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*Original article*

# PROTEASE ACTIVITY IN THE EXCRETORY-SECRETORY PRODUCTS OF NYMPHAL STAGE OF *LINGUATULA SERRATA*: FIRST DETECTION OF A METALLOPROTEASE

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**Summary**

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The aim of this study was to evaluate the presence of proteases and determine the main protease present in the excretory-secretory products (ESPs) from nymphal stage of *Linguatula serrata.* Infected mesenteric lymph nodes of goats were collected from Tabriz slaughterhouse, northwestern Iran. Recovered *Linguatula serrata* nymphs were immersed in culture medium (MEM), then ESPs were collected and protease activity in presence of specific inhibitors was assayed. Protease enzyme was further characterised by SDS-PAGE. The results of this study showed that the main protease in the ESPs from the nymphal stage of *L. serrata* was a metalloprotease that was resistant to heat. In conclusion, these data show that a major protease secreted by the larval stage of *L. serrata* exhibited properties that may play a role in the pathogenesis of *L. serrata* nymphs.

**Key words**: *Linguatula serrata,* protease, metalloprotease, zoonotic

## INTRODUCTION

*L. serrata* is a cosmopolitan zoonotic parasite whose adult form inhabits the upper respiratory system, nasal airways, frontal sinuses of dogs, foxes, cats and other carnivores as final hosts (Rezaei *et al.*, 2011; Aldemir *et al.*, 2014; Nagamori *et al*., 2019) while its immature variety resides in the mesenteric lymph nodes, liver, lungs and spleen of the herbivores and other ruminants which serve as intermediate hosts (Gul *et al.*, 2009; Hami *et al.*, 2009; Tajik & Sabet Jalali, 2010; Hajipour *et al.*, 2018; Shamsi *et al*., 2020 a,b).

Humans are occasionally infected with both adult and nymphal stages of *L. serrata*. Nasopharyngeal linguatulosis, also known as Halzoun syndrome or Marrara syndrome, is the common form of infection (Hamid *et al.*, 2012; Athari, 2013; Khalil *et al.*, 2013; Sarmadian *et al*.,

2020) which is often induced by consumption of raw or undercooked infected viscera (liver, lung and lymph nodes) of contaminated animals. The destruction of tissues in intermediate hosts happens due to migration of nymphal stage.

Proteases are interesting biomarkers for the detection of diseases (Lim & Craik, 2009), and are presumably involved in some processes such as penetration into host tissues, parasite nutrition, anti-coagulation, and evasion of host immune responses (McKerrow, 1989). It is now clear that proteases can stimulate host protective immunity and may be potent allergens (Kennedy *et al.*, 1991). There is limited information available on the proteases activity in excretory-secretory products (ESPs) of *L. serrata* (Alcala-Canto *et al.*, 2007). However, the proteases have been reported from other parasites, namely a cysteine protease in epimastigotes of *Trypanosoma cruzi and T. rangeli* (Gomes *et al*., 2009). Cysteine protease with cathepsin-L-like properties was isolated from lysosomes in blood forms of *T. congolense* (Mbawa *et al.*, 1992). It was shown that cysteine protease was separated from the mature *Schistosoma mansoni* (Horn *et al.*, 2014), *Fasciola hepatica* (Smith *et al.*, 1994) and the trophozoite form of *Entamoeba histolytica* (Luaces & Barrett, 1988). In addition, the existence of serine protease and host tissue damage have been illustrated in the bloodstream forms of *T. brucei* (Steverding *et al*., 2020), the epimastigote of *T. cruzi* (Santana *et al.*, 1992), the schizont of *Plasmodium falciparum* (Grellier *et al.*, 1989), oocyst of *Eimeria tenella* (Michalski *et al.*, 1994) and spargana of *Spirometra mansoni* (Kong *et al.*, 1994). Serine protease activity in nymphal stage of *L. serrata* has also been documented (An *et al.*, 1994).

The aim of this study was to evaluate the presence of proteases and determine the main protease present in the excretory-secretory products from nymphal stage of *L. serrata.*

## MATERIALS AND METHODS

### *Samples*

After collection of mesenteric lymph nodes of goats slaughtered in Tabriz slaughterhouse, northwest of Iran, they were transferred to laboratory. Each lymph node was cut longitudinally and put in Petri dish with distilled water for 15 minutes. The nymphs of *L. serrata* were washed three times in phosphate-buffered saline (PBS, pH 7.2; Sigma) and transferred to sterile Petri dish containing minimum essential culture medium, 25 mM of N-2-hydroxyethylpiperazine-N′-2-etane-sulfonic acid, 15% foetal calf serum, 100 U/mL of sodium penicillin, 100 U/mL of nistatin and 0.25 mg/mL of streptomycin (Sigma). The containers were incubated at 35 °C with 5% $CO_2$ for 24 h. Nymphs of *L. serrata* were removed and medium was collected, centrifuged (15 min, 5000 rpm), filtered through 0.4 μm sieves and stored at –80 °C until analysis.

### *Protease activity assays*

Protease activity was determined spectrophotometrically, using 1% casein as substrates (An *et al.*, 1994). The reaction was carried out at 37 °C for 60 min, a linear range determined from the time course of study. TCA soluble oligopeptides content was determined by the Lowry method, using tyrosine as a standard (Lowry *et al.*, 1951). Activity was defined as nmoles of tyrosine released/min/ml of crude extract. Specific activity was expressed as the amount of activity per mg protein.

*Measurement of protease activity in the presence of inhibitors*

In order to identify major protease in the ESPs released by nymphal stage of *L. serrata*, protease specific inhibitors were used. Protease inhibitors were prepared as stock solution in dimethyl sulfoxide (DMSO) and were used at various concentrations. Protease inhibitors used in this study were phenylmethylsulfonyl fluoride (PMSF; serine protease inhibitor, 10 mM), ethylenediaminetetraacetic acid (EDTA; metalloprotease inhibitor, 10 μM), *N*-[*N*-(L-3-*trans*-carboxyirane-2-carbonyl)-L-leucyl]-agmatine (E 64; cysteine protease inhibitor, 2.5 μM) and 1,10-phenanthroline (metalloprotease inhibitor, 10 mM) (Siringan *et al.*, 2006). Protease activities was determined by the modified method of An *et al.* (1994). For each of inhibitors, samples were analysed in triplicate and the mean values of data were used. The enzyme activity was considered 100% in the absence of inhibitor.

*Activity staining*

Activity staining was performed according to the method of Garcia-Carreno *et al.* (1993). Crude proteinases were separated on 10% polyacrylamide gel (Laemmli, 1970). Subsequently, gel was immersed in 1% casein, 50 mM Tris–HCl (pH 7.5) for 30 min on ice and incubated at 60 $^{o}$C for 15 min. Subsequently, gels were stained in 0.125% Coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid for 1 h. Destaining was carried out using 25% methanol and 10% acetic acid solution. A clear zone on the blue background indicated the presence of protease.

*Determination of protease thermal stability*

Thermal stability of protease in the ESPs from nymphal stage of *L. serrata* were studied by incubating aliquots of ESP's supernatants at different temperatures (40, 50, 60 and 70 $^{o}$C) up to 10 min in a thermostatic water bath and measuring their activity at room temperature after brief cooling in ice. The incubation was carried out in sealed vials to prevent any change in the sample volume which can result in the enzyme concentration due to evaporation. Assays at different temperatures were conducted in triplicate and the mean values of data were used to obtain the thermal stability. The enzyme activity in laboratory condition was considered 100%.

## RESULTS

Fig. 1 depicts the effects of different specific proteases inhibitors on the protease activity of ESPs released by nymphal stage of *L. serrata*. EDTA and 1,10-phenanthroline reduced the proteolytic activity of ESPs of nymphal stage of *L. serrata* by 12.5% and 28%, respectively. In contrast, PMSF and E64 had no effect on the proteolytic activity.

Fig. 2 shows the native PAGE of ESPs from nymphal stage of *L. serrata* stained for protease activity in the absence and presence of specific inhibitors such as PMSF, a serine protease inhibitor (10 mM), EDTA, a metalloprotease inhibitor (10 μM), E64, a cysteine protease inhibitor (2.5 μM) and 1,10-phenanthroline, a metalloprotease inhibitor (10 mM). Clear zones revealing protease activity were seen only in lanes 3, 4 and 5. These results suggested that the dominant form of protease in the experimental sample was a metalloprotease.

For the thermal stability assay, samples were incubated at 40, 50, 60 and 70 $^{o}$C for 10 minutes and then protease activity was measured (Fig. 3). The proteolytic activity of ESPs of nymphal stage

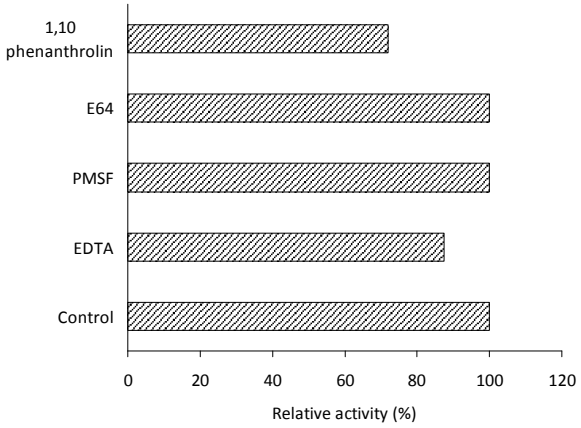


**Fig. 1.** Inhibitory effects of different specific proteases inhibitors on excretory-secretory products from nymphal stage of *L. serrata.*

of *L. serrata* was reduced by 25.6% when incubated at 70 °C, while it decreased by 19.41%, 14.4% and 9.3% after incubation at 60 °C, 50 °C and 40 °C, respectively. These results suggested the presence of a thermostable protease in the ESPs.

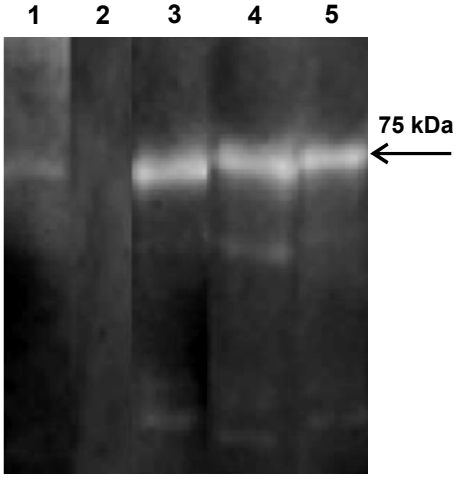


**Fig. 2.** PAGE of excretory-secretory products from nymphal stage of *L. serrata* stained for protease activity and treated with several protease inhibitors. Lane 1 (1, 10-phenanthroline); lane 2 (EDTA); lane 3 (parasitic *in vitro* released ESPs as control without inhibitor); lane 4 (E64); lane 5 (PMSF).

## DISCUSSION

Linguatulosis is an infection that mostly affects ruminants during its larval and nymphal stages, and it can also affect human beings. Proteolytic enzymes secreted by parasites are thought to be playing a key role in the processes of penetration and migration through the host tissues. It has been stated that all proteases secreted by tissue-invading parasites fall into two of the classes of proteases – serine and metallo (McKerrow, 1989). Proteases of these classes have been demonstrated in studies on the secreted products of *Schistosoma mansoni* (Maleky, 2001), *Ancylostoma caninum* (Hotez *et al.*, 1990), *Dictyocaulus viviparus* (Britton *et al.*, 1992), adult *Trichinella spiralis* (Romaris *et al.*, 2002), *Porocephalus crotali* (Buckle *et al.*, 2002), *L. serrata* (Alcala-Canto *et al.,* 2007). In this study, the proteolytic activity of the *in vitro* released ESPs of *L. serrata* nymphal stage was examined for thermal stability, activity against some protein substrates and inhibitor sensitivity. The inhibitor studies showed that metalloprotease predomi-

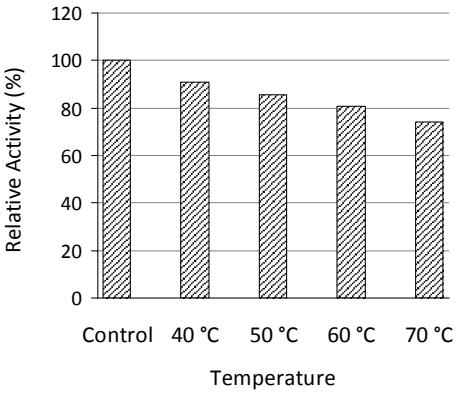


**Fig. 3.** Thermal stability of protease in the excretory-secretory products from nymphal stage of *L. serrata.*

nated in protease ESPs of *L. serrata*. Our results disagreed with the study of Alcala-Canto *et al.,* (2007) who demonstrated serine protease activity in the larval stage of *L. serrata*. The reason for this contradiction may be attributed to sampling. We used mesenteric lymph nodes infected with *L. serrata* nymphs in goats whereas Alcala-Canto *et al.,* (2007) used samples from infected sheep liver. ESPs include several proteases which may facilitate tissue migration as demonstrated by their ability to degrade a number of proteins which could relate to their development in the natural environment. Another reason for this difference could be potentially attributed to *L. serrata* strain differences, with some strains being more adapted to certain hosts (Tavassoli *et al.*, 2014). In their study on *L. serrata* isolated from different farm animals of Iran, Tavassoli *et al.* (2014) found molecular differences among the parasites by amplifying and sequencing 18S rRNA. Based on the reported results, the lowest diversity of nucleotide sequences (98.8%) was observed in sheep, cattle, and dogs, while the highest one (100%) was recorded in camels and goats. However, studies on phylogenetic relationships among *L. serrata* isolates from Iran based on 18S rRNA and mitochondrial *cox1* gene sequence by Ghorashi *et al.* (2016) showed a higher sequence diversity and intra-species variation in the *cox1* gene compared to18S rRNA sequences with no association between genetic variations and host species or geographical location, perhaps due to the small sample size.

A previous study (Jones *et al.*, 1991) had characterised a 48 kDa protease in the frontal gland extracts of VII instar of *P. crotalli* which was defined as an elastase from the metalloproteinase class. Another study showed that protease was present in ESPs, which were expressed in a stage-specific manner. These proteases are also metalloproteases being inhibited to varying degrees by EDTA and 1,10 phenanthroline (Buckle *et al.,* 2002). In conclusion, the main protease in ESPs from nymphal stage of *L. serrata* was a metalloprotease.

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