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ORIGINAL PAPER

Molecular and immunological characterization of cathepsin L-like cysteine protease of *Paragonimus pseudoheterotremus*

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Abstract Cathepsin L is a cysteine protease belonging to the papain family. In parasitic trematodes, cathepsin L plays essential roles in parasite survival and host–parasite interactions. In this study, cathepsin L of the lung fluke *Paragonimus pseudoheterotremus* (PpsCatL) was identified and its molecular biological and immunological features characterized. A sequence analysis of *PpsCatL* showed that the gene encodes a 325-amino-acid protein that is most similar to *P. westermani* cathepsin L. The *in silico* three-dimensional structure suggests that PpsCatL is a pro-enzyme that becomes active when the propeptide is cleaved. A recombinant pro-PpsCatL lacking the signal peptide (rPpsCatL), with a molecular weight of 35 kDa, was expressed in *E. coli* and reacted with *P. pseudoheterotremus*-infected rat sera. The native protein was detected in crude worm antigens and excretory–secretory products and was localized in the cecum and in the lamellae along the intestinal tract of the adult parasite. Enzymatic activity of rPpsCatL showed that the protein could cleave the fluorogenic substrate Z-Phe-Arg-AMC after autocatalysis but was inhibited with E64. The immunodiagnostic potential of the recombinant protein was evaluated with an enzyme-linked immunosorbent assay (ELISA) and suggested that rPpsCatL can detect paragonimiasis with high sensitivity and specificity (100 and 95.6 %, respectively). This supports the further development of an rPpsCatL-ELISA as an immunodiagnostic tool.

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Keywords *Paragonimus pseudoheterotremus* · Cathepsin L · Cecum · Enzyme-linked immunosorbent assay · ELISA · Recombinant protein

Introduction

Lung flukes in the genus *Paragonimus* are the causative agents of paragonimiasis and are endemic in Asia, Africa, and America (Waikagul 2007). Only 7 of more than 40 species have so far been reported to infect humans: *Paragonimus africanus*, *P. kelikotti*, *P. mexicanus*, *P. westermani*, *P. uterobilateralis*, *P. heterotremus*, and *P. pseudoheterotremus* (Intapan et al. 2012). *P. pseudoheterotremus* was first identified in the freshwater crab *Larnaudia larnaudii* from Kanchanaburi Province, Thailand (Waikagul 2007). The first human case was reported in a Thai male admitted to hospital with tuberculosis-like symptoms (Intapan et al. 2012). Fundamental information, including essential proteins, is still lacking for this novel human-infecting species, and intensive research is required to clarify the factors associated its biology and pathogenesis. In this context, the proteolytic enzyme cathepsin L of *P. pseudoheterotremus* (PpsCatL) was identified and its molecular biological and immunological features characterized here.

Cathepsin L is a cysteine protease that plays important roles in the entire life cycles of several trematodes (Collins et al. 2004; Dalton et al. 1996; Day et al. 1995; Park et al. 2002). In *P. westermani*, a variety of cathepsin L isoforms have been reported, which affect the parasite's survival and virulence (Park et al. 2002). The cathepsin L proteins of *Fasciola hepatica* and *F. gigantica* are major secretory proteases that have contributed to the development of immunodiagnostic tools (Cornelissen et al. 2001; Varghese et al. 2012) and vaccines (Villa-Mancera et al. 2014; Sansri et al. 2015). An evolutionary

study of *Fasciola* cathepsin L suggested a divergence time for *F. hepatica* and *F. gigantica* and the substrate specificities of the proteins (Irving et al. 2003). Using of *F. hepatica* cathepsins L1 and L3 to design a novel nonpeptidic inhibitors demonstrated a potential drug development against fascioliasis (Ferraro et al. 2016). Based on this information, identifying and characterizing cathepsin L of *P. pseudoheterotremus* should extend our understanding of the organism and the development of strategies to prevent and control this parasitic helminth.

Here, we amplified the cathepsin-L-encoding gene of *P. pseudoheterotremus* from its total RNA using random amplification of complementary DNA (cDNA) ends (RACE)–PCR and then analyzed the molecular biological features of the protein, including its sequence properties and in silico three-dimensional (3D) structure. The transcription level of *PpsCatL* in different developmental stages was determined with real-time RT–PCR. An expression of PpsCatL protein in the parasite was detected using western blot analysis. A recombinant pro-PpsCatL (rPpsCatL) was expressed in *Escherichia coli* and used to evaluate the immune response to the protein. The specific location of PpsCatL in the parasite tissues was analyzed with immunolocalization. The enzymatic activity of rPpsCatL was determined with fluorogenic peptide substrate, Z-Phe-Arg-AMC. To evaluate the utility of rPpsCatL in the development of immunodiagnostic tools, we reacted the protein with *Paragonimus*-infected human sera and sera infected with other parasites.

Materials and methods

Ethics statement

All animal works were conducted with the approval of the Faculty of Tropical Medicine Animal Care and Use Committee (no. FTM-ACUC 011/2012). Leftover helminth-infected human sera and healthy sera were used in this study with the permission of the Immunodiagnosis for Helminthiasis Unit, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, and the approval of the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (no. MUTM 2011-056-01).

Parasite

Natural waterfall crabs (*Demanietta* sp.) collected from Tak Province, Thailand were examined for *P. pseudoheterotremus* infection with the tissue compression technique (Sugiyama et al. 2004). To collect the metacercariae, the bodies of the *P. pseudoheterotremus*-positive crabs were separated into small pieces, homogenized in 0.85 % normal saline solution (NSS), and allowed to settle in a sediment jar with several changes of NSS (Sugiyama et al. 2004). The metacercariae of *P. pseudoheterotremus* in the sediment were collected under a stereoscopic microscope (Olympus, Japan) and used to infect the definitive host. Five 8-week-old female Wistar rats purchased from the National Laboratory Animal Center, Mahidol University were orally infected with five metacercariae each and then housed at the Animal Care Unit, Faculty of Tropical Medicine, Mahidol University. Eight weeks postinfection (wpi), a fecal examination was performed with a simple direct smear test and egg-positive rats were euthanized to isolate the adult worms from their respiratory tracts. The fresh recovered worms were washed thoroughly with NSS and stored at −80 °C until further analysis. To prepare newly excysted juvenile worms (NEJ), the metacercariae were washed thoroughly with 1× phosphate-buffered saline (PBS) and then digested with 1 % pepsin-HCl solution for 2 h at 37 °C. After the digested metacercariae were washed several times with 1× PBS, they were incubated at 37 °C for 6 h with complete medium comprising RPMI-1640 (Gibco™ Thermo Fisher Scientific, Watham, MA) supplemented with 10 % fetal bovine serum (FBS; Biowest SAS, Nuaillé, France) and 1× penicillin–streptomycin solution (Biowest SAS). The emerging NEJ were collected and incubated in complete medium at 37 °C for 3 h. The metacercariae, NEJ, and adult *P. pseudoheterotremus* were stored at −80 °C until further analysis.

Amplification of full-length PpsCatL cDNA

Degenerate primers were designed for PpsCatL by comparing the amino acid sequences of cathepsin L orthologs composed of PwCatL1, PwCatL2, PwCatL6, and PwCP7 (Table 1) using the Clustal Omega program (Goujon et al. 2010). Two highly conserved amino acid motifs with the lowest degree of degeneration, GSCWAF and WGTRWG, were selected and converted to potential nucleic acid codons. The degenerate primers designed were forward (Fwd) 5′-GGIWSITGYTGGGCITTY-3′ and reverse (Rev) 5′-ICCCCAICGIGTICCCCA-3′, where I is inosine, S is C or G, W is T or A, and Y is T or C.

The total RNA from adult *P. pseudoheterotremus* was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The total RNA (5 μg) was treated with 1 U of DNase I (Thermo Fisher Scientific) to eliminate any genomic DNA and then converted to first-strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Part of the PpsCatL cDNA was amplified with PCR in a total volume of 50 μl containing 1× *Taq* DNA polymerase buffer, 2 mM $MgCl_2$, 0.2 mM each dNTP, 200 nM each degenerate primer, and 1 U of *Taq* DNA polymerase (Thermo Fisher Scientific). The cycling conditions were 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 45 °C for 30 s, and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. The partial cDNA sequence was subcloned into the pTZ57R/T plasmid (Thermo Fisher Scientific), and the DNA sequence was analyzed with DNA

Table 1 Accession numbers of the amino acid sequences used in this study

Protein name	Type	Accession no.	Species
PpsCatL	Cathepsin L	KX139301	*P. pseudoheterotremus*
PwCatL1	Cathepsin L	AAB93494	*P. westermani*
PwCatL2	Cathepsin L	AAK35220	*P. westermani*
PwCP1	Cathepsin L	AAF21461	*P. westermani*
PwCP3	Cathepsin L	AAY81942	*P. westermani*
PwCatL5	Cathepsin L	AAY81943	*P. westermani*
PwCatL6	Cathepsin L	AAY81944	*P. westermani*
PwCP7	Cathepsin L	AAY81945	*P. westermani*
PwCatL8	Cathepsin L	AAY81946	*P. westermani*
PwCP9	Cathepsin L	AAY81947	*P. westermani*
PwCP11	Cathepsin L	AAY81948	*P. westermani*
PwWES1	Cathepsin L	AAW28151	*P. westermani*
PwWES10	Cathepsin L	AAW28152	*P. westermani*
SmCatL1	Cathepsin L	AAC46485	*Schistosoma mansoni*
SjCatL1	Cathepsin L	CAX72171	*S. japonicum*
ShCatL1	Cathepsin L	XP_012793298	*S. haematobium*
FhCatL2	Cathepsin L	BAA23743	*Fasciola hepatica*
FgCatL2	Cathepsin L	AAF44677	*F. gigantica*
CsCatB1	Cathepsin B	GAA29748	*Clonorchis sinensis*
FhCatB3	Cathepsin B	ABU62925	*F. hepatica*
FgCatB3	Cathepsin B	AAO73004	*F. gigantica*
OvCatB2	Cathepsin B	ACT99885	*Opisthorchis viverrini*
SmCatB1	Cathepsin B	AAA29865	*S. mansoni*
SjCatB1	Cathepsin B	CAA50305	*S. japonicum*

sequencing (AITbiotech Pte Ltd, Singapore). The sequence was used to design the primers with the Primer3 program (Untergasser et al. 2012) for 5′- and 3′-RACE–PCR, performed with the SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc., Mountain View, CA), according to manufacturer's instructions. The 3′-RACE- and the 5′-RACE primers were 5′-GTTTGGAATCCGAAGCTGACTATCC-3′ and 5′-CGTGCTCCTCCTCATAAGCTCCTA-3′, respectively. The PCR products were subcloned into the pTZ57R/T plasmid and sequenced (AITbiotech Pte Ltd).

Bioinformatic analysis

The nucleotide and predicted amino acid sequences were submitted to the National Center for Biotechnology Information (NCBI) database with assigned accession numbers KX139301 and used for a bioinformatic analysis. The protein's properties, including its molecular weight and isoelectric point (pI), signal peptide, and transmembrane helices, were predicted with Pepstats (Rice et al. 2000), SignalP 4.1 Server (Petersen et al. 2011), and TMHMM (Krogh et al. 2001), respectively. Any N- and O-glycosylation sites and disulfide bridges were identified with NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/), NetOGlyc 4.0 (Steentoft et al. 2013), and the DiANNA 1.1 web server (Ferre and Clote 2006), respectively. The conserved protein motifs and catalytic triad of PpsCatL were analyzed with a multiple protein alignment constructed with Clustal Omega (Goujon et al. 2010). The evolutionary relationships of PpsCatL to other orthologs were determined with a phylogenetic tree based on a maximum likelihood analysis (ML) with 100 bootstrap replications in the MEGA version 5 program (Tamura et al. 2011). All the sequences used in this experiment are shown in Table 1. The 3D structure of PpsCatL was modeled *in silico* with Swiss-Model (Biasini et al. 2014), using the crystal structure of *F. hepatica* cathepsin L (PDB ID: 2o6x.1.A) as the template. The optimality of the predicted structure was tested with a Ramachandran plot analysis implemented at the RAMPAGE server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php).

Expression of rPpsCatL in *E. coli*

The first-strand cDNA of *P. pseudoheterotremus* was used as the template to amplify the *PpsCatL* sequence without the signal sequence in a reaction volume of 25 μl, containing 2 μl of cDNA, 1× *Taq* DNA polymerase buffer, 0.2 mM each dNTP, 2 mM $MgCl_2$, 1 U of *Taq* DNA polymerase, and 100 nM PpsCatL Fwd and Rev primers. The Fwd and Rev primer sequences used to amplify pro-PpsCatL were 5′-GGGGATCCGTGAGCACTGTTCGGGTGC-3′ and 5′-AAAAAGCTTTTAACGGATGACTGCAGACG-3′, respectively. The *Bam*HI restriction site in Fwd and the *Hind*III restriction site in Rev are underlined. The PCR was performed in a C1000™ thermal cycler (Bio-Rad Laboratories, Inc., Philadelphia, PA) with cycling parameters: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. The PCR product was cloned into pQE30 vector (Qiagen GmbH, Hilden, Germany) at *Bam*HI and *Hind*III sites and then transformed into *E. coli* strain M15 (Froger and Hall 2007). The expression of rPpsCatL was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Thermo Fisher Scientific) for 4 h followed by purification it under denaturing conditions, as described previously (Pakchotanon et al. 2016). The purified rPpsCatL was used to produce a mouse polyclonal antibody and other downstream experiments.

Immunological analysis

The crude worm antigen (CWA) and the excretory–secretory product (ES) of adult *P. pseudoheterotremus* were prepared as described previously (Adisakwattana et al. 2007). CWA, ES, and rPpsCatL were size fractionated with 12 % SDS-PAGE and then transferred onto a polyvinylidene difluoride

membrane (Pall Corporation, Washington, NY). The membranes were cut into small strips for an immunoblotting analysis, as described previously (Nuamtanong et al. 2012), with some modifications. Briefly, the membranes containing rPpsCatL, CWA, or ES were blocked with blocking solution (5 % skimmed milk in 1× PBS, 0.05 % Tween 20) and then incubated in blocking solution with *P. pseudoheterotremus*-infected rat serum (diluted 1:200), collected at 0 and 8 wpi, for rPpsCatL or with anti-rPpsCatL mouse serum (diluted 1:4000) for CWA and ES. After the membranes were washed, horseradish peroxidase (HRP)-conjugated goat anti-rat immunoglobulin G (IgG; diluted 1:1000; KPL Inc., Gaithersburg, MD) or HRP-conjugated goat anti mouse IgG (diluted 1:1000; Southern Biotech, Birmingham, AL) was added, and the membrane was incubated at room temperature for 1 h. The results were visualized by incubating the membranes with the colorimetric substrate, 2,6-dichloroindophenol (Sigma-Aldrich, St. Louis, MO).

PpsCatL transcripts detected in different developmental stages with SYBR Green real-time RT–PCR

Total RNAs from metacercariae, NEJ, and adult *P. pseudoheterotremus* were isolated with TRIzol Reagent, according to the manufacturer's instructions (Invitrogen). The total RNA (1 μg) was treated with DNase I (Thermo Fisher Scientific) to eliminate any contaminating DNA. The DNA-free total RNA from each stage was used as the template to synthesize first-strand cDNA, as described above, and then to determine the level of *PpsCatL* transcription using SYBR® Green real-time PCR. The 20 μl reaction mixture contained 2 μl of first-strand cDNA, 1× iTaq Universal SYBR® Green (Bio-Rad Laboratories, Inc.), and 300 nM each of the Fwd and Rev primers. The specific primers for the PpsCatL qPCR were Fwd 5′-CTGTTCGGGTGCCAGATAAT-3′ and Rev 5′-CTCGTTCGTGCATCTGGTAG-3′. 18S ribosomal RNA (rRNA) was used as the internal control against which to normalize gene expression. The specific primers for *P. pseudoheterotremus* 18S rRNA were Fwd 5′-GATA ACGGGTAACGGGGAAT-3′ and Rev 5′-AGCCTCTGTTG AGTCCCGTA-3′. Amplification was performed with the LightCycler® 480 II Real-Time PCR System (Roche Applied Science, Mannheim, Germany), with preincubation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 20 s and 60 °C for 1 min. A melting curve analysis was performed at 65–95 °C. The level of gene expression was calculated with the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen 2001). The experiments were performed with three replicates.

Immunolocalization of PpsCatL in parasite tissues

Paraffin-embedded sections of adult *P. pseudoheterotremus* were prepared and immunolocalization was performed as described previously (Adisakwattana et al. 2007), with some modifications. In brief, 5-μm-thick paraffin-embedded sections were dewaxed, the antigenic epitopes were retrieved, and the endogenous peroxidase was neutralized. Nonspecific binding sites were blocked with blocking solution (10 % [*w*/*v*] bovine serum albumin in PBS, pH 7.4) at room temperature for 20 min, and the sections were then incubated with anti-rPpsCatL mouse serum (1:200) or preimmune serum (1:200) in blocking solution. The sections were then incubated with HRP-conjugated goat anti-mouse IgG (1:1000; SouthernBiotech, Birmingham, AL), and the color signal was developed with an aminoethyl carbazole (AEC) staining kit (Sigma-Aldrich), according to the manufacturer's instructions. The results were observed under a light microscope.

Activity assay of rPpsCatL

rPpsCatL was refolded and autocatalyzed as described previously with some modification (Hwang and Chung 2002; Kodera et al. 2005). Briefly, rPpsCatL was diluted in refolding buffer containing 5 mM EDTA, 10 mM GSH, 1 mM GSSG, 0.7 M L-arginine at 4 °C for 16 h. After dialysis and concentration, refolded rPpsCatL was mixed with 50 mM sodium acetate buffer (pH 4.0) containing 200 mM NaCl, 10 mM 2-mercaptonethanol, and then incubated at 37 °C for 16 h. The activated rPpsCatL was concentrated and subsequently used for activity assay. Enzymatic activity of rPpsCatL was performed by hydrolysis of fluorogenic peptide substrate, Z-Phe-Arg-AMC (Calbiochem® EMD Millipore Corporation, Temecula, CA) as mentioned previously (Kodera et al. 2005). The enzymatic activity was detected by a spectrofluorometer and expressed as increased fluorescence units per minute during incubation. Cysteine protease inhibitor, E64, was used as a control experiment. The assay was performed in triplicate.

Evaluation of the immunodiagnostic potential of rPpsCatL

A total of 255 serum samples from 29 diseased individuals were classified into three groups: group 1 contained sera from 28 patients with paragonimiasis, group 2 contained 197 serum samples from helminth- and protozoa-infected patients, and group 3 contained sera from 30 healthy individuals. The details are given in Table 2.

A 100-μl sample of rPpsCatL (2.5 μg/ml) or CWA (5 μg/ml) in 0.05 M carbonate buffer (pH 9.6) was coated onto each well of a 96-well microtiter plate (Nunc; Thermo Scientific, Denmark) and incubated at 37 °C for 1 h and then at 4 °C for 16–18 h. An enzyme-linked immunosorbent assay (ELISA) was performed as previously described, with some modifications. In brief, nonspecific binding was blocked with

Table 2 Parasite-infected human sera used in this study

Disease	Abbrev.	No. cases	Diagnosis				rPpsCatL-ELISA (no.)
			Stool	Immuno	Blood	Sputum	
Trematode infections							
Paragonimiasis	PRG	28	+	+		+	28
Echinostomiasis	ECS	1	+				0
Fascioliasis	FCL	3		+			1
Minute intestinal fluke	MIF	13	+				0
Opisthorchiasis	OPT	14	+	+			3
Nematode infections							
Ascariasis	ACR	10	+				2
Angiostrogyliasis	AGS	10		+			0
Malayan filariasis	MFL	10		+	+		2
Capillariasis	CPL	2	+				0
Dirofilariasis	DRF	1		+			0
Enterobiasis	ETR	1	+				0
Filariasis	FLR	3		+			0
Gnathostomiasis	GNT	10		+			0
Hookworm infection	HWI	10	+				0
Strongyloidiasis	STG	11	+	+			1
Toxocariasis	TXC	7		+			1
Trichinellosis	TCN	8		+			0
Trichostrongyliasis	TCS	16	+				0
Trichuriasis	TCR	10	+				0
Bancroftian filariasis	BFL	7		+	+		0
Cestode infections							0
Echinococcosis	ECC	3		+			
Hymenolepiasis nana	HMN	6	+				0
Neurocysticercosis	NCT	10		+			0
Sparganosis	SPG	4		+			0
Taeniasis	TAN	12	+				0
Taeniasis solium	TNS	4	+				0
Protozoan infections							0
Blastocystosis	BTC	4	+				
Amebiasis	AMB	5	+				0
Giardiasis	GAD	2	+				0
Healthy	HTH	30	+				0
Total		255					38

blocking solution (0.5 % skim milk in 1× PBS) at 37 °C for 1 h, and the sections were then washed with PBS–Tween. Human sera (1:400) were added to the wells and incubated at 37 °C for 1 h. After the wells were washed, HRP-conjugated goat anti-human IgG antibody (1:2000) was added. The samples were incubated at 37 °C for 1 h and then visualized with the addition of ABTS substrate (Sigma-Aldrich). The reaction was developed in the dark at room temperature for 30 min and then stopped with 1 % SDS. The optical density at a wavelength at 405 nm was measured with the Sunrise™ absorbance reader (Tecan Group Ltd, Männedorf, Switzerland).

Results

Sequence analysis and 3D structure prediction

The full-length PpsCatL cDNA was amplified with RACE–PCR, and the nucleotide composition was analyzed with DNA sequencing. It contained 978 bp and encoded an open reading frame encoding 325 amino acid residues (Fig. 1). A homology comparison of the deduced amino acid sequence with the available database using the protein BLAST program demonstrated its highest homology to *P. westermani* cathepsin L6

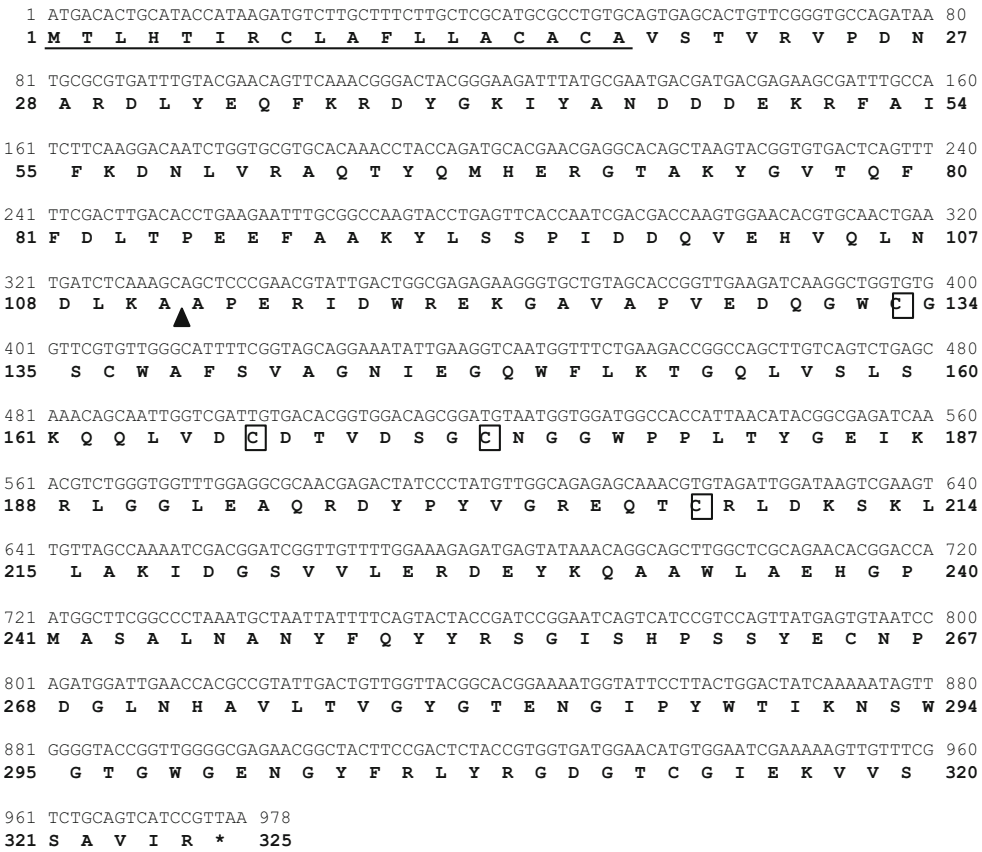

Fig. 1 Nucleotide sequence of full-length *PpsCatL* (accession no. KX139301) consists of 978 nucleotides that encode 325 amino acid residues. The signal peptide identified at amino acid residues 1–18 is *underlined*. The cleavage site between the propeptide and mature protein is indicated with an *arrowhead*. Potential disulfide bonds are *boxed*

(accession no. AAY81944.1), with 79.69 % identity. The homology shared by PpsCatL and other cysteine protease orthologues in the genus *Paragonimus* was determined and is shown with a heatmap (Fig. 2). The full-length PpsCatL cDNA and deduced amino acid sequence were submitted to the NCBI database under accession number KX139301. The protein properties were predicted with the Pepstat program, which showed that the molecular mass of the full-length PpsCatL protein is approximately 36.5 kDa, with a pI of 5.4. The potential signal peptide was predicted at amino acid positions 1–18 at the N-terminus (MTLHTIRCLAFLLACACA), but no transmembrane helix was detected. No posttranslational modification sites, including for N- or O-glycosylation, were observed in PpsCatL. Ten cysteine residues occur in the PpsCatL amino acid sequence at positions 8, 15, 17, 133, 136, 167, 174, 207, 265, and 313, with two potential disulfide bridges at Cys_{133}–Cys_{174} and Cys_{167}–Cys_{207}.

A multiple sequence alignment of PpsCatL and its orthologues suggested a putative propeptide cleavage site between Ala_{111} and Ala_{112}. The mature protease is composed of 214 amino acid residues, with a calculated molecular mass of approximately 23.7 kDa and a theoretical pI of 5.3. The conserved ERFNIN and GNFD motifs, signatures of cathepsin L, were observed in the propeptide region of PpsCatL, at amino acid positions of 48–63 and 76–82, respectively. A conserved structural motif, GCNGG, is presented in PpsCatL at positions 173–177. The catalytic triad is located at Cys_{136}, His_{272}, and Asn_{292}, and an S_1 subsite of the protease active site was identified at Gln_{130}, Cys_{136}, His_{272}, Asn_{292}, and Trp_{294}. The S_2 subsite of the active site contains Leu_{192}, Glu_{193}, Ala_{244}, and Leu_{270} (Fig. 3).

The evolutionary relationships between full-length enzymes of PpsCatL and its orthologues were analyzed with the ML method and 100 bootstrap replications. PpsCatL clustered on the same branch as *P. westermani* cathepsin L and was most closely related to PwCatL6. The *Paragonimus* cathepsins L were more closely related to *Schistosoma* cathepsins L than to *Fasciola* cathepsins L. The trematode cathepsins B were distinctly separated from the cathepsins L on the phylogenetic tree (Fig. 4).

The 3D structures of the pro- and mature PpsCatL were predicted using the crystal structure of *F. hepatica* cathepsin L (PDB ID: 2o6x.1.A) as the template. The pro-PpsCatL 3D structure showed that the propeptide region folds onto the cathepsin L surface to protect the active site from substrates (Fig. 5a). After the propeptide (amino acid residues 19–111) is removed, PpsCatL becomes a mature protease (amino acid residues 112–325), with its active site exposed to the substrate (Fig. 5b). The structure of mature PpsCatL consists of two

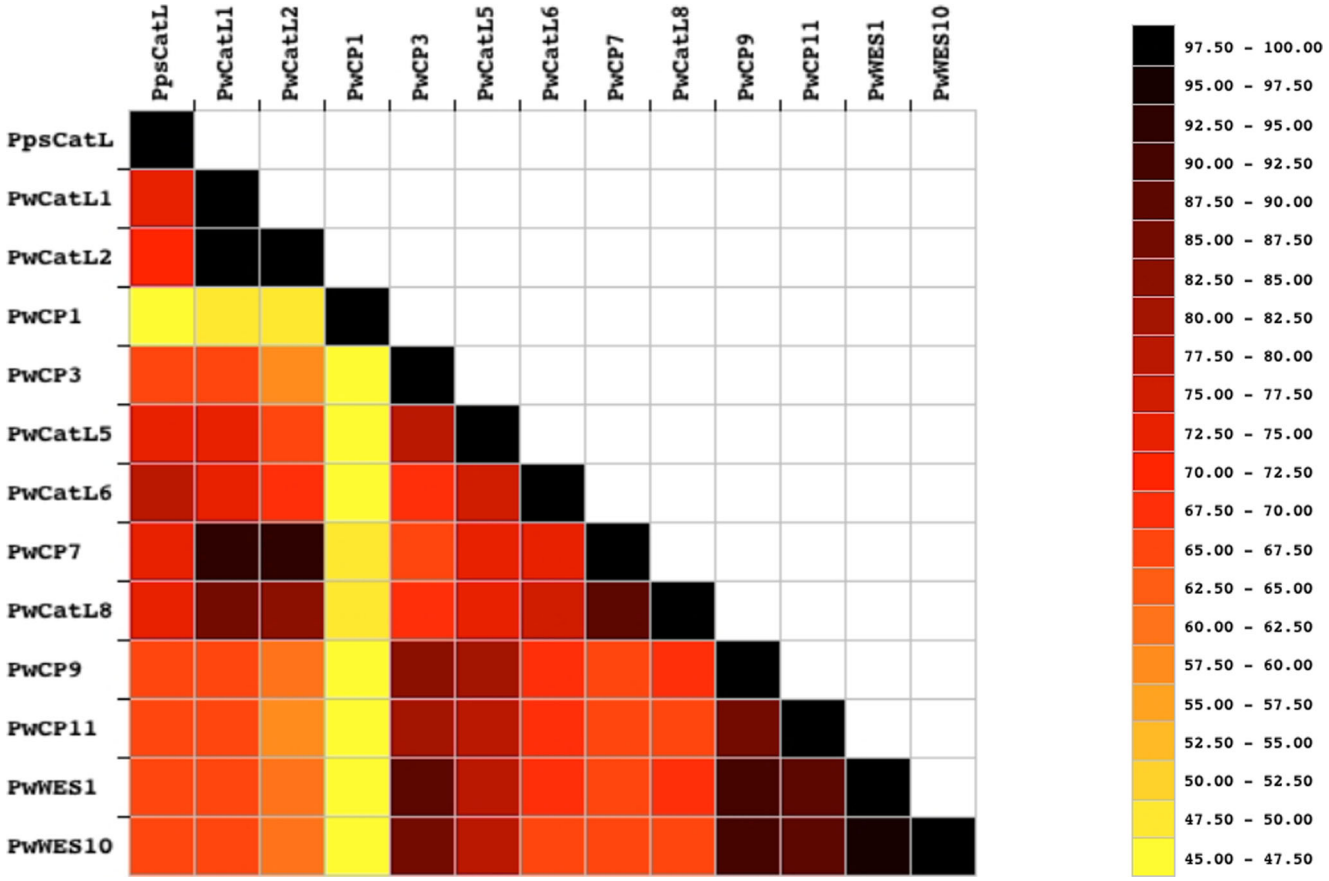

Fig. 2 Homology heatmap of cathepsin L found in *Paragonimus* species. Values of percentage identity are indicated with the *different box colors* (*right*)

Fig. 3 Multiple alignment of deduced amino acid sequences identified the conserved motifs within orthologous cathepsin L proteins. Identical and similar residues are indicated with *black and gray shadings*, respectively. Gaps are indicated with a *dash*. Putative amino acid residues of the catalytic triad (Cys [*C*], His [*H*], Asn [*N*]) are indicated with *asterisks* above the residues. *ERFNIN*, *GNFD*, and *GCNGG* motifs are labeled above the alignment with *number signs*, *pilcrow signs*, and *plus signs*, respectively. S_1 subsites are indicated with a *black arrow* below the alignment, and S_2 subsites are indicated with a *white arrow* below the alignment

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                                                                                                  #    #    ##   #
PpsCatL MTLHTIRCLAFLLACACAVSTVRVPDNARDLYEQFKRDYGKIYANDDDEKRFA-IFKDNL
PwCP6   MRLYTVSCLTFLVGCVFAVSTVQVPDSARELYEQFKRDYGKSYANDDDEKRFA-IFKDNL
SmCatL1 ------------MPVNLEYLGFKLPGNVDEKYVQFKLKYRKQYHETEDEIRFN-IFKSNI
SjCatL1 -----MKVLLLFSIITAITVAQHYDKQYDEIWRQWKLKYNKTYTSNDDEMRRKMIFMRRI
ShCatL1 ------------MPVNLEYLGFKLPANVEEKYAQFKLTYRKQYHETEDEIRFN-IFKSNI
                 #    #          ¶  ¶  ¶  ¶
PpsCatL VRAQTYQMH---ERGTAKYGVTQFFDLTPEEFAAKYLSSPIDDQV-----EHVQLNDLKA
PwCP6   VRAQNYQLQ---EQGTARYGVTQFSDLTPEEFAAKFLSSRFDDQV-----ERVQLNDLKA
SmCatL1 LKAQLYQVF---VRGSAIYGVTPYSDLTTDEFARTHLTASWVVPSSRSNTPTSLGKEVNN
SjCatL1 GKIQEHNLRHDLGLEGYTMGLNQFCDMEWEEVNRIMFPRVFGNSPLWNDDGNELELTNKP
ShCatL1 LKAQLYQVF---ERGSAIYGVTPYSDLTTDEFARTHLTASWVVSSSTNNTPISLEKEVKN
                                  *
PpsCatL APERIDWREKGAVAPVEDQGWCGSCWAFSVAGNIEGQWFLKTGQLVSLSKQQLVDCDTV-
PwCP6   APESVDWRELGAVAPVEDQGSCGSCWAFSVAGNVEGQWFLKTGQLVSLSKQQLVDCDVQ-
SmCatL1 IPKNFDWREKGAVTEVKNQGMCGSCWAFSTTGNVESQWFRKTGKLLSLSEQQLVDCDGL-
SjCatL1 VPSKWDWRDHGAVTAVKNQGMCGSCWAFSATGAIEGQLRRKHKKLISLSEQQLVDCSTPY
ShCatL1 IPKNFDWREKGAVTEVKNQGMCGSCWAFSTTGNVESQWFRKTGKLLSLSEQQLVDCDGL-
          +++++                 ^         ^
PpsCatL -DSGCNGGWPPLTYGEIKRLGGLEAQRDYPYVGREQTCRLDKSKLLAKIDGSVVLE-RDE
PwCP6   -DSGCDGGYPPTTYGEIIRMGGLEAQRDYPYVGREQPCKLDESKLLAKINSSIVLE-ANE
SmCatL1 -DDGCNGGLPSNAYESIIKMGGLMLEDNYPYDAKNEKCHLKTDGVAVYINSSVNLT-QDE
SjCatL1 GNYGCGGGYMDHAFNYL-ESHYIESENDYKYLGYDANCHYRKSKGVVKVKKFVDLPSKDE
ShCatL1 -DDGCNGGLPSNAYESIIKMGGLMLEDNYPYDAKNEKCHLKADNVAAYINSSVNLT-QDE
                                                          *
PpsCatL YKQAAWLAEHGPMASALNANY-FQYYRSGISHPSSYECNPDGLNHAVLTVGYGTE-NGIP
PwCP6   KKQAAYIAEHGPMSSGINAVT-LQFYQSGISHPSKSQCQPDWLNHGVLSVGYGTE-DGVP
SmCatL1 TELAAWLYHNSTISVGMNALL-LQFYQHGISHPWWIFCSKYLLDHAVLLVGYGVSEKNEP
SjCatL1 KTLQKAVYQYGPISVGIVALDSLIMYKSGVFE--SNDCKYADINHGVLVVGYGKE-HGKD
ShCatL1 TELAAWLYHNSAISVGMNAML-LQFYRHGISHPWWIFCSKYLLDHAVLLVGYGVSEENEP
          *                 ^         ^                       ^  ^
PpsCatL YWTIKNSWGTGWGENGYFRLYRGD-GTCGIEKVVSSAVIR
PwCP6   YWIIKNSWGTGWGEKGYFRLYRGD-GTCGIEKVVSSAIIR
SmCatL1 FWIVKNSWGVEWGENGYFRMYRGD-GSCGINTVATSAMIY
SjCatL1 YWLIKNSWGDLWGSKGYFKLRRNKHNMCGVASNASFPLLL
ShCatL1 FWIVKNSWGVEWGEKGYFRVYRGD-GTCGINTVATSALIY
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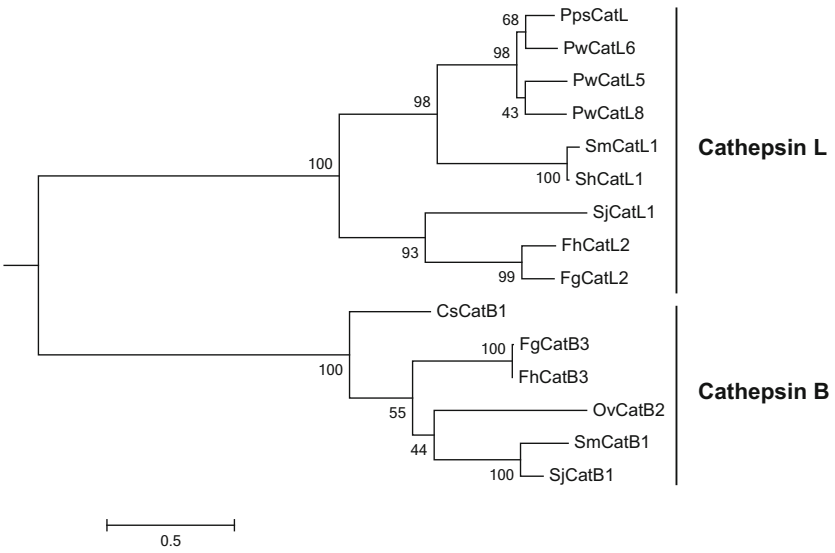


Fig. 4 Phylogenetic tree showing that *PpsCatL* is closely related to *P. westermani* cathepsin L

domains (left and right). The left domain contains three α-helices and the right domain contains a β-sheet, with a front helix that forms a coiled structure. An interaction at the top of the two domains creates the active-site cleft (Fig. 5b). Ramachandran plots suggested that this structural model is optimal (data not shown).

Expression and purification of rPpsCatL

After protein expression was induced with IPTG, rPpsCatL was predominantly expressed with an approximately molecular mass of 35 kDa (Fig. 6a). Its water solubility was tested and showed that rPpsCatL was expressed in an insoluble form in *E. coli*, which was detected with an anti-His-tag antibody (Fig. 6a). rPpsCatL was purified under denaturing condition, and a major band of 35 kDa was eluted (Fig. 6b). The purified rPpsCatL reacted with the anti-His-tag antibody (data not shown). Eluted fractions 1 to 10 (E1–E10) were pooled and then stepwise dialyzed to remove the excess urea and then used for production of mouse polyclonal antibody and further studies.

Detection of PpsCatL in native parasite antigens and the immune response to rPpsCatL

The presence of PpsCatL in the CWA and ES of *P. pseudoheterotremus* was detected on western blots probed with anti-rPpsCatL mouse serum. In the CWA, the anti-rPpsCatL serum detected the native protein at molecular weights of approximately 35, 28, and 25 kDa. In the ES, a protein band with a molecular weight of 25 kDa predominantly reacted with the anti-rPpsCatL serum, and also in the range of 10–18 kDa, but most strongly at 14 kDa (Fig. 6c).

The immune response of *P. pseudoheterotremus*-infected rats to rPpsCatL was observed with a western blotting analysis. All five infected rat sera, collected at 8 wpi, reacted with

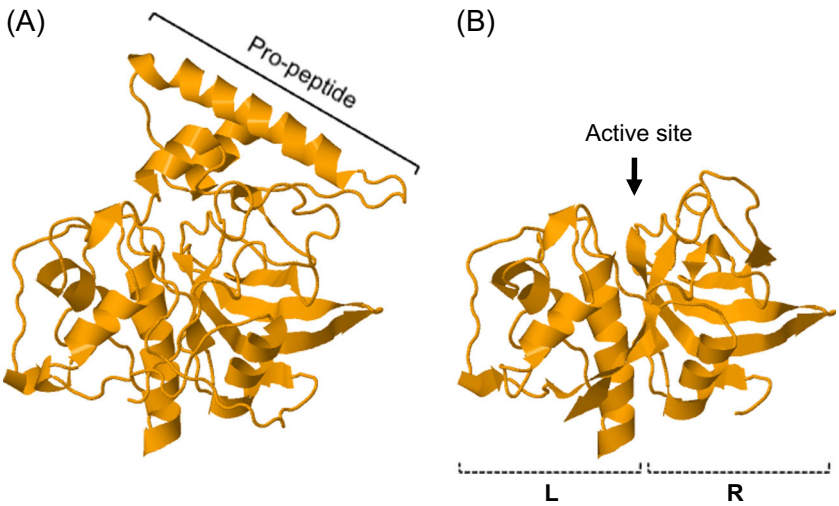


Fig. 5 *In silico* 3D structure of pro-PpsCatL (**a**) and mature-PpsCatL (**b**). The propeptide blocks the active site of the protease to prevent substrate exposure. Left (*L*) and right (*R*) domains contain three α-helices and a β-sheet, respectively

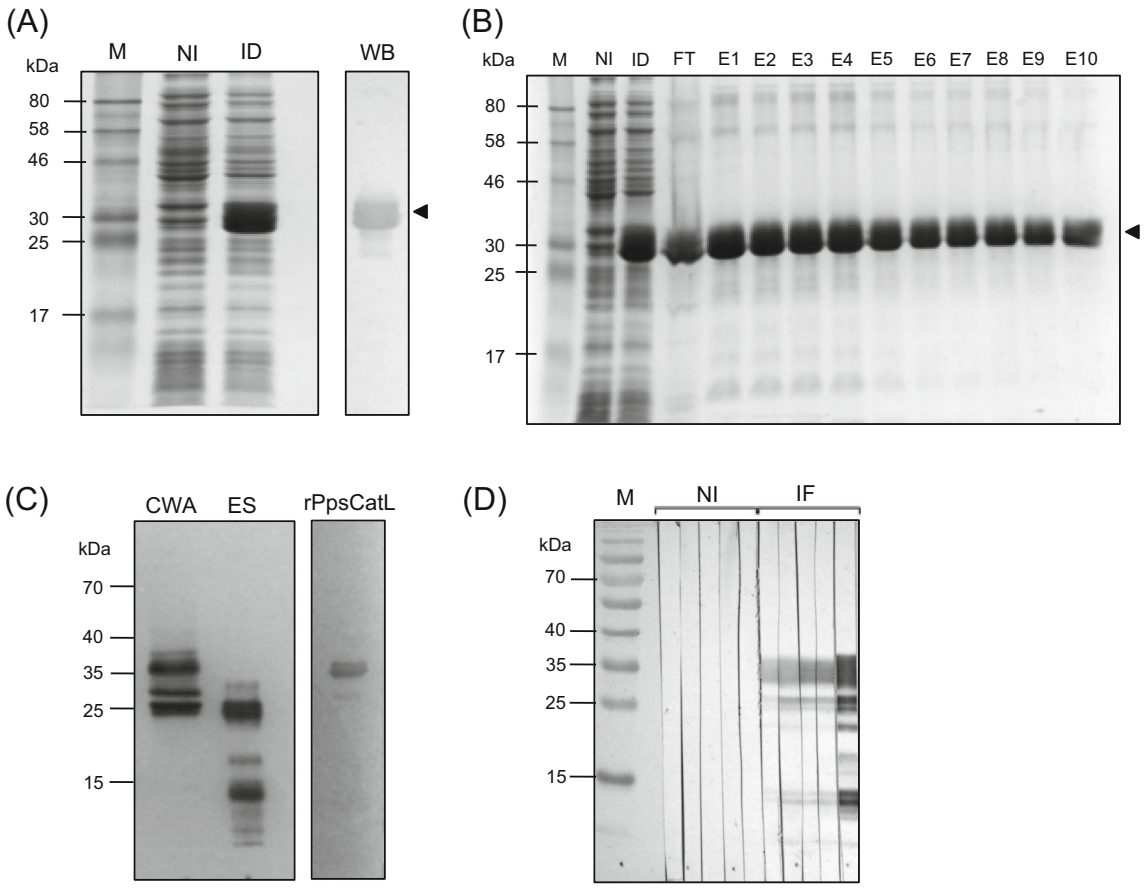


Fig. 6 rPpsCatL was heterologously expressed in *E. coli* (**a**) and then purified with a Co^{2+} affinity column (**b**). *M* broad-range prestained protein marker, *NI* noninducing, *ID* inducing, *WB* western blot analysis with anti-His-tag antibody, *FT* flow-through, *E* elution. **c** Western blotting analysis of native PpsCatL in CWA and ES using anti-rPpsCatL mouse serum. *CWA* crude worm antigen, *ES* excretory–secretory product, *rPpsCatL* recombinant *P. pseudoheterotremus* cathepsin L. **d** Detection of rPpsCatL with *P. pseudoheterotremus*-infected rat sera. *M* broad-range prestained protein marker, *NI* noninfected sera, *IF* infected sera

35 kDa rPpsCatL and other truncated proteins (Fig. 6d). Normal rat and mouse sera were used as the negative controls.

PpsCatL transcripts in different developmental stages of *P. pseudoheterotremus*

The transcription of *PpsCatL* in different developmental stages of *P. pseudoheterotremus* (metacercaria, NEJ, and adult) was determined to define its stage-specific expression. *PpsCatL* was transcribed most strongly in the adult parasite, approximately sixfold more strongly than in the metacercariae or NEJ. The expression of *PpsCatL* mRNA in the metacercariae and NEJ did not differ significantly (Fig. 7).

Tissue-specific localization of PpsCatL in adult *P. pseudoheterotremus*

PpsCatL expression was immunolocalized in the adult stage based on the transcription of the gene. *PpsCatL* was specifically expressed in the digestive system, including the

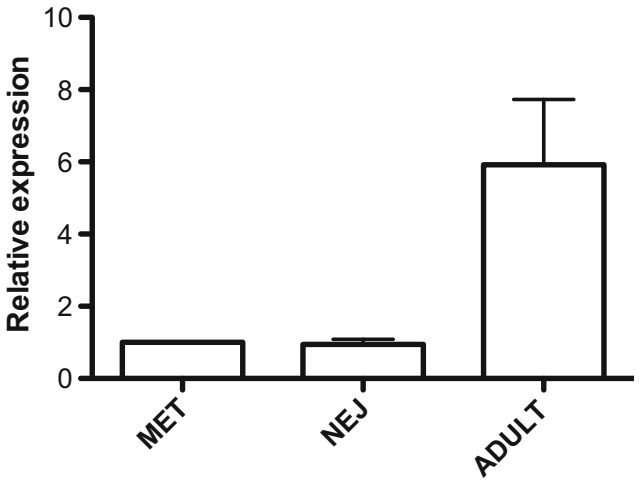


Fig. 7 Stage-specific transcription of *PpsCatL* was monitored in different developmental stages of *P. pseudoheterotremus* using SYBR Green real-time RT–PCR. *MET* metacercaria, *NEJ* newly excysted juvenile, *Adult* adult worm. Relative expression levels were calculated by comparison with the transcription levels in the metacercariae

esophagus and intestine, but not in other tissues. PpsCatL accumulated strongly at the lamellae lining the esophagus and along the intestine throughout the whole parasite body (Fig. 8a, b). Immunolocalization with preimmunized mouse serum gave negative results (Fig. 8c).

Enzymatic activity of rPpsCatL

rPpsCatL was refolded and activated by autocatalytic processes prior to determination of proteolytic activity. Hydrolysis of fluorogenic peptide substrate Z-Phe-Arg-AMC was measured, and the result demonstrated that activated rPpsCatL could cleave Z-Phe-Arg-AMC, while inactive pro-enzyme did not show proteolytic activity. The enzymatic activity of activated rPpsCatL was eliminated when incubated with cysteine protease inhibitor, E64 (Fig. 9).

ELISA

The dilutions of the human sera and the secondary antibody were optimized with checkerboard titration (data not shown). The optimal dilution of human serum and the secondary antibody were 1:400 and 1:2000, respectively. In this study, ELISAs using rPpsCatL or the CWA as the antigen were performed to compare their sensitivities and specificities. The cut-off points for CWA- and rPpsCatL-ELISAs were 0.232 (mean + 5SD) and 0.412 (mean + 6SD), respectively. The frequency distributions of the negative, positive, and samples were plotted to indicate the number of true positive results, true negative results, false positive results, and false negative results (Fig. 10a left, b left). The sensitivity and specificity of CWA were 100 and 84.1 %, respectively, and those of rPpsCatL were 100 and 95.6 %, respectively (Fig. 10a right, b right).

Discussion

In parasitic trematodes, cathepsin L is an essential cysteine protease that is involved in a variety of activities throughout their life cycles, including encystation, excystation, migration, digestion, maturation, fertilization, and immune evasion (Grams et al. 2001; Shin et al. 2001; Collins et al. 2004; Na et al. 2006; Chung et al. 2008). Several cathepsin L isoforms were previously identified in a medically important lung fluke, *P. westermani*, but not in other *Paragonimus* species (Park et al. 2002). Here, cathepsin L of the agent of zoonotic paragonimiasis, *P. pseudoheterotremus*, was identified and characterized with bioinformatic and molecular biology approaches. The amplification of *PpsCatL* using degenerate-primer-based RT–PCR combined with RACE–PCR isolated the full-length gene, which showed high homology to *P. westermani* cathepsins L, especially PwCatL6 and PwCatL5. PwCatL5 is a well-characterized cysteine protease found in the adult *P. westermani* worm and is classified in the cathepsin L family (Park et al. 2002). An amino acid analysis of PpsCatL demonstrated that the protein has a molecular weight of 36 kDa and contains a signal peptide but no transmembrane helix, indicating its excretory–secretory potential. These data suggest an extracellular role for this protein, involving digestion, migration, invasion, and host–parasite interactions (Day et al. 1995; Dalton et al. 1996; Collins et al. 2004). The presence of ten cysteine residues, with two potential disulfide bonds, in PpsCatL possibly facilitates its

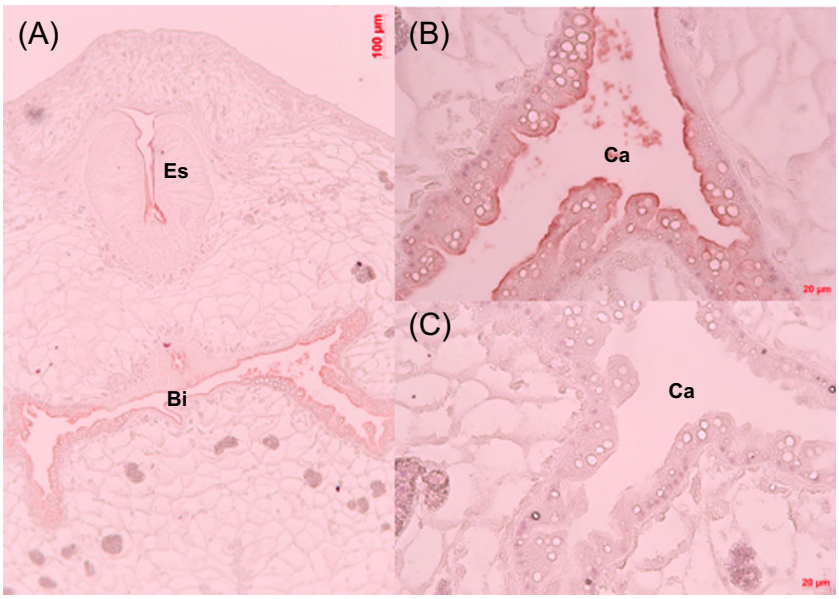


Fig. 8 Immunolocalization of PpsCatL in tissues of adult *P. pseudoheterotremus* (**a**–**c**). **a** Low magnification of adult tissue localized PpsCatL in the intestinal tissue. *Es* esophagus, *Bi* bifurcated intestine. **b** High magnification of the parasite cecum. *Ca* cecum. **c** Control incubated with preimmune mouse serum

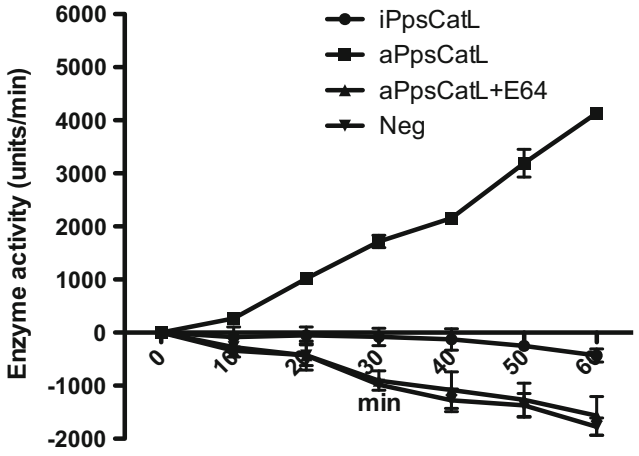
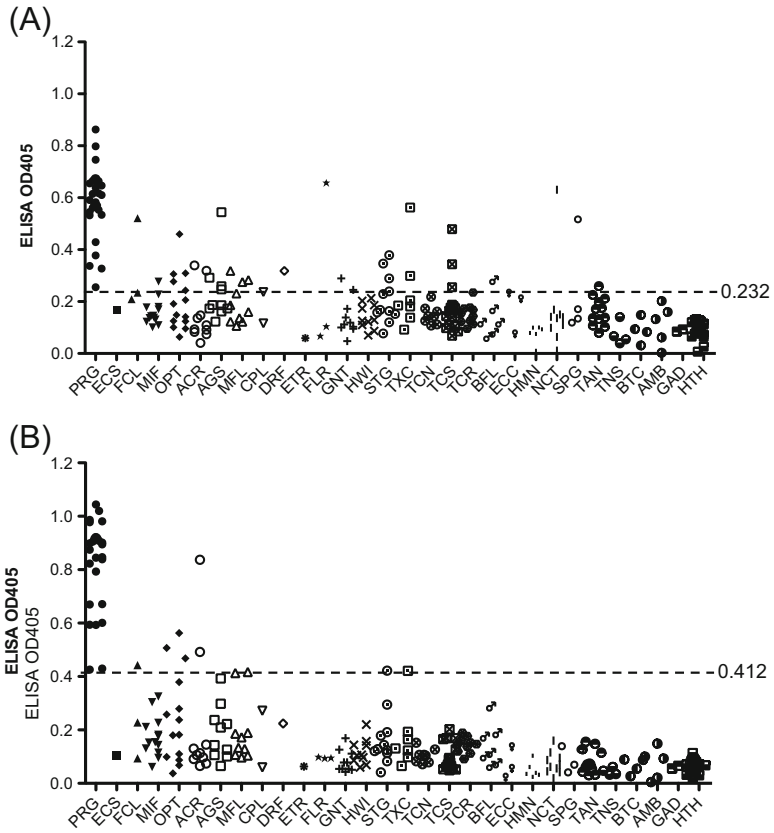


Fig. 9 Enzymatic activity of rPpsCatL was determined by hydrolysis of Z-Phe-Arg-AMC substrate. Inactivated (iPpsCatL) or activated rPpsCatL (aPpsCatL) was incubated with the substrate, and the enzymatic activity was measured as increased fluorescence units/min. Incubating activated rPpsCatL with 5 μM E64 (aPpsCatL + E64) was performed as a control. *Neg* negative control

tertiary structure, forming intermolecular interactions that create a dimeric structure, as seen in other cathepsin L orthologues. The cathepsin L found in the embryos and larvae of the brine shrimp (*Artemia franciscana*) forms a heterodimer with cathepsin L-associated protein (CLAP), which is more active and more stable than the cathepsin L monomer (Warner et al. 2004). A multiple alignment of PpsCatL with other orthologues identified the motifs ERFNIN and GNFD in the propeptide of PpsCatL, which have been described as signatures of cathepsin L-like proteases but not cathepsin B-like proteases (Kollien et al. 2004; Pandey et al. 2009). The ERFNIN and GNFD motifs are considered to facilitate the inhibition of the falcipain 2 propeptide, which was confirmed when the deletion of both motifs impaired the inhibition of falcipain 2 (Pandey et al. 2009). GCNGG is another conserved motif, found at amino acid residues 173–177 of mature PpsCatL. This motif was previously reported to be associated with the formation of the globular protein, which requires disulfide bridges (Karrer et al. 1993). The catalytic triad of mature PpsCatL is composed of Cys_{136}, His_{272}, and Asn_{292}, which are conserved within the papain family (clan CA) (Pandey and Dixit 2012). S_1 and S_2 subsites were predicted at the active site of PpsCatL, which are conserved in other cathepsin L orthologues. The S_1 and S_2 subsites of cathepsin L, especially S2, contribute to its substrate preference (Alves et al. 2001; Lecaille et al. 2007). Mutation of the S2 pocket



	STANDARD	
	Positive	**Negative**
Positive	28	36
Negative	0	191
	Sensitivity 100%	**Specificity 84.1%**

	STANDARD	
	Positive	**Negative**
Positive	28	10
Negative	0	217
	Sensitivity 100%	**Specificity 95.6%**

Fig. 10 Evaluation of CWA-ELISA (**a**) and rPpsCatL-ELISA (**b**). *Scatter plot* indicates the absorbance values for parasite-infected sera (**a** *left* and **b** *left*). *Dashed lines* indicate cutoff values for the CWA-ELISA and rPpsCatL-ELISA at 0.232 and 0.412, respectively. Sensitivity and specificity were calculated with a 2 × 2 table, which showed that the sensitivity and specificity of the CWA-ELISA were 100 and 84.1 %, respectively, and those of the rPpsCatL-ELISA were 100 and 95.6 %, respectively (**a** *right* and **b** *right*). Abbreviations on the *x*-axis are described in the Table 2

(L67Y/A205L) in human cathepsin L caused a change in its enzymatic specificity, affecting its amino acid preferences in both peptidyl substrates and small selective inhibitors (Lecaille et al. 2007).

A phylogenetic tree demonstrated that PpsCatL is closely related to the cathepsins L of *P. westermani*, PwCatL6, PwCatL5, and PwCatL8, which may suggest biological and biochemical associations of this protein in the genus *Paragonimus*. The *in silico* tertiary structure of mature PpsCatL has been reported (reviewed by Turk et al. 2012). However, a crystallographic analysis of PpsCatL and other *Paragonimus* cathepsins L is required to clearly define the structure of this protein in the genus *Paragonimus*.

In a prokaryotic expression system, pro-PpsCatL without the signal peptide was expressed as an insoluble product with an approximate molecular weight of 35 kDa, which is similar to the calculated molecular weight of pro-PpsCatL (34.5 kDa). The detection of PpsCatL in CWA and ES with anti-rPpsCatL mouse serum indicates that the pro-PpsCatL produced by *E. coli* was the same size as that found in the CWA. These data are evidence of an unglycosylated PpsCatL, which is consistent with the lack of potential glycosylation site predicted in this protein. A cathepsin L lacking potential glycosylation sites was previously observed in *F. hepatica*, procathepsin L1, and was detectable in the ES of the adult parasite (Collins et al. 2004). The lack of glycosylation in human and mouse procathepsins L does not disturb the structural folding, stability, or secretory properties of these proteases (Kane 1993; Smith et al. 1989). PpsCatL was present in the ES of *P. pseudoheterotremus*, with a predominant molecular weight of 25 kDa, which is the predicted molecular weight of mature PpsCatL. In *F. hepatica*, the mature cathepsins L1 (FhCatL1) and L2 (FhCatL2), but not the full-length proteins, were detected in the ES of the adult parasite (Collins et al. 2004). The ladder-like pattern of the protein observed in the CW and ES of adult *P. pseudoheterotremus* is attributed to catalytic of pro-enzyme or degradation. The immune response to PpsCatL was studied by treating *P. pseudoheterotremus*-infected rats with the recombinant protein. A strong signal was observed in all the infected animals, suggesting the potential utility of this protein in the development of immunodiagnostic tools. When *F. hepatica* cathepsin L was used for immunodiagnosis, fascioliasis was successfully detected in both humans and ruminants (Cornelissen et al. 2001; Gonzales Santana et al. 2013).

The transcriptional upregulation of *PpsCatL* was mainly detected in the adult stage of *P. pseudoheterotremus* and less in the other developmental stages. The differential expression of the cathepsin L isoforms in different developmental stages is frequently reported in *Fasciola* species (Dowd et al. 1994). *FgCatL1G* is mainly expressed in the metacercariae and NEJ of *F. gigantica*, but not in the adult (Norbury et al. 2011; Sansri et al. 2013). The expression of *PpsCatL* in the adult *P. pseudoheterotremus* was localized to the gastrodermal tissues along the parasite gut, especially in the gut lamellae. Secretory PpsCatL was detected in the gut lumen and ES of the parasite. These data suggest important roles for PpsCatL in the parasite itself and in the environment of the host. Cathepsin L1 secreted from *F. hepatica* is considered to be a major virulence factor, with essential roles in helminth pathogenicity, such as in nutrient acquisition by the digestion of host proteins, the cleavage of the host extracellular matrix, allowing parasite migration, and the impairment of the host immunity by destroying immunoglobulin and suppressing the Th1 immune response (Collins et al. 2004).

Enzymatic activity of rPpsCatL was determined by measuring an increment of fluorescent units after substrate cleavage. The proteolytic activity of rPpsCatL was observed after autoactivation in acidic pH but not inactive enzyme. The autocatalysis is required for activation of pro-cathepsin L to mature enzyme by removing a pro-peptide region (reviewed by Turk et al. 2012). In *Fasciola*, activation of pro-cathepsin Ls at acidic pH (approximately pH 4–5) exhibited mature enzyme that actively digested substrates (Collins et al. 2004; Sansri et al. 2013; Yamasaki et al. 2002). Inhibition of rPpsCatL activity with cysteine protease inhibitor, E64, but not other inhibitor families (data not shown) confirmed a property of PpsCatL as cysteine protease.

The rPpsCatL-ELISA was highly sensitive and specific in the diagnosis of human paragonimiasis. However, the results presented here could not confirm the species specificity of the ELISA because no species-confirmed sera were available. The cross-reaction of cathepsin L within the same genus has been reported for *F. hepatica*- and *F. gigantica*-infected ruminants. The detection of *F. gigantica* with an FhCatL-ELISA elicited similar outcome for *F. hepatica* (Cornelissen et al. 2001). Therefore, the positive results shown in this experiment may be attributable to diverse *Paragonimus* species, such as *P. westermani*, *P. heterotremus*, and *P. pseudoheterotremus*. Further evidence of cross-reactivity is the high percentage identity (>45 %) between the cathepsin L amino acid sequences within the genus *Paragonimus*. However, validation of the rPpsCatL-ELISA using sera with confirmed species-specific paragonimiasis, especially from animals infected with *P. heterotremus*, is required to clarify this issue. *P. heterotremus* and *P. pseudoheterotremus* are sister species with high genetic similarity (Intapan et al. 2012). Therefore, the high homology of the cathepsin L proteins in both species might cause cross-reactivity of the rPpsCatL-ELISA. However, cathepsin L has not yet been identified in *P. heterotremus*, and its discovery will allow the basic functions and properties of the cathepsin L protein in these sibling species to be compared, including their sequence homology, protein structures, biochemistry, and immune response. Only 10 from 197 samples of heterologous sera produced false positive results with the rPpsCatL-ELISA, including sera from individuals infected with *O. viverrini* (three cases),

Ascaris lumbricoides (two cases), *B. malayi* (two cases), *S. stercoralis* (one case), *Toxocara* sp. (one case), and *Fasciola* sp. (one case). Nine heterologous-infected sera showed weak positive with rPpsCatL, perhaps because the high antibody titers raised against these diseases affect the specificity of antibodies. As demonstrated previously, false positive HIV tests (Murex HIV Ag/Ab combination EIA) were significantly associated with increased levels of *S. haematobium* IgG1 (Everett et al. 2010). One serum from an individual infected with *A. lumbricoides* reacted strongly with rPpsCatL in our study, which may be attributable to a previous history of paragonimiasis or another closely related helminthic infection.

The high sensitivity (100 %) and specificity (95.6 %) of the rPpsCatL-ELISA suggest its potential utility in screening for paragonimiasis. The higher specificity of the rPpsCatL-ELISA (95.6 %) compared with that of the CWA-ELISA (84.1 %) suggests that CWA be replaced with rPpsCatL for clinical immunodiagnosis. However, the rPpsCatL-ELISA must be evaluated with nonparasitic infectious diseases (e.g., viral, bacterial, and fungal) and noninfectious diseases (e.g., lung cancer, lung diseases) in future studies.

In summary, a novel cathepsin L-like protease was identified in *P. pseudoheterotremus*, which is predominantly expressed in the adult stage. This cathepsin L was found in ES products and specifically localizes to the intestinal brush border of the parasite. The recombinant protein expressed in *E. coli* reacted with rat-infected sera and differentially diagnosed human paragonimiasis with high sensitivity and specificity. Further studies that evaluate the utility of the rPpsCatL-ELISA in species-specific paragonimiasis, nonparasitic infections, and some noninfectious lung diseases will further the development of an rPpsCatL-based immunodiagnostic tool in the near future.

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Compliance with ethical standards All animal works were conducted with the approval of the Faculty of Tropical Medicine Animal Care and Use Committee (no. FTM-ACUC 011/2012). Leftover helminth-infected human sera and healthy sera were used in this study with the permission of the Immunodiagnosis for Helminthiasis Unit, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, and the approval of the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (no. MUTM 2011-056-01).

Conflict of interest The authors declare that they have no conflict of interest.

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