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

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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 exretory/secretory of *A. galli* L_3 stage based on influence of inhibitor. *A. galli* L_3 were recovered from intestines of 100 heads chickens 7 days after oesophagus inoculation with 6000 L_2. L_3 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 37^0C in 5% CO_2 and culture fluid was collected after 3 days in culture. Excretory/secretory was prepared from metabolic product of L_3 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 remainded 1,8 and 0%, respectively. The results indicate that the excretory/secretory secreted by L_3 *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. Ascaridiosis 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 word (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 ascaridiosis 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 (wich act on the C- or N-terminal positions of polypeptides) and endopeptidases (wich 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 L_3 *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 L_3 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 colected under stereomicroscope. The eggs were incubated in sterile aquadestilata at room temperature for 21- 30 days developed embrionated eggs (L_2) (Tiuria et al., 2003).

A. galli L_3 were recovered from intestines of 100 heads chickens 7 days after each oesophagus inoculation with 6000 L_2. L_3 recovered in this manner were cultured (5–10 ml^{-1}) in flasks containing RPMI 1640 media, pH 6.8, without phenol red and suplemented 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 37^0C in 5% CO_2 and ES product of L_3 released in culture was collected after 3 days (Darmawi et al., 2006).

Excretory/Secretory A. galli

To prepare excretory/secretory product, culture medium L_3 *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 Incubation (37°C, 10 min)			
Trichlotoacetic (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_2CO_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 L_3 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 L_3 of *A. galli* stage is 70^oC, indicate that the temperature played an important role against enzymatic activity of protease secreted by *A. galli* L_3 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 ascaridiosis, 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 (L_3). *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 L_3 – L_4 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, Qiuhong 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* L_3 stage was able to recognized *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* L_3 satage 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 L_3 *A. galli* contained enzyme inhibited by PMSF indicated that the enzyme classified as serine protease.

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