

Enzyme activity of *Ascaridia galli* Larvae was inhibited by Phenil Methanyl Methane Sulfonyl Fluoride

Ummu Balqis¹, Darmawi² and Muhammad Hambal³

¹Laboratory of Pathology, Veterinary Medicine Faculty of Syiah Kuala University, Banda Aceh 23111, Indonesia; ²Laboratory of Microbiology, Veterinary Medicine Faculty of Syiah Kuala University, Banda Aceh 23111, Indonesia; ³Laboratory of Parasitology, Veterinary Medicine Faculty of Syiah Kuala University, Banda Aceh 23111, Indonesia. Corresponding Author: d_darmawi@yahoo.com

Abstract. Enzyme catalyse a broad spectrum of important biological reactions, including protein metabolism and immune reactions. A study was carried out to partial characterize enzyme from excretory/secretory of *A. galli* L₃ stage based on influence of inhibitor. *A. galli* L₃ were recovered from intestines of 100 heads chickens 7 days after oesophagus inoculation with 6000 L₂. L₃ recovered in this manner were cultured (5 – 10 ml⁻¹) in flasks containing rosswell park memorial institute (RPMI) 1640 media, pH 6.8, without phenol red. Cultures were incubated at 37°C in 5% CO₂ and culture fluid was collected after 3 days in culture. Excretory/secretory was prepared from metabolic product of L₃ released in culture medium. The enzyme activity was assayed against casein 2%. The amount of concentration was determined from the absorbance at 595 nm. Inhibitor sensitivity on enzyme activity was studied in phenil methanyl methane sulfonyl fluoride (PMSF) 0,5 and 1 mM. The result showed that enzyme activity of *A. galli* larvae was inhibited by PMSF 0.5 and 1 mM, which enzyme activity remained 1,8 and 0%, respectively. The results indicate that the excretory/secretory secreted by L₃ *A. galli* contained serine protease.

Key words: *Ascaridia galli*, larvae, enzyme, nematode.

Introduction

Gastrointestinal helminth infections are the most common parasites found in poultry throughout the world. Ascariidiosis caused by the nematode, *Ascaridia galli*, is an important parasitic disease of chicken, goose, guinea fowl, turkey and various wild birds in most part of the world (Permin and Nansen, 1998). Infections with *A. galli* may cause economic losses in the form of retarded growth. Previous study showed that laying hens infected by infective stage of *A. galli* could effect host-weight depression, and lowered egg production, which is directly related to the worm burden (Tiuria et al., 2003). Additionally, Chadfield et al. (2001) reported that *A. galli* eggs act as an additional risk factor in the dissemination of *Salmonella enterica* serovar Typhimurium in chicken. Although various authors indicated that *A. galli* still contribute in helminthosis, highly pathogenic intestinal nematode of poultry and can result in decreased egg production, weight loss, and death. It is poor information about host-parasite, particularly laying hen-*A. galli* interaction.

In the effort to study host-parasite interaction like *A. galli* infection caused ascariidiosis in laying hen, it is necessary to know fundamental biochemistry aspects of *A. galli*. According to Williamson et al. (2003) biochemistry aspect of nematode facilitated by enzyme such as proteases. Proteases encompass a broad class of hydrolytic enzymes that play essential roles in cellular, developmental and digestive processes, blood coagulation, inflammation, wound healing and hormone processing. Parasite proteases, some of which are in the excretory-secretory (ES) products, facilitate the invasion of host tissues, aid in the digestion of host proteins, help parasites evade the host immune response and mediate molting in parasitic nematodes. As a result, parasite proteases are considered to be potential targets for the development of novel immunotherapeutic, chemotherapeutic and serodiagnostic agents for the next generation of antiparasite interventions.

Proteases are classified as exopeptidases (which act on the C- or N-terminal positions of polypeptides) and endopeptidases (which cleave peptide bonds of more central regions of polypeptides). According to the chemical nature of the active centre, the endopeptidases are further subdivided as serine, metallo-, cysteine and aspartate proteinases (Rhoads et al., 1997). To classify the protease(s) defined in ES from L₃ *A. galli*, it is necessary to investigate the character of enzyme.

The mechanisms that underlie tissue damage and invasion by the invasive stages of the parasitic nematode *A. galli* are poorly understood, but involvement of as yet uncharacterized protease has been suggested. Proteases in ES product associated with parasite invasion, which are tightly regulated and parasite specific, are likely to be essential to the success of parasitism. To explain this opinion, additional research will be required to confirm the character of the protease activity observed at an inhibitor like phenil methanyl methane sulfonyl fluoride (PMSF), identified by enzymological studies of excretory/secretory *A. galli* larval product. Here, we employed inhibitor assays based on influence of PMSF to examine the partial character of enzyme activity in ES product released by L₃ of *A. galli*.

Materials and Methods

Parasite

Female adult worms were obtained from lumen of village chickens in a commercial abattoir in Bogor. The eggs in uteri of female adult worms were expelled, prepared and collected under stereomicroscope. The eggs were incubated in sterile aquadestilata at room temperature for 21- 30 days developed embrionated eggs (L₂) (Tiuria et al., 2003).

A. galli L₃ were recovered from intestines of 100 heads chickens 7 days after each oesophagus inoculation with 6000 L₂. L₃ recovered in this manner were cultured (5–10 ml⁻¹) in flasks containing RPMI 1640 media, pH 6.8, without phenol red and supplemented with 100 units ml⁻¹ penicillin G, 100 µg ml⁻¹ streptomycin, 5 µg ml⁻¹ gentamycin, and 0.25µg ml⁻¹ kanamycin. Cultures were incubated at 37°C in 5% CO₂ and ES product of L₃ released in culture was collected after 3 days (Darmawi et al., 2006).

Excretory/Secretory *A. galli*

To prepare excretory/secretory product, culture medium L₃ *A. galli* were centrifuged at 1000 g, temperature 4°C for 10 minutes. Supernatan was filtered with 0.45 µm Minisart® (Sartorius), poured and concentrated in vivaspin 30,000 MWCO at 1000 g, temperature 4°C for 10 minutes. Filtrate was washed in phosphate buffered saline (PBS) for three times (Balqis et al., 2007).

Assay of enzyme activity

The protease activity was assayed against casein as described by Bergmeyer and Grassl (1983). Amount 500 µl 0,6% casein mixed in Tris mM (pH 8,0) and 100 µl enzyme was incubated for 2 hours at temperature 40°C. Reaction was stopped with add trichloroacetic acid 0,4 M and incubated for 10 minutes at temperature 40°C. After centrifugation, 200 µl supernatan was mixed in 1 ml sodium carbonate and 200 µl reagent Folin-Ciocalteu and incubated for 20 minutes at temperature 40°C. The amount of degradation was determined from absorbance at 578 nm as described by Rukayadi and Suhartono (1999). For further details, please follow the step showed in Table 1.

Table 1. Procedure assayed enzyme activity

	Blank (ml)	Standard (ml)	Sample (ml)
Borat buffer (0.01 M, pH 8.0)	1.0	1.0	1.0
Casein substrate (20 mmol, pH 8.0)	1.0	1.0	1.0
Enzyme in CaCl ₂ (2 mM)	-	-	0.2
Tyrosin (standard)	-	0.2	-
Aquadest	0.2	-	-
Incubation (37°C, 10 min)			
Trichloroacetic (0.1 M)	2.0	2.0	2.0
CaCl ₂ (2 mM)	-	-	2.0
Enzyme in CaCl ₂ (2 mM)	2.0	2.0	-
Incubation (37°C, 10 min) and centrifuge (4000 rpm, 10 min)			
Filtrate	1.5	1.5	1.5
Na ₂ CO ₃ (0.4 M)	5.0	5.0	5.0
Folin Reactor (1 : 2)	1.0	1.0	1.0
Hush up (±20 min, 37°C) and read the absorbance at 578 nm			

Results and Discussion

Enzyme activity of *A. galli* larvae founded in this research was inhibited by PMSF as shown in Table 2.

Table 2. The influence of inhibitor serine against enzyme activity released by *A. galli* larvae

Inhibitor/activator	The end concentration	Activity (%)
Control		100,0
Inhibitor serin: PMSF	0.5 mM	1.8
	1 mM	0

In this study, parasite protease activity was observed. PMSF used in this manner was able to inhibit the enzyme activity, which the concentration of inhibitor influence the enzyme activity. Proteolytic inhibition occurs when a concentration between 0.5 - 1 mM PMSF is used. Concentration of inhibitor in 1 mM PMSF was able to absolutely inhibit the enzyme activity (please see Table 2). Lopez-Llorca et al., (2002) and Yang *et al.*, (2007) reported that PMSF was the most effective inhibitor for protease activity released by fungal parasites of nematode. PMSF binds specifically to the active site serine residue in a serine protease. It does not bind to any other serine residues in the protein. This is a result of the hyperactivity of that serine residue caused by the specific environmental conditions in the enzyme's active site.

Based on our finding reflect that the presence of enzyme activity of ES product released by L₃ of *A. galli* indicate that the infection process of a number of nematode organisms, including some roundworms such as *A. galli*, depends on protease. Proteases are known to play an important role in the pathogenesis of tissue-invading parasites and triggering host immune response mechanisms, including parasitic nematode such as *S. ratti* (Abe et al., 1992), *O. ostertagi* (Cock et al., 1993), *A. suum* (Rhoads et al., 1997 and 2001), and *H. contortus* (Vervelde et al., 2003). However, its proteolytic activity was highly sensitive to the serine protease inhibitor PMSF (1 mM), indicating that it belongs to the serine-type peptidase group.

Temperature influenced the enzyme activity released by nematode. Our previous study showed that temperature optimum of the protease secreted by L₃ of *A. galli* stage is 70°C, indicate that the temperature played an important role against enzymatic activity of protease secreted by *A. galli* L₃ satage (Balqis and Fahrimal, 2011). Proteases released by nematodes are recognized as biologically active molecules with important functions relating to host-parasite interaction. Proteases are involved in parasite feeding, metabolism, penetration of host tissue barriers, and they are also receiving considerable attention as potentially usefull serodiagnostic or protective antigens (Abe et al., 1992; Cock et al., 1993 and Rhoads et al., 1997).

The pathogenesis of nematode infection, especially ascariidiosis, is probably a multistep process (Permin and Nansen, 1998) that includes adhesion, degradation, and invasion. Adhesion should be the primary step, followed by invasion and degradation of extracellular matrix protein and host cells. Here, we assumed that secreted proteases of *A. galli* may be to degrade extracellular matrix protein, thereby facilitating mucosal penetration by the invasive stage (L₃). *A. galli* may likewise utilize protease to facilitate larval invasion, migration within the host tissue, and modulation of host immune response mechanisms.

Various authors have indicated that proteases played an important role in parasite survival or stimulating the host immune response. Evidence that during transition of L₃ - L₄ stage larvae of *Ascaris suum*, which are more often associated with molting (Rhoads et al., 1997), have a greater requirement for enzyme to facilitate penetration of host tissues during the extensive migration of the larval stages through the host (Rhoads et al. (2001). Proteases secreted by *Ostertagia ostertagi* may be useful target molecules for serodiagnosis designed to detect antibody and vaccination to stimulate host immune response (Cock et al., 1993). Meanwhile, Qihong et al., (2006) reported that serine protease from a *Bacillus* genus of bacteria that serves as a pathogenic factor against nematodes, an important step

in understanding the relationship between bacterial pathogen and host and in improving the nematocidal activity in biological control.

Our previous study showed that immunoglobulin *yolk* (IgY) stimulated by the excretory/secretory antigen of *A. galli* L₃ stage was able to recognize *A. galli* surface antigen on cuticle of adult worms so the ES and IgY could be applied for immunodiagnostic (Balqis et al., 2011a). Recently, Balqis et al. (2011b) showed that laying hens vaccinated with ES secreted by *A. galli* L₃ stage stimulate mucosal response against parasitic diseases caused by *A. galli* indicated by increasing significantly goblet cells in duodenum, jejunum and ileum of laying hens. This research finding appearance the new hypothesis could protease released in excretory/secretory trigger mucosal response against parasitic diseases caused by *A. galli*.

Conclusions

The excretory/secretory secreted by L₃ *A. galli* contained enzyme inhibited by PMSF indicated that the enzyme classified as serine protease.

Acknowledgements

We wish to thank the Indonesian Ministry of Research and Technology for funding of the work, grant from the Riset Unggulan Terpadu. We thank Ela Nurmalasari, Rahanto Siregar, Indra, and Janiati Karo Karo, for their help; and we thank Sulaeman and Kosasih Department of Parasitology, Veterinary Faculty; Hj. Ika Malika Department of Microbiology and Biochemistry, Pusat Antar Universitas, Bogor Agriculture Institute, for technical assistance.

References

- Abe T., Nawa Y., and Yoshimura. 1992. Protease resistant interleukin-3 stimulating components in excretory and secretory products from adult worms of *Strongyloides ratti*. *J. Helminth.* 66: 155 – 158.
- Balqis U., Darmawi, Tiuria R. 2007. Purifikasi dan karakterisasi proteinase dari substansi bioaktif stadium transisi L₃ - L₄ *Ascaridia galli* dan aplikasinya sebagai kandidat vaksin anti ascariidiosis pada ayam petelur. Laporan Riset Unggulan Terpadu, Kementerian Negara Riset dan Teknologi Republik Indonesia.
- Balqis U., Fahrimal Y. 2011. Penentuan temperatur optimum terhadap aktivitas protease serin yang dihasilkan oleh stadium L₃ *Ascaridia galli*. *Proceeding: HIMKA, FKIP Unsyiah Banda Aceh*.
- Balqis U., Darmawi., Handharyani E., and Hambal M. 2011a. Deteksi keberadaan antigen pada kutikula *Ascaridia galli* dengan imunoglobulin *yolk* melalui metode imunohistokimia. *Proceeding: CheSA, FT Unsyiah Banda Aceh*.
- Balqis U., Darmawi., and Hambal M. 2011b. Respons sel goblet terhadap penyakit parasitic pada ayam petelur yang diberikan ekskretori/sekretori stadium L₃ *Ascaridia galli*. *Proceeding: ADIC, Kuala Lumpur Malaysia*.
- Bergmeyer H.U., and Grassl M. 1983. Methods of enzymatic analysis. Vol. 2. Verlag Chemie. Weinheim.
- Chadfield M., Permin A., Nansen P., and Bisgaard M. 2001. Investigation of the Parasitic Nematode *Ascaridia galli* (Shrank 1788) as a Potential Vector for *Salmonella enterica* Dissemination in Poultry. *Parasitol. Res.* 87: 317 – 325.
- Cock H.D., Knox D.P., Claerebout E, and Graaf D.C.D. 1993. Partial Characterization of Proteolytic Enzymes in Different Developmental Stages of *Ostertagia ostertagi*. *J. Helminthol.* 67: 271 – 278.
- Darmawi, Balqis U., Tiuria R., Suhartono M.T., Soejoedono R.D., Priosoerjanto B.P., Pasaribu F.H., and Hambal M. 2006. Protease Activity of Excretory/Secretory Released by Invasive Stage of *Ascaridia galli*. *Proceeding, Enzymes: Industrial and Medical Prospects, ASEAN Biochemistry Seminar, Surabaya, February 6-7th, 2006*.
- Lopez-Llorca L.V., Olivares-Bernabeu C., Salinas J., Jansson H.B., Kolattukudy P.E. 2002. Pre-penetration events in fungal parasitism of nematode eggs. *Mycol. Res.* 106 (4): 499-506
- Permin A and Nansen J.W. 1998. Epidemiology, Diagnosis and Control of Poultry Parasites. Food and Agriculture Organization of the United Nations, Rome.

- QiuHong N., Xiaowei H., Baoyu T., Jinkui Y., Jiang L., Lin Z., and Keqin Z. 2006. *Bacillus* sp. B16 kills nematodes with a serine protease identified as a pathogenic factor. *Applied Microbiology and Biotechnology*. 69(6): 722-730. <http://www.springerlink.com/content/qrhq751133v6l111/> (October 4th 2011)
- Rhoads M.L., Fetterer R.H., and Urban Jr. J.F. 1997. Secretion an Aminopeptidase During Transition of Third-to Fourth-Stage Larvae of *Ascaris suum*. *J. Parasitol.* 83(5): 780 - 784.
- Rhoads M.L., Fetterer R.H., and Urban Jr. J.F. 2001. Release of Hyaluronidase During *in vitro* Development of of *Ascaris suum* from the Third to Fourth Larval Stage. *Parasitol. Res.* 87(9): 693 -697.
- Rukayadi Y., and Suhartono M.T. 1999. Penuntun Praktikum Biokimia (BIM.511). Program Pascasarjana Institut Pertanian Bogor.
- Tiuria R, Ridwan Y, and Murtini S. 2003. Study of Bioactive Substance from *Ascaridia galli* Adult Worm that Stimulate Intestinal Mucosal Defence Mechanism in Chicken for Medical Purpouse. *Proceeding: Toray Sci. Found., Jakarta.*
- Vervelde L, Bakker N, Kooyman F.N.J., Cornelissen A.W.C.A., Bank C.M.C., Nyame A.K., Cummings R.D., and Die I.V. 2003. Vaccination-induced Protection of Lambs Against the Parasitic Nematode *Haemonchus contortus* Correlates With High IgG Antibody Responses to the LDNF Glycan Antigen. *Glycobiol.* 13 (11): 795 - 804. <http://glycob.oupjournals.org/cgi/content/full/13/11/795> (January 18th 2005)
- Williamson A.L., Brindley P.J., Knox D.P., Hotez P.J., and Loukas A. 2003. Digestive proteases of blood-feeding nematodes (Review). *TRENDS in Parasitology*, 19(9): 417-423.
- Yang J., Liang L., Zhang Y., Li J., Zhang L., Ye F., Gan Z., and Zhang K.Q. 2007. Purification and cloning of a novel serine protease from the nematode-trapping fungus *Dactylellina varietas* and its potential roles in infection against nematodes. *Applied Microbiology and Biotechnology*. 75(3): 557-565, <http://www.springerlink.com/content/h66j21n86j564q16/> (October 4th 2011).