Partial purification and characterization of acid phosphatase from sporulated oocysts of *Eimeria tenella*

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Summary. Acid phosphatase of *Eimeria tenella* oocysts (Peak II) was purified 77-fold with a recovery of 26% using protamine sulfate precipitation, DEAE-cellulose chromatography and Sephadex G-200 gel filtration. This enzyme occurs in multiple forms as indicated by two peaks which can be separated by DEAE-cellulose chromatography and polyacrylamide gel electrophoresis. The partially purified enzyme has optimal activity at pH 4.5. With p-nitrophenyl phosphate the Km and V_{max} values for (Peak II) were 25 mM and 1.57 μmol/min/mg protein, respectively. The enzyme (Peak II) ist strongly inhibited by Hg^{++}, Cu^{++}, iodoacetamide, fluoride and molybdate. Tartrate and other divalent metal ions have no effect on enzyme activity. The partially purified Peak II phosphatase is not a glycoprotein as it is not absorbed on concanavalin-A Sepharose and its treatment with bacterial neuraminidase does not alter its elution profile through DEAE cellulose.
Key words. Eimeria tenella oocysts; acid phosphatase; phosphorylated metabolites.

Introduction

Phosphohydrolases, the enzymes catalyzing the hydrolysis of phosphate esters, have a vital function in regulating physiological levels of inorganic phosphate and phosphorylated metabolites and in some cases in regulating the activity of certain phosphorylated enzymes[1, 2].

Although cytochemical studies have indicated the presence of acid and alkaline phosphatases, β-galactosidase and enzymes of the glycolytic pathway in *Eimeria tenella*[3–7] no one has yet isolated and characterized any catabolic enzyme from this parasite. Earlier work from this laboratory has indicated the presence of several acid hydrolases in sporulated oocysts of *E. tenella*[8]. The levels of acid phosphatase were quite high in different stages of the life cycle of this parasite indicating that this enzyme may play an important role in its metabolic processes. The purpose of the present study was to isolate the acid phosphatase from sporulated oocysts of *E. tenella* and to compare its activity with that of other acid phosphatases of parasitic and host origin.

Materials and methods

p-Nitrophenyl phosphate (disodium salt), α-naphthyl phosphate, α-glycerophosphate, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, adenosine 5′-monophosphate, adenosine 5′-diphosphate, adenosine 5′-triphosphate, choline o-phosphate, and neuraminidase type VI (*Clostridium perfringens*) were purchased from Sigma Chemical, St. Louis, MO. Sephadex G-200 and concanavalin-A Sepharose were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose DE-52 was purchased from Whatman, Reeve Angle, London. Acrylamide and NN-methylene-bisacrylamide were obtained from Bio-Rad Laboratories, Richmond, CA. All other chemicals were of analytical grade.

Isolation and cleaning of oocysts: Seven days after oral inoculation of 3-week-old white leghorn chicks with 5×10^5 sporulated oocysts of a Wisconsin strain (Jeffer's Wisconsin strain) of *E. tenella,* oocysts were recovered from cecal cores using a modification of the trypsin procedure described by Shirley[6]. Oocysts were sporulated for 3 days in 2% $K_2Cr_2O_7$ with aeration. Oocyst walls were stripped with chlorox as described by Vetterling[9]. The treated oocysts were counted, then stored in 0.02 M Tris HCl buffer, pH 7.5 at −20 °C[10].

Assay of acid phosphatase. The enzyme was determined by the method of Helwig et al.[11] with slight modification using p-nitrophenyl phosphate as a substrate. The reaction mixture consisted of 0.2 ml of 250 mM sodium acetate buffer, pH 4.5, 0.2 ml of 200 mM-p-nitrophenyl phosphate and 10–50 μg enzyme protein in a total volume of 0.5 ml. The tubes were incubated for 20–30 min at 37 °C and the reaction was stopped by the addition of 2.5 ml of 0.5 M NaOH. The amount of liberated p-nitrophenol was determined spectrophotometrically at 410 nm. One unit of enzyme was defined as the amount liberating 1 μmol of p-nitrophenol/min under the above conditions. The specific activity was expressed as units/mg of protein.

In the substrate-specificity study 0.2 ml of other substrates (100 mM) were substituted for the p-nitrophenyl phosphate. The tubes were incubated at 37 °C for 1 h. The liberated Pi was estimated by the method of Fiske and Subba-Row[12]. Enzyme activity was measured in a range of concentrations in which proportionality between the reaction rate and the amount of protein was assured.

Protein estimation. Protein estimations were done by the method of Bradford[13] using bovine serum albumin as the standard. The chromatographic elution was monitored at 280 nm.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out at room temperature using the method of Brewer and Ashworth[14] with 7.5% gel in 0.03 M Tris-glycine buffer, pH 8.4, for 2½ h at a current of 2 mA/tube. Samples (20–100 μg) from various stages of purification were applied to the gels to localize proteins.

Bromophenol blue was used as a reference dye. For localization of enzyme activity, unstained gels were cut into 0.5-cm segments and incubated in the assay system for 12 h.

Purification of acid phosphatase from sporulated oocysts of E. tenella. All operations were done at 4 °C unless otherwise mentioned. Frozen oocysts (50 ml packed) were thawed and suspended in 0.02 M Tris HCl buffer, pH 7.4. The suspension was sonicated for two periods of 5 min at intervals of 3 min at 4 °C using Sonic Dismembrator Model 300 (Fisher) operating at full power. The solution was centrifuged at 13,000 g for 30 min and the turbid supernate collected. The pellet was resonicated once in the same buffer under the identical conditions and centrifuged as described above. The two supernates were combined and subjected to protamine sulfate fractionation. To the combined supernates 5 ml of 5 mg/ml protamine sulfate were added with constant mechanical stirring. The white precipitate that appeared during this process was removed by centrifugation at 12,000 g for 30 min, and the clear supernate was concentrated either by ultrafiltration in an Amicon cell (membrane PM-10) or by using polyethylene glycol compound 20 M (Union Carbide). The concentrated supernate was dialyzed against 0.02 M Tris HCl buffer, pH 7.4.

DEAE cellulose chromatography. The above solution was applied on a DEAE cellulose DE-52 column (2 × 10 cm) equilibrated in 0.02 M Tris HCl buffer, pH 7.4. The column was washed with the above buffer in 5-ml fractions at a flow rate of 0.15 ml/min. After 200 ml of the buffer had passed through the column, the elution was continued with a linear gradient formed from 100 ml of the starting buffer and 100 ml of the 0.5 M NaCl in the same buffer. The enzyme

active fractions were pooled and concentrated by ultrafiltration in an Amicon cell using membrane PM-10.

Sephadex G-200 gel filtration. The concentrated preparation was applied to a Sephadex G-200 column (3 × 3 cm) that was equilibrated with 0.02 M Tris Cl buffer, pH 7.4, containing 0.15 M NaCl. The column was eluted in 5-ml fractions with the same buffer at a flow rate of 0.2 ml/min. The fractions that contained the enzyme activity were pooled, concentrated to 3 ml by ultrafiltration.

Neuraminidase treatment. Portions of partially purified oocyst acid phosphatase Peak I and II (2.5 units or 1.59 mg protein) were incubated with neuraminidase from *Clostridium perfringens* (2.0 units) for 5½ h at 37 °C in 0.05 M sodium acetate buffer, pH 5.0. The neuraminidase was omitted from the control tubes. At the end of the incubation period the solution was dialyzed against 0.02 M Tris HCl buffer, pH 7.4. The dialyzed preparation was applied to a DEAE cellulose DE-52 column (1 × 10 cm) as described above.

Results

Eimeria tenella oocyst acid phosphatase (Peak II) was purified 77-fold (table 1) with an overall recovery of 26% by the above procedure. Sonication of oocysts under hypotonic conditions resulted in complete destruction of the oocyst wall and solubilization of acid phosphatase activity. The sporocysts and sporozoites in them remained intact. On the basis of the present study, it is not possible to determine the localization of acid phosphatase in oocysts and it remains an open question if the enzyme activity was localized in oocyts wall or leaked out from sporocysts and sporozoites. The sonicated extract was turbid and the turbidity was removed by the addition of protamine sulfate while the activity remains in the soluble fraction.

The DEAE cellulose chromatography of the oocyst extract resulted in separation of two forms of acid phosphatase (fig. 1). The enzyme activity retained on the DE-52 column (Peak II) was considered to be the acidic form, whereas the enzyme activity not absorbed in this column (Peak I) was designated the basic form. The total recovery of acid phosphatase activity from DE-52 column was 70–75%. It should be noted that Peak I did not represent the overloading of the DE-52 column; since upon rechromatography of the separated Peaks, Peak I was washed out from the DE-52 column and Peak II was retained. In sporulated oocysts Peak II accounted for the greater part of the total acid phosphatase activity. The elution profile of Peak II through Sephadex G-200 column is shown in figure 2. The enzyme appears as a sharp peak that does not coincide with the main protein peak. Since the enzyme activity of Peak I was very low, it was not studied in detail. The purity of the final preparation (Peak II) was judged by using polyacrylamide gel electrophoresis. It was noted that purified enzyme preparations had five protein bands[14]. The determination of enzyme activity in 0.5-cm segments of an unstained gel[21] indicated the presence of one form of acid phosphatase in G-200 fraction (Peak II) and this activity corresponded to the major protein band on a stained gel. The other 4 bands on the stained gel were minor and did not show any acid phosphatase activity. The determination of enzyme activity in unstained gels on which protamine sulfate fraction was applied confirmed the existence of two forms of acid phosphatase (representing Peak I and Peak II).

Concanavalin-A Sepharose chromatography under the conditions of Farooqui[15] indicated that Peak I and II enzyme were not retained on the concanavalin-A Sepharose column. The treatment of the oocyst acid phospatase (Peak I and Peak II) with neuraminidase did not alter either the proportions of these enzymes or their elution profiles through the DEAE cellulose DE-52 column.

Properties of acid phosphatase of E. tenella oocysts. The pH activity profiles of acid phosphatase (Peaks I and II) are shown in figure 3. The pH optimum for the hydrolysis of p-nitrophenyl phosphate in sodium acetate buffer was 4.5.

Table 1. Purification of acid phosphatase from the oocysts of *E. tenella*.

Fraction	Total protein (mg)	Total units (μmol/min)	Specific activity	Total yield (%)
Sonicated extract	1027	21	0.020	100
Supernate	526	18	0.034	86
Protamine-SO_4 fraction	86.5	16	0.185	76
DE-52 Chromatography				
Peak I	12.0	3.0	0.25	55
Peak II	11.0	8.5	0.77	
Sephadex G-200 gel filtration				
Peak I	3.0	1.6	0.56	8
Peak II	3.5	5.5	1.57	26

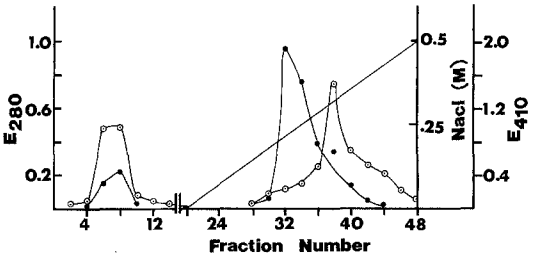

Figure 1. Elution profile of *E. tenella* acid phosphatase through a DEAE cellulose DE-52 column. The experimental conditions and analysis of fractions for protein and enzyme activity are described in Materials and methods. E280 (o); Acid phosphatase activity (●) and NaCl gradient (–).

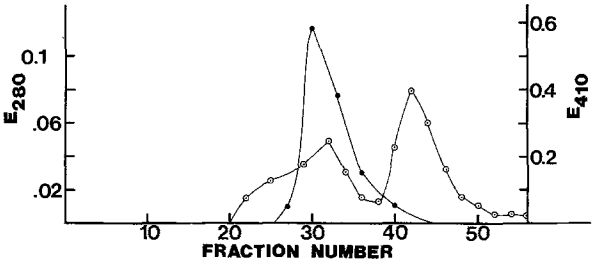

Figure 2. Elution of *E. tenella* acid phosphatase Peak II through Sephadex G-200 column. The experimental conditions and analysis of fractions for protein and enzyme activity are described in Materials and methods. E280 (o) and acid phosphatase activity (●).

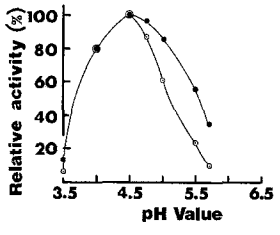

Figure 3. pH activity curve of acid phosphatase of *E. tenella*. The assay conditions are described in Materials and methods except that sodium acetate buffer of various pH values was used. Peak I (o); and Peak II (●).

Table 2. Effect of metal ions acid phosphatase[a] (Peak II) of *E. tenella*.

Addition	Conc. (mM)	Activity (%)
Control	–	100
$ZnCl_2$	0.5	98
	1.0	94
$HgCl_2$	0.5	10
	1.0	6
$CuCl_2$	0.5	60
	1.0	30
$MgCl_2$	25	100
	50	100
$MnCl_2$	25	100
	50	106
$BaCl_2$	25	96
	50	98
EDTA	10	100
	25	105

[a] The partially purified acid phosphatase was preincubated with the indicated concentrations of various metal ions at 37 °C for 15 min in 100 mM sodium acetate buffer, pH 4.5 with occasional shaking. The reaction was started by the addition of p-nitrophenyl phosphate (80 mM) in a final volume of 0.5 ml. The tubes were incubated for 30 min and the reaction was stopped as described in Materials and methods. The control reaction mixture containing all components except metal ions was treated in the same way.

Table 3. Effect of various inhibitors on acid phosphatase[a] (Peak II) of *E. tenella*.

Addition	Conc. (mM)	Acitivity (%)
Control	–	100
Sodium tartrate	50	100
	100	100
NaF	0.5	40
	1.0	21
Iodoacetamide	1.0	56
	2.0	33
	3.0	0.0
Cysteine	10.0	100
	20.0	100
Iodoacetamide + cysteine	2.0 + 20	73
Ammonium molybdate	0.05	25
	0.25	4.0
	0.50	1.5

[a] The enzyme was preincubated with indicated concentrations of various inhibitors at 37 °C for 15 min in 100 mM sodium acetate buffer, pH 4.5 with occasional shaking. The reaction was started by the addition of p-nitrophenyl phosphate (80 mM) in a final volume of 0.5 ml. The tubes were incubated for 30 min and the reaction was stopped as described in Materials and methods. The control reaction mixture containing all components except inhibitors was treated in the same way.

The time activity curve of the enzyme indicated that the hydrolysis of p-nitrophenyl phosphate was directly proportional to time for up to 1 h. The variation of substrate concentration at a fixed concentration of enzyme (Peak I and II) resulted in a double reciprocal plot indicating a Km value of 20 and 25 mM, respectively. The effects of metal ions on Peak II enzyme activity are shown in table 2. Hg^{++} and Cu^{++} at 0.5 mM produced 90% and 40% inhibition of enzyme activity. The divalent metal ions Mg^{++}, Mn^{++}, Ca^{++} and Ba^{++} had no effect on acid phosphatase activity. EDTA at 25 mM had no significant effect on enzymic activity. Sodium fluoride, iodoacetate and ammonium molybdate were potent inhibitors of enzyme activity (table 3). Cysteine alone had no effect on acid phosphatase activity. However, it protected the enzyme from inhibition with iodoacetamide. Sodium tartrate up to 100 mM had no effect on acid phosphatase of *E. tenella*.

Oocyst acid phosphatase (Peak II) was optimally active at 42 °C. The data on thermal stability of Peak I and II are presented in figure 4. It is apparent that Peak I is more stable than Peak II. Thus, at 52 °C after 30 min, Peak I displayed 40% enzyme whereas under identical conditions Peak II had only 15% enzyme activity. Attempts to restore this activity by the addition of cysteine or mercaptoethanol were unsuccessful.

The acid phosphatase of *E. tenella* oocyts (Peak II) was observed to have phosphohydrolytic activity towards several synthetic phosphate esters and phosphorylated metabolites (table 4). Adenosine 5'-monophosphate was a better substrate than adenosine 5'-triphosphate. The rate of hydrolysis

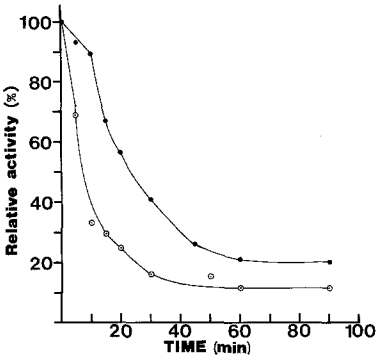

Figure 4. Thermal inactivation of acid phosphatase Peak I and II. These enzymes were heated at 52 °C in a 0.05 M Tris HCl buffer, pH 7.4. The zero time point was expressed as 100%. Samples were taken at the indicated times and the enzyme activity was determined as described in Materials and methods. The remaining activity was expressed as a relative percentage of the zero time activity. Acid phosphatase Peak I (●) and acid phosphatase Peak II (o).

Table 4. Substrate specificity of acid phosphatase[a] (Peak II) of *E. tenella*.

Substrate	Relative activity (%)
p-Nitrophenylphosphate	100
α-Napthylphosphate	60
α-Glycerophosphate	51
Adenosine 5'-monophosphate	83
Adenosine 5'-diphosphate	52
Adenosine 5'-triphosphate	15
Glucose 1-phosphate	57
Glucose 6-phosphate	65
Fructose 6-phosphate	43
Choline o-phosphate	9
Pyrophosphate	25

[a] The enzyme was assayed as described in Materials and methods.

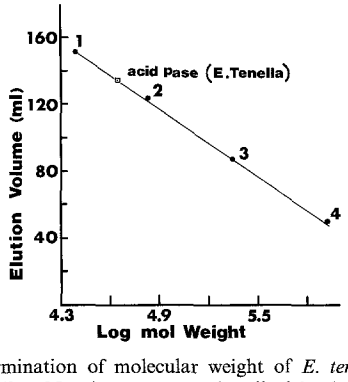

Figure 5. Determination of molecular weight of *E. tenella*. Sephadex G-200 column (2 × 35 cm) was run as described in the Materials and methods section; 1, Chymotrypsinogen A; 2, Albumin; 3, Catalase; and 4, Thyroglobulin were used as standard.

of glucose phosphate esters was higher than fructose phosphates. The lowest phosphorylatic rate was observed with choline phosphate. The enzyme also hydrolyzed sodium pyrophosphate at a rate which was 25% of that observed for p-nitrophenyl phosphate.

The molecular weight of the oocyst acid phosphatase (Peak I and Peak II) was estimated to be approximately 44,000 (fig. 5) based on the method of Andrews [16].

Discussion

The occurrence of phosphohydrolases in *Eimeria tenella* has been reported previously based on cytochemical techniques [3–5]. The present paper is the first report on the isolation and partial characterization of acid phosphorydrolases from oocysts of *E. tenella*. Protamine sulfate was used to remove the amylopectin [17] present in the oocyst extract. DEAE cellulose chromatography of the protamine sulfate fraction resulted in separation of two forms of acid phosphatase designated as Peak I and Peak II. Neither of these peaks is absorbed on a concanavalin-A Sepharose column, indicating the absence of mannose or glucose residues in these enzymes. Furthermore, the treatment of Peak I and II (acidic form) with bacterial neuraminidase does not alter its elution profile through a DEAE-cellulose DE-52 column. This is in contrast to acid phosphatases of animal origin, which are retained on concanavalin-A Sepharose columns [18]. Acid phosphatase and other acid hydrolases of chicken intestine are retained on concanavalin-A Sepharose column [19]. Furthermore, the treatment of animal acid phosphatases with bacterial neuraminidase converts the acidic forms of these enzymes into basic forms [20].

The properties of the *E. tenella* oocyst acid phosphatase indicate that it can be best classified as a relatively nonspecific acid phosphomonoesterase [1]. While oocyst acid phosphatase has many properties such as inhibition by fluoride and molybdate in common with the acid phosphatases of animal and other parasitic origin [2], differences such as its insensitivity to sodium tartrate were noted.

Oocyst acid phosphatase is inhibited by iodoacetamide and Hg^{++} indicating the presence of a sulfhydryl group in the enzyme. We have shown that *sarcocystis suicanis* bradyzoites also contain an acid phosphatase which has a sulfhydryl group in its active site [21].

The partially purified acid phosphatase of *E. tenella* hydrolyzes a wide variety of nonphysiological and physiological phosphate esters. Among the nucleotide phosphates used, adenosine 5′-monophosphate was hydrolyzed at the highest rate, followed by adenosine 5′-diphosphate and triphosphate. This is in contrast to the tartrate-resistant acid phosphatase of animal sources which does not hydrolyze adenosine 5′-monophosphate but dephosphorylate adenosine di- and triphosphates at an appreciable rate [22].

Glucose phosphates are better substrates for *E. tenella* acid phosphatase than fructose phosphate. The oocyst acid phosphatase has a molecular weight of about 44,000 compared to the phosphohydrolases of *S. suicanis* which has a molecular weight of 170,000 [21].

The physiological significance of oocyst acid phosphatase is not understood at present. However it may be possible that 1) this enzyme hydrolyzes the phosphate esters of the oocyst matrix and provides nutrition to the sporozoites which are living in it, 2) the enzyme is involved in the formation of oocyst wall and 3) acid phosphatase may be involved in protecting the parasite from the host. It is well known that phosphorylation and dephosphorylation of certain host enzymes results in alteration of their enzyme activities [23, 24].

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1 Roche, J., Phosphatases, in: Enzymes, vol. 1, pp. 473–510. Eds J. B. Sumner and K. Myrback. Academic Press, New York 1950.
2 Hollander, V. P., Acid phosphatases, in: Enzymes, vol. 4, pp. 449–497. Ed. P. Boyer. Academic Press, New York 1971.
3 Gill, B. S., and Ray, H. N., Phosphatases and their significance in *Eimeria tenella*. Ind. J. Vet. Sci. Ani. Husb. *24* (1954) 239–244.
4 Ray, H. N, and Gill, B. S., Preliminary observations on alkaline phosphatase in experimental *Eimeria tenella* infections in chicks. Ann. trop. Med. Parasitol. *48* (1954) 8–10.
5 Frandsen, J. C., *Eimeria stiedae:* cytochemical identification of acid and alkaline phosphatases, carboxylic ester hydrolases and succinate, lactate and glucose 6-phosphate dehydrogenases in endogenous stages or rabbit tissues. Exp. Parasitol. *23* (1968) 398–411.
6 Shirley, M. W., Enzyme variation in *Eimeria* species of chicken. Parasitology *71* (1975) 369–376.
7 Rollinson, D., Electrophoretic variation of enzymes in chicken coccidia. Tran. R. Soc. trop. Med. Hyg. *69* (1975) 436–437.
8 Farooqui, A. A., Lujan, R., and Hanson, W. L. Acid hydrolases of the coccidian, *Eimeria tenella*. Experientia *39* (1983) 1368–1370.
9 Vetterling, J. M., Continuous-flow differential density flotation of coccidial oocysts and a comparison with other methods. J. Parasitol. *55* (1969) 412–417.
10 Roberson, E. L., Hanson, W. L., and Chapman Jr, W. L., *Trypanosoma cruzi*. Effect of antithymocyte serum in mice and neonatal thymectomy in rats. Exp. Parasitol. *34* (1973) 168–180.
11 Helwig, J. J., Farooqui, A. A. Bollack, C., and Mandel, P., Distribution of lysosomal hydrolases in glomerular and tubular fractions of rabbit kidney cortex. Int. J. Biochem. *8* (1977) 323–327.
12 Fiske, C. H., and Subba Row, Y., The colorimetric determination of phosphorus. J. biol. Chem. *66* (1925) 375–400.
13 Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. *72* (1976) 248–254.
14 Brewer, J. M., and Ashworth, R. B., Disc electrophoresis. J. chem. Educ. *46* (1969) 41–45.
15 Farooqui, A. A., Purification and properties of arysulfatase A from human placenta. Archs int. Physiol. Biochem. *84* (1976) 479–492.
16 Andrews, P., The gel filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J. *96* (1965) 595–606.
17 Ryley, J. F., Bentley, M., Manners, D. J., and Stark, J. R., Amylopectin, the storage polysaccharide of the coccidia *Eimeria brunetti* and *E. tenella*. J. Parasitol. *55* (1969) 839–845.
18 Helwig, J. J., Farooqui, A. A., Bollack, C., and Mandel, P., Purification and some properties of tartrate-sensitive acid phosphatase from rabbit kidney cortex. Biochem. J. *175* (1978) 321–329.
19 Farooqui, A. A. and Hanson, W. L., Comparison of arylsulphatases from *Eimeria tenella* (parasite) and chicken caecum (host). Biochem. J. *242* (1987) 97–102.
20 Patel, A., and Koenig, H., Brain lysosomal hydrolases I. Solubilization and electrophoretic behavior of acid hydrolases in nerve-ending and mitochondrial-lysosomal fractions from rat brain. Effect of autolysis, neuraminidase and storage. Neurochem. Res. *1* (1976) 275–298.
21 Farooqui, A. A., Adam, D. D., Hanson, W. L., and Prestwood, A. K., Studies on the enzymes of *Sarcocystis suicanis*: Purification and characterization of an acid phosphatase. J. Parasitol. *73* (1987) 681–688.
22 Lam, K., Lai, L., Burkart, P. T., and Yam, L. T., Kinetic properties of tartrate resistant acid phosphatase isolated from human spleen with leukemic reticuloendotheliosis. J. biol. Chem. *252* (1977) 3371–3373.
23 Krebs, E. G., and Beavo, J. A., Phosphorylation-dephosphorylation of enzymes. A. Rev. Biochem. *48* (1979) 923–959.
24 Cohen, P., The role of protein phosphorylation in neural and hormonal control of cellular activity. Nature *296* (1982) 613–620.

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