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Investigation of protease activity in the excretory-secretory release from nymphal stage of *Linguatula serrata*

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ABSTRACT

Objective: To evaluate the presence of proteases and determine the main protease present in the excretory-secretory release from nymphal stage of *Linguatula serrata* (*L. serrata*). **Methods:** Infected mesenteric lymph nodes of suspected goats were collected from Tabriz slaughterhouse, northwestern of Iran. Recovered nymphs of *L. serrata* immersed in culture medium, then E-S products were collected and protease activity in presence of specific inhibitors was assayed and protease enzyme was further characterized by SDS-PAGE. **Results:** The results of this study showed that the main protease in the E-S products release from nymphal stage of *L. serrata* was metalloproteas that is resistant to temperature. **Conclusions:** these data show that a major protease secreted by the larval stage of *L. serrata* exhibits properties to consider as a role in long survival time of *L. serrata* nymphs

1. Introduction

Linguatula serrata (*L. serrata*) is a cosmopolitan zoonotic parasite whose adult form inhabits in the upper respiratory system, nasal airways, frontal sinuses of dogs, foxes, cats and other carnivores as final hosts[1-3] while its immature variety resides in the mesenteric lymph nodes, liver, lungs and spleen of the herbivorous and other ruminants which serve as intermediate hosts[4-9]. Human is 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 in human[10-14] which is often 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 the migration of nymphal stage. Proteases are interesting biomarkers for the detection of diseases[15], and presumabley they are also involved in Some processes such as penetration into host tissues, parasite nutrition, anti-coagulation , and evasion of host immune response[16]. It is now clear that proteases can stimulate host protective immunity and may be potent allergens[17]. There is limited information available on the proteases activity in E-S products in *L. serrata*[18]. However, the proteases have been reported are reported from other parasites, such as cysteine protease in epimastigotes of *Trypanosoma cruzi (T. cruzi)* and *Trypanosoma rangeli*[19]. Cysteine protease with cathepsin-L-like properties was isolated from lysosomes in blood forms of *Trypanosoma congolense*[20]. It was shown that cysteine protease was separated from the mature *Schistosoma mansoni*[21], *Fasciola hepatica*[22] and trophozoite form of *Entamoeba histolytica*[23]. In addition, the existence of serine protease and host tissue damage have been illustrated in the bloodstream forms of *Trypanosoma brucei*[24], the epimastigote of *T. cruzi*[25], the schizont of *Plasmodium falciparum*[26], oocyst of *Eimeria tenella*[27] and spargana of *Spirometra mansoni*[28]. Serine protease activity in nymphal stage of *L. serrta* has also been documented[18]. The aim of this study was to evaluate the presence of proteases and determine the main protease

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present in the excretory-secretory release from nymphal stage of *L. serrata*.

2. Material and methods

2.1. Sampling

After collection of mesenteric lymph nodes of goats slaughtered in Tabriz slaughterhouse, northwest of Iran, they were transferred to laboratory and each lymph node was cut longitudinally and put in the Petri dish with distilled water for 15 minutes. The nymphs of *L. serrata* were washed three times in saline phosphate-buffered (PBS = 7.2; Sigma) and transferred to the strilesterile Petri dish containing minimum essential culture medium, 25 mM of N-2-hydroxyethylpiperazine-N′-2-etanes-sulfonic acid, 15% fetal calf serum, 100 U/mL of sodium penicillin,100 U/mL of nistatin and 0.25 mg/mL of streptomycin (sigma). Containers were incubated at 35 ℃ with 5% CO_2 for 24 h. Nymphys of *L. serrata* were removed and medium was collected, centrifuged (15 min, 5 000 rpm), filtered through 0.4 μm sieves and stored at -80 ℃ until analysis.

2.2. Proteases activity assays

Protease activity was determined spectrophotometrically, using 1% casein as substrates[29]. The reaction was carried out at 37 ℃ 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[30]. Activity was defined as nmole of tyrosine released/min/mL of crude extract. Specific activity was expressed as the amount of activity per mg protein.

2.3. Measurement of protease activity in the presence of inhibitors

In order to to identify major major protease in the E.S release from 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 phenyl methyl sulfonyl fluoride (PMSF; serine protease inhibitor,10 mM), ethylene diamine tetra acetic 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)[31]. The enzyme activity assay was performed according to the protocol 2-2. The enzyme activity was considered 100% in the absence of inhibitor.

2.4. Activity staining

Activity staining was performed according to the method of Garcia-Carreno *et al*[32]. 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 ℃ 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.

2.5. Determination of protease thermal stability

Thermal stability of protease in the E.S release from nymphal stage of *L. serrata* were studied by incubating aliquots of supernatant E.S products at temperatures (40 ℃, 50 ℃, 60 ℃ and 70 ℃) 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 con concentration due to evaporation .Assays at different temperatures were conducted at least in 3 separate experiments and the mean values of data were used to obtain the thermal stability. The enzyme activity in laboratory condition was considered 100%.

3. Results

Figure 1 depicts the effects of different specific proteases inhibitors on the protease activity of E.S release from nymphal stage of *L. serrata*. As shown in this figure, EDTA and1, 10-phenanthroline inhibitors reduced the proteolytic activity of E.S release from nymphal stage of *L. serrata* by 12.5% and 28%, respectively. In contrast, PMSF and E_{64} didn't have any effect on the proteolytic activity.

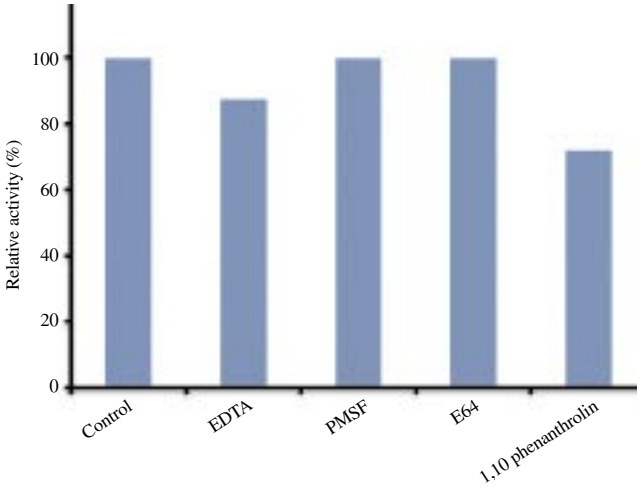

Figure 1. Inhibitory effects of different specific proteases inhibitors on E.S release from nymphal stage of *L. serrata*.

Figure 2 shows the native PAGE of excretory-secretory release from nymphal stage of *L. serrata*, stained for protease activity in the absence and presence of specific inhibitors such as PMSF; serine protease inhibitor (10 mM), EDTA; metalloprotease inhibitor (10 μM), E64; cysteine protease inhibitor (2.5 μM) and 1, 10-phenanthroline; metalloprotease inhibitor, (10 mM). As shown in this figure, clear zones revealing the protease activity can be seen only in lanes 3, 4 and 5. These results suggest that the dominant form of protease in the experimental sample was metalloproteas.

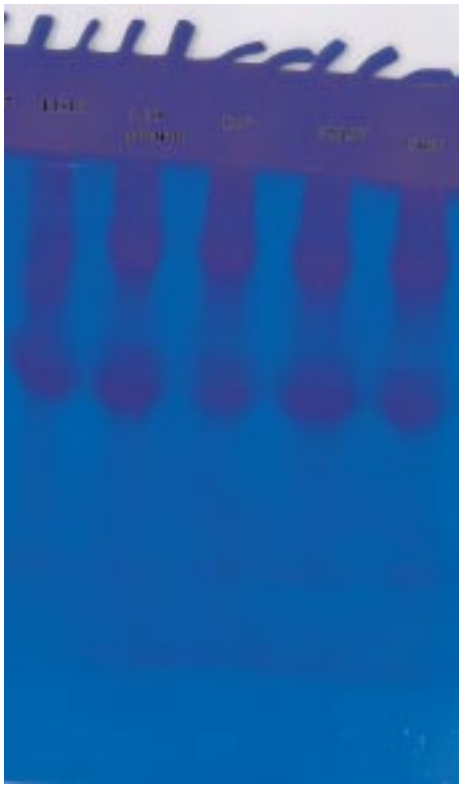

Figure 2. Native PAGE of E.S release from nymphal stage of *L. serrata* stained for protease activity treated with several protease inhibitors.
Lane 1, EDTA; lane 2, 1, 10-phenanthroline; lane 3, E 64; lane 4, PMSF; lane 5, control without inhibitor.

For thermal stability assay, sample was incubated at 40℃, 50℃, 60℃ and 70℃ for 10 minutes and then protease activity was measured (Figure 3). The proteolytic activities in E.S release from nymphal stage of *L. serrata* reduced 25.6% when incubated at 70℃ while it decreased 19.41%, 14.4% and 9.3% when incubated at 60℃, 50℃ and 40℃, respectively. These results suggest that a thermostable protease enzyme existed in the E.S release from nymphal stage of *L. serrata*.

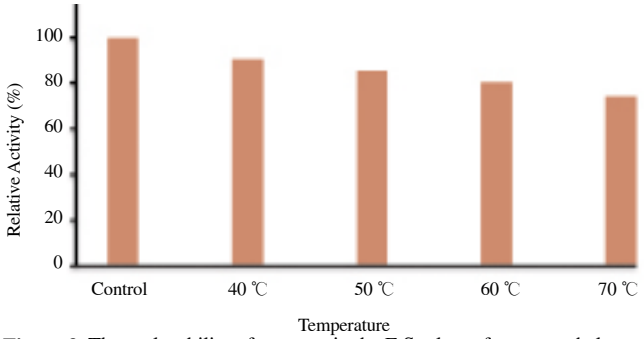

Figure 3. Thermal stability of protease in the E.S release from nymphal stage of *L. serrata*.

4. 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 of the proteases secreted by tissue-invading parasites fall into two of the classes of proteases serine and metallo[16]. Proteases of these classes have been demonstrated in studies on the secreted products of *Schistosoma mansoni*[33], *Ancylostoma caninum*[34], *Dictyocaulus viviparus*[35], adult *Trichinella spiralis*[36], *Porocephalus crotali*[37], *L. serrata*[18]. In this study, the proteolytic activity of the *in vitro* released ES of nymphal stage of *L. serrata* was examined according to their thermal stability, activity against some protein substrates and inhibitor sensitivity. The inhibitor studies showed that metalloprotrase predominated of protease in E-S product in *L. serrata*. Our results were in disagreement with that study by Alcala-Canto *et al*[18]. He demonstrated serine protease activity in the larval stage of the *L. serrata*. The reason for this contradiction may be attributed to sampling. We used mesenteric lymph nodes infection with *L. serrata* nymphs in goats but Alcala sampled from infected sheep liver. E.S products included several protease 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. A previous study[38] had characterized a 48 kDa protease in the frontal gland extracts of Ⅶ instar of *P. crotalli* which was defined as an elastase of the metalloproteinase class. Another study showed that protease was present in E-S products, which were expressed in a stage-specific manner. These proteases are also metalloprotease being inhibited to varying degrees by EDTA and 1,10 phenanthroline[37]. In conclusion, the main protease in excretory-secretory release from nymphal stage of *L. serrata* is metalloprotease.

Conflict of interest statement

The authors declare no conflict of interest.

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