



ACTIVITY OF SERINE PROTEASES FROM *FASCIOLA HEPATICA* EGGS IN RELATION TO PH AND TEMPERATURE

L. KIANIFARD¹, M. YAKHCHALI¹ & M. IMANI²

¹Department of Pathobiology, ²Department of Basic Sciences;
Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

Summary

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This study was conducted to analyse the serine protease of *Fasciola hepatica* eggs by specific substrates and inhibitors, and investigation of the effects of pH and temperature on proteases' activity and stability. Adult worms were isolated from infected livers. After homogenisation, their protein levels were measured with the Bradford method. Total proteolytic activity of the *Fasciola hepatica* extract was evaluated with azocasein substrate at pH values from 2 to 12. N-benzoyl-arginine-p-nitroanilide (BAPNA) trypsin and N-succinyl-alanine-alanine-prolin-phenylalanine-p-nitroanilide (SAAPFpNA) chymotrypsin substrates were used to measure specific protease activities. The effect of protease inhibitors phenylmethane sulfonyl fluoride (PMSF), pepsin, and ethylenediaminetetraacetic acid (EDTA) on these enzymes was evaluated. Estimation of optimum temperature and pH was performed in the temperature range of 10–90 °C and pH values from 2–12. The optimum pH activities for trypsin and chymotrypsin were at alkaline pH and for total proteolytic activity at acidic pH. The results using protease inhibitors showed that the eggs had serine protease activity. The optimum temperature activity of trypsin and chymotrypsin was 50 °C. These proteases were stable up to 40 °C. Due to the importance of pH and temperature in life cycle of *Fasciola hepatica*, these findings can be used for induction of some modifications in pH and preventing the activity of the enzyme for decrement of the efficacy of embryonic development and egg hatching of this zoonotic parasite.

Key words: *Fasciola hepatica*, pH, serine protease, stability, temperature

INTRODUCTION

Fasciolosis is a worldwide disease in humans and animals caused by the digenean trematode *Fasciola hepatica* (Yakhchali & Bahramnejad, 2016). The World Health Organization recognises fasciolosis as a major food-borne disease, with up to 17 million human cases in 61 countries and 180 millions at risk for fasciolosis (Mas-

Coma, 2005). Fasciolosis is also known as a common parasitic infection which leads to significant loss in growth and meat production in livestock in Europe, Africa, Asia, the Americas and Australasia and annual economic losses of more than USD 3 billion (Mas-Coma, 2005).

Proteases are essential for parasites and facilitate important activities such as invasion, hatching, excystment, nutrition and helminth fecundity and immune evasion (Horn *et al.*, 2014; Dvorak *et al.*, 2016). They are a target for vaccine and drug production (Tallima *et al.*, 2017). Most studies are concentrated on cysteine and aspartic proteases of Fasciolidae (McVeigh *et al.*, 2012). The largest family of proteases are serine proteases and their function is critical in many occurrences (Rawlings *et al.*, 2016). They are important in the life cycles of a wide range of helminths (Yang *et al.*, 2015). Serine proteases are expressed in newly excysted larvae, immature, mature flukes and are involved in penetration and tissue migration (Sajid & McKerrow, 2002). Dipeptidylpeptidase (DPP) has been isolated from *F. hepatica* (Carmona *et al.*, 1993). DPP is secreted by newly excysted juveniles, immature and mature flukes. It was recognised as a serine protease by the specific substrate and a slightly acidic pH optimum. The prolyl carboxypeptidase was identified from the proteins secreted by 21 days old immature and adult *F. hepatica* (Robinson *et al.*, 2009). Environmental factors such as pH and temperature are critical for *F. hepatica*'s life cycle including penetration, invasion, tissue migration, embryonic development and egg hatching (Hurtrez-Boussès, 2001; Yakhchali & Bahramnejad, 2016).

Most studies of *Fasciola* proteases were concentrated on *F. hepatica* and *F. gigantica* proteases from adult and newly excysted juveniles and very little information was reported on the proteases from *F. gigantica* eggs. The laboratory assessment of the impact of pH and temperature on the serine proteases activity in the extract of *F. hepatica* eggs can be useful in further understanding of the degree of their

effects in natural environments. This study was aimed at evaluating the serine proteases in the extract of *F. hepatica* eggs with substrates and determination of the optimum pH for enzyme activity. The inhibitory patterns of proteases were evaluated by protease inhibitors.

MATERIALS AND METHODS

Helminth sampling and egg collection

A number of 250 *F. hepatica* adult worms were isolated from naturally infected livers of the slaughtered cattle. The livers were cut into small pieces and the adult helminths were removed from the bile ducts. *F. hepatica* was saved directly in an ice box during transportation to the laboratory. The isolated *Fasciola* were examined for the presence of eggs by microscopic inspection. The fertile helminths were crushed in a mortar containing 10 mL of distilled water and sieved to gather the eggs. The eggs were washed several times using 0.086% Ringer's solution and centrifuged at 445×g for 5 min to remove debris. The supernatant was discarded and the precipitated eggs were washed three times (Yakhchali & Bahramnejad, 2016).

Protease activity assays

The eggs were homogenised by grinding in liquid nitrogen eight times to access total cytosolic proteins, then centrifuged at 16,000 ×g for 10 min at 4 °C, and the supernatant was collected as the extract. The protein concentration of the supernatant was determined by the Bradford method (Bradford, 1976). The pH optimums were determined for the extracts of egg's protease activities using pH values from 2 to 12. General proteolysis was assessed with azocasein as substrate (Elpidina *et al.*, 2001). The reaction mix-

ture including 30 µL azocasein 2%, 90 µL universal buffer (acetate-phosphate-sodium borate 50 mM) at different pH from 2 to 12, 15 µL extract was incubated at 36 °C for 60 min. Afterward, protein digestion was stopped by adding 30 µL of 30% trichloroacetic acid. Then, non-hydrolysed azocasein was precipitated by incubating the reaction mixture at 4 °C for 1 h and supernatant was collected by centrifugation in 16,000 ×g for 10 min. Seventy-five µL of supernatant and seventy-five µL of 1N NaOH were mixed and measured at 450 nm with ELISA plate reader. For all assays, appropriate blanks were run.

Serine protease assays (trypsin and chymotrypsin)

In order to evaluate the trypsin and chymotrypsin (serine proteases), 10 µL of extract and 5 µL of substrate solution were added (1 mM concentration) to 85 µL of universal buffer (pH 2 to 12) and was evaluated using ELISA plate reader at 405 nm. N-benzoyl-arginine-p-nitroanilide (BAPNA) and N-succinyl-alanine-alanine-prolin-phenylalanine-p-nitroanilide (SAA PFpNA) (final concentration of 1 mM) were used to evaluate the trypsin and chymotrypsin, respectively. The substrates containing p-nitroanilide (pNA) released after enzyme digestion was recorded by ELISA plate reader at 405 nm. Since the substrates had low solubility, dissolution of possible precipitants was performed by using 20 mM dimethyl sulfoxide. Estimation of optimum temperature and pH were made within the temperature range from 10 to 90 °C and pH range from 2 to 12. The thermal stability was investigated by measuring the residual activity of the enzymes after 15 min of incubation at different temperatures prior to substrate addition (Erlanger *et al.*, 1961; 1964).

Protease inhibitors assay

The type of protease was determined with inhibitors for definition of protease classes and azocasein as substrate (da Silva-Lopez & Giovanni-De-Simone, 2004). The proteases inhibitors, *i.e.* phenylmethylsulfonyl fluoride (PMSF, 5 mM), pepstatin (10 µM) and ethylenediaminetetraacetic acid (EDTA, 10 mM) were added to 15 µL extract separately (the inhibitors were added to the extract 15 min before incubation with azocasein) with 30 µL 2% azocasein and 90 µL universal buffer and incubated at 36°C for 60 min in parallel with a control group. Protein digestion was stopped by addition of 30 µL 30% trichloroacetic acid. Non-hydrolysed azocasein was also precipitated at 4 °C for one hour and the supernatant collected after centrifugation at 16,000×g for 10 min. Light absorbance of the mixture of supernatant (75 µL) and 1N NaOH (75 µL) was measured at 450 nm.

Statistical analysis

All data are reported as mean±SD. GraphPad PRISM® software (version 5.04, GraphPad Software, Inc. USA) was used for evaluation of the findings.

RESULTS

Protease activity

The total protein of the extract of the eggs was 0.233 ± 0.03 mg/mL. Due to the concentration of the total protein in the extract, we had approximately 1,000 *Fasciola* eggs per sample ground in liquid nitrogen. The proteolytic activity and the pH dependence using azocasein as substrate is demonstrated on Fig. 1. The azocasein was hydrolysed in a wide range of pH 2 (5.5 %) to pH 12 (17 %). The maximum proteolytic activity of the eggs ex-

tract was at pH 5. The highest activity of the enzyme was approximately preserved at pH 4–6 (76% at pH 4) (Fig. 1).

On all following figures, the maximum OD is considered as 100% and data are expressed relative to maximum OD.

Serine proteases (trypsin, chymotrypsin)

The content of the eggs extract showed trypsin and chymotrypsin activities. Maximum activity toward BApNA was at pH 10 (pH 7–11) and 95.53% activity at pH 9 (Fig. 2). The extract also had the highest activity toward SAAPFpNA at pH 8 (pH 7–10) and more than 95% activity at pH 9 and 10 (Fig. 3).

Fig. 4 shows the optimum temperature curves for trypsin and chymotrypsin protease activities. The two proteases had the same maximum temperature activity at 50 °C, and the activity of trypsin quickly declined at a higher extent than the activity of chymotrypsin as temperature increased. The effect of temperature on the stability of trypsin and chymotrypsin was examined (Fig. 5). The two proteases were stable up to 40 °C, followed by a decrease in activity with increasing the temperature. Also, chymotrypsin was more resistant to high temperatures compared to trypsin.

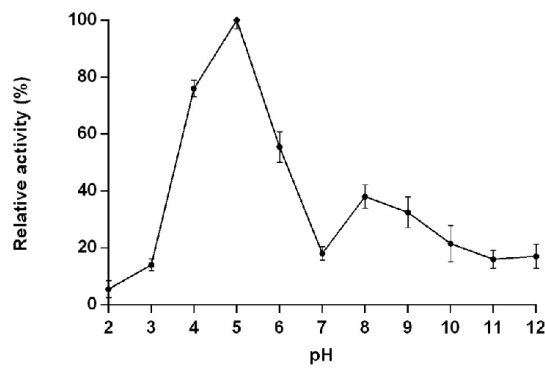


Fig. 1. Total proteolytic activity (mean ± SD) of *Fasciola hepatica* eggs extract at different pH levels by use of azocasein.

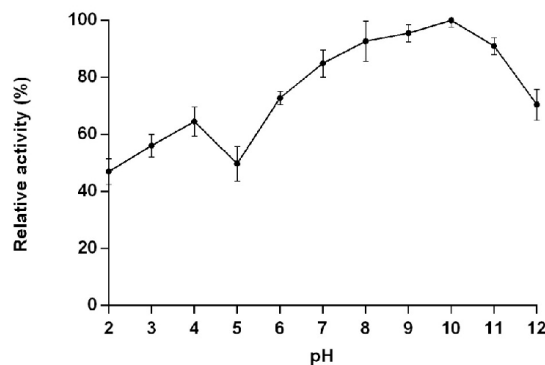


Fig. 2. Trypsin activity (mean ± SD) of *Fasciola hepatica* eggs extract at different pH levels by use of BapNA.

Activity of serine proteases from Fasciola hepatica eggs in relation to pH and temperature

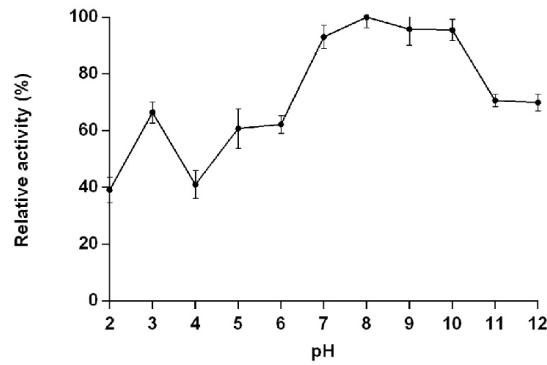


Fig. 3. Chymotrypsin activity (mean \pm SD) of *Fasciola hepatica* eggs extract at different pH levels by use of SAAPFpNA.

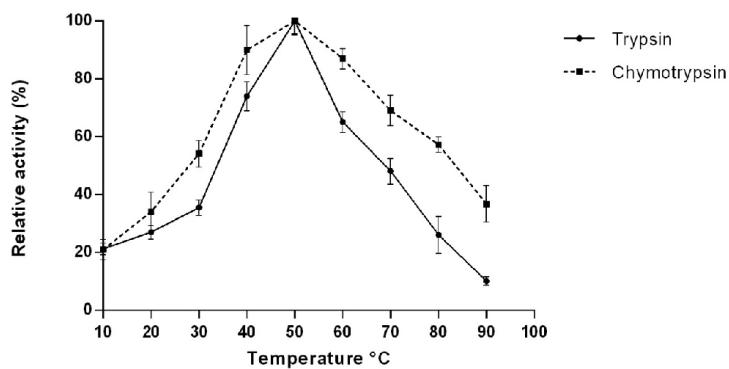


Fig. 4. Optimum temperature of trypsin and chymotrypsin protease activities of *Fasciola hepatica* eggs extract (mean \pm SD).

Protease inhibitors

Table 1 shows the effect of protease inhibitors on protease activity. The inhibitory effect was 53.3%, 9.1 % and 3.5% for PMSF, pepstatin and EDTA, respectively. PMSF demonstrated good inhibitory potential for serine protease with over 50% reduction in activity. The aspartate protease inhibitor, pepstatin and EDTA, which inhibits metallopeptidases had a poor effect.

DISCUSSION

In this study, the proteolytic activity of the extract of *F. hepatica* eggs was investigated and its inhibitory pattern was described by protease inhibitors. The results of this study showed that the proteases from eggs extract hydrolysed azocasein in a wide range of different pH values, and

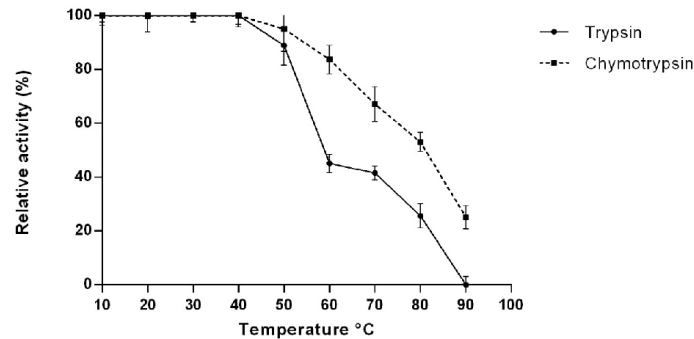


Fig. 5. Temperature stability of trypsin and chymotrypsin protease activities of *Fasciola hepatica* eggs extract (mean \pm SD).

Table 1. The effect of protease inhibitors on the protease activity from the extract of the eggs of *F. hepatica* (mean \pm SD)

Type of target protease	Inhibitors	Concentration	Residual activity (%)
Serine protease	PMSF	5 mM	46.7 \pm 2.4
Aspartic protease	Pepstatin	10 μ M	90.9 \pm 4.9
Metalloprotease	EDTA	10 mM	96.5 \pm 3.1

PMSF=phenylmethylsulfonyl fluoride; EDTA= ethylenediaminetetraacetic acid

the maximum proteolytic activity was in acidic pH. However, substrates BApNA and SAAPFpNA were more specialised for demonstrating trypsin and chymotrypsin protease activities respectively. In this study, by use of the above mentioned specific substrates and protease inhibitors, it seemed that eggs of *F. hepatica* contained trypsin and chymotrypsin proteases.

The results of this study showed that the optimum pH of trypsin and chymotrypsin proteases was in alkaline pH, which was near to the pH optimum for serine protease from *Fasciola gigantica* (Mohamed *et al.*, 2005). In another study on adult *F. gigantica*, the maximum proteolytic activity was observed at pH 4–6 while, some activities were also shown at alkaline pH 10 (Fagbemi & Hillyer, 1992). Accordingly, cysteine protease

inhibitors had the highest inhibitory effect on proteolytic activity. The PMSF had a 37% and the pepstatin had 14% inhibitory effect on aspartate protease while the EDTA did not inhibit proteolytic activity. The study in *F. gigantica* with serine protease inhibitors, the PMSF and pepstatin, an aspartate protease inhibitor, demonstrated that some of the serine protease and aspartate protease activity existed in *F. gigantica* (Fagbemi & Hillyer, 1992). It has been reported that the purified protease from adult *F. hepatica* had homogeneity at 14.5 kDa (Rege *et al.*, 1989). The inhibition of proteases activity by leupeptin and PMSF indicated that most of them were cysteine or serine proteases. This was in agreement with Dalton & Hefernan (1989). The results of this study suggested that, while the extract of eggs

was inhibited by PMSF, pepstatin and EDTA showed poor effect in inhibiting the proteolytic activity.

Determination of the type of proteases was studied by the use of specific inhibitors. Serine proteases are inhibited by serine protease inhibitors (da Silva-Lopez & Giovanni-De-Simone, 2004). In the current study, we demonstrated serine protease activity in the extract of *F. hepatica* eggs. In contrast, previous reports demonstrated that all released proteases from *F. hepatica* were cysteine proteases (Dalton & Heffernan, 1989). Both serine proteases and aspartate proteases were reported in *F. hepatica* (Rupova & Keilova, 1979). The study on adult *F. hepatica* showed that the PMSF has a less inhibitory effect on proteolytic activity, and EDTA didn't reduce protease activity (Wijffels, 1994).

The results of the current study demonstrated that trypsin and chymotrypsin had the same maximum temperature activity at 50 °C in agreement with findings related to *F. gigantica* (Mohamed *et al.*, 2005). The activity of trypsin quickly declined at a higher extent than that of chymotrypsin when the temperature increased. Trypsin and chymotrypsin proteases were stable up to 40 °C, followed by a decrease in activity with increasing of temperature that was near to the thermal stability of serine proteases from *F. gigantica* (Mohamed *et al.*, 2005). In addition, chymotrypsin was more resistant toward high temperatures compared to trypsin. The lower optimal temperature (28 °C) was reported for serine protease from *L. amazonensis* and the thermal stability indicated that 50% of the enzymatic activity was preserved after 4 min of pre-treatment at 42 °C and after 24 h at 37 °C, both in the absence of substrate (da Silva-Lopez & Giovanni-De-Simone, 2004).

The study of the cathepsin-L1 stability in *F. hepatica* demonstrated that the enzyme retained 100% of its activity at 37 °C for 24 h (Dowd *et al.*, 2000).

The results of this study showed that the eggs of *Fasciola hepatica* had trypsin and chymotrypsin activity. Future studies may elucidate their specific role in fasciolosis. According to the importance of pH and temperature in the life cycle of *F. hepatica*, determination of the optimum pH and temperature of activity of the eggs of this parasite could help to increase the effectiveness of protease inhibitors for reduction of embryonic development and egg hatching. Also, considering that the transmission of fasciolosis is favoured by the contamination of pastures with viable eggs, the possible effects of the anti-fertility effect that a given protease inhibitor could provide might prevent disease transmission and therefore decrease the prevalence of fasciolosis in endemic areas.

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Correspondence:

Leila Kianifard
Department of Pathobiology, Faculty of Veterinary Medicine,
Urmia University, Urmia, Iran
PO Box: 5756151818
tel: +98 4432752741
e-mail: leilakianifard@gmail.com