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Molecular Cloning and Expression of *Giardia* Cathepsin B

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Abstract: ***Giardia lamblia, a protozoan parasite, is a leading cause of waterborne diarrheal disease globally. Cysteine proteases (CPs), particularly cathepsin B-like enzymes, have been implicated in Giardia's pathogenicity, yet their specific roles remain underexplored. The study highlights on the cloning, expression, and functional characterization of CP14019, a cathepsin B-like CP. The CP14019 gene is successfully amplified, cloned into the pET28a vector, and expressed in Escherichia coli BL21 cells. The recombinant protein, predominantly found in the insoluble fraction, was purified via Ni^{2+}-affinity chromatography. Western blot analysis confirmed the antibody's specificity, detecting a ~28kDa band corresponding to CP14019 in both recombinant samples and Giardia trophozoite lysates. Functional assays demonstrated that CP14019 exhibits proteolytic activity characteristic of cathepsin B enzymes, effectively degrading tight junction proteins and chemokines such as IL-8, thereby compromising epithelial barrier integrity and modulating host immune responses. These findings underscore CP14019's role in Giardia's virulence and highlight its potential as a target for therapeutic intervention. The generated antibodies provide valuable tools for further investigations into CP14019's function during Giardia infections.***

Keywords: *Giardia*, Diarrhoea, Cathepsin B, Cloning, Protein expression

1. Introduction

Giardia lamblia is a protozoan parasite that colonise in the upper small intestines and causing waterborne diarrhoea worldwide (Ankarklev et al. 2010). Around 280 million of the giardiasis cases have been reported worldwide (Haldar et al. 2024). Within eight distinct *Giardia* assembles specifically assemblages A and B affecting humans (Certad et al. 2017). *Giardia* infection begins with the ingestion of cysts via the fecal-oral route, upon reaching the duodenum and jejunum, these cysts release excyzoites that differentiate into trophozoites. The trophozoites attach to upper small intestinal, leading to diarrhoea, malabsorption and weight loss (Einarsson et al. 2016). These effects are especially severe in immunocompromised individuals, young children and the elderly in developing countries (Mmbaga et al. 2017). Recent studies highlight the role of *Giardia* CPs in disease progression and pathogenesis (Cotton et al. 2015). CP secretion increases during host-parasite interactions in vitro (Rodriguez-Fuentes et al. 2006) and these enzymes can disrupt tight junctions, weakening the intestinal epithelial barrier (Chin et al. 2002).

Recent findings reveal that CPs secreted by *Giardia* can cleave the microvillus protein villin, compromising epithelial integrity. These enzymes also degrade the prionflammatory chemokine IL-8, thereby dampening intestinal inflammation (Cotton et al. 2014). Moreover, CPs modulate gut microbial communities by influencing biofilm formation and inhibiting the growth of pathogenic intestinal bacteria (Manko et al. 2017). These studies collectively highlight that CPs actively mediate *Giardia*'s interactions with the host. However, most research has relied on cell extracts or CP inhibitors, emphasizing the need to investigate the distinct roles of individual CPs in *Giardia*'s pathogenic mechanisms. In the *Giardia* WB genome, CPs are the most abundant proteases with 26 genes, including cathepsin B, C and K/L-like types (DuBois et al. 2008). Among these, cathepsin B-like proteases are highly expressed, especially during differentiation and interactions with intestinal epithelial cells. Specific CPs such as CP2, CP1, and CP3 are involved in excystation, CP14109 in encystation, and CP14019 in degrading endocytosed proteins.

Our study focused on cloning and expression of the cathepsin cysteine protease CP14019, a clan CA protease featuring a catalytic cysteine-histidine dyad; notably, cathepsin B proteases possess an occluding loop with two histidines that enhance their endopeptidase and exopeptidase functions. In *Giardia*, many cathepsin protease genes remain uncharacterized, although several are upregulated upon interaction with intestinal epithelial cells.

2. Methodology

Parasite Culture

Giardia trophozoites (ATCC, Portland 1) were cultured at 37°C in TYI-S-33 medium with penicillin, streptomycin, and 10% adult bovine serum, adjusted to pH 6.8 via 0.22 µm filtration (Haldar et al. 2024). After ~2 days reaching log phase, cells were transferred to fresh 15 ml media tubes and routinely maintained the culture.

RNA Isolation

Giardia trophozoites (~2×10^8) in logarithmic growth phase were lysed using 1 ml of Trizol reagent (Invitrogen). After rapid pelleting and a 5-minute room temperature incubation, 0.2 ml of chloroform per ml of Trizol was added, followed by vigorous shaking for 15 seconds (Haldar et al. 2024). The samples were incubated for 5 minutes at RT and then centrifuged at 12,000g for 20 minutes at 2-8°C. This procedure led to the formation of three distinct layers: a lower phenol-chloroform phase, a middle interphase, and a transparent upper aqueous phase. The aqueous phase was carefully collected and mixed with 0.5 ml isopropanol, incubated at 15-30°C for 10 minutes to precipitate RNA. This

was followed by centrifugation at 12,000g for 10 minutes at 2-8°C to pellet the RNA. The pellet was washed with 75% ethanol, centrifuged again at 7,500g for 5 minutes, air-dried lightly, and finally dissolved in DEPC-treated water at 55-60°C for 5 minutes to preserve RNA integrity for downstream applications.

cDNA Preparation

Prepare the reaction mixture by combining 1 µl oligo (dT) 12-18 (500 µg/ml), 1 µl dNTP mix, and 5-10 ng total RNA in a 12 µl volume adjusted with nuclease-free water. Heat to 65°C for 5 min, then chill on ice. Centrifuge briefly, and then add 4 µl 5X First Strand Buffer, 2 µl 0.1 M DTT, and 1 µl RNase inhibitor (Haldar et al. 2024). Incubate at RT for 5 min, add 1 µl Super RT (2000 U/µl), mix gently, and incubate at 42-52°C for 50 min. Terminate the reaction at 70°C for 15 min.

PCR Amplification

A PCR reaction was performed in 0.5 mL sterile tubes by mixing 10X buffer (5 µL), $MgCl_2$ (2 µL), dNTPs (3 µL), forward primer (5'-TAAGCAGAATTCATGAAGCTCTTTCTCCTC-3', BamHI site) and reverse primer (5'-TGCTTAAAGCTTTTACTCATCGAAGAAGCC-3', HindIII site) (each primer1 µL), 3 µL template, 0.5 µL Ex Taq polymerase, and water to 50 µl total. PCR was performed with an initial denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s, ending with a final extension at 72°C for 7 min. The ~1 kb PCR product was gel-purified, end-filled, digested with BamHI, and ligated into the Pet vector digested with HindIII and BamHI. Positive clones were selected by transforming *E. coli* and plating on kanamycin-containing LA plates.

Restriction digestion & Ligation

Restriction digestion was carried out using BamHI and HindIII enzymes, which recognize specific DNA sequences and cleave the phosphate backbone. Total 25 µl reaction was prepared on ice using 2 µl buffer, 1 µl each of BamHI and HindIII (Takara Bio™), 12 µl water, and DNA. A negative control lacking DNA was also set up. The mixtures were incubated at 37°C for 1 hour, then enzymes were inactivated at 65°C for 10 minutes.

For ligation, 100 ng of vector DNA and 17 ng of insert were combined with 2 µl of 10X ligase buffer, 1 µl T4 DNA ligase and distilled water to a final volume of 10 µl. The mixture was gently mixed and incubated overnight at 16°C. Five microliters of the ligation product were used to transform 50 µl of competent *E. coli* DH5α cells.

Preparation of Competent Cells

Chemically *E. coli* DH5α competent cells were prepared using a calcium chloride method. Single colonies grown on Luria broth agar were used to inoculate overnight cultures, which were diluted and grown to mid-log phase. Cells were harvested, sequentially treated with ice-cold $MgCl_2$ and $CaCl_2$, then resuspended in $CaCl_2$ (100 mM) containing 15% glycerol. Aliquots were stored at -80 °C. Transformation efficiency was evaluated using 1-100 ng of an ampicillin-resistant plasmid on LBM + ampicillin plates.

Transformation of pET28a-Cathepsin B in *E. coli* BL21 (DE3)

To begin the transformation, thaw competent cells and keep the ligation DNA on ice. Add 50 µl of cells to the DNA and mix gently by pipetting or flicking (avoid vortexing). Incubate on ice for 30 minutes without disturbance, then heat shock at 42 °C for 45-60 seconds. Immediately add 950 µl of RT LB or SOC medium and incubate at 37 °C for 1 hour with shaking. After incubation, plate 50-100 µl of the mixture and incubate overnight at 37 °C.

Expression of Gene in *E. coli* BL21 (DE3)

Competent *E. coli* cells were subjected to transformation using pET28a-CathB plasmid DNA and subsequently cultivated in LB supplemented with kanamycin. The cells underwent induction with IPTG and were then centrifuged to form pellets. The total SDS-solubilized proteins were examined using a 10% SDS polyacrylamide gel, and the results were compared with the lane containing SDS-solubilized proteins from uninduced cells.

SDS-PAGE

Approximately 100-200 pg of protein sample was mixed with sample buffer, boiled for 3 minutes, vortexed, and briefly centrifuged at 1000g before loading into gel wells. Electrophoresis was conducted using a BioRad Protean II vertical slab gel apparatus. The run began at 90 volts until the samples entered the resolving gel, after which the voltage was increased to 120 volts. Electrophoresis continued until the dye front was 1–2 cm from the bottom of the gel. The gel was then carefully removed, immersed in a staining solution for one hour, and subsequently destained in a destaining solution for 2-3 hours.

Purification of Cathepsin B

The sonicated insoluble fraction from cathepsin B overexpression was applied to a Ni^{2+}-charged HisTrap column pre-equilibrated with binding buffer. Unbound proteins were washed away using ten column volumes of binding buffer with 5 mM imidazole, followed by an additional wash with 20 mM imidazole. The desired protein was subsequently eluted with a buffer containing 250 mM imidazole. Imidazole and NaCl were subsequently removed by dialysis using a 30 kDa cut-off Centricon. Purified fractions were analyzed by SDS-PAGE.

3. Results

Electrophoresis of RNA

The electrophoresis pattern of the isolated RNA, visualized in **Fig. 1** using a Bio-Rad Gel Documentation System, revealed three distinct bands corresponding to the 5.8S, 16S, and 23S rRNA of *Giardia.*

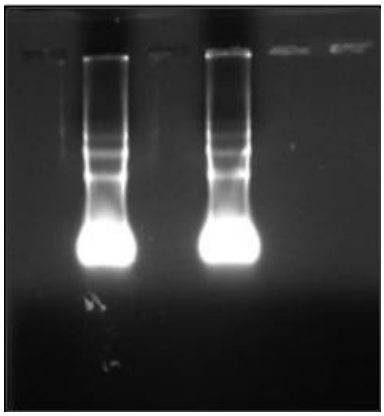

Figure 1: *Giardia lambia* total RNA ran in MOPS gel.

Agarose gel electrophoresis of DNA

In the agarose gel electrophoresis results, lanes 1 and 2 show a positive cathepsin B PCR band, size 1038 bp, while lane 3 serves as the negative control.

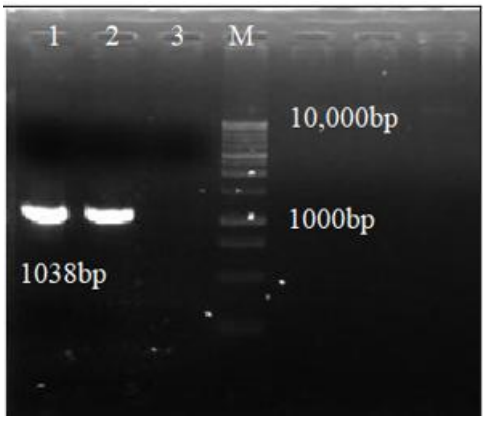

Figure 2: PCR amplification of cathepsin B gene. Lane 1 and 2 contain the samples, lane 3 is the negative control, and the marker was loaded ranging from 250 to 10,000 bp.

SDS-PAGE of Cathepsin B after expression

Identification and purification of cathepsin B involved SDS-PAGE analysis, which indicated that both the supernatant and precipitate contained the target protein. However, the supernatant indicated that the recombinant proteins mainly existed in the insoluble form (**Fig. 3**). Western blot analysis revealed that the mouse anti-His tag monoclonal antibody, along with the HRP-conjugated goat anti-mouse IgG antibody, specifically detected a ~28 kDa protein, which corresponds to the expected molecular weight of cathepsin B. The target protein was successfully eluted using 250 mM imidazole.

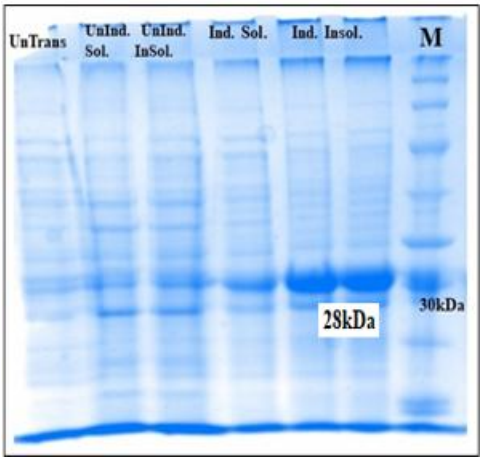

Figure 3: The results shows SDS-PAGE of cathepsin B gene. Lane 1 shows the untransformed sample. Lanes 2 and 3 are uninduced soluble and insoluble fractions respectively. Lane 4 represents the induced soluble fraction. Lanes 5 and 6 display induced insoluble fractions. A standard protein marker is shown in lane 7.

Specificity of anti-Cathepsin B antibody

The specificity of the Cathepsin B antibody was evaluated. ELISA detected the antibody titer, demonstrating reactivity with recombinant cathepsin B expressed in *E. coli* as a polyhistidine fusion protein (**Fig. 4**). Furthermore, Western blot assays with total trophozoite lysates revealed specificity to a 28kDa protein band. These results confirm that the antibody specifically targets Cathepsin B in trophozoites.

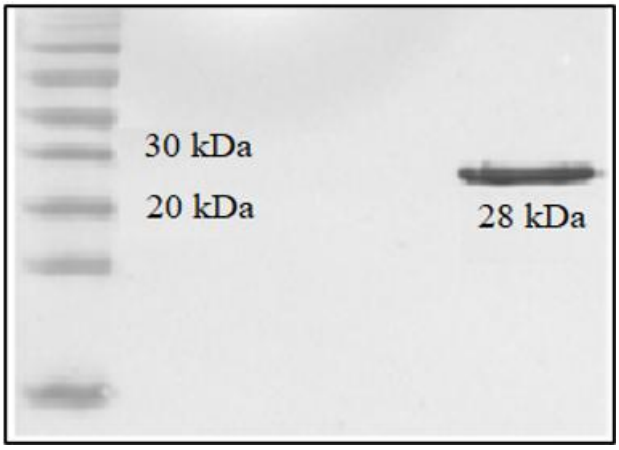

Figure 4: Confirmation of the specificity of anti-cathepsin B antibodies: Western blot analysis of recombinant cathepsin B proteins. The result depicts the reactivity of the anti-cathepsin B antibodies with the recombinant proteins.

4. Discussion

Cathepsin B-like CPs in *Giardia* have been recognized as key players in host-pathogen interactions (Bhargava et al. 2015; Cotton et al. 2014), yet their specific identities remain unclear. This study focuses on the cloning and expression of cathepsin B, a CP implicated in *Giardia*-host interactions. Prior studies report upregulation of CP14019 at both transcript and protein levels during in vitro interactions, along with its release into co-culture media (Ringqvist et al. 2011). The presence of secretion signal peptides and localization to vesicle-like structures and the ER suggests roles in protein trafficking and release (Mach et al. 1994).

Cathepsin B-like CPs released by *Giardia* during host-cell interactions exhibit proteolytic activity that disrupts epithelial junctional complexes, including tight and adherens junctions. These CPs can traverse the epithelial barrier, degrading host-derived chemokines secreted basolaterally in response to infection. CPs become active following proteolytic maturation of pro-cathepsins, a process facilitated either spontaneously or under acidic conditions, where the pro-peptide acts as a chaperone for folding and activity regulation (Pungercar et al. 2009). Recombinant CPs show optimal enzymatic activity at pH 5.5-6.0 and preferentially cleave the fluorescent substrate FR-AMC (Kissoon-Singh et al. 2011). The cooperative effects of CPs during *Giardia*-host interactions require further investigation, particularly in the context of barrier integrity. Disruption of epithelial junctional architecture, including the mislocalization of proteins such as ZO-1, occludin, α-actinin, and F-actin, has been linked to increased permeability in vitro. Additionally, reduced claudin-1 expression in chronic giardiasis highlights the role of CPs in compromising intestinal barrier function.

Giardia CPs play a crucial role in pathogenesis by degrading key host components such as collagen I, a major structural element of the intestinal extracellular matrix and immune mediators like chemokines. During early infection, intestinal epithelial cells (IECs) upregulate chemokine transcripts, notably CCL20, CCL2 and CXCL1-3, which are involved in recruiting dendritic cells, macrophages, neutrophils, and T cells to the infection site. However, despite strong transcriptional induction, secreted levels of CCL20 remain low, suggesting regulation at the post-transcriptional or protein degradation level (Roxström-Lindquist et al. 2005). Previous studies have shown that *Giardia* CPs can degrade IL-8, thereby reducing neutrophil chemotaxis. Our study demonstrated that recombinant CPs exhibit cathepsin B-like activity, degrading junctional proteins and chemokines, thereby compromising epithelial barrier function and dampening immune responses. These findings highlight the virulence potential of *Giardia* CPs and support further investigation into their specific roles in disease progression using in vivo models.

5. Conclusion

This study reports the successful cloning and expression of the *Giardia* cathepsin B gene, along with the generation of a specific antibody against the expressed protein. These findings lay the groundwork for future investigations focused on proteomic-level analysis of gene expression dynamics during *Giardia* pathogenesis.

References

[1] Ankarklev J, Jerlström-Hultqvist J, Ringqvist E, Troell K, Svärd SG. Behind the smile: cell biology and disease mechanisms of *Giardia* species. *Nat Rev Microbiol*. 2010; 8(6):413-22.
[2] Haldar T, Sardar SK, Ghosal A, Das K, Saito-Nakano Y, Dutta S, Nozaki T, Ganguly S. Unveiling the anti-giardial properties of Andrographis paniculata leaf extract through in vitro Studies. *Trop Biomed*. 2024; 41(3): 1-9.
[3] Certad G, Viscogliosi E, Chabe M, et al. Pathogenic mechanisms of Cryptosporidium and *Giardia*. *Trends Parasitol*. 2017; 33:561–76.
[4] Einarsson E, Ma'ayeh S, Svard SG. An up-date on *Giardia* and Giardiasis. *Cur Opin Microbiol*. 2016; 34:47–52.
[5] Mmbaga BT, Houpt ER. Cryptosporidium and *Giardia* infections in children: A REview. *Pediatr clin North Am*. 2017; 64:837–50
[6] Cotton JA, Amat CB, Buret AG. Disruptions of host immunity and inflammation by *Giardia Duodenalis*: Potential consequences for co-infections in the Gastro Intestinal Tract. *Pathogens*. 2015; 4:764–92
[7] Rodriguez-Fuentes GB, Cedillo-Rivera R, Fonseca-Linan R, et al. *Giardia duodenalis*: analysis of secreted proteases upon trophozoite-epithelial cell interaction in vitro. *Mem do Inst Oswaldo Cruz*. 2006; 101:693–6
[8] Chin AC, Teoh DA, Scott KG, et al. Strain-dependent induction of enterocyte apoptosis by *Giardia lamblia* dis rupts epithelial barrier function in a caspase-3-dependent manner. *Infect Immun*. 2002; 70:3673–8
[9] Cotton JA, Bhargava A, Ferraz JG et al. *Giardia duodenalis* cathepsin B proteases degrade intestinal epithelial interleukin-8 and attenuate interleukin-8-induced neu trophil chemotaxis. *Infect Immun.* 2014;82(7):2772–87
[10] Manko A, Motta JP, Cotton J, Feener T, Oyeyemi A, Vallance BA, et al. *Giardia* co-infection promotes the secretion of antimicrobial peptides beta-defensin 2 and trefoil factor 3 and attenuates attaching and effacing bacteria-induced intestinal disease. *PloS ONE*. 2017; 12: e0178647.
[11] DuBois KN, Abodeely M, Sakanari J, et al. Identification of the major cysteine protease of *Giardia* and its role in encystation. J Biol Chem. 2008; 283:18024–31
[12] Haldar T, Sardar SK, Ghosal A, Prasad A, Nakano YS, Dutta S, Nozaki T, Ganguly S. Andrographolide induced cytotoxicity and cell cycle arrest in *Giardia* trophozoites. *Exp Parasitol*. 2024 Jul; 262:108773.
[13] Bhargava A, Cotton JA, Dixon BR, et al. *Giardia duodenalis* surface cysteine proteases induce cleavage of the intestinal epithelial cytoskeletal protein villin via myosin light chain kinase. *Plos One*. 2015; 10:e0136102
[14] Ringqvist E, Avesson L, Söderbom F, Svärd SG. Transcriptional changes in *Giardia* during host-parasite interactions. *Int J Parasitol*. 2011 Mar; 41(3-4):277-85.
[15] Mach L, Mort JS, Glossl J. Maturation of human proca thepsin B. Proenzyme activation and proteolytic process ing of the precursor to the mature proteinase, in vitro, are primarily unimolecular processes. *J Biol Chem*. 1994; 269:13030–5.
[16] Pungercar JR, Caglic D, Sajid M, et al. Autocatalytic proc essing of procathepsin B is triggered by proenzyme activity. *Febs J*. 2009; 276:660–8
[17] Kissoon-Singh V, Mortimer L, Chadee K. Entamoeba his tolytica cathepsin-like enzymes: Interactions with the host gut. *Adv exp med biol*. 2011; 712:62–83
[18] Roxström-Lindquist K, Ringqvist E, Palm D, Svärd S. *Giardia lamblia*-induced changes in gene expression in differentiated Caco-2 human intestinal epithelial cells. *Infect Immun*. 2005 Dec; 73(12):8204-8.