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Serine proteases activity in miracidia of *Fasciola hepatica* and effects of chemical and herbal inhibitors

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Abstract

Fasciolosis is a zoonotic parasitic disease caused by the trematode *Fasciola hepatica*. The proteases are essential for the survival of parasites. The present study was aimed to determine serine proteases activities in miracidia of *F. hepatica* and evaluate the effects of pH and different inhibitors on the serine proteases activities. Adult *F. hepatica* helminths were removed from naturally infected livers of the slaughtered cattle and crushed. The eggs were incubated at 28.00 ˚C for 16 days. The released miracidia were homogenized and total proteolytic activity of the extract of miracidia at different pH values were evaluated. Serine proteases activities were determined using specific substrates. The inhibitory effects of chemical and herbal inhibitors on the enzymes were also assessed. The extract of miracidia hydrolyzed azocasein with optimum activity at pH 8.00. The optimum pH effect on serine proteases activities was found at alkaline pH. Phenylmethylsulfonyl fluoride and Bowman-Birk inhibitors inhibited and decreased the proteases activities in the miracidia extract. It was concluded that there were proteases activities in miracidia of *F. hepatica* which were inhibited by chemical and herbal inhibitors.

Introduction

Fascioliasis is a cosmopolitan disease caused by *Fasciola hepatica* in both humans and animals.[1] The World Health Organization recognizes fascioliasis as a major food-borne disease, with up to 17 million human cases in 61 countries with 180 million at risk.[2,3] Fascioliasis is also a common parasitic infection in livestock causing significant losses in growth and production with annual economic losses of more than 3 billion US$ worldwide.[2,4]

Proteolysis is a crucial activity for physiological operations and facilitate important activities of parasites like invasion, egg hatching, excystment, nutrition, helminths fecundity, immune evasion and modulation of the host or fluke physiology.[5-12] This makes proteases key targets for vaccine and drug production.[13,14] The largest family of proteases is serine proteases which are important in penetration and tissue migration of a wide range of helminths larvae and are expressed in newly excysted larvae, both immature and mature flukes.[15,16] Most earlier studies on proteases in *Fasciola* and *Schistosoma* species were, respectively, focused on cysteine and aspartic proteases, and serine proteases.[17-20]

In 1947, the possible role of proteases inhibitors in plant protection revealed that the insect larvae were unable to develop normally on soybean products. Trypsin inhibitors were expressed in soybeans and were toxic to the larvae of flour beetle.[21] So far, many proteases inhibitors with activities against insects are reported.[22,23] The role of serine proteases inhibitors as defensive compounds against predators was particularly well established.[24] Environmental factors like pH are critical for life cycle of *F. hepatica*, specifically miracidia penetration into the aquatic snails, *i.e.* lymnaeid snails. Dalton *et al*. noted that serine proteases with "trypsin-like" activities from secretions of cercariae of *S. mansoni* were involved in host invasion.[25] Accordingly, chemical and herbal inhibitors of serine proteases may probably be useful to inhibit miracidia penetration into the body of snails. Thus, the present study was carried out to determine serine proteases activities in miracidia of *F. hepatica* and to assess the effects of pH and different inhibitors on the serine proteases activities.

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Materials and Methods

Helminths sampling and eggs collection. Adult *Fasciola* was isolated from naturally infected livers of the slaughtered cattle. The livers were cut into small pieces and adult *Fasciola* helminths were removed. They were washed several times in 0.01 M phosphate buffer saline (PBS; pH = 7.20) (Merck, Darmstadt, Germany) and stained using Asetokarmin (Shahrazma Co., Tehran, Iran). *Fasciola* species were examined under light microscope at 100× magnification and identified using key identification described by Soulsby.[26] The identified helminths, *F. hepatica* were microscopically examined for the presence of eggs and crushed in a mortar containing 10.00 mL of distilled water and sieved to gather the eggs.[1] The eggs were washed several times using 0.086% Ringer's solution (Samen Co., Mashhad, Iran) and centrifuged at 445 *g* for 5 min. The supernatant was discarded and the precipitated eggs were washed three times. The collected eggs were incubated at 24.00 ˚C for 16 days. On the last day, they were exposed to the light with density of 100 Watts for 6 hr to stimulate miracidia release as previously described.[1]

Total protein and enzymes assay. To access total cytosolic proteins, miracidia were ground in liquid nitrogen eight times and then centrifuged at 16,000 *g* for 10 min at 4.00 ˚C. The supernatant was collected as the extract of miracidia and its protein content was determined using Bradford method.[27] The pH optimum of proteases activities was determined and general proteolytic activities was also assessed using azocasein substrate.[28] The reaction mixture including azocasein (Sigma-Aldrich, Taufkirchen, Germany) 2.00% (30.00 µL), universal buffer (90.00 µL, containing acetate, phosphate, borate (Merck, Darmstadt, Germany) 50.00 mM at pH 2.00-12.00) and miracidia extract (15.00 µL) was incubated at 36.00 ˚C for 60 min. Protein digestion was stopped using trichloroacetic acid (Merck) 30.00% (30.00 µL, v/v). To precipitate non-hydrolyzed azocasein, reaction mixture was incubated at 4.00 ˚C for 1 hr. The supernatant was collected and signal was measured by absorbance (450 nm) on ELISA plate reader (Nowingostar, Tehran, Iran).

Serine proteases assays. Serine proteases, *i.e.* trypsin and chymotrypsin were evaluated by adding miracidia extract (10.00 µL) and substrate solution 1.00 mM (10.00 µL) to universal buffer (85.00 µL; pH 2.00 - 12.00) and light absorbance was measured in wavelength of 405 nm on ELISA plate reader (Nowingostar). The substrates of BApNA (1.00 mM; Nα-benzoyl-DL-arginine 4-nitroanilide; Sigma-Aldrich) and SAAPFpNA (1.00 mM; N-succinyl-alanine-alanine-prolin-phenylalaninc-p-nitroanilide; Sigma-Aldrich) were, respectively, applied to do trypsin and chymotrypsin assays.[29-31]

Serine proteases, chemical and herbal inhibitors assays. The chemical inhibitors of serine proteases, *i.e.* phenylmethylsulfonyl fluoride (PMSF; 5.00 mM; Sigma-Aldrich), pepstatin (10.00 µM; Sigma-Aldrich) and ethylenediaminetetraacetic acid (EDTA; 10.00 mM, Sigma-Aldrich) was separately added to the extract of miracidia (15.00 µL), azocasein 2.00% (30.00 µL; Sigma-Aldrich) and universal buffer (90.00 µL) in parallel with control group. The mixture was incubated at 36.00 ˚C for 60 min and trichloroacetic acid 30.00% (30.00 µL; Merck) was added to stop protein digestion. Non-hydrolyzed azocasein was precipitated at 4.00 ˚C for 1 hr. The supernatant was collected and signal was measured by absorbance (450 nm) on ELISA plate reader (Nowingostar).

The herbal inhibitor, soybean Bowman-Birk inhibitor (SBBI; Sigma-Aldrich) activity was determined following described method with minor modification.[32] Briefly, miracidia extract of *F. hepatica* (50.00 µg mL^{-1}) as treatment groups along with SBBI (500 µg mL^{-1}; Sigma-Aldrich) and control group were incubated at room temperature for 2 hr and centrifuged.[33] Sediments were homogenized several times by grinding in liquid nitrogen and centrifuged at 16,000 *g* for 10 min at 4.00 ˚C. The supernatant was collected and serine proteases activities were measured using a mixture composed of the substrates (5.00 µL) and extract (10.00 µL) which added to universal buffer (85.00 µL; pH = 2.00 - 12.00) and signal was measured by absorbance (450 nm) on ELISA plate reader (Nowingostar).

Results

Serine proteases activities. The proteolytic activity and pH effects are shown in Figure 1A. The total protein content of miracidia was 0.47 ± 0.08 mg mL^{-1}. The azocasein hydrolysis exhibited proteolytic activity in a wide range of pH 2.00 (36.00%) to pH 12.00 (29.00%). The maximum proteolytic activity in the extract of miracidia was at pH 8.00. The highest activity of the enzyme was approximately preserved at pH 7.00 (98.49%).

Serine proteases assay. Data pertaining to the enzymes responsible for hydrolysis of the substrate is tabulated in Table 1. The miracidia extract demonstrated the maximum activity toward BApNA at pH 10.00 (pH = 6.00 - 11.00) and 98.20% activity at pH 8.00 (Fig. 1B). The miracidia extract also had the highest activity toward SAAPFpNA at pH 8.00 (pH = 6.00 - 11.00) and 99.73% activity at pH 6.00 (Fig. 1C).

The chemical and herbal inhibitors assays. The chemical inhibitors effects on serine proteases were 71.80% for PMSF, 40.70% for pepstatin, and 20.20% for EDTA. Of those, PMSF demonstrated the highest inhibitory effect with 70.00% reduction in serine proteases activities. The inhibitory effect of herbal inhibitor, SBBI on serine proteases activities in the extract was decreased and revealed effective inhibitory action against examined serine proteases toward BApNA (43.10% in 50.00 µg mL^{-1}

and 50.20% in 500µg mL^{-1}) and SAAPFpNA (53.00% in 50.00 µg mL^{-1} and 60.10% in 500 µg mL^{-1}), (Table 2). The inhibitory effect of SBBI on serine proteases was found to be more effective in 500 µg mL^{-1}. Both concentrations of SBBI, 50.00 µg mL^{-1} and 500 µg mL^{-1} had more inhibitory effect on chymotrypsin activity than trypsin activity.

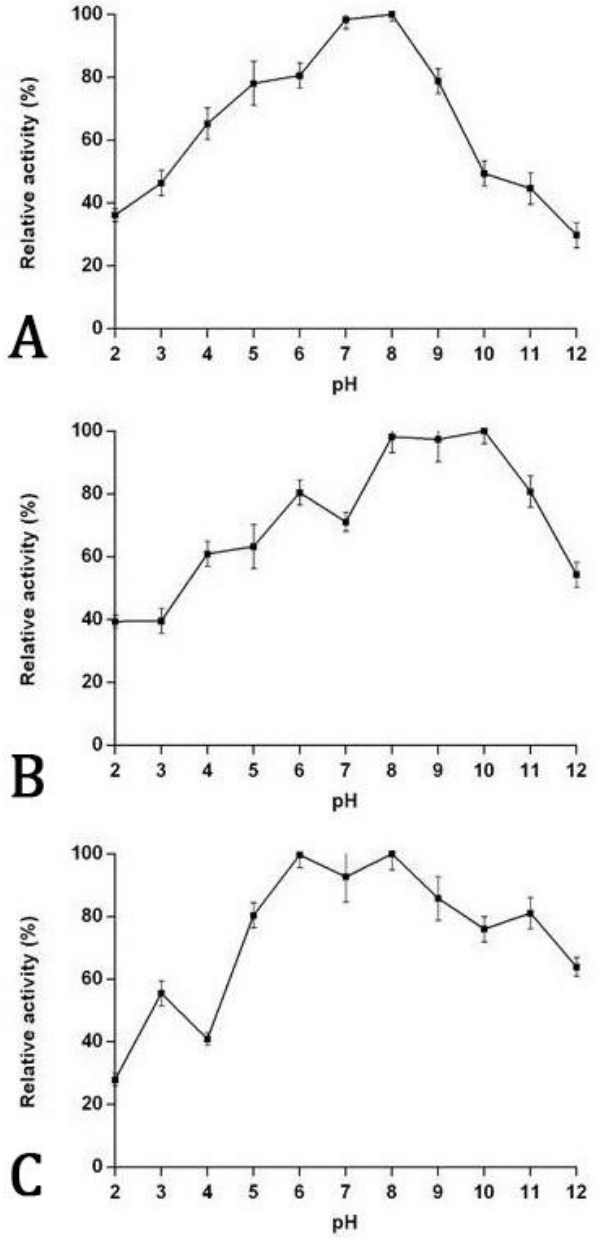

Fig. 1. The proteolytic activity **(A)**, trypsin activity **(B)**, and chymotrypsin activity **(C)** of the extract of miracidia of *Fasciola hepatica* in different pH values using azocasein and SAAPFpNA.

Table 2. The effects of soybean Bowman-Birk inhibitor (SBBI) on the extract of miracidia of *Fasciola hepatica* protease activity.

SBBI	Relative activity (%)	
	BApNA (1.00 mM)	SAAPFpNA (1.00 mM)
50.00 µg mL^{-1}	43.10	53.00
500 µg mL^{-1}	50.20	60.10

BApNA: Nα-benzoyl-DL-arginine 4-nitroanilide, and SAAPFpNA: N-succinyl-alanine-alanine-prolin-phenylalaninc-p- nitroanilide.

Discussion

Many parasites penetrate into host tissues through proteolytic enzymes secretion which play an important role in migration of miracidia of *F. hepatica* in the intermediate hosts.[34] In the current study, the extract of miracidia of *F. hepatica* hydrolyzed azocasein in different levels of pH and serine proteases activities. The optimum pH for serine proteases was in agreement with findings related to *Leishmania amazonensis* and *Eimeria tenella.*[35,36] Geiger and Fritz demonstrated trypsin activity in larvae stage of *Chrysomya bezziana* was at pH 5.00 - 7.00.[37] Johnston *et al*. noted trypsin-like and chymotrypsin enzymes in *Heliothis virescens* hydrolyzed synthetic substrates.[38] Serine proteases activities are also reported from *F. gigantica* at pH 7.50.[39] While Dalton and Heffernan demonstrated that all released proteases from *F. hepatica* were cysteine proteases.[6]

In the present study, serine proteases activities in miracidia of *F. hepatica* were inhibited with PMSF and pepstatin. In other studies, serine proteases were inhibited with inhibitors.[31,38] In earlier studies, chemical inhibitors like thiol also inhibited proteases activities from *F. hepatica*, *F. gigantica*, *S. mansoni*, *H. virescens*, and *Paragonymus westemani*.[38,40-45] Rege *et al.* reported serine proteases from adult stage of *F. hepatica* which were inhibited with leupeptin and PMSF in accordance with Dalton and Heffernan.[6,46] Herbal inhibitors were competitive inhibitors of proteinases with a similar mode of action.[47] Many studies were focused on the effects of herbal inhibitors, *i.e.* SBBI and soybean trypsin inhibitor (SBTI) in larval stages of parasites.[48,49] In the present work, SBBI and PMSF inhibited proteases activities of the miracidia extract which indicated trypsin and chymotrypsin activities. In another study, cysteine proteinases from adult stage of *F. hepatica* were sensitive to the inhibitors.[7] Wijffels *et al.* reported that maximum proteolytic activity in adult stage of *F. gigantica* was at pH 4.00 - 6.00 which inhibited with chemical inhibitors, *i.e.* PMSF and pepstatin.[7] Broadway and Duffey demonstrated the effects of purified SBTI and potato inhibitor II on

Table 1. The effect of protease inhibitors on the extract of miracidia of *Fasciola hepatica* protease activity.

Type of target protease	Inhibitor	Concentration (mM)	Residual activity (%)
Serine	PMSF	5.00	28.20
Aspartic	Pepstatin	10.00	59.30
Metallo	EDTA	10.00	79.80

PMSF: Phenylmethylsulfonyl fluoride; EDTA: Ethylenediaminetetraacetic acid.

growth and digestive physiology of larvae of *H. zea* and *Spodoptera exigua*.[50]

The present work was the first report of proteases activities in miracidia extract of *F. hepatica* at different pH levels. The optimal pH probably could be used to alter pH for reducing miracidium penetration into intermediate host snails. It was also demonstrated that the chemical and herbal inhibitors of serine proteases might be useful for interfering with the penetration into the tissues of snails. Therefore, further studies are recommended to do investigation on bioassay and effectiveness of SBBI for determination of its role in restriction of miracidia penetration into the intermediate hosts.

Conflict of interests

The authors declare that there is no conflict of interests.

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