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RESEARCH ARTICLE

Immunochemical studies of *Toxocara canis* proteases

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ABSTRACT

Proteases of nematodes play a crucial role in larval molting and, in addition to their active role in egg hatching, proteases are also considered a crucial factor in tissue invasion and connective tissue remodeling. In *Toxocara canis*, proteases play important roles throughout the complex life cycle. They can degrade components of a model of extracellular matrix, basement membranes and different physiological substrates. In the present study, measurements of the proteolytic activity of the perivitelline fluid (PF) surrounding *Toxocara canis* embryos at different stages of development, the hatching fluid (HF) surrounding the infective larvae, as well as the excretory secretory (ES) products of the larvae in the culture media were performed. Measurements were made using casein as substrate following the Sigma non-specific protease activity assay. The results showed that enzyme activity increased as the embryo matured. The infective larvae were found to continuously produce proteases in the surrounding HF and ES products after *in vitro* cultivation indicating that *Toxocara canis* proteases might be important for the worm in the egg and the host. Optimal enzymatic activity was found at pH 8. Incubation of the antiserum from infected mice with the HF and ES products decreased their proteolytic activities, suggesting that there may be a link between the proteases present in these fluids and the immune response.

Keywords: Egg shell; excretory secretory products; hatching fluid; perivitelline fluid; proteases.

INTRODUCTION

Toxocara canis (*T. canis*) worms live in the small intestines of dogs, foxes and other canid species. Immature eggs have a very tough thick egg shell and are excreted with the feces of infected definitive hosts. Under favorable environmental conditions, the eggs mature in the soil and can remain infectious for a long time (Overgaauw *et al*., 2009). Developing larvae secrete their products into the surrounding perivitelline fluid (PF) and continue to develop until the infective 3rd stage larvae. Infective larvae are surrounded by hatching fluid (HF) and undergo developmental arrest until ingested by a suitable host (Geenen *et al*., 1999).

Man, rodents and other animals are the paratenic hosts of *T. canis*. Humans, especially children, become infected after accidental ingestion of mature eggs. Infective 3rd stage larvae in undercooked or raw meat tissues of other paratenic hosts are other sources of infection (Strube *et al*., 2013). After ingestion, the infective larvae penetrate the intestinal wall and migrate widely, invading different tissues. Infection of the paratenic host give rise to two variants of the disease; the disseminated form that include covert toxocariasis and visceral larva migrans or the compartmentalized form that manifest as ocular larva migrans or neurotoxocariasis (Rubinsky-Elefant *et al*., 2010; Macpherson, 2013). Eosinophilia is a prominent feature of the tissue-invasive helminths (el Zawawy *et al*., 1995). A full life cycle of *T. canis* is completed only in young dogs. In these puppies, *T. canis* worms go through a pulmonary larval phase and live as adults in the small intestine. In adult dogs, the larvae do not usually reach the mature stage but remain alive in different tissues (Dubey, 1978).

T. canis has many biological characteristics that allow the worm to complete its life cycle. The larvae can invade a wide variety of paratenic hosts which may, through predation, allow the parasite to reach its final canid definitive host species (Strube *et al*., 2013). Within the paratenic host, the larvae migrate through the tissues and enter a markedly arrested state for a long time, but remain metabolically active and produce excretory secretory (ES) products. The main components the ES products of *T. canis* are the secretion of the esophageal gland, the excretion of the excretory cells along with the outer surface coat that is continuously shed off (Page *et al*., 1992; el Naga, 2000). These products allow life cycle progression by evading immune destruction (Badley *et al*., 1987). Throughout this complex life cycle, proteases perform important physiological functions. Infective larvae can also release their ES products *in vitro* into culture media (de Savigny, 1975). The proteolytic enzymes of HF of *T. canis* are of aspartic type while those of the ES products are serine proteases. The enzymes in the PF of various nematodes include leucine aminopeptidase, pseudocollagenase and chitinases (Ward & Fairbairn 1972; Rogers 1982; Mkandawire *et al*., 2022). Proteases of nematodes play a crucial role in larval molting as they digest the old cuticle, degrade the cuticular anchor proteins or activate molting enzymes by processing their proenzymes (Page *et al*., 2014). In addition to their active role in egg hatching, proteases are also considered to be a key factor in tissue invasion

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and connective tissue remodeling (Dzik, 2006; Shyu *et al*., 2019). Proteases of *T. canis* can degrade components of a model of extracellular matrix, basement membranes and various physiological substrates (Robertson *et al*., 1989; González-Páez *et al*., 2007; González-Páez *et al*., 2014).

HF and ES products of *T. canis* can be recognized by the serum of infected animals (Smith *et al*., 1982; Badley *et al*., 1987). ES products of *T. canis* are used in the serodiagnosis of toxocariasis (Iddawela *et al*., 2017) and their proteomic study revealed potential candidates for vaccines (da Silva *et al*., 2018). Furthermore, in immunosuppressed pregnant and lactating bitches, the dominant larvae are activated to migrate to the fetus through the placenta and the mammary gland, although this could be due to the response of the larvae to hormones produced during these two conditions (Lloyd *et al*., 1983; Robertson *et al*., 1989). These findings suggest a link between the proteases present in these fluids and the immune response.

In this work we measured the proteolytic activities of the PF of *T. canis* in different periods of the intra ovular development. Proteolytic activity was also measured in the HF after development of infective larvae and in the ES products released into the media of 3rd stage larvae cultured *in vitro*. The pH-dependent activity of the proteases produced by the infective larvae in both the HF and ES products was determined, as well as the effect of the immune serum evoked in infected mice, on the enzymatic activity.

MATERIALS AND METHODS

Ethics approval and consent to participate

All animal studies were performed in accordance with the regulations of the Ethics Committee of Faculty of Medicine, Alexandria University in accordance with the Egyptian regulations for animal experimentation (00012098).

Collection of *T. canis* worms and embryonation of eggs

Adult female *T. canis* worms were collected from the intestine of pups after euthanasia. The worms were washed with sterile physiological saline solution. The eggs were collected from the vagina and the distal third of the uterus. Embryonation of the eggs was carried out as previously described by Abou-El-Naga (2018). Briefly, the eggs were cultured in 1% formalin at 28°C at a concentration of 4000 eggs/ml and aerated for half an hour/day for 28 days with continuous observation of the larval development. Examination of the eggshell layers was performed using the light microscope.

Collection of the perivitelline fluid (PF) and the hatching fluid (HF)

The PF was collected from the eggs at day zero, seven, 12, 16 and 21 of culture. Infective 3rd stage larva developed at the 21st day of embryonation and HF was collected at 28th day. Eggs were filtered through gauze, centrifuged and washed three times in sterile physiological saline to remove the formalin. They were concentrated by centrifugation at 1500xg for 15 min at 4°C. Concentrated eggs were incubated for 12 hours in 1% sodium hypochlorite solution at room temperature to remove the outer shell of the egg (el Naga, 2000). The decorticated eggs were washed five times with 20 ml RPMI 1640 medium containing 1% (w/v) glucose. Eggs in the RPMI medium were subjected to rotatory mixing for 5 min to induce hatching. The PF and HF in the medium (15 ml) were collected directly after centrifugation.

Collection of the ES products

The hatching of mature eggs containing infective larvae at the 28th day of culture was done (Abou-El-Naga, 2018). Viable larvae were harvested using the Baermann's technique and washed with saline to remove any HF. Larvae were cultured in RPMI 1640 medium containing streptomycin (1 mg/mL), penicillin (1000 Units/mL), gentamicin (0.2 mg/mL) and amphotericin (2.5 µg/mL), and incubated in 5% CO2 incubator at 37°C at 10000 larvae/ml. The supernatant was collected after seven days of culture (de Savigny, 1975).

Preparation of the immune serum

Six albino mice of the Swiss strain were infected orally with 1000 *T. canis* eggs containing 3rd stage larvae. Mice were anesthetized and bled from the retro orbital plexus on day 15 post infection. Serum was frozen at -20°C until use. Serum from three uninfected mice was included as a control.

Determination of the protease activity in the PF, the HF and the ES products

The PF collected from the eggs at different culture periods (zero, seven. 12, 16, 24 and 30), the HF and ES products were dialyzed four times against deionized water at 4÷C and the protein content was determined (Lowry *et al*., 1951). The protease activity in the different samples was measured using casein as substrate following the Sigma non-specific protease activity assay. Briefly, casein (10 µg) was dissolved in two ml 50 mM potassium phosphate buffer pH 8. The substrate was added to one mg of the lyophilized PF, HF or ES products dissolved in one ml of deionized water and incubated at 38°C for exactly 30 min. Five ml trichloroacetic acid (0.3 N) was used to stop the reaction. Tyrosine was released after digestion of casein by the protease. The mixture was centrifuged at 2 000 rpm for 15 min and then the absorbance was recorded spectrophotometrically at 660 nm. Tyrosine values were obtained by interpolation from a standard curve established for a known concentration of tyrosine. Enzyme activity was expressed as the amount in micromoles of tyrosine equivalents released per mg protein per minute (Cupp-Enyard, 2008). The substrate and the lyophilized PF, HF or ES products were included as controls. Each experiment was repeated three times and the mean value was determined.

Effect of the pH on the protease activity of the HF and the ES products

The effect of the pH on the protease activity was determined by adding one mg of the lyophilized HF or the ES products, each dissolved in one ml of deionized water to a series of reaction tubes containing two ml of casein in 0.05 M phosphate buffer over the pH range 6-10. Separate controls were included for each pH tested. Protease activity in the different samples was measured as mentioned above (Cupp-Enyard, 2008). The experiment was performed in triplicate and the mean value was determined.

Effect of the serum on the protease activity of the HF and the ES products

The effect of the serum on the proteases of the HF and the ES products was performed by preincubation one mg of the lyophilized HF or the ES products, each dissolved in one ml of deionized water, with 0.1 ml serum from infected mice for 30 min in two separate tubes. Two ml of casein in 0.05 M phosphate buffer were added to each tube and monitoring of protease activity was performed as mentioned above (Cupp-Enyard, 2008). The same procedure was performed after preincubation of the samples with 0.1 ml serum from non-infected mice for 30 min in two separate tubes. The experiment was performed in triplicate and the mean value was determined. The effect of the serum on the proteases of the HF and the ES products was compared with the activity of the corresponding proteases in the HF and the ES products without preincubation with serum (control group).

Statistical analysis

All the experiments were performed in triplicate and the values were expressed as the mean ± standard deviation (SD). Significant differences between groups were analyzed by t-test and the level of significance was set at 0.05.

RESULTS

Observation of the developmental stages of *T. canis* inside the eggs

During egg culture, observation of embryo development stages revealed the appearance of the 1st, 2nd and 3rd larval stages at the 12th, 16th and 21st days of cultivation (Figure 1a-c). Examination of *T. canis* egg showed that the outer surface was pitted (Figure 1d) and that the egg shell consisted of four layers (lipid, chitinous, vitelline and an outer fibrous protein layer) (Figure 1e).

Protease activity of the PF

The results of the protease activity of the PF at different culture periods were illustrated in Figure 2. No obvious enzyme activity was detected in non-embryonated eggs. The activity increased as the embryo matured to 0.13±0.007 micromoles of tyrosine/mg protein/minute at the 7th day. A notable increase in proteolytic activity was observed with the development of the larval stages until reaching 1.992±0.038 micromoles of tyrosine/mg protein/minute with the appearance of the 1st stage larva at the 12th day of culture, 5.43±0.01 micromoles of tyrosine/mg protein/minute when the 2nd stage larva emerged at the 16th day and reached 8.918±0.119 micromoles of tyrosine/mg protein/minute at the 21st day when the 3rd larval stage developed.

Protease activity of the HF and the ES products

The enzyme activity of the HF collected at the 28th day of egg cultivation was 12.75±0.052 micromoles of tyrosine/mg protein/minute. Examination of the ES products produced by the 3rd stage larvae revealed that these larvae continue to produce the proteolytic enzymes with an activity of 11.771±0.01 micromoles of tyrosine/mg protein/minute, although the activity was slightly lower than that of the HF.

Effect of the pH on the protease activity of the HF and the ES products

The proteolytic activities of the HF and ES products had a wide pH range. Optimal activity was at an alkaline pH 8 for the HF and ES products as shown in Figure 3.

Effect of the serum on the protease activity of the HF and the ES products

Incubation of the HF and ES products with the serum from orally infected mice collected at the 15th day post infection resulted in a decrease in the proteolytic activity compared to the corresponding proteases activity in the HF and the ES products without preincubation with serum (12.75±0.052 and 11.771±0.01 micromoles of tyrosine/mg protein/minute, respectively) (control group) to reach 7.65 ± 0.031 micromoles of tyrosine/mg protein/minute for the HF and 5.492 ± 0.001 micromoles of tyrosine/mg protein/minute for the ES products. The reduction was statistically significant ($P< 0.05$). An insignificant reduction in the protease activity was detected when the HF and the ES products were preincubated with normal serum ($P> 0.05$) (Figure 4, 5).

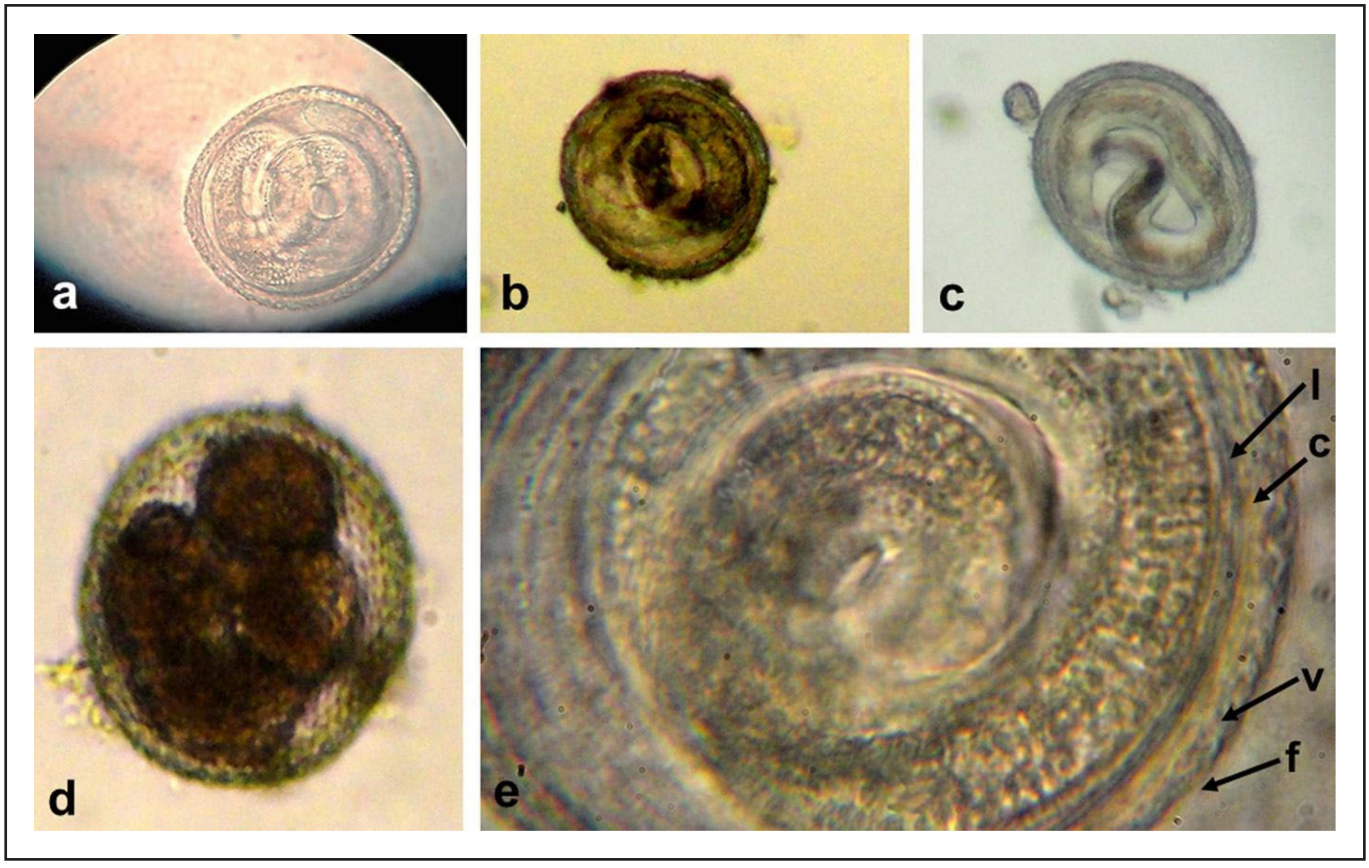

Figure 1. Larval stages inside the eggs of *Toxocara canis* and the structure of the egg shell. a: first larval stage (x40), b: second larval stage (x40), c: third larval stage (x40), d: egg contains a four-stage embryo showing the pitted outer surface of the egg (x40), e: *Toxocara canis* egg shell layers (l. lipid layer, c. chitinous layer, v. vitelline layer, f. fibrous protein layer) (x100).

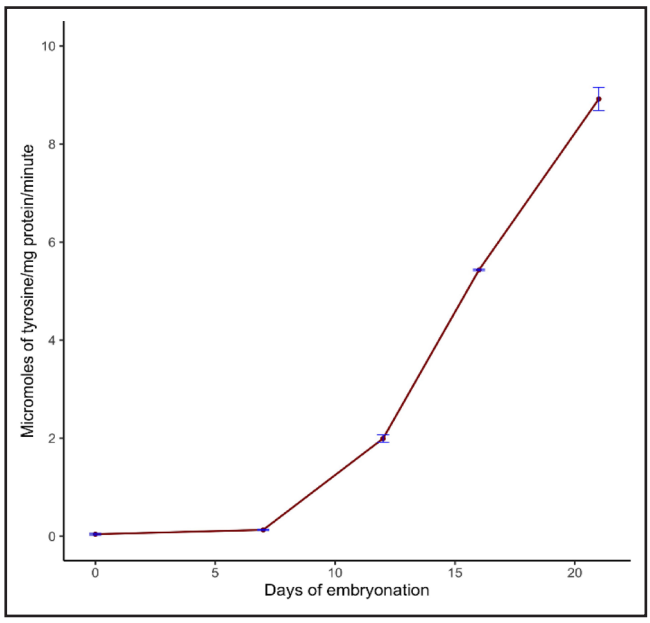

Figure 2. Protease activity in micromoles of tyrosine/mg protein/minute of the perivitelline fluid (PF) from *Toxocara canis* eggs at different periods of embryonation using casein as a substrate, following Sigma's non-specific protease activity assay.

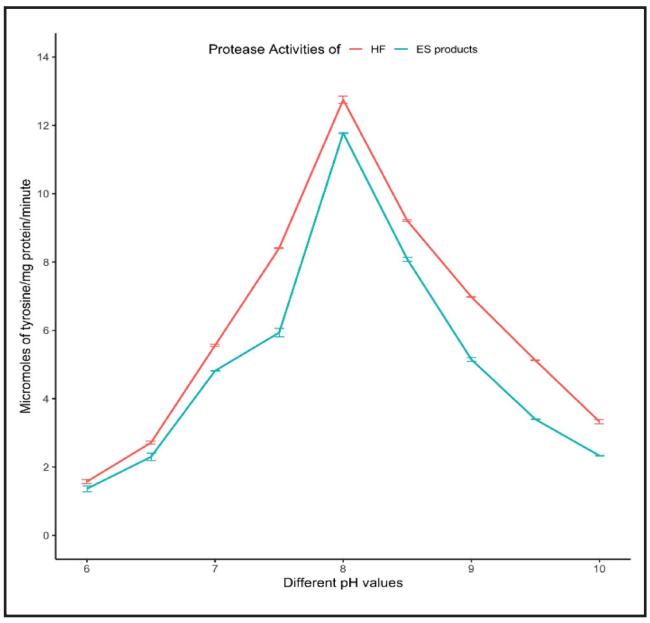

Figure 3. Protease activity in micromoles of tyrosine/mg protein/minute of the hatching fluid (HF) and excretory secretory products (ES) of *in vitro* cultivated *Toxocara canis* 3rd stage larvae at different pH values using casein as a substrate, following Sigma's non-specific protease activity assay.

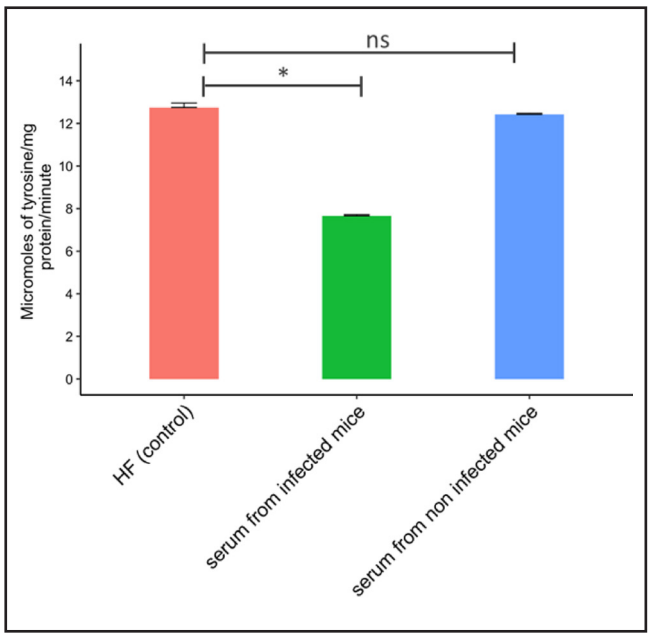

Figure 4. Effect of the serum on the protease activity in micromoles of tyrosine/mg protein/minute of the HF using casein as a substrate, following Sigma's non-specific protease activity assay.

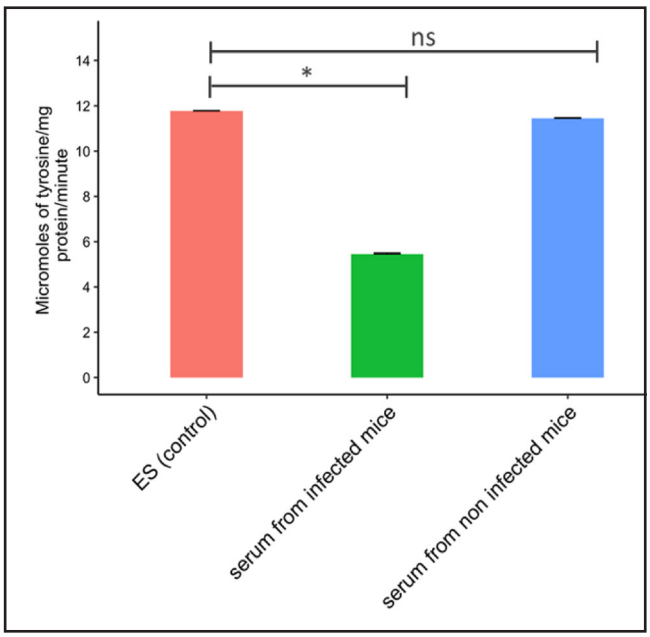

Figure 5. Effect of the serum on the protease activity in micromoles of tyrosine/mg protein/minute of the ES products using casein as a substrate, following Sigma's non-specific protease activity assay.

DISCUSSION

Embryogenesis, molting and hatching of *T. canis* eggs and larval migration involve regulated signaling pathway as well as the production of certain enzymes (Ma *et al*., 2019). In this work we measured the proteolytic activity in the PF along the intra ovular developmental stages of *T. canis*. The results revealed no evident proteolytic activity in the early embryonation period. The increase in the enzyme activity was detected after embryo maturation and the beginning of molting. The first definitive ascent was observed at 12th day of culture, coinciding with the development of the first larval stage. From this time on, the proteolytic activity of the PF continuously increased precisely after the development of the 2nd and the 3rd larval stages. Therefore, the increase in proteolytic activity paralleled the maturation and molting of *T. canis* larvae. Nematode proteases have been found to play a crucial role in molting process, digesting old cuticle and degrading cuticular anchor proteins or activating molting enzymes by processing their proenzymes (Page

et al., 2014). Brunaská *et al*. (1995) suggested that the first and the second cuticles of *T. canis* larvae were disrupted by the effect of the proteolytic enzymes.

The egg shell layers of *T. canis*, as shown in this study, were found to be similar to those described by Brunanská (1997). The shell of the egg is thick helping to withstand harsh environmental conditions similar to the related nematode *Ascaris lumbricoides* (Azam *et al*., 2012). The thickness of the egg shell layers varies in *Toxocara* species depending on the environmental conditions in which they survive. *Toxocara pteropodis* eggs are exposed to mangrove branches and leaves in tropical areas, therefore, for successful transmission, the egg shell is much thicker compared to that of *T. canis* which develops in the soil and are less exposed to desiccation (Prociv, 1989).

The present work showed that proteases accumulated after the development of the infective larva at the 21st day until they were measured in the HF at the 28th day of cultivation. This suggests that the inner egg shell layer prevents the proteases from exerting their effect on the outer egg shell layers until the hatching process begins. In *Ascaris lumbricoides* egg hatching is induced by the dramatic increase in the larval motility which mechanically disrupts the lipid layer, allowing proteases and other hatching enzymes to reach the different layers of the egg shell, leading to their degradation and thinning (Rogers, 1958). Co-culturing of *Ascaris suum* eggs with certain fungi led to lytic destruction of the egg shell layers by a combined effect of fungal proteases and chitinases (Huang *et al*., 2004; Blaszkowska *et al*., 2014). The continued increase in proteolytic activity after the development of the 3rd stage larvae can aid in the hatching process and increase the infectivity of the eggs. Brunaská *et al*. (1995) and el Naga (2000) found that the 3rd stage larva reached its maximum infectivity after a period of time approximately seven days following its development.

The proteases in the HF and ES products detected in the present study had an optimal level of activity at an alkaline pH (pH 8). This could help the emerging larvae to take advantage of the circumstances encountered during penetration of the gastrointestinal tract where egg hatching is induced precisely under the alkaline pH of the intestine. Furthermore, the maximum activity of the proteases in ES products at alkaline pH is consistent with their ability to migrate through the blood and various tissues of their hosts. Robertson *et al*. (1989) and Gonzɔlez-Pɔez *et al*. (2014) also found that the optimal activity of the proteolytic enzymes of the ES products was at pH 9, although González-Páez *et al*. (2007) detected the optimal activity of the HF proteases at an acidic pH $\leq$5.5.

The effect of the serum on *T. canis* proteolytic enzymes present in the HF and ES products was demonstrated in the current work by the reduction of the enzymatic activity after incubation with serum from infected mice. The inhibition of the HF proteases by the antiserum was greater than the inhibition of those in the ES products. In this context, Smith *et al*. (1982) found that the HF from *T. canis* was also excretory-secretory in nature like the ES products of the infective larvae. They also found that HF was highly reactive to the antiserum collected in the early periods of infection up to the 21 days post-infection of the animal (Smith *et al*., 1982). Protease activities of the larval stages of *Ascaris suum* and *Schistosoma mansoni*; are inhibited by antibodies elicited by infection (Knox & Kennedy, 1988). *Trichinella spiralis* protease antibodies partially inhibit the corresponding *in vitro* larval invasion of intestinal epithelial cells (Song *et al*., 2018). Proteases from *Babesia bovis* infected erythrocytes were able to elicit protective response (Commins *et al*., 1985).

Host antibodies can recognize both the mucin and the lectins of the ES products of *T. canis*. Mucins have multiple glycan side-chains consisting of trisaccharide with one or two *O*-methylation modifications. *O*-methylation of the dominant glycan acts as a highly specific target for host antibody recognition (Schabussova *et al*., 2007). Among the mucin members, MUC4 shows serine protease activity and is predominantly recognized by IgM. Anti-lectin antibodies show specific diagnostic potential (Schabussova *et al*., 2007; González-Páez *et al*., 2014; Długosz & Wisniewski, 2016). In paratenic hosts, the humoral and cellular immune responses directed against larval ES products upon repeated infection limit larval tissue migration (Revajová *et al*., 2006). The inhibitory effect of the serum on the proteases activities of the HF and ES products in the current study suggested that there may be a link between the proteases present in these fluids and the immune response. It is worth mentioning that not all enzymes secreted by parasites that can bind to antibodies should be protective. Although antibodies stimulated by infection with *Trichostrongylus* species bind to acetylcholine enzyme, they are unable to inhibit enzyme activity (Rothwell *et al*., 1973).

In conclusion, the results of the present study indicated that *T. canis* proteases were synthesized in the PF during embryonic stages and increased with larval development and molting. Proteases from the infective larvae accumulated in the HF and these larvae continued to produce these enzymes in ES products after *in vitro* culture. The optimal activity of theses enzymes was at an alkaline pH 8. The results also indicated that *Toxocara canis* proteases could be important for the worm inside the egg and the host. Inhibition of proteases by serum from infected mice suggested that there may be a link between the proteases present in these fluids and the immune response.

Conflicts of interests

The authors declare that they have no conflict of interests.

REFERENCES

Abou-El-Naga, I.F. (2018). Developmental stages and viability of *Toxocara canis* eggs outside the host. *Biomɪdica* **38**: 189-197. https://doi.org/10.7705/biomedica.v38i0.3684

Azam, D., Ukpai, O.M., Said, A., Abd-Allah, G.A. & Morgan, E.R. (2012). Temperature and the development and survival of infective *Toxocara canis* larvae. *Parasitology Research* **110**: 649-656. https://doi.org/10.1007/s00436-011-2536-8

Badley, J.E., Grieve, R.B., Bowman, D.D., Glickman, L.T. & Rockey, J.H. (1987). Analysis of *Toxocara canis* larval excretory-secretory antigens: physicochemical characterization and antibody recognition. *Journal of Parasitology* **73**: 593 600. https://doi.org/10.2307/3282142

Blaszkowska, J., Kurnatowski, P., Wojcik, A., Goralska, K. & Szwabe, K. (2014). *In vitro* evaluation of the ovistatic and ovicidal effect of the cosmopolitan filamentous fungi isolated from soil on *Ascaris suum* eggs. *Veterinary Parasitology* **199**: 165-171. https://doi.org/10.1016/j.vetpar.2013.10.026

Brunaská, M., Dubinský, P. & Reiterová, K. (1995). *Toxocara canis*: ultrastructural aspects of larval moulting in the maturing eggs. *International Journal for Parasitology* **25**: 683-690. https://doi.org/10.1016/0020-7519(94)00183-O

Brunanská, M. (1997). *Toxocara canis* (Nematoda:Ascaridae): the fine structure of the oviduct, oviduct-uterine junction and uterus. *Folia Parasitologica* **44**: 55-61.

Commins, M.A., Goodger, B.V. & Wright, I.G. (1985). Proteinases in the lysate of bovine erythrocytes infected with *Babesia bovis*: initial vaccination studies. *International Journal for Parasitology* **15**: 491 495. https://doi.org/10.1016/0020-7519(85)90042-6

Cupp-Enyard, C. (2008). Sigma's non-specific protease activity assay - Casein as a substrate. *Journal of Visualized Experiments* **19**: 899. https://doi.org/10.3791/899

da Silva, M.B., Urrego, A.J.R., Oviedo, Y., Cooper, P.J., Pacheco, L.G.C., Pinheiro, C.S., Ferreira, F., Briza, P. & Alcantara-Neves, N.M. (2018). The somatic proteins of *Toxocara canis* larvae and excretory-secretory products revealed by proteomics. *Veterinary Parasitology* **259**: 25-34. https://doi.org/10.1016/j.vetpar.2018.06.015

de Savigny, D.H. (1975). *In vitro* maintenance of *Toxocara canis* larvae and a simple method for the production of *Toxocara* ES antigen for use in serodiagnostic tests for visceral larva migrans. *Journal of Parasitology* **61**: 781-782. https://doi.org/10.2307/3279492

Długosz, E. & Wisniewski, M. (2016). *Toxocara canis* glycans influence antigen recognition by mouse IgG1 and IgM antibodies. *Acta Parasitologica* **61**: 191-194. https://doi.org/10.1515/ap-2016-0026

Dubey, J.P. (1978). Patent *Toxocara canis* infection in ascarid-naive dogs. *Journal of Parasitology* **64**: 1021-1023. https://doi.org/10.2307/3279714

Dzik, J.M. (2006). Molecules released by helminth parasites involved in host colonization. *Acta Biochimica Polonica* **53**: 33-64. https://doi.org/10.18388/abp.2006_3361

el Naga, I.F. (2000). *Toxocara canis*: Determination of the origin of antigenic materials released from infective larvae. *Journal of the Egyptian Society for Parasitology* **30**: 669-678.

el Zawawy, L.A., el Nassery, S.F., al Azzouni, M.Z., Abou el Naga, I.F., el Temsahi, M.M. & Awadalla, H.N. (1995). A study on patients with eosinophilia of suspected parasitic origin. *Journal of the Egyptian Society for Parasitology* **25**: 245-255.

Geenen, P.L., Bresciani, J., Boes, J., Pederson, A., Erikesn, L., Fagerholm, H.P. & Nansen, P. (1999). The morphogenesis of *Ascaris suum* to the infective third-stage larvae within the egg. *Journal of Parasitology* **85**: 616-622. https://doi.org/10.2307/3285733

González-Páez, G.E., Alba-Hurtado, F., García-Tovar, C.G. & Arg ello-García, R. (2014). Proteinases in excretory-secretory products of *Toxocara canis* second-stage larvae: zymography and modeling insights. *BioMed Research International* **2014**: 418708. https://doi.org/10.1155/2014/418708

González-Páez, G.E., Arg ello-García, R. & Alba-Hurtado, F. (2007). *Toxocara canis*: proteinases in perivitelline fluid from hatching eggs. *Veterinary Parasitology* **147**: 332-335. https://doi.org/10.1016/j.vetpar.2007.03.036

Huang, X., Zhao, N. & Zhang, K. (2004). Extracellular enzymes serving as factors in nematophagous fungi involved in infection of the host. *Research in Microbiology* **155**: 811-816. https://doi.org/10.1016/j.resmic.2004.07.003

Iddawela, D., Ehambaram, K. & Bandara, P. (2017). Prevalence of *Toxocara* antibodies among patients clinically suspected to have ocular toxocariasis: A retrospective descriptive study in Sri Lanka. *BMC Ophthalmology* **17**: 50. https://doi.org/10.1186/s12886-017-0444-0

Knox, D.P. & Kennedy, M.W. (1988). Proteinases released by the parasitic larval stages of *Ascaris suum* and their inhibition by antibody. *Molecular and Biochemical Parasitology* **28**: 207-216. https://doi.org/10.1016/0166-6851(88)90005-9

Lloyd, S., Amerasinghe, P.H. & Soulsby, E.J.L. (1983). Periparturient immunosuppression in the bitch and its influence on infection with *Toxocara canis*. *Journal of Small Animal Practice* **24**: 237-247. https://doi.org/10.1111/j.1748-5827.1983.tb00437.x

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry* **193**: 265 275.

Ma, G., Wang, T., Korhonen, P.K., Nie, S., Reid, G.E., Stroehlein, A.J., Koehler, A.V., Chang, B.C.H., Hofmann, A., Young, N.D. *et al*. (2019). Comparative bioinformatic analysis suggests that specific dauer-like signalling pathway components regulate *Toxocara canis* development and migration in the mammalian host. *Parasites and Vectors* **12**: 32. https://doi.org/10.1186/s13071-018-3265-y.

Macpherson, C.N. (2013). The epidemiology and public health importance of toxocariasis: a zoonosis of global importance. *International Journal for Parasitolology* **43**: 999-1008. https://doi.org/10.1016/j.ijpara.2013.07.004

Mkandawire, T.T., Grencis, R.K., Berriman, M. & Duque-Correa, M.A. (2022). Hatching of parasitic nematode eggs: a crucial step determining infection. *Trends in Parasitology* **38**: 174-187. https://doi.org/10.1016/j.pt.2021.08.008

Overgaauw, P.A., van Zutphen, L., Hoek, D., Yaya, F.O., Roelfsema, J., Pinelli, E., van Knapen, F. & Kortbeek, L.M. (2009). Zoonotic parasites in fecal samples and fur from dogs and cats in the Netherlands. *Veterinary Parasitology* **163**: 115-122. https://doi.org/10.1016/j.vetpar.2009.03.044.

Page, A.P., Hamilton, A.J. & Maizels, R.M. (1992). *Toxocara canis*: monoclonal antibodies to carbohydrate epitopes of secreted (TES) antigens localize to different secretion-related structures in infective larvae. *Experimental Parasitology* **75**: 56-71. https://doi.org/10.1016/0014-4894(92)90122-Q

Page, A.P., Stepek, G., Winter, A.D. & Pertab, D. (2014). Enzymology of the nematode cuticle: A potential drug target? *International Journal for Parasitolology. Drugs and Drug Resistance* **4**: 133-141. https://doi.org/10.1016/j.ijpddr.2014.05.003

Prociv, P. (1989). Intraovular development and moulting of *Toxocara pteropodis*. *International Journal for Parasitology* **19**: 749 755. https://doi.org/10.1016/0020-7519(89)90062-3

Revajová, V., Levkut, M., Aldawek, A.M., Herich, R., Dvorožňáková, E. & Krupicer, I. (2006). Immunological changes after multiple *Toxocara canis* infection of lambs. *Helminthologia* **43**: 69. https://doi.org/10.2478/s11687-006-0014-0

Robertson, B.D., Bianco, A.T., McKerrow, J.H. & Maizels, R.M. (1989). *Toxocara canis*: proteolytic enzymes secreted by the infective larvae in vitro. *Experimental Parasitology* **69**: 30-36. https://doi.org/10.1016/0014-4894(89)90168-9

Rogers, W.P. (1958). Physiology of the batching of eggs of *Ascaris lumbricoides*. *Nature* **181**: 1410-1411. https://doi.org/10.1038/1811410a0

Rogers, W.P. (1982). Enzymes in the exsheathing fluid of nematodes and their biological significance. *Intsrnational Journal for Parasitology* **12**: 495-502. https://doi.org/10.1016/0020-7519(82)90043-1

Rothwell, T.L., Ogilvie, B.M. & Love, R.J. (1973). Acetylcholinesterase secretion by parasitic nematodes. II. *Trichostrongylus spp*. *International Journal for Parasitology* **3**: 599-608. https://doi.org/10.1016/0020-7519(73)90084-2

Rubinsky-Elefant, G., Hirata, C.E., Yamamoto, J.H. & Ferreira, M.U. (2010). Human toxocariasis: diagnosis, worldwide seroprevalences and clinical expression of the systemic and ocular forms. *Annals of Tropical Medicine and Parasitology* **104**: 3-23. https://doi.org/10.1179/136485910X12607012373957

Schabussova, I., Amer, H., van Die, I., Kosma, P. & Maizels, R.M. (2007). O-methylated glycans from *Toxocara* are specific targets for antibody binding in human and animal infections. *International Journal for Parasitology* **37**: 97-109. https://doi.org/10.1016/j.ijpara.2006.09.006

Shyu, L.Y., Chen, K.M. & Lai, S.C. (2019). Matrix metalloproteinase-2 and matrix metalloproteinase-9 in mice with ocular toxocariasis. *Parasitology Research* **118**: 483-491. https://doi.org/10.1007/s00436-018-06196-4

Smith, H.V., Quinn, R., Bruce, R.G. & Girdwood, R.W. (1982). Development of the serological response in rabbits infected with *Toxocara canis* and *Toxascaris leonina*. *Transaction of the Royal Society of Tropical Medicine and Hygiene* **76**: 89-94. https://doi.org/10.1016/0035-9203(82)90028-1

Song, Y.Y., Wang, L.A., Ren, H.N., Qi, X., Sun, G.G., Liu, R.D., Jiang, P., Zhang, X., Cui, J. & Wang, Z.Q. (2018). Cloning, expression and characterisation of a cysteine protease from *Trichinella spiralis*. *Folia Parasitologica (Praha)* **65**: 007. https://doi.org/10.14411/fp.2018.007

Strube, C., Heuer, L. & Janecek, E. (2013). *Toxocara spp*. infections in paratenic hosts. *Veterinary Parasitology* **193**: 375-389. https://doi.org/10.1016/j.vetpar.2012.12.033

Ward, K.A. & Fairbairn, D. (1972). Chitinase in developing eggs of *Ascaris suum* (Nematoda). *The Journal of Parasitology* **58**: 546-549. https://doi.org/10.2307/3278202