Acid hydrolases of the coccidian *Eimeria tenella*[1]

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Summary. Activities of acid hydrolases were higher in sporozoites of *Eimeria tenella* than in unsporulated and sporulated oocysts. These enzymes along with proteinases may be involved in the penetration of epithelial cells of chicken cecum by sporozoites.

Because of the location of intracellular parasites, they must interact in some manner with the cell membrane of the host cell at the molecular level when entering the cell. These molecular interactions, which occur at the host-parasite interface, are of great importance in understanding the host parasite relationship[2,3]. The parasitic protozoan, *Eimeria tenella*, infects the cecal epithelial cells of the domestic chicken and causes the disease, cecal coccidiosis. This parasite has a complex life cycle involving both asexual and sexual multiplication. The parasites multiply by mechanisms yet unknown within the cecal epithelial cells[4]. Although some cytochemical information is available on the occurrence of acid hydrolases in sporulated oocysts of *E. tenella*[5-7] nothing is known about the activities of these enzymes in various life cycle stages of this parasite. In the present report, we describe the activities of acid hydrolases in unsporulated oocysts, sporulated oocysts, and sporozoites of *E. tenella*.

Materials and methods. Unsporulated oocysts and sporulated oocysts of *E. tenella* (WIS) were isolated as described earlier[8]. Excystation of sporocysts[9] and isolation of sporozoites were carried out by the method of Wagenbach[10] using a glass bead column (2.5 cm × 16 cm). Sonication of unsporulated oocysts resulted in disintegration of the oocyst wall as well as the zygotes themselves. Sonication of sporulated oocysts resulted in disintegration of the oocyst wall with a freeing of intact sporocysts. The latter were then removed by centrifugation. Sporozoite sonicates were prepared from fresh sporulated oocysts and maintained frozen (−20 °C) until used. The following enzymatic activities were determined:

Arylsulfatase: The enzyme acitivity was assayed by measuring the formation of p-nitrocatechol from p-nitrocatechol sulfate[11]. The assay mixture consisted of 100 µmoles sodium acetate buffer pH 5.2, 4 µmoles p-nitrocatechol sulfate, and up to 50 µg enzyme protein in a total volume of 0.4 ml. The reaction mixture was incubated for 30 min at 37 °C, and the reaction terminated by the addition of 2.5 ml of 1 M sodium hydroxide. The amount of 4-nitrocatechol formed was determined spectrophotometrically at 510 nm. 1 unit of the enzyme activity was defined as the amount liberating 1 nmole of 4-nitrocatechol per min under the above conditions.

β-N-Acetylhexosaminidase: The enzyme was determined by measuring the production of p-nitrophenol from p-nitrophenyl N-acetyl β-D-glucosaminide[12]. The assay mixture consisted of 10 µmoles sodium citrate pH 4.0, 2.4 µmoles p-nitrophenyl N-acetyl β-D-glucosaminide, and up to 50 µg enzyme protein in a final volume of 0.5 ml. The tubes were incubated for 30 min at 37 °C, and the reaction stopped by the addition of 2.5 ml of 1 M sodium hydroxide. The amount of p-nitrophenol liberated was determined spectrophotometrically at 405 nm. 1 unit of the enzyme was defined as the amount liberating 1 nmol of p-nitrophenol per min under the above conditions.

β-Galactosidase: The enzyme activity was determined by the method of Helwig et al.[13] using p-nitrophenyl β-D-galactoside as the substrate. The reaction was stopped by the addition of 2.5 ml of 1 M sodium hydroxide and the liberated p-nitrophenol was determined spectrophotometrically as described above. 1 unit of the enzyme was defined as the amount liberating 1 nmole of p-nitrophenol per min under the above conditions.

β-Glucuronidase: The enzyme was determined by measuring the liberation of p-nitrophenol from p-nitrophenyl β-D-glucuronide. The assay mixture consisted of 50 µmoles sodium acetate buffer pH 5.0; 4 µmoles p-nitrophenyl β-D-glucuronide and up to 50 µg enzyme protein in a total volume of 0.5 ml. The tubes were incubated for 60 min at 37 °C and the reaction terminated by the addition of 2.5 ml of 1 M sodium hydroxide. The liberated p-nitrophenol was determined spectrophotometrically as described above. 1 unit of the enzyme was defined as the amount liberating 1 nmole p-nitrophenol per min under standard assay conditions.

Acid and alkaline phosphatases: For acid phosphatase the assay mixture consisted of 50 µmol sodium citrate buffer pH 4.8, 30 µmoles p-nitrophenyl phosphate and up to 50 µg enzyme protein in a total volume of 0.5 ml. The tubes were incubated for 60 min at 37 °C and the reaction terminated by the addition of 2.5 ml of 1 M sodium hydroxide. The yellow coloration of p-nitrophenol was determined spectrophotometrically at 405 nm. The assay conditions for alkaline phosphatase were the same essentially except that 50 µmol Tris HCl buffer, pH 8.2, was used[14].

Hyaluronidase: The enzyme was assayed by the method of Srivastava and Farooqui[15] using hyaluronic acid as the

Table 1. Activities of acid hydrolases in various stages of the life cycle of *Eimeria tenella*

Enzyme	Specific activity (nmoles/min/mg protein)		
	Unsporulated oocyst	Sporulated oocyst	Sporozoites
Acid phosphatase	6.0 ± 1.23	7.77 ± 1.31	50.52 ± 7.95
Alkaline phosphatase	0.0	3.02 ± 0.92	20.21 ± 4.03
Arylsulfatase	0.0	0.0	0.21 ± 0.027
β-N-Acetylhexosaminidase	0.0	0.085 ± 0.031	2.88 ± 0.48
β-Galactosidase	0.0	0.055 ± 0.024	2.56 ± 0.44
β-Glucuronidase	0.0	0.0	1.57 ± 0.157
Neuraminidase	0.0	0.0	6.05 ± 1.03
Hyaluronidase	0.0	0.0	1.83 ± 0.32
Azocoll proteinase	9676 ± 2073	12920 ± 1972	13976 ± 2784

Results are means ± SD, for 3 determinations.

substrate. 1 unit of the enzyme equals 1 nmole N-acetylglucosamine released per min.

Neuraminidase: The enzyme was assayed by the method of Srivastava and Farooqui[16] using fetuin as a substrate. 1 unit of neuraminidase was defined as 1 nmol of sialic acid liberated from fetuin per min under the conditions described elsewhere[16].

Azocoll proteinase assay: Azocollegen (Calbiochem) hydrolysis was determined by the method described in Calbiochem Document No.3805. 1 unit of the azocoll proteinase was equal to an absorbance of 0.001 at 520 nm in 1 min at 37 °C. Total protein was determined by the method of Bradford[17] using bovine serum albumin as the standard. Specific activity of enzymes was expressed as nmoles of product formed per min per mg protein.

Preparation of enzyme extract: Frozen unsporulated oocysts, sporulated oocysts, or sporozoites were thawed and suspended in 0.02 M Tris HCl buffer, pH 7.4. The suspensions of unsporulated oocysts and sporulated oocysts were sonicated for 2 periods of 5 min at intervals of 3 min at 4 °C using a sonic Dismembrator Model 300 (Fisher) operating at full power. The suspension of sporozoites was sonicated for 2 periods of 2 min at intervals of 5 min at 4 °C as described above. The preparations were centrifuged at 13,000 × g for 30 min at 4 °C and the resultant supernatant was used as a source of enzyme after 13 h dialysis against 0.02 M Tris HCl buffer, pH 7.4 at 4 °C.

Results and discussion. Activities of acid hydrolases were different in the different stages of *E. tenella* life cycle (table 1). Acid phosphatase and azocoll proteinase were the only enzymes present in extracts of unsporulated oocysts, while activities of azocoll proteinase, acid and alkaline phosphatases, β-N-acetylhexosaminidase and β-galactosidase were detected in extracts of sporulated oocysts. Arylsulfatase, β-glucuronidase, neuraminidase and hyaluronidase were absent from extracts of sporulated oocysts. All these enzymes were detected in the extract of sporozoites. The specific activities of acid and alkaline phosphatases were 7–8 times higher in sporozoite extracts than in sporulated oocyst extracts. Furthermore, in sporozoites the activities of β-N-acetylhexosaminidase and β-galactosidase were 30–58-fold greater than that in the extract from sporulated oocysts. The activities of azocoll proteinase were similar in extracts of all three stages of this parasite.

To study the localization of these enzymes in sporozoites of *E. tenella*, the crude extract of freshly prepared sporozoites was fractionated into membrane and soluble fractions by ultracentrifugation at 60,000 × g for 60 min. The specific activity of acid phosphatase, β-N-acetylhexosaminidase, β-galactosidase and neuraminidase were demonstrated in the membrane fraction and was about 4 times higher than the sporozoite extract (table 2). In contrast, proteolytic activity (azocoll proteinase) was twice as great in the soluble fraction as was observed in the extract of sporozoites. The recovery of protein from the membrane and soluble fractions was about 14 and 57%, respectively. Acid hydrolases generally are considered as mammalian lysosomal (marker) enzymes both from the biochemical[18] and cytochemical points of view[19]. Various acid hydrolases also have been reported to occur in plasma membranes of various cell types[20]. It has been reported recently that the parasitic protozoan, *Leishmania donovani*[21,22] and other trypanosomatids[23], have acid phosphatase located in their plasma membranes.

It is well known that the membranes of animal cells are composed of glycoproteins, glycolipids and glycosaminoglycans. The sugar moieties of the glycoconjugates play a key role in cell recognition processes such as a) specific cellular adhesion, b) sexual mating of gametes and c) contact inhibition and cellular migration[24–27]. Further, acid hydrolases, arylsulfatase, β-N-acetylhexosaminidase, β-galactosidase, β-glucuronidase, neuraminidase and hyaluronidase are undoubtedly involved in the catabolism of glycolipids, glycoproteins and glycosaminoglycans[28–31]. The question arises in regard to the function of these enzymes in various stages of *E. tenella*. While we have no data to prove it, the occurrence of hydrolytic enzymes in sporozoites suggests that these enzymes along with proteinases could be involved in the degradation and penetration of the membrane structures of the epithelial cells of the chicken cecum, or the action of parasitic enzymes on the host cell surface may expose certain receptors which are necessary for the parasite-host interaction. In this regard, it should be recalled that the coccidian, *Toxoplasma gondii*, uses a 'penetration-enhancing factor' to invade the HeLa cell in culture[32–36]. The exact nature of this factor has not yet been investigated but lysozyme, hyaluronidase, β-glucuronidase and β-galactosidase have been reported to have a penetration-promoting effect[34] on the infection of the HeLa cell culture by *T. gondii*. In contradistinction, studies on surface membranes of *Plasmodium* species indicated that sporozoites and merozoites contain receptors which are involved in the penentration of the parasite into the host cell[37]. The high activities of acid and alkaline phosphatases in *E. tenella* sporozoites suggest that these enzymes may provide orthophosphate for the metabolic processes of the parasite

Table 2. Distribution of acid hydrolases in membrane and soluble fractions of sporozoites of *Eimeria tenella*

Enzyme	Fraction	Specific activity (nmoles/min/mg)	Recovery (%)	Relative specific activity
Acid phosphatase	Extract	53.5	100	1
	Membranes	206.0	53	3.8
	Supernatant	97.0	25	1.8
β-N-Acetylhexosaminidase	Extract	3.0	100	1
	Membranes	12.0	49	4
	Supernatant	6.0	27	2
β-Galactosidase	Extract	3.0	100	1
	Membranes	6.0	48	2
	Supernatant	3.5	31	1
Neuraminidase	Extract	6.0	100	1
	Membranes	24.0	59	4
	Supernatant	9.0	18	1.5
Azocoll proteinase	Extract	15,037	100	1
	Membranes	9,032	9	0.6
	Supernatant	27,097	59	1.8

by hydrolysing the phosphate esters of the host cell[8]. It remains an open question whether these enzymes are located on the outer surface of the sporozoite as in the case of ectoacid phosphatase of *L. donovani*[21,22,38] or whether they are embedded into the subcellular structures of the parasitic cell. To date the occurrence of lysosomes or lysosome-like structures in *E. tenella* has not yet been reported[39]. However, the secretory structures termed 'rhoptries' reportedly release their contents during penetration of embryonic bovine tracheal cells by *E. magna*[40,41]. Although the mechanisms involved in the penetration of host cells by *Eimeria* are not understood[42], available evidence from other related parasitic intracellular protozoans suggests that biochemical mechanisms should be carefully considered. In addition, the demonstration of hydrolytic enzymes in the various stages of *E. tenella* along with proteinases, in our opinion, enhances the possibility that cell recognition and penetration by these parasites may involve biochemical specificities at the molecular level. Additional work will be required to determine if this is true.

1 We are grateful to Professor Moises Agosin, Department of Zoology, for useful discussion. Cultures of *Eimeria tenella* (WIS) were provided by Drs L. McDougald and P. Long of this University. The authors were supported in part by grant No. 901-15-174 from the United States Department of Agriculture.

2 Trager, W., Science *183* (1974) 269.
3 Joyner, L.P., in: The Biology of the Coccidia, p.35. Ed. P.L. Long. University Park Press, Baltimore 1982.
4 Hammond, D.M., in: The Coccidia, p.45. Eds D.M. Hammond and P.L. Long. University Park Press, Baltimore 1973.
5 Ray, H.N., and Gill, B.S., Ann. trop. Med. Parasit. *48* (1954) 8.
6 Gill, B.S., and Ray, H.N., Indian J. vet. Sci. *24* (1954) 239.
7 Frandsen, J.C., Exp. Parasit. *23* (1968) 398.
8 Farooqui, A.A., and Hanson, W.L., Z. ParasitKde (1983) in press.
9 Patton, H.W., and Brigman, G.P., J. Parasit. *65* (1979) 526.
10 Wagenbach, G.E., J. Parasit. *55* (1969) 833.
11 Farooqui, A.A., and Srivastava, P.N., Biochem. J. *181* (1979) 331.
12 Farooqui, A.A., and Srivastava, P.N., Biochem. J. *191* (1980) 827.
13 Helwig, J.J., Farooqui, A.A., Bollack, C., and Mandel, P., Int. J. Biochem. *8* (1977) 323.
14 Farooqui, A.A., Adams, D.D., Hanson, W.L., and Prestwood, A.K., J. Parasit. (1983) in press.
15 Srivastava, P.N., and Farooqui, A.A., Biochem. J. *183* (1979) 531.
16 Srivastava, P.N., and Farooqui, A.A., Biol. Reprod. *22* (1980) 858.
17 Bradford, M.M., Analyt. Biochem. *72* (1976) 248.
18 De Duve, C., in: Lysosomes in Biology and Pathology, vol. 1, p.3. Ed. J.T. Dingle and H.B. Fell. North-Holland, Amsterdam 1969.
19 Novikoff, A.B., Essner, E., Goldfischer, S., and Heus, M., in: Interpretation of Ultrastructure, p.149. Ed. R.J.C. Harris. Academic Press, New York 1962.
20 Stefanovic, V., Mandel, P., and Rosenberg, A., J. biol. Chem. *251* (1976) 493.
21 Gottlieb, M., and Dwyer, D.M., Science *212* (1981a) 939.
22 Gottlieb, M., and Dwyer, D.M., Exp. Parasit. *52* (1981b) 117.
23 Brooker, B.E., Z. Zellforsch, *116* (1971) 532.
24 Spiro, R.G., Adv. Protein Chem. *27* (1973) 349.
25 Jeanloz, R.W., and Codington, J.F., in: Biological Roles of Sialic Acid, p.201. Eds A. Rosenberg and C.L. Schengrund. Plenum Press, New York 1976.
26 Kobata, A., Cell Struct. Funct. *4* (1979) 169.
27 Luft, J.H., Int. Rev. Cytol. *45* (1976) 291.
28 Davidson, E.A., in: Metabolic Conjugation and Metabolic Hydrolysis, vol. 1, p.327. Ed. W.H. Fishman, Academic Press, New York 1970.
29 Hayashi, S., J. Biochem., Tokyo *82* (1977) 1287.
30 Hayashi, S., J. Biochem., Tokyo *83* (1978) 149.
31 Kobata, A., Analyt. Biochem. *100* (1979) 1.
32 Lycke, E., Lund, E., and Strannegard, O., Br. J. exp. Path. *46* (1965) 189.
33 Lycke, E., and Norrby, R., Br. J. exp. Path. *47* (1966) 248.
34 Norrby, R., and Lycke, E., J. Bact. *93* (1967) 53.
35 Norrby, R., Londholm, L., and Lycke, E., J. Bact. *96* (1968) 916.
36 Norrby, R., Infect. Immun. *2* (1970) 250.
37 Kilejian, A., Trends biochem. Sci. *7* (1982) 5.
38 Gottlieb, M., and Dwyer, D.M., Molec. Cell Biol. *2* (1982) 76.
39 Aikawa, M., and Sterling, C.R., in: Intracellular Parasitic Protozoa, p.42. Eds M. Aikawa and C.R. Sterling. Academic Press, New York 1974.
40 Scholtyseck, E., and Chobotar, B., Z. ParasitKde *49* (1975) 91.
41 Jensen, J.B., and Edgar, S.A., J. Parasit. *62* (1978) 988.
42 Long, P.L., in: Cues that Influence Behavior of Internal Parasites, p.127. Ed. W.D.S. Bailey. Agricultural Research Service (Southern Region), U.S. Department of Agriculture, New Orleans 1982.

0014-4754/83/121368-03$1.50 + 0.20/0

Metallothionein induced in the frog *Xenopus laevis*[1]

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Summary. A low molecular weight cadmium- and copper-binding protein, induced in the liver of the frog, *Xenopus laevis*, by the administration of cadmium, was shown to consist of a single isoprotein and was characterized as an amphibian metallothionein based on its high cysteine and metal content, its low molecular weight, and the lack of aromatic amino acids.

Metallothionein is a low molecular weight, cysteine-rich protein (mol. wt = 6000–7000) binding heavy metals such as cadmium, zinc, copper and mercury. Metallothionein was first isolated as a cadmium- and zinc-binding protein from equine renal cortex by Margoshes and Vallee in 1957[2]. Thereafter, many investigators have reported the induction of metallothionein following the administration of heavy metals to a variety of organisms, both animals and plants. Although metallothionein has been well documented in vertebrates, especially in mammals, the protein in amphibia has been reported thus far only in the frog, *Xenopus laevis*[3,4]. Amphibia hold a phylogenetical position between