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ORIGINAL PAPER

Characterization of cysteine proteases from the carcinogenic liver fluke, *Opisthorchis viverrini*

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Abstract Protease activities in extracts of *Opisthorchis viverrini* were investigated using gelatin zymography and fluorogenic peptide substrates. Using gelatin-impregnated X-ray film, 2 μg of *O. viverrini* excretory–secretory products (*Ov*-ES) and adult somatic extract (*Ov*-SE) showed proteolytic activity. Zymography of both *O. viverrini* extracts revealed bands at ~30 kDa. Using fluorogenic peptide substrates, the majority of *O. viverrini* activity was determined to be cathepsin L-like cysteine protease (cleaved Z–Phe–Arg–aminomethylcoumarin (AMC)) whereas little or no activity was ascribable to other classes of proteases. The *O. viverrini* cysteine protease activity was greatest at pH 6.0 and the activity was inhibited by the class-specific inhibitors, E-64 and Z–Ala–CHN_2. Chromatographic purification of *O. viverrini* cysteine proteases on thiol-sepharose enriched for protein(s) of ~30 kDa from *Ov*-ES and *Ov*-SE. The activity profile of the purified enzyme was similar to that of the cathepsin L-like activity characterized in *Ov*-SE and *Ov*-ES**.** Furthermore, determination of cysteine protease activity in several developmental stages of the parasite revealed the highest protease activity in metacercariae soluble extract, followed by *Ov*-ES, egg soluble extract, and *Ov*-SE. These findings demonstrated that *O. viverrini* has a cathepsin L-like cysteine protease(s) and suggested that abundant cysteine protease activity was present in metacercariae where the hydrolase might be involved in cyst excystation during mammalian infection.

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Introduction

Opisthorchiasis caused by *Opisthorchis viverrini* remains a major public health problem in many parts of Southeast Asia including Thailand, Lao People's Democratic Republic, Vietnam, and Cambodia (IARC 1994). The infection is associated with a number of hepatobiliary diseases, including cholangitis, obstructive jaundice, hepatomegaly, chole-

cystitis, and cholelithiasis (Harinasuta et al. 1984; Sripa 2003). Moreover, both experimental and epidemiological evidence strongly implicate liver fluke infection as the major risk factor in cholangiocarcinoma, cancer of the bile ducts (Thamavit et al. 1978; IARC 1994; Sripa et al. 2007).

Proteases (peptide hydrolases) of parasites, aside from known catabolic functions and protein processing, play diverse roles in parasites, including excystment–encystment, immunoevasion, digestion of host tissue, and activation of inflammation leading to pathology (Sajid and McKerrow 2002; Williamson et al. 2003; Donnelly et al. 2006; McKerrow et al. 2006; Knox 2007). Proteases have been reported in a number of liver flukes (e.g., Cordova et al. 1999; Smooker et al. 2000; Park et al. 2001; Dalton et al. 2003). Cysteine proteases have been purified from the oriental liver fluke of humans and close relative of *O. viverrini*, *Clonochis sinensis* (Park et al. 1995, 2001; Chung et al. 2000), and a 24-kDa cysteine protease from *C. sinensis* exhibits cytotoxic effects on Chinese hamster ovary cells (Park et al. 1995). In addition, cysteine proteases are promising antigens for immunodiagnosis (Kim et al. 2001) and vaccination against (Lee et al. 2006) clonorchiasis. However, to our knowledge, protease activity has not yet been reported from *O. viverrini*. In this study, we showed that the major catalytic activity detected in *O. viverrini* extracts and secreted proteins belongs to the cysteine protease class, specifically ~30-kDa cathepsin L-like enzymes.

Materials and methods

Parasite preparations

O. viverrini metacercariae were obtained from naturally infected cyprinoid fish captured from an endemic area of Khon Kaen province, Thailand. The fish were digested with pepsin–HCl at 37°C for 2 h. After several washes in normal saline and sedimentations, the metacercariae were collected and identified under a dissecting microscope. Moving viable metacercariae were used for infecting hamsters. Adult *O. viverrini* worms obtained from the livers and bile ducts of infected hamsters were washed several times in cold normal saline containing penicillin (200 U/ml) and streptomycin (200 μg/ml) to remove any debris and residual blood. After washing, viable worms were used for collection of excretory–secretory (*Ov*-ES) products (see below), and inactive worms were snap frozen in liquid nitrogen and stored at −80°C. For somatic extracts (*Ov*-SE), frozen worms were crushed and ground in liquid nitrogen. The ground powder was then solubilized in sterile phosphate-buffered saline (PBS), sonicated on ice, centrifuged at 10,000 rpm for 30 min at 4°C and the supernatant stored at −80°C. *Ov*-ES products were prepared by in vitro culture of viable flukes in Roswell Park Memorial Institute medium containing penicillin (100 U/ml) and streptomycin (100 kg/ml) and the spent medium was collected every 6 h for up to 5 days. Dead worms were periodically removed. The culture media were centrifuged and the supernatant and pellets were stored at−80°C for preparing *Ov*-ES and egg soluble extract (*Ov*-EG), respectively. For *Ov*-ES, the pooled supernatant was concentrated by membrane ultrafiltration (Amicon Ultra-15, Millipore, MA, USA), dialyzed in PBS several times, and then aliquoted at −80°C. For *Ov*-EG, the eggs were washed several times in normal saline to remove any residual *Ov*-ES products. After centrifugation, the egg pellets were crushed in liquid nitrogen as above and centrifuged and the supernatant was aliquoted at −80°C. Protein concentrations of all *O. viverrini* preparations were determined by the Bradford method (Bio-Rad, Hercules, CA, USA).

Gelatinolytic activity

The X-ray film method described by Cheung et al. (1991) was initially used to detect gelatinolytic activity. The technique relies on the presence of a gelatin emulsion which coats commercial X-ray film; the gelatin in this emulsion can serve as the substrate to assay for protease activity. Briefly, *Ov*-ES or *Ov*-SE (10 μg protein/10 μl) were twofold serially diluted in an equal volume of PBS (pH 6.0) and then 2 μl of the extracts were spotted onto unprocessed X-ray film (Kodak). The experiment was conducted in a moisture chamber at 37°C overnight. The X-ray film was developed in Kodak D-19 Developer (Sigma P-5670) and fixed in Kodak Fixer (Sigma, P-6557) before washing in tap water. The gelatinolytic activity was visualized as a zone of clearance, using a dissecting microscope. Papain (1 μg/ml, Sigma) and PBS served as the positive and negative controls, respectively.

Zymography (Gelatin–SDS–PAGE)

O. viverrini protein preps diluted 1:1 in non-denaturing sample buffer were applied to a sodium dodecyl sulfate (SDS)–polyacrylamide gel containing (PAGE) 0.1% porcine gelatin (Sigma) in the separating gel and electrophoresis was performed to resolve the proteins. To activate the proteases by regeneration of the enzyme in situ, the gel was incubated in 0.1 M sodium phosphate, pH 6.0, 0.1% Triton X-100 and 5 mM dithiothreitol for 1 h to remove SDS. Subsequently, the gel was stained with Coomasie Brilliant Blue and destained according to standard procedures. Activity of proteases was detected as bands of clearance of the gelatin against the blue background representing Coomasie-stained (undigested) gelatin.

Fluorometric assay for protease activity

Protease activity(ies) in *O. viverrini* metacercariae soluble extract (*Ov*-MC), *Ov*-SE, *Ov*-EG and *Ov*-ES were measured fluorometrically using peptide-7-amino-4-methylcoumarin substrates (Hill and Sakanari 1997). The following buffers were used for pH activity profiles: 0.1 M sodium acetate (pH 4.0–5.0), 0.1 M sodium phosphate (pH 5.5–7.0), 0.1 M Tris-HCl (pH 7.5–8.0), and 0.1 M glycine (pH 8.5–11.0). Class-specific substrates assessed included Z–Arg–AMC (cysteine protease, cathepsin H), Z–Arg–Arg–AMC (cysteine protease, cathepsin B), Z–Phe–Arg–AMC (cysteine protease, cathepsin L), *o*-aminobenzoyl–Ile–Glu–Phe–nPhe–Arg–Leu–NH_2 (aspartic protease), Z–Gly–Gly–Arg–AMC (serine protease). Protease inhibitors tested were Z–Ala–CHN_2 (cysteine proteases), E-64 (cysteine proteases), pepstatin (aspartic protease), and soy bean trypsin (serine protease). The substrates and inhibitors were purchased from Sigma. The assay conditions used were as follows: 50 μl of appropriate AMC substrate (10-μM final concentration) was incubated with 50 μl of *Ov*-ES, *Ov*-SE, *Ov*-EG, or *Ov*-MC (1-μg of final amount–well) for 60 min at 37°C in 96-well black clear-bottom plates (Corning-Costar, NY, USA) in appropriate buffer. Inhibition assays were carried out by incubating class-specific protease inhibitors with parasite extracts for 10 min prior to the addition of substrate. Cleavage of AMC was detected using a fluorescence plate reader (Fluorostar Optima, BMG) with excitation at 360 and emission at 460. The amount of AMC released was determined from a standard curve generated using free AMC (Sigma), and one unit (U) of enzyme activity was defined as the amount that catalyzed the release of 1 nM of AMC per minute per milligram of protein at 37°C.

Purification of *O. viverrini* cysteine protease

Proteases from *Ov*-SE and *Ov*-ES were purified by affinity chromatography using thiol-sepharose (Knox et al. 1999). Briefly, *Ov*-SE or *Ov*-ES were dialyzed into 0.1-M sodium phosphate buffer, pH 6.0. Thiol-sepharose 4B resin (Sigma) was equilibrated with the same buffer, then 2.0 mg of each protein extract in a final volume of 2 ml was loaded onto the thiol-sepharose column equipped with Bio-Rad Econo chromatographic system at a flow rate of 1 ml/min. After thoroughly washing with the same buffer, bound material was eluted from the column by washing with equilibration buffer containing 25 mM cysteine. The peak fractions were pooled and the cysteine removed by passage through a Sephadex-G25 column which was equilibrated in 10 mM Tris, 0·1% reduced Triton X-100, 0·1% sodium azide, pH 7·4. The protein peak fractions were pooled and then concentrated using Amicon Ultra-15 (Millipore, MA, USA) before protein determination by using the Bradford method. The thiol-sepharose-purified protein was analyzed by SDS–PAGE and assayed for protease activity as described above.

Results

Ov-ES and *Ov*-SE digested gelatin impregnated in X-ray film, visualized as a clear zone similar to that generated when the gelatin was digested with papain. The PBS negative control did not digest gelatin. Proteolytic activity was detected in *Ov*-ES at a titer as low as 1:64 (~0.0156 μg), whereas activity in *Ov*-SE was detected at a titer as low as 1:32 (~0.0313 μg; Fig. 1a). *O. viverrini* protease activity was then assessed by gelatin acrylamide gel electrophoresis (zymography) to determine the molecular mass of the proteases responsible for this activity. Ten micrograms of *Ov*-SE and *Ov*-ES showed detectable protease activity with clear zones on the gel corresponding to molecular mass of approximately 30 kDa for both extracts (Fig. 1b).

The mechanistic classes of enzymes responsible for this activity were further explored using specific fluorogenic peptide substrates. *Ov*-SE showed highest protease activity per milligram of protein when Z–Phe–Arg–AMC was used as a substrate. The protease activity of cysteine, serine, and aspartic protease was 59.83, 5.70 and 6.8 U/μg protein, respectively. Class-specific cysteine protease activity was shown to be mainly cathepsin L (59.83 U) but not cathepsin H (6.55 U) or cathepsin B (7.43 U; Table 1). The cathepsin L activity was inhibited with E-64 and Z–Ala–CHN_2 while weaker activity of the other classes of enzyme inhibited by their class-specific inhibitors (Table 2).

The greatest cysteine protease activity was found in metacercariae extract followed by excretory–secretory products, egg extract, and adult worm extract. The protease activities using Z–Phe–Arg–AMC of *Ov*-MC, *Ov*-ES, *Ov*-EG, and *Ov*-SE were 97.54, 81.17, 77.37 and 64.19 U, respectively. Cleavage of Z–Phe–Arg–AMC by *Ov*-SE was minimal at pH 4.0, was increased sharply at pH 5.0, was optimal at pH 6.0, and was dropped off at pH 6.5. Cleavage of the substrate was inhibited by 98% with E-64 and 86% with Z–Ala–CHN_2; pepstatin-A and soybean trypsin inhibitor did not inhibit cleavage (Table 2).

O. viverrini cysteine protease(s) was purified from *Ov*-SE and *Ov*-ES using thiol-sepharose, and purified eluate proteins are referred to as p*Ov*-CP-SE and p*Ov*-CP-ES, respectively. Both eluates migrated with approximate molecular mass of 30 kDa (Fig. 2). Both purified proteases digested gelatin X-ray film at a dilution of 1:256 (Fig. 3a), indicating that the specific activity had been enhanced compared with *Ov*-SE (1:32) and Ov-ES (1:64) by affinity purification. The pH profiles of cleavage of Z–Phe–Arg–

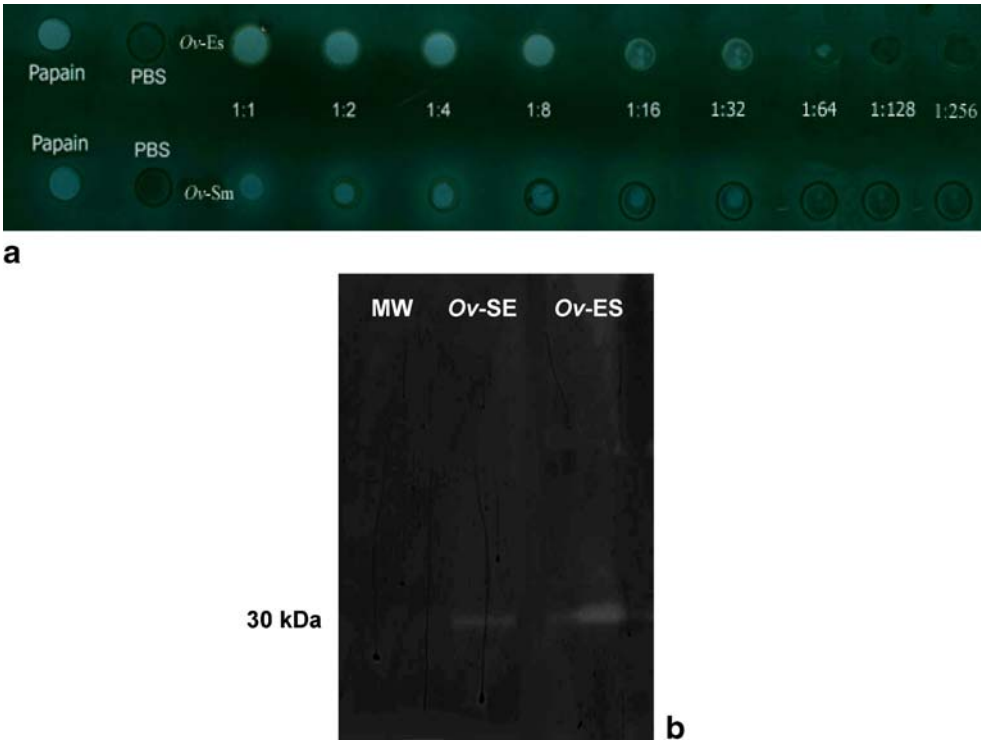

Fig. 1 Gelatinolytic activity of *Opisthorchis viverrini* protease (s) as detected using X-ray film emulsion (**a**) and in gelatin SDS–PAGE gels (zymography; **b**). Both the somatic extract (*Ov-SE*) and excretory–secretory products (*Ov-ES*) exhibited protease activity as revealed by clear zones of gelatin digestion up to a titer of 1:64 for a serially diluted *Ov*-ES (**a**). At pH 6.0 in the presence of the reducing agent DTT, gelatinolytic activity ascribable to a protease of ~30 kDa in size was readily apparent (**b**)

AMC by p*Ov*-CP-ES and p*Ov*-CP-SE was the same as that observed with the crude extract, *Ov*-SE (Fig. 3b), and the activity was inhibited by the cysteine protease inhibitors, E-64 and Z–Ala–CHN_2, but not by inhibitors for serine, aspartic proteases, and metalloproteases (Table 2). Details of the protease activities in different *Opisthorchis* protein extracts and thiol-purified products are shown in Table 3.

Discussion

Proteases of a number of parasites have been investigated to elucidate their roles in parasitism, pathogenesis, and pathology (Auriault et al. 1982; Tamashiro et al. 1987; McKerrow 1989; Sakanari et al. 1989; Carmona et al. 1993). Accordingly, cysteine proteases of parasites are known to play roles in diverse developmental processes including egg hatching and subsequent stage transitions, invasion, and migration through host tissues. They also participate in nutrient acquisition and immunological modulation (Chung et al. 1995; Michel et al. 1995; Ward et al. 1997; Kong et al. 1998; Mottram et al. 1998; Syfrig et al. 1998). These enzymes might also be developed as targets for vaccines (Jankovic et al. 1996; Piacenza et al. 1999) and chemotherapy (Coombs et al. 1997; Engel et al. 1998; Abdulla et al. 2007). Whereas numerous reports of

Table 1 Protease activities in *O. viverrini* somatic extract (*Ov*-SE) against the synthetic peptides Z–Arg–AMC (diagnostic for cathepsin H), Z–Arg–Arg–AMC (cathepsin B), Z–Phe–Arg–AMC (cathepsin L), AB–NH2 [*o*-aminobenzoyl–Ile–Glu–Phe–nPhe–Arg–Leu–NH2 (aspartic protease)] and Z–Gly–Gly–Arg–AMC (serine protease)

Substrates	Enzyme activity (units)
Z–Arg–AMC (cathepsin H)	6.55
Z–Arg–Arg–AMC (cathepsin B)	7.43
Z–Phe–Arg–AMC (cathepsin L)	59.83
AB–NH2 [*o*-aminobenzoyl–Ile–Glu–Phe–nPhe–Arg–Leu–NH2 (aspartic protease)]	6.80
Z–Gly–Gly–Arg–AMC (serine protease)	5.70

Activity ascribable to cathepsin L, cysteine protease(s) was the prominent protease activity detected in *Ov*-SE. One unit of enzyme activity catalyzes the release of 1 nM of AMC per minute per milligram of protein

Table 2 Inhibition of protease activities of *Ov*-SE, p*Ov*-CP-ES, and p*Ov*-CP-SE

Inhibitors	Percentage of inhibition (%)		
	Ov-SE	p*Ov*-CP-ES	p*Ov*-CP-SE
Control	1.13	1.09	1.77
Z–Ala–CHN_2 (1 mM)	85.73	93.12	91.93
E-64 (1 mM)	98.12	95.41	95.93
Pepstatin A (1 mM)	12.84	21.06	19.24
Soy bean trypsin (1 mM)	23.96	20.19	24.85

The protease activities against the cysteine protease cathepsin L fluorogenic substrate using various inhibitors. Conditions for protease activity assays are detailed in the "Materials and methods" section.

trematode proteases are available, none has focused on the proteolytic enzymes of the Oriental liver fluke, *O. viverrini*. In the present study, we have demonstrated and characterized protease activities in the developmental stages of *O. viverrini*. A major band of protease of ~30 kDa was enriched from both *Ov*-ES and *Ov*-SE. On activated thiol-sepharose 4B, class-specific substrate and inhibition profiles demonstrated this thiol-sepharose-enriched enzyme to be an *O. viverrini* cathepsin L. Cathepsin L is a papain-like cysteine protease, an endopeptidase belonging to clan CA of the cysteine proteases according to the MEROPS classification, ID C01.032 (http://merops.sanger.ac.uk/cgi-bin/make_frame_file?id=C01.032). The enzymological profile of the cathepsin L activity from *O. viverrini* is similar to cathepsin-L-like proteases reported from related fluke species. In particular, the *O. viverrini* cysteine protease displayed optimal catalytic activity at pH 6.0 similar to that of *Fasciola gigantica* protease which has optimal activity at pH 5.5 to 7.5 (Mohamed et al. 2005). The 27-kDa cysteine protease of *Paragonimus westermani* exhibited endodipeptidolytic activity at pH 5–8.5 and remained active and stable at neutral pH for 3 days (Yamakami and Hamajima 1987). *P. westermani* cysteine protease is inhibited by the cysteine protease inhibitor E-64 (Chung et al. 1995). Moreover, the proteases efficiently hydrolyzed collagen, fibronectin, and myosin at pH 8 (Chung et al. 1997).

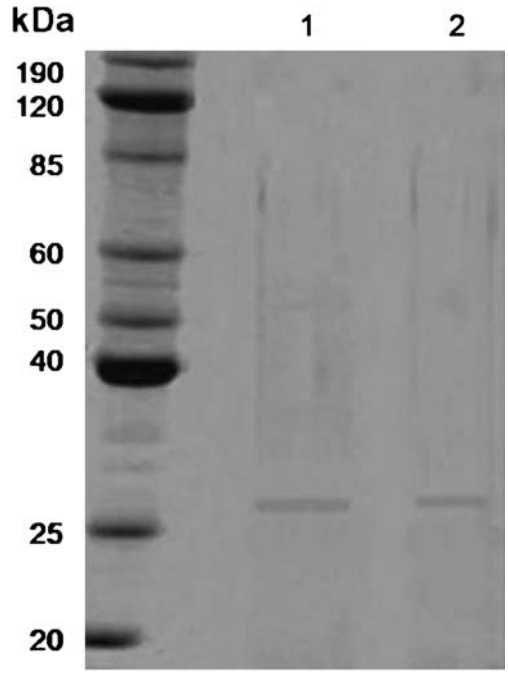

Fig. 2 SDS–PAGE–Coomassie-stained gel analysis of thiol-sepharose-4B-purified *O. viverrini* proteins from excretory–secretory product (p*Ov*-CP-ES; *lane 1*) and somatic extract (p*Ov*-CP-SE) (*lane 2*). Molecular masses in kilodaltons are shown at the *left*

Furthermore, the *O. viverrini* activity profile was in general similar to the protease activity profiles of other trematode parasites reported to date, including for *C. sinensis*, *P. westermani*, *Fasciola hepatica* and the human schistosomes (e.g., Song et al. 1990; Chung et al. 1995; Thorsell et al. 1965; Tort et al. 1999; Bogitsh et al. 2001; Delcroix et al. 2006). Minor activities ascribable to serine and aspartic proteases also were apparent in the *O. viverrini* extracts. Metacercariae of *O. viverrini* displayed highest activity against Z–Phe–Arg–AMC, followed by excretory–secretory product, egg, and adult worm, respectively. This is a similar developmental expression of protease activity to *C. sinensis* (Song and Rege 1991). Protease activities are also present in *P. westermani* excretory–secretory products of newly excysted metacercariae, and in a similar fashion to *O. viverrini*, mature adult *P. westermani* lung flukes show less specific activity of cysteine protease than do the metacercariae (Chung et al. 1995). In *P. westermani* metacercariae, much of the cysteine protease is localized in the excretory bladder, in excretory granules, and in newly excysted juvenile worms (Chung et al. 1995). Early release of the proteases from the excretory bladder of the encysted larva is thought to accelerate excystation of

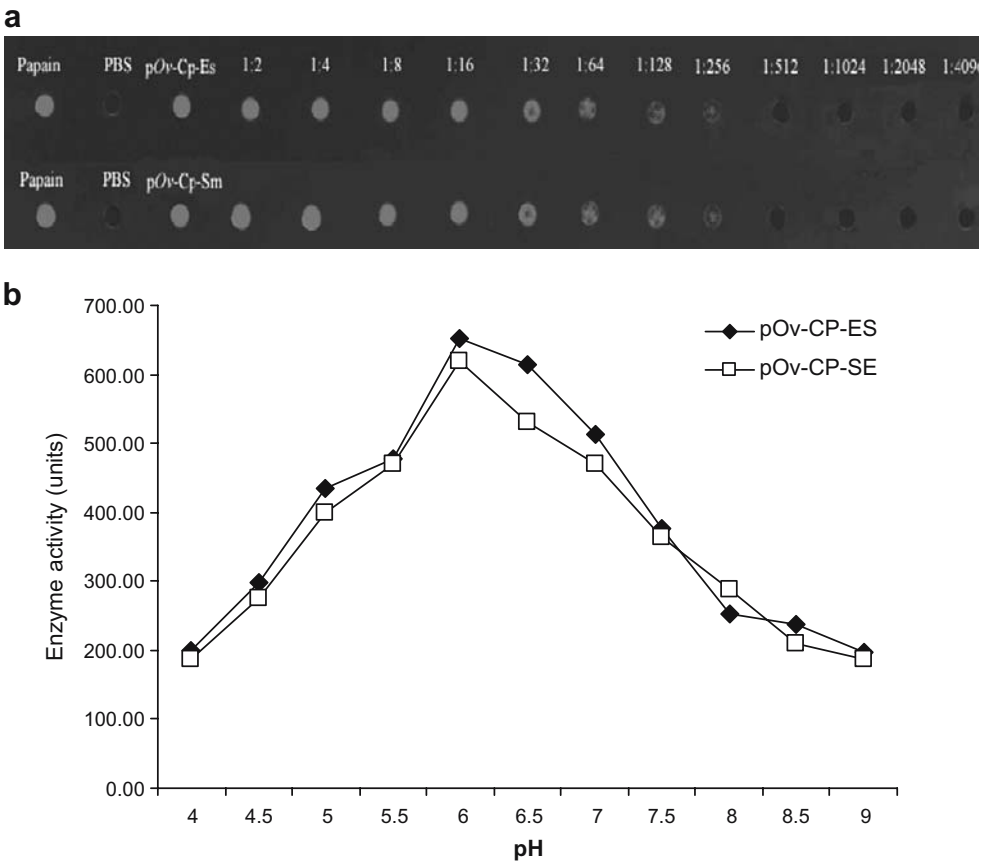

Fig. 3 Gelatinolytic activity of thiol-sepharose purified O. viverrini protease(s) from excretory–secretory product (pOv-CP-ES) and somatic extract (pOv-CP-SE): **a** activity detected on X-ray film substrate; b pH optima for enzyme activity of the two parasite extracts against Z–Phe–Arg–AMC. In **b**, enzyme activities of 1-μg *Opisthorchis* extract per reaction were determined in duplicate and mean values are presented. One unit of enzyme activity catalyzes the release of 1 nM of AMC per minute per milligram of protein

P. westermani metacercariae (Chung et al. 2005). A pioneering investigation of hydrolases in the cyst wall of metacercariae of the western liver fluke *F. hepatica* identified cysteine activity (Thorsell et al. 1965); these earlier findings along with the findings presented here implicate cathepsin L or other papain-like cysteine proteases in the escape of the immature adult fluke from the metacercarial cyst. Cathepsin-L-like cysteine protease activities have been well characterized in *Schistosoma mansoni* and *Schistosoma japonicum* where they participate in the parasite gut in digestion of ingested blood (e.g., Bogitsh et al. 2001; Delcroix et al. 2006). Schistosome cathepsin L activity has been characterized in the penetration glands of the parasite eggs and miracidia, where it likely functions in penetration of the host snail (Sung and Dresden 1986; Yoshino et al. 1993) and in the preacetabular glands of the cercariae (Dalton et al. 1997).

In conclusion, this report provides the first biochemical characterization of protease activities of *O. viverrini* and its developmental stages. More specifically, the present findings demonstrated that *O. viverrini* has a cathepsin-L-like cysteine protease(s), and its elevated developmental expression in metacercariae suggested that hydrolase might participate in larval excystation during mammalian infection. Given the remarkable link between infection with *O. viverrini* and induction of cholangiocarcinoma (see Parkin 2006; Sripa et al. 2007), an enhanced understanding of the enzymes and other proteins secreted by *O. viverrini* can be expected to enhance our understanding of the pathogenesis of liver-fluke-induced liver cancer.

Table 3 Purification by affinity chromatography of cysteine protease activity from extracts and secretions of *Opisthorchis viverrini*

Purification step[a]	Total protein (mg/ml)	Specificity activity (U)
Ov-SE (crude)	2	64.19
Ov-ES (crude)	2	81.17
Ov-SE (purified)	0.5	500.9
Ov-ES (purified)	0.4	642.7

[a] The purification conditions are described in the Materials and methods section.

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