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Proteolytic Activity of Secretions from Newly Excysted Metacercariae of *Paragonimus westermani*

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Abstract

The possibility that secretions from newly excysted metacercariae of *Paragonimus westermani* possess proteolytic activity was studied. The incubation media of the larvae expressed a constant level of proteolytic activity at each hourly interval for up to 24 hours. The protease was found to have an optimum pH of 7.5 in the hydrolysis of azocoll and to require reducing agents for the full expression of its activity. The pH profile, catalytic properties and inhibitory sense of the protease resembled those of a member of a neutral thiol protease family. The present results showed that the newly excysted larvae secreted a neutral thiol protease capable of hydrolyzing several proteins and chromogenic substrates.

Key words: *Paragonimus westermani,* excysted metacercariae, secretions, proteolytic activity

Introduction

Larval mammalian trematode parasites have been shown to appear in proteolytic systems to degrade tissue proteins for invasion to mammalian host (Keene *et al.*, 1983). During infection by cercariae of *Schistosoma mansoni,* such extracellular matrices as collagen, elastin and glycoproteins are hydrolyzed by cercarial proteinases (McKerrow et al., 1985a,b). Like the other trematode parasites, the encysted form of *P. westermani* metacercariae also possesses a proteolytic system in its extracts that degrades azocoll and fluorescein isothiocyanate labeled-collagen (Yamakami and Hamajima, 1986, 1987). It is thus possible that the metacercarial secretions include protease(s) capable of disrupting connective tissue proteins to facilitate the larval invasion of the mammalian host and its migration.

The ability of larval parasites in infected host to hydrolyze tissue proteins evokes important considerations related to parasitic diseases. Studies of the physiological and immunological functions of proteases secreted from larval *Paragonimus* need to progress in close association with the development of serological diagnosis, such as that performed by enzyme-linked immunosorbent assay (Ruitenberg and van Knapen, 1977). Studies of enzymes secreted from the larvae are thus important in elucidating parasitic diseases. To investigate one aspect of the above problems, proteolytic activities were surveyed in materials secreted from newly excysted metacercariae of *Paragonimus.* The present report describes the initial studies on protease secreted from the excysted larvae, and provides a preliminary characterization of its activity.

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Materials and Methods

Preparation of secretions

To prepare secretions, metacercariae of *P. westermani* (triploid type) were obtained from crabs, *Eriocheir japonicus.* After being washed with 0.6% HCl, the metacercariae were excysted for 10 hours under the stimulus of mild heat treatment at 40°C in RPMI 1640 (Whittaker M.A. Bioproducts) with 100 i.u./ml of penicillin G and 0.1 mg/ml of streptomycin (Meiji Seika, Japan) as bacteriostats. About 2,000 of the newly excysted metacercariae were placed in a sterile Petri dish filled with 2.5

ml of the above medium that had been filtered (0.45 μm). To obtain secretions, the excysted larvae were incubated at 37°C under 5% CO_2 in air for up to 24 hours. The medium was replaced every hour, immediately passed through a filter (0.45 μm), then assayed for proteolytic activity and protein content. Control experiments were performed under the same conditions but without the larvae.

Assay of proteolytic activity

Enzymatic activity in the secretions was determined at each time interval by assaying azocoll hydrolysis as described previously (Yamakami and Hamajima, 1987). Samples (20 μl) were incubated at 37°C for one hour with 1 mg of azocoll (Sigma) in a total volume of 220 μl of 0.1 M imidazole-HCl, pH 7.5, and 5 mM cysteine. The reaction was terminated by adding 1 ml of 0.1 mM leupeptin (Protein Research Foundation, Japan). After centrifugation at 2,000 *g* for 10 min, azodye in the supernatant was measured at an absorption of 540 nm. The optimum pH of the protease was determined by the above assay procedure with the exception that imidazole-HCl was replaced by universal buffer, ranging from pH 3.0 to 10.0. Protease activity was also assayed using peptidyl-4-methyl-coumaryl-7-amide (peptidyl-MCA; Protein Research Foundation), casein, hemoglobin and fluorescein isothiocyanate labeled-bovine atelo collagen (FITC-collagen) as described previously (Yamakami and Hamajima, 1987). The designations of relative activity against the proteins were based on the total amounts of azodye, fluorescence or absorption at 280 nm of each substrate.

The assays were performed in triplicate. All results were representative of two similar experiments using different preparations of the excysted metacercariae.

Determination of protein concentration

The protein concentration was measured by the procedure of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Results

The incubation conditions of the newly excysted metacercariae of *P. westermani* were investigated first, because the excysted larvae did not survive well under the conditions tested. When the larvae were incubated in RPMI 1640 containing penicillin G and streptomycin at 37°C under 5% CO_2, they survived for more than 8 days.

To detect the activity of the protease, the incubation medium in which the larvae were maintained was assayed for azocoll hydrolysis. Thiol-dependent activity against the substrate was observed at a neutral pH. As shown in Fig. 1, activity increased gradually for the first 3 hours. A relatively constant amount of activity was detected in the medium from 3 hours up to 24 hours. During the course of incubation, the amount of protein secreted paralelled the level of azocoll hydrolysis. The results indicated that the proteolytic activity was due to the continued release of a constant amount of enzyme from the larvae. In the absence of the reducing agent, activity was scarcely detectable in the medium. On the other hand, the addition of 2 mM of calcium, magnesium or manganese

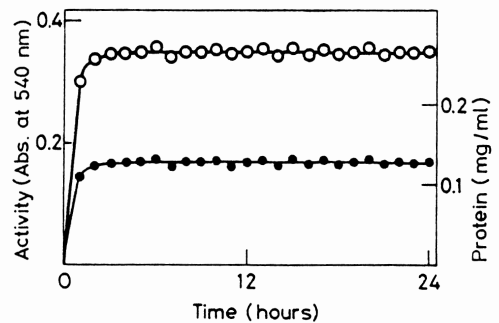

Fig. 1. Time course of proteolytic activity in the incubation medium of newly excysted metacercariae of *P. westermani.* Approximately 2,000 larvae were incubated in 2.5 ml of RPMI 1640 containing penicillin G and streptomycin at 37°C under 5% CO_2 for up to 24 hours. The medium was replaced every hour, and the harvested medium was immediately passed through a membrane filter and assayed for activity against azocoll (O) and protein content (•). Each assay were performed in triplicate.

ion prevented the appearance of hydrolysis in both the experimental and control media. There was no detectable activity or protein at time zero, indicating that there was no leakage of any substance from damaged larvae. The living larvae used in the experiments were intact and motile at the end of incubation. In the control media without the larvae, proteolytic activity and protein were completely undetectable.

The general properties of proteolytic activity in pooled media were studied. Since 5 mM of cysteine was an effective activator, the pH dependence of the activity was measured in its presence. A high level of the azocoll hydrolysis was observed at pH 7.5 (Fig. 2). Proteolytic activity was expressed, at a decreasing rate, against azocoll, casein, FITC-collagen and hemoglobin under the above conditions (Table 1). However, there was no proteolytic activity against elastin or bovine serum albumin. Cleaving activity against peptidyl-MCA, Boc-Val-Leu-Lys-MCA and Z-Phe-Arg-MCA (Boc, *t*-butyloxycarbonyl; Z, carboxybenzoyl), was also observed in the presence of cysteine, but the splitting level of Pro-Phe-Arg-MCA was lower than those of the above peptides. The enzyme, therefore, preferentially cleaved *N*-substituted peptidyl substrates with a basic amino acid at the carboxyl terminus. In contrast, no activity against the above substrates was detected in the control incubation media. The activity against azocoll was almost completely inhibited by [*L*-3-*trans*-carboxy-

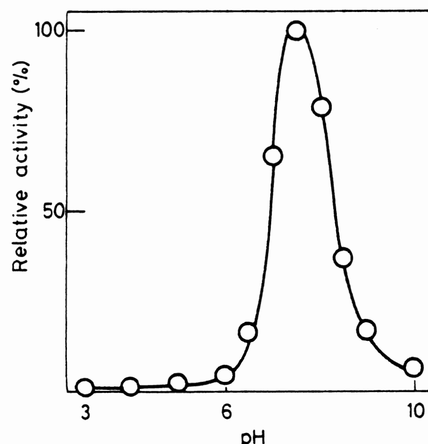

Fig. 2. pH activity curve of the activity secreted from newly excysted metacercariae of *P. westermani*. Assays were employed on the hydrolysis of azocoll with universal buffer, ranging from pH 3.0 to 10.0, in the presence of 5 mM cysteine. The point of maximal activity was taken as 100%. Each point is the mean for two experiments.

Table 1 Properties of the proteolytic activity in secretions from newly excysted metacercariae of *P. westermani*

Substrate specificity

Substrate	Activity
Azocoll	(100)
Casein	85
FITC-collagen	42
Hemoglobin	14
Boc-Val-Leu-Lys-MCA	(100)
Z-Phe-Arg-MCA	65
Pro-Phe-Arg-MCA	4

Effect of inhibitor

Inhibitor	Concentration	Inhibition
	(M)	(%)
None		0
E-64	10^{-6}	99.5
Antipain	10^{-6}	87.5
Leupeptin	10^{-6}	96.6
Chymostatin	10^{-6}	91.7
TLCK	10^{-5}	98.0
TPCK	10^{-5}	92.2
SBTI	(0.1 mg/ml)	1.4
DFP	10^{-3}	0

Measurements of substrate specificity in the hydrolysis of proteins were performed for 60 min at 37°C, while those in the peptidyl-MCA cleavages were performed for 10 min at 27°C in 0.1 M imidazole-HCl, pH 7.5, containing cysteine. Relative activities on the substrates are expressed as percentages of azocoll or Boc-Val-Leu-Lys-MCA hydrolysis. For assays of the effects of inhibitors, the enzyme solutin was preincubated with the inhibitor for 5 min at 27°C in 10 mM acetate buffer, pH 6.0, then residual activities on the hydrolysis of azocoll were determined. The rates of suppression were based on activity in the absence of inhibitor. Values are means for triplicate determinations.

oxyran-2-carbonyl]-*L*-leucyl agmatin (E-64), antipain, leupeptin, chymostatin, tosyl-*L*-lysine chloromethylketone (TLCK) and tosyl-*L*-phenylalanine chloromethylketone (TPCK). In contrast, proteolysis was not suppressed by serine or metallo protease inhibitors like diisopropyl fluorophosphate (DFP), soybean trypsin inhibitor (SBTI), phosphoramidon and EDTA. The optimum pH profile, catalytic properties and inhibitory sense of the secretions, thus resembled those of a neutral thiol protease.

Discussion

As shown in the present study, the product secreted into the incubation medium from newly excysted metacercariae of *Paragonimus* contained neutral proteolytic activity capable of hydrolyzing the tissue protein FITC-collagen. Judging from its substrate specificity, the function of the enzyme may be to facilitate histolysis of host tissues during invasion of the larvae. Our results on optimum pH profile showed that no acidic activity was observed to be isolated from encysted metacercariae or the adult stages of *Paragonimus* (Yamakami, 1986; Yamakami and Hamajima, 1987). A neutral thiol protease was also found in encysted larvae (Yamakami and Hamajima, 1987). Therefore, similar proteolytic systems should be present in mammalian host at the infection stages of the larvae. The secreted protease has also been found in the invasive form of *S. mansoni*, cercariae, which is active against collagen, elastin and glycoproteins (McKerrow *et al.*, 1985b). However, there were crucial differences in properties between the enzyme secreted from the excysted larvae of *Paragonimus* and that from larval *Schistosoma*. The major difference was that the *Schistosoma* enzyme was defined as a serine protease from its sensitivity to the typical inhibitors (McKerrow *et al.*, 1985b), whereas the *Paragonimus* enzyme was activated by reducing agens and was significantly suppressed by the thiol protease inhibitors E-64 and antipain. Moreover, proteolysis by larval *Paragonimus* was totally unaffected by classical serine protease inhibitors like SBTI and DFP, suggesting that the excysted metacercariae did not secrete a serine protease in the present experiments. These differences may be due to differences in the process of infecting to the mammalian host between *Paragonimus* and *Schistosoma*: *Paragonimus* invades the host intestinal wall, whereas the *Schistosoma* penetrates the mammalian skin.

The present results showed that the proteolytic enzyme continued to be secreted by newly excysted metacercariae of *Paragonimus in vitro*. One possible explanation for the function of the protease is that the enzyme may be involved in the invasion of the larvae to the mammalian host. The biological functions of the protease secreted from the cercariae of *S. mansoni* have also been investigated for their contribution to the hydrolysis of connective-tissue and basement-membrane macromolecules in its infection of the host (McKerrow *et al.*, 1985a). The occurence of the protease characterized in this report implies that it performs an indispensable function in larval invasion and migration by causing the histolysis of host tissue proteins.

References

1) Hamajima, F., Yamakami, K. and Tsuru, S. (1987): Anthelmintic effects of bithionol-antibody complex on *Paragonimus westermani* juveniles. Jpn. J. Parasitol., 36, 355–357.
2) Keene, W. E., Jeong, K. H., McKerrow, J. H. and Werb, Z. (1983): Degradation of extracellular matrix by larvae of *Schistosoma mansoni*. II. Degradation by newly transformed and developing schistosomula. Labo. Invest., 49, 201–207.
3) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265–275.
4) McKerrow, J. H., Keene, W. E., Jeong, K. H. and Werb, Z. (1983): Degradation of extracellular matrix by larvae of *Schistosoma mansoni*. I. Degradation by cercariae as a model for initial parasite invasion of host. Labo. Invest., 49, 195–200.

5) McKerrow, J. H., Jones, P., Sage, H. and Pino-Heiss, S. (1985a): Proteinases from invasive larvae of the trematode parasite *Schistosoma mansoni* degrade connective-tissue and basement-membrane macromolecules. Biochem. J., 231, 47–51.
6) McKerrow, J. H., Pino-Heiss, S., Lindquist, R. and Werb, Z. (1985b): Purification and characterization of an elastinolytic proteinase secreted by cercariae of *Schistosoma mansoni*. J. Biol. Chem., 260, 3703–3707.
7) Ruitenberg, E. J. and Knapen, F. van (1977): The enzyme-linked immunosorbent assay and its application to parasitic infections. J. Infect. Diseas., 136, S267–S273.
8) Yamakami, K. (1986): Purification and properties of a thiol protease from lung fluke adult *Paragonimus ohirai*. Comp. Biochem. Physiol., 83B, 501–506.
9) Yamakami, K. and Hamajima, F. (1986): Proteolysis of collagen by a thiol protease from metacercariae of *Paragonimus westermani*, Jpn. J. Parasitol., 35, 145–148.
10) Yamakami, K. and Hamajima, F. (1987): Purification and properties of a neutral thiol protease from larval trematode parasite *Paragonimus westermani* metacercariae. Comp. Biochem. Physiol., 87B, 643–648.