Available online at www.sciencedirect.com
SCIENCE DIRECT
Comparative Biochemistry and Physiology, Part B 142 (2005) 192 – 200
CBP
www.elsevier.com/locate/cbpb

Proteases in egg, miracidium and adult of *Fasciola gigantica*. Characterization of serine and cysteine proteases from adult

Saleh A. Mohamed [a,*], Afaf S. Fahmy [a], Tarek M. Mohamed [b], Soha M. Hamdy [c]

[a] *Molecular Biology Department, National Research Centre, Cairo, Egypt*
[b] *Chemistry Department, Faculty of Science, Tanta University, Tanta, Egypt*
[c] *Biochemistry Division, Chemistry Dept., Faculty of Science, Cairo University (Fayoum Branch), Fayoum, Egypt*

Received 7 April 2005; received in revised form 26 June 2005; accepted 5 July 2005
Available online 15 August 2005

Abstract

Proteolytic activity of 0–12 day old eggs, miracidium and adult worm of *Fasciola gigantica* was assessed and proteases were partially purified by DEAE-Sepharose and CM-cellulose columns. Four forms of protease were separated, PIa, PIb, PIc and PII. Purifications were completed for PIc and PII using Sephacryl S-200 chromatography. A number of natural and synthetic proteins were tested as substrates for *F. gigantica* PIc and PII. The two proteases had moderate activity levels toward azoalbumin and casein compared to azocasein, while gelatin, hemoglobin, albumin and fibrin had very low affinity toward the two enzymes. Amidolytic substrates are more specific to protease activity. PIc had higher affinity toward BAPNA–HCl (*N*-benzoyl–arginine–*p*-nitroanilide–HCl) and BTPNA–HCl (*N*-benzoyl–tyrosine–*p*-nitroanilide–HCl) at pH 8.0 indicating that the enzyme was a serine protease. However, PII had higher affinity toward BAPNA at pH 6.5 in the presence of sulfhydryl groups (β-mercaptoethanol) indicating that the enzyme was a cysteine protease. The effect of specific protease inhibitors on these enzymes was studied. The results confirmed that proteases PIc and PII could be serine and cysteine proteases, respectively. The molecular weights of *F. gigantica* PIc and PII were 60,000 and 25,000, respectively. *F. gigantica* PIc and PII had pH optima at 7.5 and 5.5 and K_M of 2 and 5 mg azocasein/mL, respectively. For amidolytic substrates, PIc had K_M of 0.3 mM BAPNA/mL and 0.5 mM BTPNA/mL at pH 8.0 and PII had K_M of 0.6 mM BAPNA/mL at pH 6.5 with reducing agent. *F. gigantica* PIc and PII had the same optimum temperature at 50 °C and were stable up to 40 °C. All examined metal cations tested had inhibitory effects toward the two enzymes. From substrate specificity and protease inhibitor studies, PIc and PII could be designated as serine PIc and cysteine PII, respectively.
© 2005 Elsevier Inc. All rights reserved.

Keywords: *F. gigantica*; Development; Cysteine protease; Serine protease; Purification; Characterization

1. Introduction

Fasciola hepatica and *F. gigantica* are the parasites of liver fluke disease (fasciolosis). It is not only an important human disease but also affects cattle and sheep. Infection causes worldwide economic losses of approximately two billion dollars per year (Torgerson and Claxton, 1999). Fasciolosis is also an emerging pathogen of humans, particularly in South America (Bolivia, Peru and Equador), in Egypt and Iran (O'Neill et al., 1998; Mas-Coma et al., 1999; Rokni et al., 2002). It is estimated that 2.4 million people are infected with liver flukes worldwide (Mas-Coma et al., 1999).

Parasitic and microbial organisms utilize the digestive actions of proteases on proteins of cells, tissues and organs for purposes of invasion and migration in host tissues. Like many other parasitic helminths, including the related trematode *Schistosoma mansoni*, *F. hepatica* liver flukes release many proteolytic enzymes that belong to the group of cysteine proteases (Dalton and Heffernan, 1989). Most of these proteases resemble mammalian liver cathepsin L (CL) proteases both in amino acid sequence and substrate specificity (Brady et al., 1999; Tort et al., 1999). Liver fluke CL-like proteases are important for

* Corresponding author. Tel.: +202 3669980; fax: +202 3370931.
E-mail address: saleh38@hotmail.com (S.A. Mohamed).

1096-4959/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpc.2005.07.008

immune evasion (Carmona et al., 1993; Smith et al., 1993), tissue invasion (Dalton and Heffernan, 1989; Berasain et al., 1997), nutrition (Yamasaki et al., 1989; Smith et al., 1993) and egg production (Wijffels et al., 1994a; Dalton et al., 1996). Two CL subclasses with different substrate specificities have been identified that have been termed CL1 and CL2 (Dowd et al., 1994, 1995). Both are synthesized by cells lining the fluke's gut (Smith et al., 1993). Southern blot analysis revealed that *F. hepatica* contains at least five CL genes (Heussler and Dobbelaere, 1994). Many CL cDNAs have been isolated from adult flukes (Yamasaki and Aoki, 1993; Heussler and Dobbelaere, 1994; Wijffels et al., 1994b) and several were functionally expressed in the yeast *Saccharomyces cerevisiae* (Dowd et al., 1997; Roche et al., 1997). Vaccination of cattle or sheep with *F. hepatica* CL1 or CL2 proteins resulted in a significant reduction in both fluke burden and production of viable eggs (Wijffels et al., 1994a; Dalton et al., 1996; Mulcahy et al., 1999; Piacenza et al., 1999). The transcriptional products of *F. gigantica* genes encoding cathepsin B proteases were cloned from adult, newly excysted juvenile and metacercarial stages (Meemon et al., 2004). *F. gigantica* cathepsin proteases are used for diagnosis of fasciolasis (Dixit et al., 2002; Tantrawatpan et al., 2005) and immune evasion (Dixit et al., 2004).

On the other hand, the parasitic insects of mammals have used the serine proteases in establishing suitable environments upon or within their hosts (Bowles et al., 1988; Sandeman et al., 1990). Commonly, the same group of proteases is used for nutrient digestion within and without the gut (Casu et al., 1994; Bowles et al., 1990; Johnston et al., 1995), invasion of host cells and tissue (Braun-Breton et al., 1992; Roggwille et al., 1996; da Silva-Lopez and Giovanni-De-Simone, 2004), and modification of host proteins (Burleigh et al., 1997) detrimental to parasite survival. These proteases are thought to be potential targets for vaccines or pesticides that disrupt establishment of larval stages on or within the host and interfere with the digestion of food in the gut of larval and adult stages (Tellam and Bowles, 1997). Also, animal and plant parasitic nematodes express a number of metallo- and serine proteases that are essential for penetration and tissue migration (Sajid and McKerrow, 2002). In addition, serine proteases are found in all organisms studied and participate in blood clotting, complement activation cascade reactions and a vast number of other biological phenomena as well (Rawling and Barret, 1994).

As mentioned above the most studies of proteases in *Fasciola* were concentrated on the *F. hepatica* proteases. Very little information was reported on the proteases from *F. gigantica.* In the present study, we screened the proteases during development, eggs, miracidium and adult of sheep *F. gigantica*, in addition to the purification and characterization of serine and cysteine proteases from the adult.

2. Materials and methods

2.1. Fasciola material

F. gigantica was obtained from sheep liver in the Cairo slaughter house. *F. gigantica* was saved directly in an ice box during transportation to the Lab, lyophilized and stored at −20 °C till use. Also, *F. gigantica* were used as a source of eggs in this study. In the laboratory the eggs were dissected from the uteri of flukes and washed by sedimentation and decantation several times to remove debris. Eggs were cultured in coloured-glass bottles containing distilled water and kept at 24 to 28 °C. Eggs were collected and frozen at −40 °C at intervals of three days. Miracidia were obtained when eggs were removed from incubation to light after 15 days.

2.2. Enzyme activity assays

2.2.1. Azocasein assay

Protease activity with azocompounds as substrates was determined according to Dominguez and Cejudo (1996). Up to 50 μL of crude extract were incubated with 500 μL 100 mM sodium acetate buffer, pH 5.5, and 100 μL 3% azocasein and distilled water to a total volume of 1 mL. Assays were carried out at 37 °C for 1 h, then stopped by the addition of 200 μL of 20% (*v*/*v*) trichloroacetic acid. After removal of precipitated protein by centrifugation (12,000 ×*g* for 5 min at room temperature) the absorbance of the supernatant at 366 nm was determined. One unit of protease activity was defined as the amount of enzyme hydrolyzing 1 μg azocasein per hour under standard assay conditions.

2.2.2. Ninhydrin assay

Protease activity with natural substrates namely gelatin, casein, bovine, fibrin, albumin and hemoglobin was determined according to Moore (1968) by measuring the liberated α-amino nitrogen. Up to 50 μL of crude extract were incubated with 500 μL of 100 mM sodium acetate buffer, pH 5.5, and 100 μL of 3% of each substrate and adjusted to 1 mL with distilled water. Assays were carried out at 37 °C for 1 h and then stopped by the addition of 200 μL of 20% (*v*/*v*) trichloroacetic acid. After centrifugation, 0.5 mL of the supernatant was added to 1.0 mL of ninhydrin reagent (0.5 mL of 1% ninhydrin in 0.5 M citrate buffer, pH 5.5, 0.2 mL of the same buffer, and 1.2 mL glycerol) (Lee and Takahashi, 1966) and heated for 10 min at 100 °C. Four milliliters of distilled water were added to each sample and the absorbance at 570 nm was measured and the increase in free amino groups was determined. Isoleucine was used for standard. One unit of proteolytic activity was defined as microgram of α-amino acid liberated per hour under standard assay conditions.

2.2.3. Trypsin and chymotrypsin (serine protease) assays

Trypsin and chymotrypsin were determined according to the method of Erlanger et al. (1961, 1964). The substrates

BAPNA–HCl (*N*-benzoyl–arginine–*p*-nitroanilide–HCl) and BTPNA (*N*-benzoyl–tyrosine–*p*-nitroanilide–HCl) were dissolved in dimethylsulfoxide at a concentration of 10 mM. The reaction mixture contained in 1.0 mL total volume 880 μL of 0.1 M Tris–HCl buffer, pH 8.0 and 20 μL of the enzyme. The reaction was initiated by the addition of 100 μL of the substrate with final concentration of 1 mM.

2.2.4. Cysteine protease assay

Cysteine protease was assayed according to the method of Arnon (1970). The substrate BAPNA–HCl was dissolved in dimethylsulfoxide at a concentration of 10 mM. The assay reaction mixture contained in 1.0 mL total volume 880 μL of 50 mM sodium phosphate buffer, pH 6.5 containing 5 mM β-mercaptoethanol and 2 mM EDTA and 20 μL of the enzyme. The reaction was initiated by the addition of 100 μL of the substrate with a final concentration of 1 mM.

The activity of trypsin, chymotrypsin or cysteine protease was measured by following the increase in absorbance at 405 nm at 25 °C. One unit of proteolytic activity was defined as the amount of enzyme releasing 1 μmol *p*-nitroaniline per hour under standard assay conditions.

2.3. Enzyme purification

The complete purification procedure was performed at 4 °C. Lyophilized *F. gigantica* (200 mg) were homogenized in 3 mL 50 mM Tris–HCl buffer, pH 7.0. The homogenate was centrifuged at 10,000 ×*g* for 15 min and the supernatant was designated as crude extract. The crude extract was applied directly to a DEAE-Sepharose column (6 × 1 cm i.d.) pre-equilibrated with the same buffer. The adsorbed material was eluted with a stepwise gradient of NaCl ranging from 0.0 to 0.4 M prepared in the same buffer at a flow rate of 40 mL/h and 2 mL fractions were collected. Fractions exhibiting protease activity were eluted at 0.0 and 0.1 M NaCl and designated proteases PI and PII, respectively, according to their elution order. The pooled negatively charged PI fraction was dialyzed against 50 mM sodium acetate buffer, pH 5.5, overnight and concentrated by sucrose then analyzed on CM-cellulose column (6 × 1 cm i.d.) previously equilibrated with the same buffer. The exchanged material was eluted with a stepwise gradient of NaCl ranging from 0.0 to 0.4 M prepared in the same buffer at a flow rate of 40 mL/h and 2 mL fractions were collected. Fractions exhibiting protease activity were eluted at 0.0, 0.1, and 0.3 M NaCl and designated proteases PIa, PIb and PIc, respectively, according to their elution order. Proteases PIc and PII were concentrated by sucrose and separately applied on a Sephacryl S-200 column (90 × 1.6 cm i.d.) previously equilibrated with the same buffer and developed at a flow rate of 20 mL/h and 3 mL fractions were collected. The two proteases were eluted with the same buffer.

2.4. Protein determination

Protein was quantified by the method of Bradford (1976) with bovine serum albumin as standard.

2.5. Polyacrylamide gel electrophoresis

Electrophoresis under nondenaturing conditions was performed in 10% (*w*/*v*) acrylamide slab gel according to the method of Davis (1964) using a Tris–glycine buffer, pH 8.3. Protein bands were stained with Coomassie Brilliant Blue R-250.

2.6. Molecular weight determination

Molecular weight was determined by gel filtration on Sephacryl S-200. The column (90 × 1.6 cm i.d.) was calibrated with cytochrome *C* (12,400), carbonic anhydrase (29,000), bovine serum albumin (67,000), alcohol dehydrogenase (150,000) and β-amylase (200,000). Dextran blue (2,000,000) was used to determine the void volume (V_o). Subunit molecular weight was estimated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). SDS-denatured phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and α-lactalbumin (14,200) were used for the calibration curve.

2.7. Enzyme characterization

Estimates of optimal temperature and pH were made by using a temperature range of 10–90 °C and a pH range of 5.0–8.5. 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. The K_M values were determined from Lineweaver–Burk plots by using different substrate concentrations. The effect of metal cations was performed by incubating the enzyme for 15 min at 37 °C with 2 mM of cations prior to substrate addition. The effect of protease inhibitors was determined by incubation of assay reaction mixture in the presence of inhibitors.

3. Results

3.1. Developmental changes

The total units and specific activities of protease in *F. gigantica* eggs increased gradually from day 0 (1715 units/g tissue, 25.2 units/mg protein) to day 12 (8886 units/g tissue, 130.6 units/mg protein) during development (Table 1). A significant increase in protease activity was detected on day 15, miracidium (12,680 units/g tissues) and adult (27,770 units/g tissues). Although the specific activities for miracidium (176 units/mg protein) and adult (180 units/mg

Table 1
Changes in protease activity during development of *F. gigantica*

Days after oviposition	Units/g tissues	Protein (mg)	S.A
Day 0	1715±85	68.05±3.1	25.2±1.6
Day 3	2762±150	68.02±3.7	40.6±1.9
Day 6	4203±270	73.1±4.5	57.5±2.1
Day 9	8045±340	71.0±4.1	113.3±3.8
Day 12	8886±310	68.03±3.5	130.6±4.8
Day 15 (Miracidium)	12,680±420	72.04±4.8	176±5.1
Adult	27,770±1220	154.2±9.8	180±7.3

Each value represents the mean of 3 runs for each developmental stage±S.E. S.A: specific activity in units/milligram protein.

protein) were approximately equal, the units of adult were 2.2 times higher than those for miracidium. This was attributed to the high level of protein content for adult compared to miracidium.

3.2. Purification of protease from F. gigantica

The purification of protease from *F. gigantica* adult is summarized in Table 2. The elution profile of the chromatography on DEAE-Sepharose (Fig. 1) appeared that protease activity was detected in two peaks, the negative adsorbed fractions and fractions eluted with 0.1 M NaCl and designated as PI and PII. Protease PI was applied to a CM-cellulose column, where it separated into three peaks, PIa, PIb and PIc (Fig. 2). PIc and PII were separately applied to a Sephacryl S-200 (Fig. 3a and b). The two proteases PIc and PII are proved to be pure after Sephacryl S-200 column as judged by polyacrylamide gel electrophoresis (Fig. 4a). The specific activities of Sephacryl proteases PIc and PII were 3795 and 2282 units/mg protein, which represented 21- and 12.6-fold purification over the crude extract, respectively.

3.3. Molecular weight of PIc and PII

The native molecular weights of PIc and PII were estimated to be 60,000 and 25,000, respectively, using Sephacryl S-200 column. These values were confirmed by SDS-PAGE (Fig. 4b), where the molecular weights of PIc and PII had the same values.

3.4. Characterization of PIc and PII

A study of substrate specificity for PIc and PII was made by using two types of different substrates (Table 3). Firstly, azo-proteins and natural proteins were used as substrates, where the activity with azocasein is regarded as 100% activity. PIc and PII had moderate activity levels toward azoalbumin and casein compared to azocasein, while gelatin, hemoglobin, albumin and fibrin had very low affinity toward the two enzymes. Secondly, the specific substrates for serine and cysteine proteases were used. The data in Table 3 appears that PIc had higher affinity toward BAPNA and BTPNA at pH 8.0 indicating that the enzyme had trypsin- and chymotrypsin-like activities (serine protease). However, PII had higher affinity toward BTPNA at pH 6.5 in the presence of SH (β-mercaptoethanol) indicating that the enzyme is cysteine protease. The K_m values of PIc and PII were estimated to be 2 and 5 mg azocasein/mL, respectively. For amidolytic substrates, PIc had K_m of 0.3 mM BAPNA/mL and 0.5 mM BTPNA/mL at pH 8.0 and PII had K_m of 0.6 mM BAPNA/mL at pH 6.5 with reducing agent.

PIc and PII had optimum pHs of 7.5 and 5.5, respectively (Fig. 5). Fig. 6a shows the temperature optima curves for PIc and PII activities. The two enzymes had the same optimum temperature at 50 °C, where the activity of PIc is quickly declined more than the activity of PII with increasing of temperature. The effect of temperature on the stability of PIc and PII was examined (Fig. 6b). The two enzymes were stable up to 40 °C, followed by a decrease in activity with increasing of the temperature. Also, PII had more resistance toward the high temperatures compared to PIc.

All examined cations had inhibitory effects on PIc and PII (Table 4). The effectiveness of metal cations as inhibitors for PIc was $Mg^{2+}>Fe^{3+}>Ca^{2+}>Co^{2+}>Mn^{2+}>Ni^{2+}>Hg^{2+}$ with 100%, 91%, 84%, 70%, 67%, 58%, and 50% inhibition, respectively, while for PII was $Mg^{2+}>Fe^{3+}>Ca^{2+}>Hg^{2+}>Mn^{2+}>Co^{2+}>Ni^{2+}$ with 90%, 87%, 83%, 74%, 66%, 48%, and 46% inhibition, respectively. The effect of different protease inhibitors on PIc and PII was examined (Table 5). While PIc was inhibited by PMSF and trypsin inhibitor (serine protease inhibitors), PII was

Table 2
Purification scheme for *F. gigantica* protease

Step	Total units*	Total protein (mg)	S.A. (units/mg protein)	Fold purification	Recovery (%)
Crud extract	5554	30.8	180.3	–	100
DEAE-Sepharose 0.0 M NaCl (PI)	3243	7.8	415.7	2.3	58.3
0.1 M NaCl (PII)	2027	2.5	810	4.5	36.4
CM-cellulose 0.0 M NaCl (PIa)	486	3.3	147.2	0.81	8.7
0.1 M NaCl (PIb)	243	1.8	135	0.74	4.3
0.3 M NaCl (PIc)	2432	1.285	1892	10.5	43.7
Gel filtration on Sephacryl S-200 PIc	1632	0.43	3795	21.0	29.3
PII	1278	0.56	2282	12.6	23.0

* One unit of protease activity was defined as the amount of enzyme hydrolyzing 1 μg azocasein per hour under standard assay conditions.

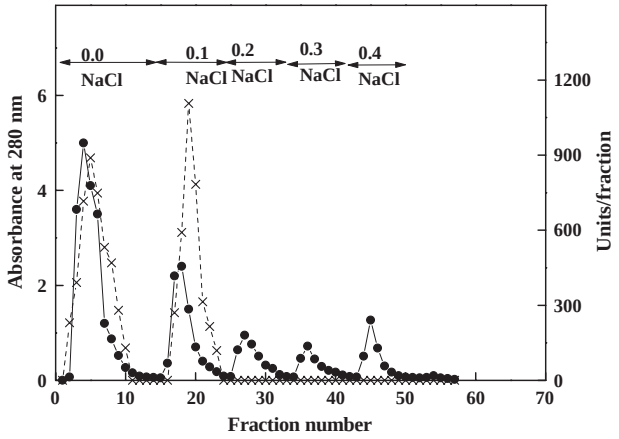

Fig. 1. Elution profile for the chromatography of *F. gigantica* adult protease on DEAE-Sepharose column (6 × 1 cm i.d.) previously equilibrated with 50 mM Tris–HCl buffer, pH 7.0 at a flow rate of 40 mL/h and 2 mL fractions. Absorbance at 280 nm (●-----●) and protease activity (x-----x).

inhibited by *p*-CMB, *N*-EM and iodoacetic acid (cysteine protease inhibitors). These results indicated that proteases PIc and PII could be serine and cysteine proteases, respectively.

4. Discussion

In the present study, the proteolytic activity was screened during the development of *F. gigantica*. The results appeared that the proteolytic activity increased during embryogenesis (1715–8886 units/g tissues), followed by miracidium (12,680 units/g tissues) and adult (27,770 units/g tissues). The increase of protease activity levels during development of *F. gigantica* would have to be of greater physiological roles. Several investigations studied the proteases during development of parasites such as: the serine proteases from larvae of *Heliothis virescens* (Johnston et al., 1995), the neutral proteases of the three developmental stages of *S. mansoni* (Auriault et al., 1982), serine proteases from *Chrysomya bazziana* larvae (Muharsini et al., 2000), a neutral thiol protease from *Paragonimus westemani* metacercariae (Yamakami and

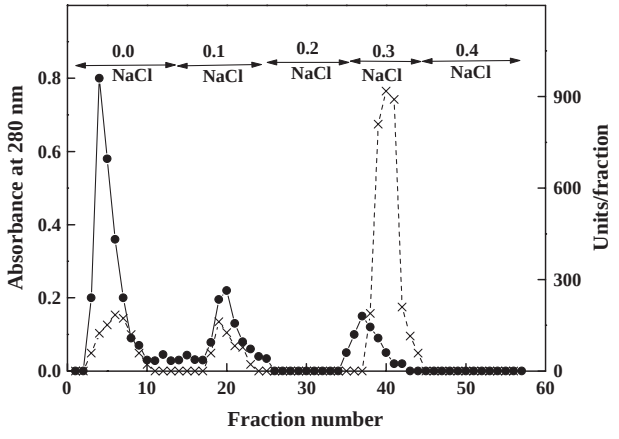

Fig. 2. Elution profile for the chromatography of *F. gigantica* adult protease DEAE-Sepharose fraction PI on CM-cellulose column (6 × 1 cm i.d.) previously equilibrated with 50 mM acetate buffer, pH 5.5 at a flow rate of 40 mL/h and 2 mL fractions. Absorbance at 280 nm (●-----●) and protease activity (x-----x).

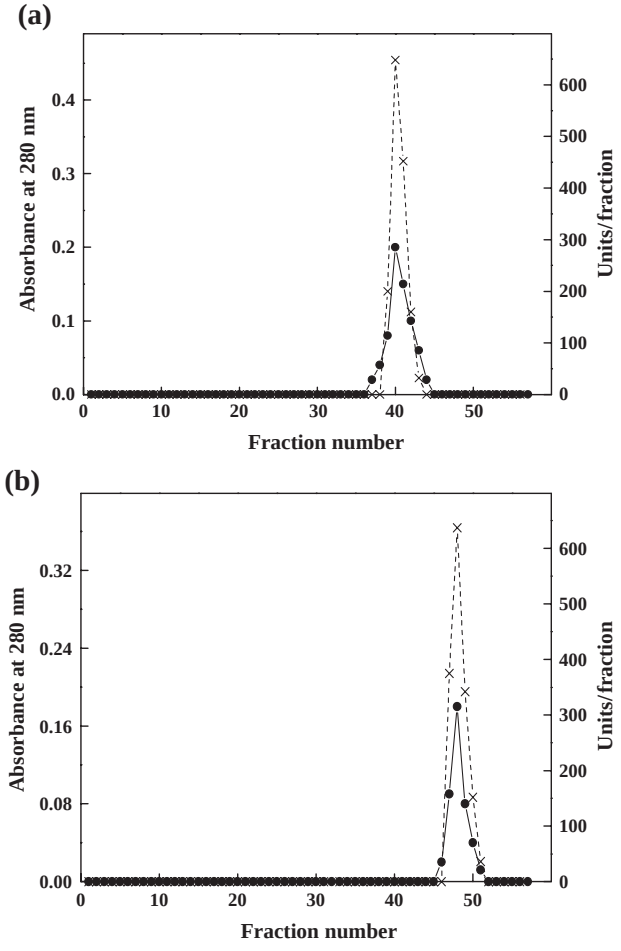

Fig. 3. Elution profile for *F. gigantica* adult protease CM-cellulose fraction PIc (a) and DEAE-Sepharose fraction PII (b) on Sephacryl S-200 column (90 × 1.6 cm i.d.) previously equilibrated with 50 mM Tris–HCl buffer, pH 7.0 at a flow rate of 20 mL/h and 3 mL fractions. Absorbance at 280 nm (●-----●) and protease activity (x-----x).

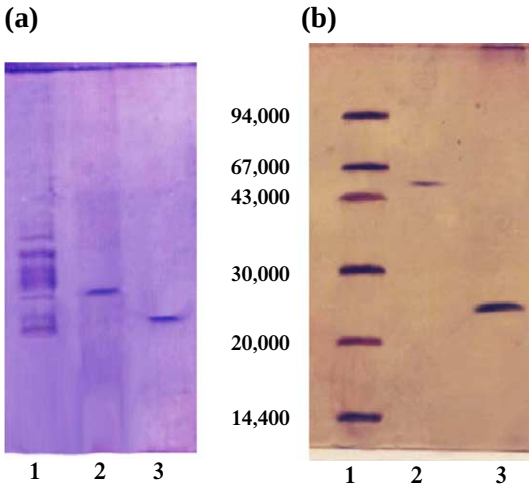

Fig. 4. (a) Polyacrylamide gel electrophoresis for *F. gigantica* adult protease during purification steps. 1—crude extract; 2 and 3—Sephacryl S-200 PIc and PII, respectively. (b) SDS-PAGE for molecular weight determination of *F. gigantica* protease. 1—Standard proteins; 2 and 3—Sephacryl S-200 *F. gigantica* PIc and PII, respectively.

Hamajima, 1990) and serine and cysteine-type proteases from *Eimeria tenella* oocytes (Michalski et al., 1994). Enzymatic assays have shown that *F. hepatica* cathepsin L activity is expressed in different developmental stages (Carmona et al., 1993; Smith et al., 1993).

During purification of protease from *F. gigantica*, using DEAE-Sepharose and CM-cellulose columns, four forms of protease were separated, PIa, PIb, PIc and PII. The purification was completed for PIc and PII with the highest proteolytic activity using Sephacryl S-200 column. Fagbemi and Hillyer (1992) purified one form of cysteine protease from *F. gigantica*. Generally, different forms of proteases were demonstrated in different stages of parasites (Siddiqui et al., 1993; Ghoneim and Klinkert, 1995; Johnston et al., 1995; Hawthorne et al., 2000; Muharsini et al., 2000; Harmsen et al., 2004).

A number of natural and synthetic proteins were tested as substrates for *F. gigantica* PIc and PII. The two proteases had moderate activity levels toward azoalbumin and casein

Table 3
Substrate specificity of *F. gigantica* proteases PIc and PII

Substrate	Relative activity (%)	
	PIc	PII
Azocasein	100	100
Azoalbumin	30	40
Casein	22	25
Gelatin	9.2	7.3
Hemoglobin	6.5	5.3
Albumin	8.9	4.6
Fibrin	3.2	2.5
BAPNA–HCl (pH 8.0)	100	8.5
BTPNA–HCl (pH 8.0)	80	6.5
BAPNA–HCl (pH 6.5)	10	100

BAPNA–HCl, *N*-benzoyl–arginine–*p*-nitroanilide–HCl and BTPNA–HCl, *N*-benzoyl–tyrosine–*p*-nitroanilide–HCl.

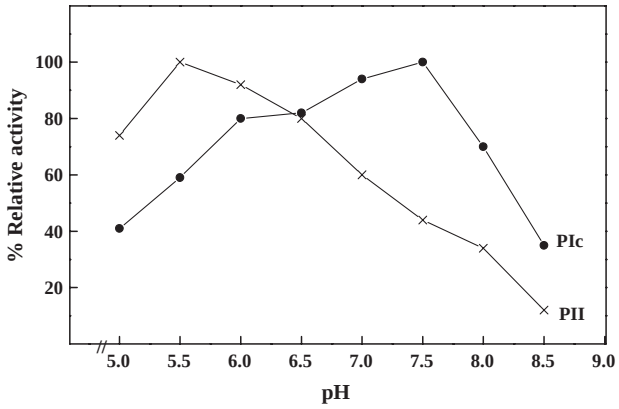

Fig. 5. pH optimum of *F. gigantica* adult proteases PIc and PII. The reaction mixture contained in 1.0 mL: 1, 100 μl of 3% azocasein, suitable amount of enzyme and 50 mM acetate buffer (pH 5.0–5.5), sodium phosphate buffer (pH 6.0–7.5) and Tris–HCl (8–8.5).

compared to azocasein, while gelatin, hemoglobin, albumin and fibrin had very low affinity toward the two enzymes. Hemoglobin, bovine serum albumin, ovalbumin and both gelatin and peptide substrates containing arginine in ester bound were hydrolyzed by *Leishmania amazonensis* serine protease (da Silva-Lopez and Giovanni-De-Simone, 2004). *P. westemani* neutral thiol protease hydrolyzed protein

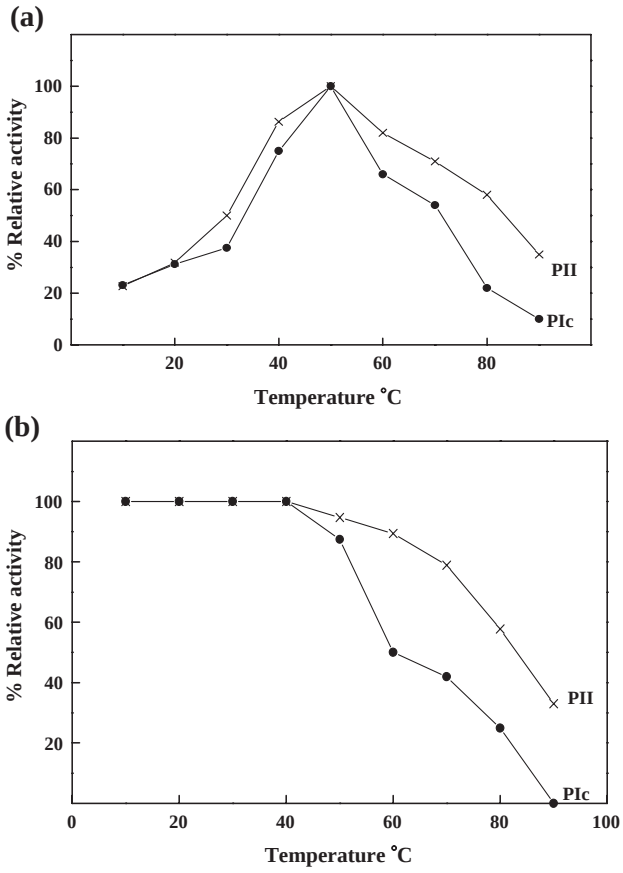

Fig. 6. Temperature optimum (a) and temperature stability (b) of *F. gigantica* adult proteases PIc and PII. Each point represents the average of two experiments.

Table 4
Effect of metal cations on *F. gigantica* proteases PIc and PII

Metal cation	Relative activity (%)	Relative activity (%)
	PIc	PII
Hg^{2+}	50	26
Ni^{2+}	42	54
Mn^{2+}	33	34
Co^{2+}	30	52
Ca^{2+}	16	17
Fe^{3+}	9	13
Mg^{2+}	0	10

The enzyme was preincubated for 15 min at 37 °C with 2 mM of listed metal cations prior to substrate addition. Activity in absence of cations was taken as 100%. Each value represents the average of two experiments.

substrates, casein and azocol and showed low specificity toward hemoglobin, but no activity with bovine serum albumin (Yamakami and Hamajima, 1990). However, amidolytic substrates, BAPNA and BTPNA, are more specific to protease activity. Using these specific substrates it appeared that *F. gigantica* PIc and PII are serine (trypsin and chymotrypsin) and cysteine proteases, respectively. Using the trypsin substrate, BAPNA, (Geiger and Fritz, 1984), the larval *C. bezziana* trypsins are active over pH 5.0–7.0 in the sodium acetate buffers. In this buffer, the tryptic activity was 30% higher than in Tris–HCl buffer over the same range (6.5–7.0), indicating that the tryptic activities were buffer ion dependent (Muharsini et al., 2000). Two major protease activities were present in *H. virescens*, a trypsin-like enzyme, hydrolyzing BAPNA and an enzyme which hydrolyzed synthetic chymotrypsin substrates containing more than one amino acid, but not BTPNA (Johnston et al., 1995).

For confirming that *F. gigantica* PIc and PII are serine and cysteine proteases, respectively, as concluded above, we studied the effect of some specific protease inhibitors on these enzymes. The results appeared that while PIc was inhibited by PMSF and trypsin inhibitor (serine protease inhibitors), PII was inhibited by *p*-CMB, *N*-EM and iodoacetic acid (cysteine protease inhibitors). These results indicated that proteases PIc and PII could be serine and cysteine proteases, respectively. Several articles studied the inhibitory effect to determine the type of the protease. Thiol proteases from *F. gigantica* (Fagbemi and Hillyer, 1992), *P. westemani* (Yamakami and Hamajima, 1990) and *S. mansoni* (Dresden and Deelder, 1979; Ghoneim and Klinkert, 1995) were inhibited by thiol protease inhibitors. Serine proteases were also inhibited by serine protease inhibitors (Johnston et al., 1995; da Silva-Lopez and Giovanni-De-Simone, 2004).

The molecular weights of *F. gigantica* serine PIc and cysteine PII were 60,000 and 25,000, respectively, using Sephacryl S-200 column. These values were confirmed by SDS-PAGE and represented monomer subunits. The molecular weight of cysteine PII was near to the molecular weights of cysteine proteases from *F. gigantica* (26,000–28,000) (Fagbemi and Hillyer, 1992), *F. hepatica* (30,000) (Siddiqui et al., 1993), and *P. westemani* (22,000) (Yamakami and Hamajima, 1990). *L. amazonensis* had two serine proteases, relatively high molecular weight (68,000) (da Silva-Lopez and Giovanni-De-Simone, 2004) and higher molecular weight (101 kDa) (Ribeiro de Andrade et al., 1998).

F. gigantica serine PIc had pH optimum at 7.5. This was near to the pH optimum for serine protease from *L. amazonensis* (Ribeiro de Andrade et al., 1998) and *E. tenella* (Michalski et al., 1994). *F. gigantica* cysteine PII had acidic pH optimum at 5.5. Fagbemi and Hillyer (1992) reported that *F. gigantica* cysteine protease had pH optimum at 4.5. *F. hepatica* cathepsin L-like proteases were active over a wide pH range (pH 3.0–8.0) (Dowd et al., 2000).

Although, *F. gigantica* serine PIc and cysteine PII had the same optimum temperature at 50 °C, the two enzymes were stable up to 40 °C. 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 of temperature at 37 °C, both in the absence of substrate (da Silva-Lopez and Giovanni-De-Simone, 2004).The stability study of *F. hepatica* cathepsin L1 demonstrated that the enzyme retained 100% of its activity at 37 °C for 24 h (Dowd et al., 2000). *E. tenella* serine protease was stable at room temperature for 24 h and storage at −20 °C for 3 months resulted in no loss of activity. The dependence of enzymatic activity on environment temperature could also be observed for serine protease of *Acanthamoeba healyi*, which causes granulomatous encephalitis and pneumonitis (Kong et al., 2000). All examined metal cations tested (Mg^{2+}, Fe^{3+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, and Hg^{2+}) had inhibitory effects toward *F. gigantica* serine PIc and cysteine PII. The enzyme activity of serine protease from *L. amazonensis* was reduced 40% and 20% by $Ca^{2+}$and Mg^{2+}, while Mn^{2+} positively modulated the enzyme (about 15% higher than the control) (da Silva-Lopez and Giovanni-De-Simone, 2004). Michalski et al. (1994) reported that the stability of *E. tenella* serine protease and its activity were not modified by either

Table 5
Effect of protease inhibitors on *F. gigantica* proteases PIc and PII

Inhibitors*	Relative activity (%)	Relative activity (%)
	PIc	PII
p-CMB	92	16
N-EM	90	66
Iodoacetic acid	93	45
PMSF	9	92
Trypsin inhibitor	23	88
1,10-Phenanthrolin	95	98

The protease activity was measured in the presence of 2 mM inhibitors listed. Activity in the absence of inhibitors was taken as 100%. Each value represents the average of two experiments.
* *p*-CMB, *p*-chloromercuribenzoic acid; *N*-EM, *N*-ethylmaleimide; PMSF, phenylmethylsulphonyl fluoride.

divalent cations or chelating agents. In conclusion, from substrate specificity and protease inhibitor studies, *F. gigantica* PIc and PII could be designated as serine PIc and cysteine PII, respectively.

References

Arnon, R., 1970. Papain. Methods Enzymol. 19, 226–244.

Auriault, C., Pierce, R., Cesari, I.M., Capron, A., 1982. Neutral protease activities at different developmental stages of *Schistosoma mansoni* in mammalian hosts. Comp. Biochem. Physiol., B 72, 377–384.

Berasain, P., Goni, F., McGonigle, S., Dowd, A., Dalton, J.P., Frangione, B., Carmona, C., 1997. Proteinases secreted by *Fasciola hepatica* degrade extracellular matrix and basement membrane components. J. Parasitol. 83, 1–5.

Bowles, V.M., Carnegie, P.R., Sandeman, R.M., 1988. Characterization of proteolytic and collagenolytic enzymes from the larvae of *Lucilia cuprina*, the sheep blow fly. Aust. J. Biol. Res. 41, 269–278.

Bowles, V.M., Feehan, J.P., Sandeman, R.M., 1990. Sheep plasma inhibitors influencing protease activity and growth of *Lucilia cuprina* larvae in vitro. Int. J. Parasitol. 20, 169–174.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brady, C.P., Dowd, A.J., Brindley, P.J., Ryan, T., Day, S.R., Dalton, J.P., 1999. Recombinant expression and localization of *Schistosoma mansoni* cathepsin L1 support its role in the degradation of host hemoglobin. Infect. Immun. 67, 368–374.

Braun-Breton, C., Blisnick, T., Jouin, H., Barale, J.C., Rabilloud, T., Langsley, G., Pereira da Silva, L.H., 1992. *Plasmodium chabaudi* p68 serine protease activity required for merozoite entry into mouse erythrocyte. Proc. Natl. Acad. Sci. U. S. A. 88, 9647–9651.

Burleigh, B.A., Caler, E.V., Webster, P., Andrews, N., 1997. A cytosolic serine endopeptidase from *Trypanosoma cruzi* is required for the generation of Ca^{+2} signaling in mammalian cells. J. Cell Biol. 136, 609–620.

Carmona, C., Dowd, A.J., Smith, M., Dalton, J.P., 1993. Cathepsin L proteinase secreted by *Fasciola hepatica* in vitro prevents antibody mediated eosinophil attachment to newly excysted juveniles. Mol. Biochem. Parasitol. 62, 9–18.

Casu, R.E., Jarmey, J.M., Elvin, C.M., Eisemann, C.H., 1994. Isolation of a trypsin-like serine protease gene family from the sheep blow fly *L. cuprina*. Insect Mol. Biol. 3, 159–170.

Dalton, J.P., Heffernan, M., 1989. Thiol proteases released in vitro by *Fasciola hepatica*. Mol. Biochem. Parasitol. 35, 161–166.

Dalton, J.P., McGonicle, S., Rolph, T.P., Andrews, S.J., 1996. Induction of protective immunity in cattle against infection with *Fasciola hepatica* by vaccination with cathepsin L proteinases and with hemoglobin. Infect. Immun. 64, 5060–5074.

da Silva-Lopez, R.E., Giovanni-De-Simone, S., 2004. *Leishmania* (*Leishmania*) *amazonensis*: purification and characterization of a promastigote serine protease. Exp. Parasitol. 107, 173–182.

Davis, B.J., 1964. Disc electrophoresis. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121, 404–427.

Dixit, A.K., Yadav, S.C., Sharma, R.L., 2002. 28 kDa *Fasciola gigantica* cysteine protease in the diagnosis of prepatent ovine fasciolosis. Vet. Parasitol. 109, 233–247.

Dixit, A.K., Yadav, S.C., Sharma, R.L., 2004. Experimental bubalian fasciolosis: kinetics of antibody response using 28 kDa *Fasciola gigantica* cysteine proteinase as antigen. Trop. Anim. Health Prod. 36, 49–54.

Dominguez, F., Cejudo, F.J., 1996. Characterization of the endoproteases appearing during wheat grain development. Plant Physiol. 112, 1211–1217.

Dowd, A.J., Smith, A.M., McGonigle, S., Dalton, J.P., 1994. Purification and characterization of a second cathepsin L proteinase secreted by the parasitic trematode *Fasciola hepatica*. Eur. J. Biochem. 223, 91–98.

Dowd, A.J., McGonigle, S., Dalton, J.P., 1995. *Fasciola hepatica* cathepsin L proteinase cleaves fibrinogen and produces a novel type of fibrinogen clot. Eur. J. Biochem. 232, 241–246.

Dowd, A.J., Tort, J., Roche, L., Ryan, T., Dalton, J.P., 1997. Isolation of cDNA encoding *Fasciola hepatica* cathepsin L2 and functional expression in *Saccharomyces cerevisiae*. Mol. Biochem. Parasitol. 88, 163–174.

Dowd, A.J., Dooley, M., Fagain, C., Dalton, J.P., 2000. Stability studies on the cathepsin L proteinase of the helminth parasite, *Fasciola hepatica*. Enzyme Microb. Technol. 27, 599–604.

Dresden, M.H., Deelder, A.M., 1979. *Schistosoma mansoni*: thiol proteinase properties of adult worm hemoglobinase. Exp. Parasitol. 48, 190–197.

Erlanger, B.F., Kokowsky, N., Cohen, W., 1961. Preparation and properties of two chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95, 271–278.

Erlanger, B.F., Cooper, A.G., Bendisch, A.J., 1964. The heterogeneity of three times crystallized α-chymotrypsin. Biochemistry 3, 1880–1883.

Fagbemi, B.O., Hillyer, G.V., 1992. The purification and characterization of a cysteine protease of *Fasciola gigantica* adult worms. Vet. Parasitol. 43, 223–232.

Geiger, R., Fritz, H., 1984. Trypsin. In: Bergmeyer, H.U (Ed.), Methods of Enzymatic Analysis, 5. Verlag-Chemie, Weinheim, pp. 119–129.

Ghoneim, H., Klinkert, M.-Q., 1995. Biochemical properties of purified cathepsin B from *Schistosoma mansoni*. Int. J. Parasitol. 25, 1515–1519.

Harmsen, M.M., Cornelissen, J.B.W.J., Buijs, H.E.C.M., Boersma, W.J.A., Jeurissen, S.H.M., van Milligen, F.J., 2004. Identification of a novel *Fasciola hepatica* cathepsin L protease containing protective epitopes within the propeptide. Int. J. Parasitol. 34, 675–682.

Hawthorne, S.J., Pagano, M., Halto, D.W., Walker, B., 2000. Partial characterization of a novel cathepsin L-like protease from *Fasciola hepatica*. Biochem. Biophys. Res. Commun. 277, 79–82.

Heussler, V.T., Dobbelaere, D.A.E., 1994. Cloning of a protease gene family of *Fasciola hepatica* by the polymerase chain reaction. Mol. Biochem. Parasitol. 64, 11–23.

Johnston, K.A., Lee, M.J., Brough, C., Hilder, V.A., Gatehouse, A.M.R., Gatehouse, J.A., 1995. Protease activities in the larval midgut of *Heliothis virescens*: evidence for trypsin and chymotrypsin-like enzymes. Insect Biochem. Mol. Biol. 25, 375–383.

Kong, H.H., Kim, T.H., Chung, D., 2000. Purification and characterization of a secretory serine protease of *Acantamoeba healyi* isolated from GAE. J. Parasitol. 86, 12–17.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227, 680–685.

Lee, Y.P., Takahashi, 1966. An improved colorimetric determination of amino acids with the use of ninhydrin. Anal. Biochem. 14, 71–77.

Mas-Coma, S., Bargues, M.D., Esteban, J.G., 1999. Human fasciolosis. In: Dalton, J.P. (Ed.), Fasciolosis. CAB international, Wallingfords, UK, pp. 411–434.

Meemon, K., Grams, R., Vichasri-Grams, S., Hofmann, A., Korge, G., Viyanant, V., Upatham, E.S., Habe, S., Sobhon, P., 2004. Molecular cloning and analysis of stage and tissue-specific expression of cathepsin B encoding genes from *Fasciola gigantica*. Mol. Biochem. Parasitol. 136, 1–10.

Michalski, W.P., Crooks, J.K., Prowse, S.J., 1994. Purification and characterization of a serine-type protease from *Eimeria tenelia* oocytes. Int. J. Parasitol. 24, 189–195.

Moore, S., 1968. Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. J. Biol. Chem. 243, 6281–6283.

Muharsini, S., Sukarsih, S., Riding, G., Partoutomo, S., Hamilton, S., Willadsen, P., Wijffels, G., 2000. Identification and characterization of the excreted/secreted serine proteases of larvae of the old world screw worm fly, *Chrysomya bezziana*. Int. J. Parasitol. 30, 705–714.

Mulcahy, G., O'Connor, F., Clery, D., Hogan, S.F., Dowd, A.J., Andrews, S.J., Dalton, J.P., 1999. Immune responses of cattle to experimental anti-*Fasciola hepatica* vaccines. Res. Vet. Sci. 67, 27–33.

O'Neill, S.M., Parkinson, M., Strauss, W., Angles, R., Dalto, J.P., 1998. Immunodiagnosis of *Fasciola hepatica* infection (fascioliasis) in a human population in the Bolivian altiplano using purified cathepsin L cysteine protease. Am. J. Trop. Med. Hyg. 58, 417–423.

Piacenza, L., Acosta, D., Basmadjian, I., Dalton, J.P., Carmona, C., 1999. Vaccination with cathepsin L proteinases and with leucine aminopeptidase induces high levels of protection against faciolosis in sheep. Infect. Immun. 67, 1954–1961.

Rawling, N.D., Barret, A.J., 1994. Families of serine peptidases. Methods Enzymol. 244, 19–45.

Ribeiro de Andrade, A., Santoro, M.M., Melo, N.M., Mares-Guia, M., 1998. *Leishmania* (*Leishmania*) *amazonensis*: purification and characterization of a soluble serine oligopeptidase from promastigotes. Exp. Parasitol. 89, 153–160.

Roche, L., Dowd, A.J., Tort, J., McGonigle, S., McSweeney, A., Curley, G.P., Ryan, T., Dalton, J.P., 1997. Functional expression of *Fasciola hepatica* cathepsin L1 in *Saccharomyces cerevisiae*. Eur. J. Biochem. 245, 373–380.

Roggwille, E., Betoulle, M.E., Blisnick, T., Braun-Breton, C., 1996. A role for erythrocytes band 3 degradation by the parasite gp 76 serine protease in the formation of the parasitophorus vacuole during invasion of erythrocytes by *Plasmodium falciparum*. Mol. Biochem. Parasitol. 82, 13–24.

Rokni, M.B., Massoud, J., O'Neill, S.M., Parkinsonb, M., Dalto, J.P., 2002. Diagnosis of human fasciolosis in Gilan province of northern Iran: application of cathepsin L-ELISA. Diagn. Microbiol. Infect. Dis. 44, 175–179.

Sajid, M., McKerrow, J.H., 2002. Review. Cysteine proteases of parasitic organisms. Mol. Biochem. Parasitol. 120, 1–21.

Sandeman, R.M., Feehan, J.P., Chandler, R.A., Bowles, V.M., 1990. Tryptic and chymotryptic proteases released by larvae of the blow fly *Lucilia cuprina*. Int. J. Parasitol. 20, 1019–1023.

Siddiqui, A.A., Zhou, Y., Podesta, R.B., Karcz, S.R., Tognon, C.E., Strejan, G.H., Dekaban, G.A., Clarke, M.W., 1993. Characterization of Ca-dependent neutral protease (calpain) from human blood flukes, *Schistosoma mansoni*. Biochim. Biophys. Acta 1181, 37–44.

Smith, A.M., Dowd, A.J., Heffernan, M., Robertson, C.D., Dalton, J.P., 1993. *Fasciola hepatica*: a secreted cathepsin L-like proteinase cleaves host immunoglobulin. Int. J. Parasitol. 23, 977–983.

Tantrawatpan, C., Maleewong, W., Wongkham, C., Wongkham, S., Intapan, P.M., Nakashima, K., 2005. Serodiagnosis of human faschioliasis by a cystatin capture enzyme-linked immunosorbent assay with recombinant *Fasciola gigantica* cathepsin L antigen. Am. J. Trop. Med. Hyg. 72, 82–86.

Tellam, R.L., Bowles, V.M., 1997. Control of blow fly strike in sheep: current strategies and future prospects. Int. J. Parasitol. 27, 261–273.

Torgerson, P.R., Claxton, J.R., 1999. Epidemiology and control. In: Dalto, J.P. (Ed.), Fasciolosis. CAB International, Oxford, pp. 465–525.

Tort, J., Brindley, P.J., Knox, D., Wolfe, K.H., Dalton, J.P., 1999. Proteinases and associated genes of parasitic helminths. Adv. Parasitol. 43, 161–166.

Wijffels, G.L., Salvatore, L., Dosen, M., Waddington, J., Wilson, L., Thompson, C., Campbell, N., Sexton, J., Wicker, J., Bowen, F., Friedel, T., Spithill, T.W., 1994a. Vaccination of sheep with purified cystein proteinases of *Fasciola hepatica* decreases worm fecundity. Exp. Parasitol. 78, 132–148.

Wijffels, G.L., Panaccio, M., Salvatore, L., Wilson, L., Walker, I.D., Spithill, T.W., 1994b. The secreted cathepsin L-like proteinases of the trematode *Fasciola hepatica*, contain 3-hydroxyproline residues. Biochem. J. 299, 781–790.

Yamakami, K., Hamajima, F., 1990. A neutral thiol protease secreted from newly excysted metacercariae of trematode parasite *Paragonimus westemani*: purification and characterization. Comp. Biochem. Physiol., B 95, 755–758.

Yamasaki, H., Aoki, T., 1993. Cloning and sequence analysis of the major cysteine protease expressed in the trematode parasite *Fasciola* spp. Biochem. Mol. Biol. Int. 31, 442–537.

Yamasaki, H., Aoki, T., Oya, H., 1989. A cystein proteinase from the liver fluke *Fasciola* spp.: purification, characterization, localization and application to immunodiagnosis. Jpn. J. Parasitol. 38, 373–384.