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Identification and characteristics of a cathepsin L-like cysteine protease from *Clonorchis sinensis*

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Abstract

Cathepsin L-like protease is an important member of the papain-like cysteine protease and plays numerous indispensable roles in the biology of parasitic organisms. In a previous study, we identified a gene encoding a cathepsin L-like protease of *Clonorchis sinensis* (CsCPL) that was detected in the cercaria, metacercaria, and adult worm stages by immunolocalization, suggesting that this cysteine protease may be important and involved in the development of *C. sinensis*. In this study, the mature domain of CsCPL (CsCPL-m) was cloned and expressed in the form of inclusion bodies in *Escherichia coli*. After refolding, the recombinant CsCPL-m displayed optimal protease activity towards Z-Phe-Arg-AMC substrates but not towards Z-Arg-Arg-AMC, and the activity of the protease was inhibited completely by the cysteine protease-specific inhibitors E-64 and IAA, which further demonstrated that CsCPL belongs to the cathepsin L-like cysteine protease family. Recombinant CsCPL-m exhibited considerable activity at temperatures ranging from 28 to 42 °C, with the highest activity observed at 42 °C. Furthermore, recombinant CsCPL-m exhibited activity across a broad range of pH values (pH 4.0–8.0), with an optimal pH of 5.5. The *Km* and *Vmax* of the recombinant CsCPL-m towards Z-Phe-Arg-AMC were determined to be 5.71×10^{-6} M and 0.6 μM/min, respectively, at 37 °C and pH 5.5. The recombinant CsCPL-m could degrade BSA and gelatine, but could not degrade human hemoglobin and human immunoglobulin G. These results implied that CsCPL might participate in the catabolism of host proteins for nutrition during the parasitic life cycle of *C. sinensis*; thus, CsCPL could be used as a potential vaccine antigen and drug target against *C. sinensis* infection.

Keywords *Clonorchis sinensis* · Cathepsin L-like cysteine protease · Enzymatic characteristics

Introduction

Clonorchis sinensis infection can result in clonorchiasis characterized by dysfunction of the hepatobiliary system. Clonorchiasis is one of the most important food-borne parasitic diseases. Persons are infected by consuming raw or uncooked freshwater fish or shrimp containing metacercaria of *C. sinensis*. It is estimated that more than 200 million people are threatened by *C. sinensis* infection, and over 15 million people are infected worldwide. At present, China has the largest portion of infected individuals, with an estimated 13 million people infected with the parasite (Qian et al. 2016; Fürst et al. 2012).

Cysteine proteases from most parasitic helminths belong to the C1 family, which comprises mainly cathepsin L-like and cathepsin B-like proteases. Cathepsin L-like protease from parasites has been functionally characterized in nutrient uptake (Dvorák et al. 2009), molting (Guiliano et al. 2004), tissue invasion and migration (Larson et al. 2009;Tsubokawa et al. 2017), pathogenesis (Hernández et al. 2014), immune evasion (Berasain et al. 2000, 2003), and excystment (Zawistowska-Deniziak et al. 2013). Cathepsin L-like protease has also been valuable in the development of immunodiagnosis (Wongkham et al. 2005; León et al. 2013), vaccines, and drug targets (Wesołowska et al. 2018; Larson et al. 2009). As components of excretory-secretory products (ESPs) in both adult worms and metacercaria, the cysteine protease

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family of *C. sinensis* (CsCP) plays an important role in the parasite life cycle and parasite-host interactions (Lv et al. 2012; Li et al. 2004). The CsCP participates in the excystment of metacercariae (Chung et al. 2004) and the catabolism of host proteins for nutrition (Na et al. 2008). Therefore, the CsCP may be a potential target for effective vaccines and drug development.

In our previous study, a gene encoding CsCPL was identified from a cDNA library of *C. sinensis* adults. CsCPL contains a deduced signal peptide and a proprotein (zymogen). It has been shown that the recombinant propeptide of CsCPL is a promising antigen for immunodiagnosis of human clonorchiasis (Li et al. 2012). In addition, immunolocalization studies have shown that CsCPL is expressed in the cercaria, the metacercaria, and the adult stages. These findings indicate that CsCPL might play a role in the developmental stages of *C. sinensis* for cercaria invasion, metacercaria excystment, adult nutrition uptake, and pathogenesis (Li et al. 2009). The current study aimed to acquire recombinant CsCPL with enzymatic activity and to characterize its biochemical functions for the purpose of further understanding its role in the *C. sinensis*-host relationship. In the present study, we cloned the mature (catalytic) domain of CsCPL (CsCPL-m). The expression and biochemical properties of recombinant CsCPL-m were also assessed.

Materials and methods

Identification of the structure of CsCPL

The structures of the full-length cDNA sequence of CsCPL (GenBank Accession number: DQ902583) were analyzed by software provided by http://www.ncbi.nlm.nih.gov/ and https://www.expasy.org/tools/. The CsCPL ORF is from 80 to 1191 bp in length and encodes 371 amino acids that contain a typical signal peptide sequence (amino acids 1–18) , a prodomain (amino acids 19–127), and a mature domain (amino acids 128–371).

Expression, purification, and refolding of the recombinant mature domain of CsCPL (rCsCPL-m)

The entire mature domain of CsCPL was amplified by polymerase chain reaction (PCR) using the pET-28a-CsproCPL plasmid as the template (Li et al., 2009). The primers were as follows: 5′-GGCATATGGATGTATTACGCGGCTT-3′ and 5′-GGCTCGAGCTACATAAGAGGGTAG-3′ with *NdeI* /*XhoI* (Promega) restriction enzyme sites (underlined), respectively. PCR was carried out for 35 cycles at 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 30 s, followed by an extension period of 10 min at 72 °C. The purified PCR product was cloned into the corresponding restriction sites of a prokaryotic expression vector pET28a (+) (Novagen) for expression of the recombinant protein with an N-terminal 6×his tag. After confirmation by DNA sequencing, the recombinant plasmid was transformed into *Escherichia coli* (*E. coli*) BL21 (DE3) (Promega).

The expression of the recombinant protein was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM for 5 h at 37 °C. Subsequently, the bacterial cells were harvested at 6,000*g* × 15 min at 4 °C and sonicated on ice. After centrifugation, the pellet of cells was solubilized completely in 6 M urea lysis buffer (20 mM Tris-HCl buffer, pH 8.0) and purified by a His Bind Purification kit (Novagen) in denatured conditions. Refolding was followed with dialysis through stepwise dilution of urea (6 to 1 M) in 20 mM Tris-HCl and 5 mM EDTA buffer (pH 8.0). Finally, the urea was removed by dialysis using phosphate-buffered saline (PBS, pH 7.4). The quality of the purification was analyzed by 12% SDS-PAGE, and the product concentration was determined using the BCA protein assay kit (Novagen).

Western blotting analysis

The purified rCsCPL-m (10 μg) was subjected to 12% SDS-PAGE, and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Qbiogene), and the membrane was probed with anti-6×his tag antibody (1:1000 dilution) (Novagen) over night at 4 °C. After washing procedures, the membrane was incubated with peroxidase horseradish–conjugated anti-rat immunoglobin G (1:2000 dilution) (Boster) for 1 h at room temperature. After washing again, the membrane was visualized using diaminobenzidine (DAB) solution (Boster) as the substrate.

Enzyme activity assays

The enzyme activity of rCsCPL-m was assayed as the hydrolysis substrates of carbobenzoxy-phenylalanyl-arginyl 7-amino-4-methyl coumarin (Z-Phe-Arg-AMC) (Sigma) and carbobenzoxy-arginyl-arginyl 7-amino-4-methyl coumarin (Z-Arg-Arg-AMC) (Sigma) (Barrett and Kirschke 1981). Briefly, the method was modified as follows: the total reaction volume was 1 ml and rCsCPL-m (1.45 μg) was incubated with a substrate (20 μM) in reaction buffer (6 mM DTT, 0.05% Brij 35, 1 mM $EDTANa_2$, 100 mM sodium phosphate solution, pH 5.5) for 20 min at 37 °C. The enzyme reaction was terminated by adding stop buffer (100 mM sodium monochloroacetate, 30 mM sodium acetate, 70 mM acetic acid, pH 4.3). The fluorescence intensity of the AMC label (excitation 360 nm, emission 460 nm) was measured with a Spectra MAX Gemini fluorometer (Molecular Devices, TECAN, Switzerland). The standard curve was set up according to the fluorescence intensity of different concentrations (0,

2.0, 4.0, 8.0, 12.0, 16.0 μM) of the AMC label. The activity of the rCsCPL-m was then calculated as the amount of fluorescence units according to the standard curve.

For *Km* determinations, Z-Phe-Arg-AMC was used as a substrate at different concentrations (4.0, 8.0, 12.0, 16.0, 20.0, 24.0, 28.0, 32.0, 36.0, 40.0, and 44.0 μM). The fluorescence intensity of AMC was measured continuously every 3 min. The data were analyzed by SPSS 13.0 software.

To determine the optimal pH of the enzyme activity of rCsCPL-m, the protein (0.87 μg) and Z-Phe-Arg-AMC (20 μM) were incubated at 37 °C for 20 min in 100 mM sodium acetate buffer (pH 3.5, 4.0, 4.5, 5.0, 5.5), 100 mM sodium phosphate buffer (pH 6.0, 6.5, 7.0, 7.5), or 100 mM Tris-HCl buffer (pH 8.0, 8.5). The thermal activity of rCsCPL-m was investigated by incubating rCsCPL-m (0.87 μg) with Z-Phe-Arg-AMC (20 μM) at 0, 4, 16, 28, 37, and 42 °C in 100 mM sodium acetate buffer (pH 5.5) for 20 min. Then, the enzyme activity was evaluated as described above.

Inhibitory effects of rCsCPL-m on enzyme activity

To confirm the specificity of enzyme activity, we performed enzymatic inhibition experiments by using different enzyme inhibitors. Briefly, rCsCPL-m (0.87 μg) was preincubated with protease inhibitors, including trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64, 10 μM, Sigma), iodoacetic acid (IAA, 20 μM, Sigma), N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK, 100 μM, Sigma), phenylmethanesulfonyl fluoride (PMSF, 1 mM, Amresco), and $EDTANa_2$ (2 mM, AMRESCO) for 30 min at 37 °C. Then, Z-Phe-Arg-AMC (20 μM) was added to the reactions which were incubated for another 20 min. Each assay was performed in triplicate, and enzyme activity was measured as described above.

Degradation of the host protein

The rCsCPL-m protein was mixed with 4 × SDS-PAGE loading buffer (0.1 mM 2-mercaptoethanol, 4% SDS, 10% glycerol, and 0.1% bromophenol blue, 0.1 mM Tris–HCl, pH 6.8). Then, the mixture (10 μg of rCsCPL-m) was run on a 10% SDS gel containing 0.1% gelatine (*w*/*v*) (Sigma). After electrophoresis at 4 °C, the gel was rinsed three times in 0.25% Triton X-100 for 20 min to remove excess SDS and to allow protein renaturation. Then, the gel was incubated in zymography developing buffer (100 mM sodium phosphate, 2 mM DTT, pH 6.0, or pH 7.5) at 37 °C for 18 h. Bands of gelatine degradation were visualized by staining the gels with 0.5% Coomassie brilliant blue R-250 in methanol/acetic acid (45:10) and destaining in 10% acetic acid (*v*/*v*).

To test whether the CsCPL could degrade host proteins, the rCsCPL-m (40 μg) was incubated with host proteins for 1 h at 37 °C. Bovine serum albumin (BSA, 200 μg), human hemoglobin (Hb, 200 μg), and human immunoglobulin G (IgG, 200 μg) were used as substrates. The assays were performed in a 300-μl mixture buffer (6 mM DTT, 1 mM $EDTANa_2$, 100 mM sodium phosphate, pH 6.0, and pH 7.5). The reaction mixtures were analyzed by 10% SDS-PAGE.

Results

Expression, purification, and refolding of the rCsCPL-m

The rCsCPL-m was expressed as a fusion protein with a 6×his tag in *E. coli* after induction by IPTG. SDS-PAGE demonstrated that the rCsCPL-m had a molecular weight of approximately 28.8 kDa, which was consistent with bioinformatic prediction (Fig. 1a, lane 2) and was highly expressed in inclusion bodies (Fig. 1a, lane 4). The rCsCPL-m was purified by affinity chromatography under denaturing conditions (Fig. 1a, lane 5). The rCsCPL-m (1 mg/ml) was soluble in PBS after refolding. The refolded protein could be identified by anti-6×his monoclonal antibodies (Fig. 1b, lane 1).

Enzyme activity assay and inhibitory test

To confirm the cathepsin activity of the rCsCPL-m, a conventional enzyme assay was carried out using fluorogenic peptide substrates. The family of cathepsin L-like proteases preferentially hydrolyses the typical substrate Z-Phe-Arg-AMC, whereas the family of cathepsin B-like proteases hydrolyses Z-Arg-Arg-AMC (Barrett and Kirschke 1981). The rCsCPL-m displayed protease activity towards Z-Phe-Arg-AMC but not towards Z-Arg-Arg-AMC. E-64 and IAA, as cysteine protease-specific inhibitors, completely inhibited the enzyme. Incubation with TPCK only partially reduced the catalytic activity of the rCsCPL-m, whereas incubation with PMSF and EDTA did not influence the rCsCPL-m activity (Fig. 2a). At least 40% of the total activity was detected between pH values of 4.0 and 8.0, and the optimum pH value was 5.5 (Fig. 2b). The thermal activity analysis of the rCsCPL-m indicated that the activity increased with increasing temperature and reached a maximum value at 42 °C (Fig. 2c). The *Km* and *Vmax* of the rCsCPL-m against Z-Phe-Arg-AMC were determined to be 5.71×10^{-6} M and 0.6 μM/min at 37 °C and pH 5.5, respectively (Fig. 2d).

Degradation of host proteins

rCsCPL-m could hydrolyze gelatine and BSA as the substrates were more readily degraded by rCsCPL-m at acidic pH 6.0 than at pH 7.5, indicating that catalytic activation occurred efficiently in a weak acidic environment. Hb and IgG were not degraded at pH 6.0 or 7.5 (Fig. 3).

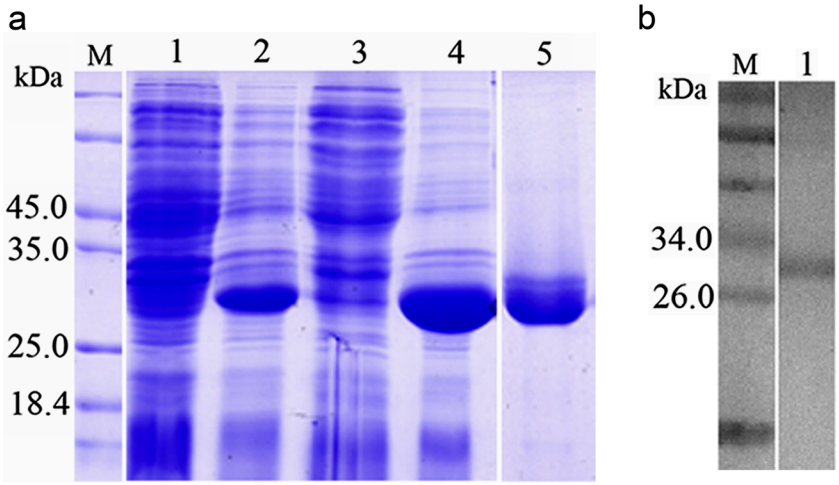

Fig. 1 **a** Analysis of the expression, purification, and refolding of rCsCPL-m by 12% SDS-PAGE stained with Coomassie brilliant blue. Lane M, molecular mass standards; lane 1, BL21 cell containing pET28a-CsCPL-m without IPTG induction; lane 2, BL21 cell containingpET28a-CsCPL-m with IPTG induction; lanes 3 and 4, supernatant and precipitant of lysate of BL21 containing pET28a-CsCPL-m with IPTG induction, respectively; lane 5, the purified rCsCPL-m. **b** Analysis of Western blotting. Lane M, molecular mass standards; lane 1, rCsCPL-m probed with anti-6×his tag monoclonal antibody

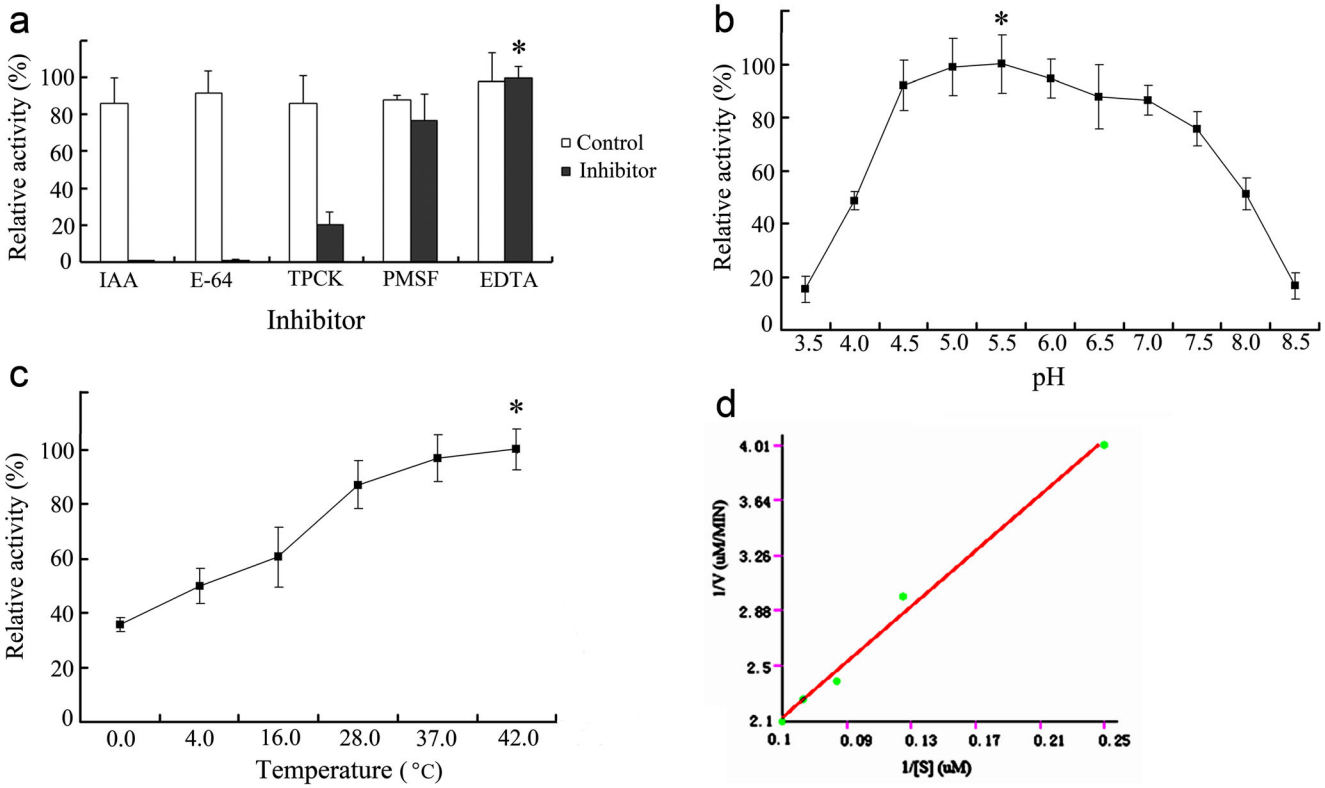

Fig. 2 Enzymatic activity of the rCsCPL-m against Z-Phe-Arg-AMC. **a** Enzymatic activity was measured after rCsCPL-m preincubation with inhibitors, including IAA (20 μM), E-64 (10 μM), TPCK (100 μM), PMSF (1 mM), and EDTA (2 mM) at a pH of 5.5 and 37 °C. **b** Enzyme activity of the rCsCPL-m was measured at pH values of 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 at 37 °C. **c** Enzyme activity was measured at 0, 4, 16, 28, 37, and 42 °C at a pH of 5.5. **d** Enzymatic kinetics parameters of the rCsCPL-m were detected at a pH of 5.5 and at 37 °C. Fluorescent intensity was measured to calculate relative enzyme activity. Asterisk indicates the maximum activity as 100%. Errors represent data from triplicate samples

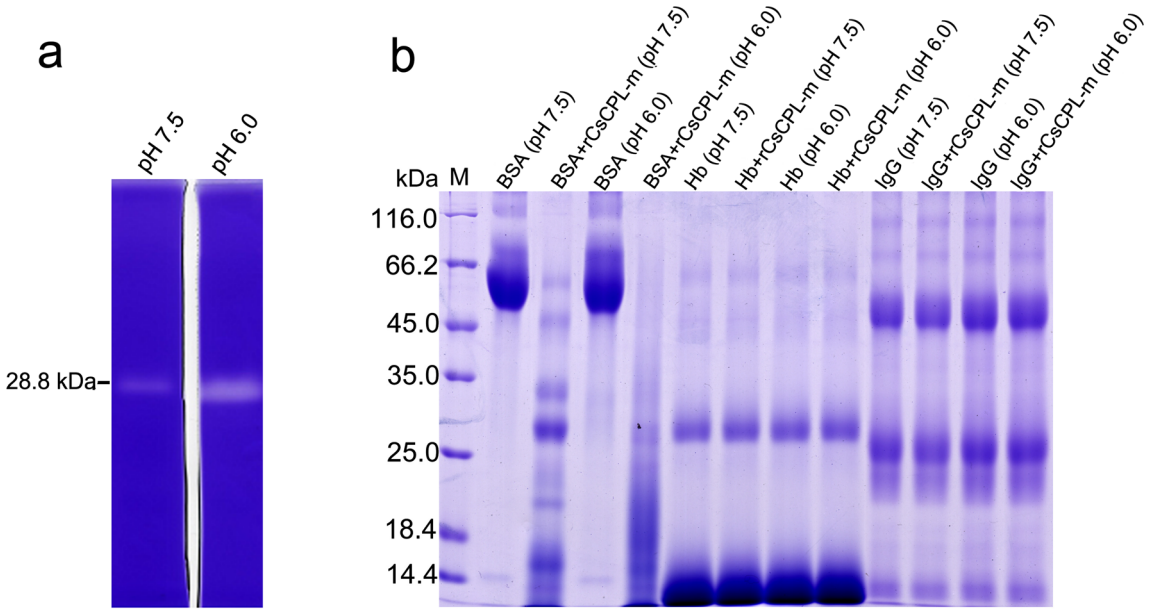

Fig. 3 Host protein degradation by rCsCPL-m. **a** Gelatine hydrolysis assay in 10% SDS-PAGE containing 0.1% (*w*/*v*) gelatine. **b** The assays were performed in a 300-μl mixture buffer (6 mM DTT, 1 mM EDTANa_2, 100 mM sodium phosphate, pH 6.0 and pH 7.5) containing rCsCPL-m (40 μg) and various host proteins (200 μg). The reactions were analyzed by 10% SDS-PAGE. Degradation assay using BSA, Hb, and IgG as the substrate

Discussion

In our previous study, the CsCPL was predicted by bioinformatic analysis to contain a signal peptide sequence, a prodomain (109 amino acids), and a mature domain (244 amino acids). The C-terminal segment of the prodomain of CsCPL included two motifs (ERFNIN and GNFD) that are conserved in cathepsin L-like sub-family papain family proteases as the mediator of prodomain inhibitory activity, and the catalytic triad of Cys177-His318-Asn338, which is highly conserved among the papain-like cysteine proteases, is responsible for the catalytic activity of the enzyme in the mature domain. These data revealed that CsCPL belongs to the C1 family of cathepsin L-like cysteine proteases (Hu et al. 2008).

Cysteine proteinase is synthesized as an inactive precursor (zymogen), and then, the protein is converted into an enzymatically active form after the removal of the prodomain, which is achieved by other enzymes' catalysis or autocatalysis in vivo (Sajid and Mckerrow 2002). Due to limited knowledge and the difficulty of simulating the activation of cysteine proteinases happening in vivo, the activity of recombinant cysteine proteinases is also difficult to obtain in vitro. Active enzymes have been obtained mainly by trying to induce auto-activation of recombinant proteins. Recombinant cysteine proteinases expressed in the prokaryote system were previously able to undergo further maturation induced by pH changes (e.g., acidic pH values) (Franta et al. 2011; Sanderson et al. 2000). Other studies showed that the cathepsin L-like cysteine proteases from *Taenia solium* and *Gnathostoma spinigerum*, the two recombinant proproteins (rproproteins) expressed in *E. coli*, did not show enzymatic activity; however, the rproproteins expressed in *Pichia pastoris* showed typical biochemical characteristics of cysteine proteases (Li et al. 2006; Kongkerd et al. 2008), suggesting that auto-maturation of zymogens to mature enzymes could be carried out due to the correct folding of rproproeins in the eukaryotic system. In our study, we also attempted to refold the recombinant proprotein of CsCPL expressed in *E. coli*, but the refolded protein displayed little proteolytic activity (Li et al. 2009). The 3D structural analysis of CsCPL revealed that the catalytic active domain exhibits a V-cleft shape, the catalytic triad of Cys177-His318-Asn338 is located at the bottom of the cleft, and the prodomain covers the cleft, impeding substrates from binding to the catalytic site; thus, the prodomain plays a role in inhibiting enzyme activity through spatial configuration. The prodomain must be removed to expose the site of enzyme activity and to allow the substrate to bind so that the enzyme activity can be initiated. In this study, the goal of hydrolyzing the prodomain was achieved by directly cloning the active enzyme instead of employing auto-activation methods. However, in addition to inhibiting enzyme activity, the prodomain also participates in the correct folding of the spatial structure of enzyme molecules. Ramón et al. also tried to express the mature domain of a cathepsin L-like proteinase from *Trichomonas vaginalis* in *E. coli*. The recombinant enzymes did not display activity, probably due to the absence of the prodomain, which is essential for the correct folding and activation of papain-like CPs in vitro (Ramón-Luing Lde et al. 2011; Coulombe et al. 1996). Intriguingly, the rCsCPL-m displayed hydrolytic properties after refolding, suggesting that the prodomain of CsCPL is not required to guide proper

folding. The results of the enzyme activity assay and inhibitory test showed that the specific substrate of CsCPL-m was Z-Phe-Arg-AMC, and rCsCPL-m enzymatic activity was completely inhibited by E-64 and IAA, which confirmed that CsCPL belongs to the cathepsin L-like cysteine proteases, and this conclusion is consistent with the results of bioinformatic analysis.

Previous studies showed that CsCPL is expressed in the cercaria, metacercaria, and adults of *C. sinensis*, suggesting that CsCPL may be a pivotal cysteine protease involved in the development of *C. sinensis*. The rCsCPL-m exhibited high activity across a broad range of appropriate temperatures from 28 to 42 °C, which agrees with the optimal temperatures for cercaria and metacercaria development in the intermediate hosts of freshwater snails and fish and the adult development in the definitive host vertebrates. The high activity expressed by CsCPL across a broad temperature range might be a consequence of selection for CsCPL function in different parasitic environments.

The proteolytic activities of cysteine proteases have been well characterized, and these proteases are regarded as the key digestive enzymes for parasites. The rCsCPL-m showed enzymatic activity across a wide range of pH values, while the peak activity was measured at a pH value of 5.5. Furthermore, the rCsCPL-m could hydrolyze gelatine and BSA more completely at a pH value of 6.0 than at a pH value of 7.5, suggesting that CsCPL-m is more active under acidic conditions and is an acid proteinase. CsCPL is localized to the intestinal wall and lumen, and the gut lumen of parasites is an acidic environment (Delcroix et al. 2006), which is an optimal environment for CsCPL to fully realize enzymatic hydrolysis activity. The experimental results also show that CsCPL can hydrolyze macromolecule proteins of gelatine and BSA, indicating that CsCPL could be involved in the digestion of host proteins in vivo.

Cysteine proteases have been proposed to catalyze hemoglobin degradation in parasites, especially in blood-feeding helminths (Williamson et al. 2003). Recombinant cathepsin L-like protease from schistosomes was capable of degrading human hemoglobin directly (Brady et al. 1999; Dvorák et al. 2009). The inability to hydrolyze intact hemoglobin did not indicate that CsCPL could not act as a digestive enzyme involved in hemoglobinolysis. There is evidence that cathepsins B and L of *Schistosoma mansoni* further degrade the fragments released following the initial cleavage of hemoglobin produced by cathepsin D, suggesting that hemoglobin digestion is modulated through multi-regulated cascade and protease network (Delcroix et al. 2006). We suspect that CsCPL may be involved in the subsequent digestion of hemoglobin in vivo, even though CsCPL cannot directly degrade hemoglobin in vitro.

In summary, we cloned the sequence encoding only the mature domain of the cathepsin L-like cysteine protease from *C. sinensis* (CsCPL) and characterized its catalytic activity. CsCPL could act as a key protease in the digestion of host proteins to supply nutrition for the development of *C. sinensis*. Further research should focus on evaluating the role of CsCPL in pathogenesis and the potential value of CsCPL as a drug target against *C. sinensis* infection.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflicts of interest.

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