. vaginalis*

Substrate	Sub. added	Relative rate of the activity
α-glycerophosphate 8 mM	—	1.000
	$MgCl_2$ 4 mM	1.052
	$MgCl_2$ 40 mM	1.058
α-glycerophosphate 40 mM	—	2.753
	$MgCl_2$ 4 mM	2.900
	$MgCl_2$ 40 mM	2.700
α-glycerophosphate 8 mM	—	1.000
	$CaCl_2$ 1 mM	1.010
	$CaCl_2$ 10 mM	1.072
α-glycerophosphate 40 mM	—	1.691
	$CaCl_2$ 1 mM	1.762
	$CaCl_2$ 10 mM	1.619
α-glycerophosphate 8 mM	—	1.000
	$CdCl_2$ 5 mM	0.988
α-glycerophosphate 40 mM	—	2.258
	$CdCl_2$ 5 mM	2.290
α-glycerophosphate 8 mM	—	1.000
	$CoCl_2$ 5 mM	1.048
α-glycerophosphate 40 mM	—	1.727
	$CoCl_2$ 5 mM	1.727
α-glycerophosphate 8 mM	—	1.000
	$ZnSO_4$ 4 mM	1.038
α-glycerophosphate 40 mM	—	1.639
	$ZnSO_4$ 4 mM	1.721
α-glycerophosphate 8 mM	—	1.000
	KCN 5 mM	1.009
α-glycerophosphate 40 mM	—	1.435
	KCN 5 mM	1.639

Table 2-B Effect of some inhibitors for acid phosphatases of some other kinds of origins on the enzyme in *T. vaginalis*

Substrate	Sub. added	Relative rate of the activity
α-glycerophosphate 8 mM	—	1.000
	PCMB 0.01 mg	0.894
α-glycerophosphate 40 mM	—	1.643
	PCMB 0.01 mM	1.547
α-glycerophosphate 8 mM	—	1.000
	formaldehyde 0.1%	0.939
α-glycerophosphate 40 mM	—	2.150
	formaldehyde 0.1%	2.135
β-glycerophosphate 8 mM	—	1.000
	p-nitrophenol 0.1%	1.079
β-glycerophosphate 40 mM	—	2.197
	p-nitrophenol 0.1%	1.997
β-glycerophosphate 8 mM	—	1.000
	EDTA 0.5 mM	1.023
	EDTA 2 mM	1.050

hate in both fractions. With regard to glycerophsphate, β-isomer was hydrolyzed slightly more rapidly than α-isomer in both of the fractions. On the contrary, Pi was released in the lowest level when ATP was used as the substrate.

On the whole, no distinct difference of the amount of Pi released was observed between the cytoplasm fraction and the particle fraction other than the case in which glucose-6-phosphate was employed as the substrate.

(3) effect of some inorganic ions and well-known inhibitory substances on the enzyme activity.

None of the inorganic ions added to the assay mixtures affected the activity of acid phosphatase at any substrate concentrations examined (Table 2-A). Even magnesium chloride which is known as an activatory cofactor of acid phosphatase of some other origins brought no effect on the activity of the enzyme.

Regarding the inhibitory substances for acid phosphatases of some other origins, no distinct inhibition of the enzyme was recognized in the present experiment (Table 2-B).

On the contrary, in the inhibition experiment by sodium fluoride or L-tatrate, the former inhibited the activity of acid phosphatase in both cytoplasm and particle fraction. Among the fractions, the activity in the particle fraction was inhibited a little more than that in the other fraction. Although 45% inhibition of the enzyme was observed in the particle fraction when the substrate was used in 40 mM, only 12% inhibition was recognized in the other fraction (Table 2-C).

(4) distribution of the enzyme

The apparent specific activity of the enzyme was recognized in the cytoplasm fraction, almost seven times as much as in the partice fraction (Table 3-A). Whereas, in another series of the experiment shown in Table 3-B, the most prominent specific activity was observed in Sed. 3 fraction.

Discussion

With regard to the studies on acid phosphatase in Mastigophora, cytochemical experiments have been carried out by several investigators, whereas biochemical ones have

appeared very few. Nomura (1956) performed cytochemical studies on acid phosphatase in *Trichomonas muris* by means of the procedure described by Gomori (1941). Lehman (1963) reported that both alkaline and acid phosphatases were found out in the cultured form of *Trypanosoma ranarum*. One of the present authors (Ohashi 1971, 1972) studied localization of acid phosphatase in special ralation to the physiological function of dense body and phagosome of *Trichomonas vaginalis* and *T. tenax* by light and electron microcopy. In his studies, almost complete process of autophagocytosis in *T. vaginalis* was observed, and acid phosphatase activity associated in part with this process was found

Table 2-C Effect of fluoride and L-tartrate on the activity of acid phosphatase in *T. vaginalis*

(1) In the case of addition of fluoride

Fraction used	Substrate	NaF (200 mM)	Relative rate of the activity
	β-glycerophosphate		
	8 mM	−	1.000
	20 mM	−	1.611
Particle fr.	40 mM	−	2.616
	8 mM	+	0.619
	20 mM	+	0.951
	40 mM	+	1.426
	β-glycerophosphate		
	8 mM	−	1.000
	20 mM	−	1.685
Cytoplasm fr.	40 mM	−	2.480
	8 mM	+	0.696
	20 mM	+	1.258
	40 mM	+	2.173

(2) In the case of addition of L-tartrate

Fraction used	Substrate	L-tartrate	Relative rate of the activity
	α-glycerophosphate		
	8 mM	−	1.000
	20 mM	−	1.315
Particle fr.	40 mM	−	1.750
	8 mM	+	1.007
	20 mM	+	1.375
	40 mM	+	1.815
	α-glycerophosphate		
	8 mM	−	1.000
	20 mM	−	1.443
Cytoplasm fr.	40 mM	−	1.921
	8 mM	+	1.064
	20 mM	+	1.411
	40 mM	+	1.883

Table 3-A Distribution of acid phosphatase in the cytoplasm and the particle fraction

	Cytoplasmic fr.	Particle fr.
Pi released (μM)	0.049	0.041
Protein (mg/ml)	0.200	1.100
Specific activity	0.244	0.038

Table 3-B Localization of acid phosphatase in the fractions prepared by centrifugation

	Sed. 1	Sed. 2	Sed. 3	Sup.
Pi released (μM)	0.050	0.045	0.044	0.040
Protein (mg/ml)	8.000	0.750	0.390	0.850
Specific activity	0.006	0.060	0.112	0.047

in autophagolysosome, lysosome, endoplasmic reticulum, and Golgi apparatus. Among the organellas positive to the reaction, autophagolysosome showed the most prominent activity.

As to the biochemical characteristics of acid phosphatase in Mastigophora, only a few reports are available. Seed et al. (1967) performed the study on acid phosphatase in *Trypanosoma gambiense* by means of biochemical techniques and electron microscopy. They reported the presence of relatively specific acid phoosphatase activity for pyrophosphate and *p*-nitrophenyl phosphate at pH 3.6. Furthermore, they found out the activity of non-specific acid phosphatase which hydrolyzed purine and pyrimidine 5′-triphosphate as well as adenosine di- and mono-5′-phosphate. In addition, they recognized the glucose-6-phosphate hydrolyzing activity in which Mg^{++}, Ni^{++} or Co^{++} ion was required for the full activity between pH 6.0 and 7.2.

In the present study, biochemical characteristics of acid phosphatase in cultured form *T. vaginalis* was evaluated on the following aspects: (1) optimal pH of acid phosphatase activity (2) substrate specificity (3) effect of several substances on the activity (4) distribution of the enzyme.

With regard to optimal pH of the enzyme activity, the most prominent activity was recognized at pH 5.0. However, effect of the more minute difference of pH on the reaction was not examined in the present experiment. Therefore, it might be more accurate to conclude that optimal pH of the enzyme activity is about 5.0.

Folley and Kay (1936) classified acid phosphatase into 4 subclasses on the basis of the difference of optimal pH, substrate specificity especially for α- or β-glycerophosphate, and activation by Mg^{++} ion. Furthermore, Sumner and Somers (1953) also classified phosphomonoesterase into 9 subclasses.

In the present study, the activity of acid phosphatase in *T. vaginalis* was not enhanced by addition of magnesium chloride and the most prominent activity was recognized when pH of the assay mixture was adjusted to 5.0. In addition, the enzyme hydrolyzed β-glycerophosphate a little more than α-glycerophosphate. Therefore, it may be concluded that, so far as the present study concerns, acid phosphatase in *T. vaginalis* is mainly so-called non-specific acid phosphatase of class A II type in Folley and Kay s' classification, or class II type in the classification by Sumner and Somers (1953) taking account of the results obtained in the assay of substrate specificity of the enzyme at the same time. However, it never means that specific phosphatase such as glucose-6-phosphatase is not operative in *T. vaginalis*. Asami and Kawamura (1967) detected glucose-6-phosphatase in crude homogenate of *T. vaginalis*. Judging from the result of their study on localization of glucose-6-phosphatase (Asami and Kawamura 1967) and acid phosphatase (Ohashi 1972), these two enzyme seem to be different ones, despite that the former workers did not take account of the operation of non-sprcific acid phosphatase in understanding of glucose-6-phosphatase observed in phagosome or plasma membrane. However, the reaction of gluchose-6-phosphatase in their study was run at pH 6.7–6.8, while the activity of non-specific acid phosphatase over pH 6.0 was low as observed in the present experiment.

With respect to substrate specificity of the

enzyme, many reports have appeared until now using the enzyme obtained from various kinds of cells and organs. Igarashi and Hollander (1968) investigated acid phosphatase in rat liver, and demonstrated the allosteric nature of the enzme. Furthermore, they reported that both P II enzyme which was obtained by DEAE cellulose colum chromatography and crystalline enzyme showed the most prominent activity in the case of using *p*-nitrophenyl phosphate as the substrate. However, in comparison with this substrate, only 10–30% of acid phosphatase activity was recognized when AMP, β-glycerophate, D-glucose-6-phosphate, or fructose-6-phosphate was used as the substrate. According to Brightwell and Tappel's report (1968) on acid phosphatase of rat liver, *p*-nitrophenyl phosphate was hydrolyzed most rapidly. However, β-glycerophosphate, phosphoenol pyruvate as well as some kinds of adenine nucletides such as 5′-AMP, 3′AMP or 2′-AMP were also hydrolyzed to rather high extent. Heinrikson(1969) who purified a low molecular weight acid phosphatase III from bovine liver, stated in the report that *p*-nitrophenyl phosphate was hydrolyzed most rapidly by the enzyme, although flavin monoucleotide as well as galactose-6-phosphate was also hydrolyzed. Furthermore, he reported that α-glycerophosphate, β-glycerophosphate, and some sorts of nucleotides such as AMP or GMP were hydrolyzed at negligible degree in comparison with *p*-nitrophenyl phosphate. As regards substrate specificity of the enzyme in *Trypanosoma gambiense*, Seed et al. (1967) reported that *p*-nitrophenyl phosphate was more appropriate substrate for the enzyme than β-glycerophosphate, fructose-1, 6-diphosphate or glucose-6-phosphate.

Among the substrates investigated in the present experiment, glucose-1-phosphate and fructose-1, 6-diphosphate were hydrolyzed more rapidly than α- or β-glycerophosphate. Although it is not decided whether this fact is due to the coexistence of some other kinds of phosphatases specific for glucose-1-phosphate or fructose-1, 6-diphosphate in the enzyme solution or not, it appears to be reasonable at least that some specific phosphatases such as fructose-1, 6-diphosphatase are operative in the organism, because glucose-6-phosphatase which is one of the enzymes in gluconeogenetic pathway was already detected. Furthermore, acid phosphatase purified from various sources hydrolyzed glycerophosphate more rapidly than glucose-1-phosphate or fructose-1, 6-diphosphate in most cases.

In the experiment in which the effect of several metal ions on acid phosphatase activity was examined, none of them enhanced the activity. As regards inhibition of acid phosphatase, it has been widely known that acid phosphatase is inhibited by fluoride (Fenton and Richardson 1967, Chersi *et al.* 1966, Nelson 1966). In addition, Barron (1951) reported that prostatic acid phosphatase was inhibited by PCMB, Cu^{++} and Ferric ion. Abul-Fadl and King (1948) showed the inhibitory effect of formaldehyde (0.1 %) on the activity of red blood cell acid phosphatase. However, they stated that formaldehyde did not inhibit prostatic acid phosphatase. The enzyme in *T. vaginalis* was not inhibited by the addition of PCMB, EDTA, *p*-nitrophenol or formaldehyde. According to Nelson (1966), among the enzymes in lysosomal and in supernatant fractions prepared from rat liver, only the latter was inhibited by fluoride or L-tartate. In the present study, acid phosphatase activity in the cytoplasm fraction as well as in the particle fraction prepared from the cultured form of *T. vaginalis* was inhibited by the addition of fluoride. On the other hand, the addition of L-tartrate did not show any inhibitory effect on the enzyme activity in these two kinds of fractionated preparations.

In fluoride inhibition, the enzyme in the particle fraction was inhibited a little more than the other fraction. These findings might be derived from the partial contamination of the cytoplasmic enzyme with that originally existed in the particle fraction on account of disruption of some particles during the process of preparation of crude homogenate. *T. vaginalis* is considerably labile

against mechanical action such as homogenation, sonic vibration orfreezing and thawing. Even if so, however, it seems to be reasonable to say that there are at least two kinds of acid phosphatases which are different with regard to the mode of action of fluoride in the organism.

Judging from the results of cytochemical analysis by electron microscope (Ohashi 1972), acid phosphatase is located on some organellas such as lysosome, Golgi apparatus or endoplasmic reticulum. In the present study, the most prominent specific activity was observed in Sed. 3 fraction in which some kinds of particles seem to be present. The activity observed in Sup. fraction might indicate the enzyme in endoplasmic reticulum, and the activity in Sed. 2 fraction might be derived from large granules like autophagolysosome. However, some further analyses, for instance by electron microscopy, are needed to identify the organellas contained in these fractionated preparations.

Summary

In the present study, the biochemical characteristics of acid phosphatase in *Trichomonas vaginalis* were investigated in the following sapects: (1) optimal pH (2) substrate specificity (3) effect of several substances on the enzyme activity (4) distribution of the enzyme. The results obtained were as follows.

(1) Optimal pH of acid phosphatase activity was about 5.0.

(2) The most prominent activity of the enzyme was recognized when glucose-1-phosphate was used as the substrate. ATP was hydrolyzed to the lowest degree so far as the present study concerns.

(3) None of the substances examined in the present experiment showed stimulatory effect on the enzyme activity. With regard to inhibition of the enzyme, fluoride inhibited the activity of acid phosphatase in the particle fraction (15,000×g, 20 minutes sediment) a little more than that in the cytolasm fraction (15,000×g, 20 minutes supernetant). L-tartrate, PCMB, *p*-nitrophenol, formaldehyde, or EDTA did not inhibit the enzyme activity.

(4) The more prominent specific activity was observed in Sed. 3 fraction (24,000×g, 60 minutes sediment) comparing with those in Sed. 2 fraction (11,000×g, 60 minutes sediment) and Sup. fraction (24,000×g, 60 minutes supernatant).

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腟トリコモナスにおける酸フォスファターゼの生化学的諸特質について

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腟トリコモナスの酸フォスファターゼの諸生化学的性質について，(1)，酵素に対する至適 pH，(2)，基質特異性，(3)，無機イオンを含む種々の物質の酵素作用に及ぼす影響，および (4)，酵素の細胞内局在について調べ，次のような結論を得た．(1)，酵素作用に対する至適pH は大体5.0であつた．(2)，15,000×g，20分の沈渣，上清分画ともグルコース-1-リン酸が最も速やかに分解された．ついでフルクトース-1･6-二リン酸が速やかに分解された．ATP の分解速度は最もおそかつた．グリセロリン酸では β 異性体の方が α 異性体よりやや強く分解された．(3)，マグネシウムイオンなど用いられた物質は全て酵素活性を促進しなかつた．阻害実験においてはフルオライドのみに阻害効果が見られた．これは15,000×g，20分間の沈渣の分画の酵素に対してその上清分画の酵素より強い阻害効果を示した．PCMB，フォルムアルデハイド，パラニトロフェノール，EDTA，L-酒石酸などは阻害作用を示さなかつた．(4)，15,000×g，20分間の遠沈では上清に強い比活性が見られた．更に細かく遠沈分画した場合は24,000×g，60分間の沈渣に多く比活性が集中した．11,000×g，60分間の沈渣，24,000×g，60分間の上清ではほぼ同程度の比活性が見られた．