Published in final edited form as:

Mol Biochem Parasitol. 2013 September ; 191(1): . doi:10.1016/j.molbiopara.2013.07.003.

Characterization of the Phytochelatin Synthase from the Human Parasitic Nematode *Ancylostoma ceylanicum*

Coraline Rigouin^{1,†}, Jon J. Vermeire², Elyse Nylin¹, and David L. Williams^{1,*}

¹Department of Immunology/Microbiology, Rush University Medical Center, 1735 West Harrison Street, Chicago, IL 60612, United States

²Program in International Child Health and Department of Pediatrics, Yale University School of Medicine, New Haven, CT, United States

Abstract

Hookworm disease is a debilitating worm infection that affects hundreds of millions of people. Despite the existence of anthelmintic drugs, reports have testified of a decrease in efficacy of these drugs. Therefore, it is imperative to find new drugs and drug targets for hookworm disease treatment. In this study we identify the gene encoding the phytochelatin synthase in the human hookworm, *Ancylostoma ceylanicum* (AcePCS). Phytochelatin synthase catalyzes the production of metal chelating peptides, the phytochelatins, from glutathione (GSH). In plants, algae, and fungi phytochelatin production is important for metal tolerance and detoxification. Phytochelatin synthase proteins also function in the elimination of xenobiotics by processing GSH *S*-conjugates. We found that *in vitro* AcePCS could both synthesize phytochelatins and hydrolyze a GSH *S*-conjugate. Interestingly, the enzyme works through a thiol-dependant and, notably, metal-independent mechanism for both transpeptidase (phytochelatin synthesis) and peptidase (hydrolysis of GSH *S*-conjugates) activities. AcePCS mRNAs are expressed *in vivo* throughout the life cycle of *A. ceylanicum*. Mature adult male hookworms isolated from the small intestines of their hosts displayed significantly enhanced expression of AcePCS with transcript levels 5-fold greater than other developmental forms. Although the role of AcePCS in *A. ceylanicum* biology has yet to be fully investigated the results reported here provide encouraging evidence of the potential that this enzyme holds as a target for new chemotherapeutic intervention.

Keywords

Glutathione; Hookworm; Metal toxicity; Neglected tropical disease; Phytochelatin; Xenobiotic metabolism

© 2013 Elsevier B.V. All rights reserved.

*To whom correspondence can be addressed: Telephone: 1-312-942-1375; david_l_williams@rush.edu.

†Current address: Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, IL 60607, United States

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The parasite *Ancylostoma ceylanicum* expresses a phytochelatin synthase throughout its life cycle. AcePCS is capable of synthesizing phytochelatins and hydrolyzing GSH *S*-conjugates through a thiol-dependant and metal-independent mechanism.

1. Introduction

Phytochelatin synthase (PCS) proteins are γ -glutamylcysteine dipeptidyltranspeptidases (EC 2.3.2.15). These transpeptidases belong to the papain superfamily of cysteine proteases and function through a mechanism of deglycination of glutathione (γ -Glu-Cys-Gly; GSH) involving a catalytic amino acid triad Cys-His-Asp [1]. Phytochelatins are synthesized from GSH with the general structure (γ -Glu-Cys) $_n$ -Gly (where PC₂ is a polymer with $n=2$; PC₃, $n=3$; etc.). Phytochelatin synthases can also hydrolyze glutathione *S*-conjugates to produce the corresponding γ -Glu-Cys-*S*-conjugates. PCS proteins have been broadly described and characterized in plants, yeasts, algae, fungi and bacteria, as well as nematodes and trematodes [2–13].

Curiously, while PCS genes have been described from a multitude of metazoan organisms they are not found in any vertebrate species. Metazoan organisms produce metal-binding ligands such as metallothioneins and phytochelatins in order to sequester and physiologically respond to the cellular uptake and accumulation of metals from their environment [5, 7]. Phytochelatins are produced in response to exposure to a range of metal ions, especially cadmium, endowing the organisms with metal-tolerant phenotypes. Organisms deficient in PCS display hypersensitivity to cadmium or become unable to tolerate cadmium-induced toxicity [7, 12]. In addition to their established role in metal ion detoxification, PCS proteins are also involved in xenobiotic metabolism and detoxification in plants and fungi [14, 15]. After conjugation of GSH to electrophilic compounds by GSH *S*-transferases, GSH *S*-conjugates are excreted or further metabolized by PCS and/or other enzymes of the phase II xenobiotic degradation pathway. Recently, we reported the first description of a metazoan PCS capable of xenobiotic detoxification [16].

Hookworm disease in humans is caused by infection with the soil-transmitted nematodes (STNs) *Necator americanus* and *Ancylostoma* spp. Hookworm infection is counted among the ‘neglected tropical diseases’ (NTDs) along with several other maladies so named because they afflict the poorest global populations living predominantly in the developing world [17]. Historically, NTDs have received little attention in terms of preclinical research investment due in part to a lack of profitability for pharmaceutical companies in developing effective chemotherapeutics [17–19]. Hookworm disease is a debilitating infection that leads to blood loss and a resulting anemia in infected individuals. A conservative estimate suggests that nearly 600–900 million people suffer from hookworm infection around the world. Hookworm infection is a disease of morbidity, having a debilitating effect on those who are heavily infected. In terms of disability-adjusted life years (DALYs) hookworm disease accounts for approximately half of the global burden incurred by all NTDs combined [20]. The gold standard of hookworm disease treatment is chemotherapy with albendazole or mebendazole, both members of the benzimidazole class of anthelmintic drugs. Despite their availability, there is an inadequacy in the variety of drugs available to treat hookworm and other STN nematode infections. This fact coupled with reports of decreased efficacy of the drugs currently in use further exacerbates this situation [21, 22]. New parasite targets and candidate chemotypes are needed.

The development of novel therapies that selectively target essential metazoan pathogen proteins represents a unique challenge due to high levels of conservation amongst host and pathogen cellular and metabolic processes. As a result, compounds proposed to combat metazoan pathogen infections, including anthelmintics, have the potential to cause deleterious side effects due to a lack of target specificity [23]. Therefore, the identification of pathogen-specific targets is essential for the progression towards the development of less harmful and more effective chemotherapies. In this paper, we identified and characterized the PCS of *A. ceylanicum*. It shows high sequence identity with PCS proteins from

nematodes, trematodes, and cestodes with conservation of the catalytic triad of Cys, His, and Asp. Comparative analyses of mRNA abundance throughout hookworm development confirmed the presence of AcePCS transcripts in all hookworm life cycle stages investigated. Significantly higher AcePCS transcript levels were observed in adult male worms relative egg/L1 and L3 larval stages as well as mature female worms. The expression of the recombinant protein in *Escherichia coli* and its purification enabled us to obtain data on the catalytic mechanism of AcePCS. Most interestingly, we have demonstrated that AcePCS is capable of both transpeptidase and hydrolytic activities, through a thiol-dependent but metal-independent mechanism. These data establish a fundamental basis for understanding the functional role of AcePCS in hookworm biology that will contribute to future studies aimed at evaluating its' potential as a drug target.

2. Materials and methods

2.1. Cloning the *A. ceylanicum* PCS coding sequence

The amino acid sequence of *Caenorhabditis elegans* PCS (NP_496475) was queried against the National Center for Biotechnology (NCBI) expressed sequence tag (EST) database using the tBLASTn algorithm. Two *A. caninum* ESTs with significant similarity were identified: EY459888 with 73% identity to amino acids 7 to 131 and EY469621 with 54% identity to amino acids 223–369 of *C. elegans* PCS and including a potential termination codon. An *A. ceylanicum* L3 cDNA library was used as a PCR template for the amplification the *A. ceylanicum* (Ace) PCS open reading frame using Taq DNA polymerase with the forward primer (5'-CCAACATATACTTCAAGCTAGC-3') from EY459888 and a reverse primer (5'-CACAGCACTGCTCGAAAACAC-3') from EY469621. The start of the *A. ceylanicum* PCS open reading frame was obtained by 5 RACE using the M13 forward primer of pBlueScript II and a reverse, gene-specific primer (5'-GAGTCCACAATATGCGG-3') from the sequence obtained from the first PCR. In both cases, PCR products were cloned into pCRII TOPO TA vector (Invitrogen) and TOP 10 *Escherichia coli* were transformed following the manufacturer's protocol. Plasmids were isolated using the Qiagen mini plasmid isolation kit and sequenced at DNA service facility of University of Illinois at Chicago (www.uic.edu/depts/rrc/dnas/).

2.2. Real-time quantitative PCR (qPCR) analyses of AcePCS mRNA transcripts

For qPCR experiments, AcePCS mRNA transcript levels were quantified in *A. ceylanicum* cDNA samples obtained from mixed populations of eggs and first stage larvae (Eggs/L1), infectious third stage larvae (L3), and adult male and female worms. First strand cDNA was synthesized from hookworm total RNA for use in real-time quantitative PCR (qPCR) reactions. RNA was extracted directly from *A. ceylanicum* life cycle stages using the TRIzol® (Invitrogen) reagent according to the manufacturer's instructions and the resulting RNA pellet was resuspended in diethylpyrocarbonate-treated water. cDNA was synthesized using the iScript™ cDNA kit (Bio-Rad). Primers amplifying a 160 base pair segment of the AcePCS open reading frame and a portion of the 60S ribosomal RNA gene from *A. ceylanicum* were generated utilizing Primer Quest Software (<http://www.idtdna.com/Scitools/Applications/Primerquest/>). The decision to use 60S rRNA as a reference gene was based on previous work identifying 60S rRNA as a valid housekeeping gene, being expressed equally in all hookworm life cycle stages [24]. Briefly, adult male and female hookworms were harvested from the small intestines of Syrian hamsters 18 days post-infection as previously described [25]. The egg/L1 stage larvae were collected and purified from the feces of hookworm infected Syrian hamsters 18 days post-infection with *A. ceylanicum* L3 larvae. The L3 stage larvae were produced by collecting feces of infected Syrian hamsters containing eggs and incubating them in bone charcoal coprocultures at 27°C for 2 weeks followed by isolation of L3 on a Behrman apparatus. All of the parasites were extensively

washed with PBS prior to RNA extraction in Trizol reagent to remove contaminating host or culture material. A no RT control was run in all qRT PCR experiments which produced no detectable amplification product, indicating the absence of primer cross-reaction with contaminating from parasite, host or culture genomic DNA. We also performed PCR reactions using the hookworm-specific AcePCS primers and hamster pooled tissue (kidney, liver, spleen, small intestine) cDNA prepared from Syrian hamsters and observed no cross-reactive amplification or PCR product detectable by gel electrophoresis or qPCR.

PCR amplification was performed in a 96-well format using optical tube strips and caps in an MJ Research DNA Engine Opticon 2 PCR apparatus (Bio-Rad). Reaction mixtures contained 1 μ L cDNA, 200 nM of each primer and iQ SYBR Green Supermix (Bio-Rad). Fold differences were calculated using the 2^{-Ct} method [26]. 60s rRNA-normalized AcePCS data were statistically analyzed using a Student's t-Test. The maintenance and care of experimental animals complied with the National Institutes of Health guidelines for the humane use of laboratory animals and were approved by the Yale University Animal Care and Use Committee.

2.3. Recombinant AcePCS Protein Expression and Biochemical Characterization

The L3 cDNA library was used as a PCR template to amplify the complete AcePCS open reading frame using Pfu DNA polymerase (Stratagene) and gene-specific primers (forward 5'-CACCATGGTCTCTAGAGTCTTCGA3' and reverse 5'-TTACAATTTGATATCATT-3'). The PCR product was cloned into pET100 (Invitrogen). The sequence of recombinant plasmid construct was verified as described above.

The pET100 plasmid containing AcePCS was transformed into BL21 StarTM (DE3) *E. coli* (Invitrogen). An overnight culture in Luria broth containing 50 μ g/mL carbenicillin was used to inoculate a 1 L culture and the cells were grown at 37°C to an optical density at 600 nm of 0.5, at which time isopropyl 1-thio- β -D-galactoside was added to 0.2 mM and the culture was incubated overnight at 24°C to express the recombinant protein. Cells were collected by centrifugation at 4500 $\times g$ for 25 min at 4°C. The cells were resuspended in lysis buffer (50 mM potassium phosphate (pH 7.8), 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 30 mM imidazole, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mg/mL lysozyme) and disrupted after 3 freeze-thaw cycles by sonication. After centrifugation at 14000 $\times g$ for 40 min at 4°C, the supernatant was filtered and applied to a 1 mL HisTrapTM HP column (GE Healthcare Life Sciences) first equilibrated with binding buffer (lysis buffer without dithiothreitol and lysozyme). The column was washed first with 20 mL of binding buffer, then with 10 mL of buffer A (20 mM sodium phosphate (pH 7.5), 500 mM NaCl, 100 mM imidazole, 10 mM 2-mercaptoethanol (-ME) and the recombinant protein was eluted with 3 mL of elution buffer B (20 mM sodium phosphate (pH 7.5), 500 mM NaCl, 5% glycerol, 500 mM imidazole, 10 mM -ME). The elution fraction containing the recombinant AcePCS protein was dialyzed against elution buffer B without imidazole. Homogeneity of the purified proteins was confirmed by SDS PAGE. The concentration of proteins was measured using the Bio-Rad Protein Assay with BSA as the standard.

2.4. Enzyme assays

PCS activity was measured according to the method of Oven *et al.* [27] and as previously described [16]. The reaction mixture (180 μ L) contained 200 mM Tris-HCl (pH 8), 10 mM -Me, 10 mM GSH, 0.1 mM CdCl₂ and 3 μ g of AcePCS. PCS activity for PC₂ synthesis was measured, with the same conditions but in a final volume of 60 μ L. PCS activity was expressed as the amount of PC₂ synthesized by 1 mg of protein per minute and was measured in the first 5 min when the synthesis of PC_(n>2) was negligible. To measure PCS

activity with GSH *S*-conjugates, the GSH bimane conjugate (GSH *S*-bimane, 1 mM) was used in a reaction mixture (60 μ L) containing 200 mM Tris-HCl (pH 8), 10 mM γ -Me, 0.1 mM CdCl₂ and 3 μ g of AcePCS and incubated for 30 min. Synthesis of GSH *S*-bimane was done as previously described [16]. PCS activity was expressed as the amount of γ -Glu-Cys-*S*-bimane synthesized by 1 mg of protein per min. All the reactions were incubated at 37°C and were stopped by addition of an equal volume of 0.2 N HCl. After centrifugation at 12000 $\times g$ for 5 min, the supernatants were analyzed by LC-MS.

2.5. LC-MS

Ten μ L of each sample was injected onto a reverse-phase column (Eclipse Plus C18, Agilent) attached to an Agilent 1946A LC-MSD system, an Agilent 1100 HPLC LC system coupled to a single quadrupole LC-mass spectrometer equipped with electrospray ionization. Thiol compounds were separated by using solvent (A) 0.1% TFA and (B) 100% acetonitrile at a flow rate of 0.5 mL/min. The gradient was from 2% to 20% of solvent (B) over 20 min. Before injecting a new sample, the column was washed (4 min 98% B) and equilibrated (4 min 2% B). The integrated peak area was used to quantify the PC levels after calibration with chemically synthesized PC₂, PC₃, PC₄ (AnaSpec) and γ -Glu-Cys-*S*-bimane.

3. Results and Discussion

3.1 Identification of the AcePCS sequence

Using the *C. elegans* PCS sequence we identified two *A. caninum* ESTs encoding partial PCS sequences. Using these EST sequences the entire AcePCS open reading frame was successfully amplified from an *A. ceylanicum* L3 cDNA library. The sequence was deposited at NCBI with the accession number KC914882. The AcePCS sequence encodes a predicted protein of 425 amino acids with a theoretical pI of 7.77 and Mw of 47,657 Daltons. Alignment of the PCS domain of AcePCS (the N-terminal half of the protein) with PCS domains from nematodes, trematodes and cestodes indicates that there is high sequence identity and conservation of the catalytic triad of Cys, His, and Asp found in other PCS proteins (Fig. 1). The four Cys residues in the N-terminal portion of eukaryotic PCS proteins are present in AcePCS. The sequence displays an additional 15 Cys residue in the C-terminal domain (approximately amino acids 220–425). This domain is generally less well conserved between PCS proteins and is present only in eukaryotic, but not prokaryotic, PCS proteins. Although the function of this domain is not completely understood, it was suggested to function in metal regulation of protein activity in the *Arabidopsis thaliana* PCS model [28]. However, recent data investigating the PCS protein from *S. mansoni* showed that the C-terminal domain is neither involved in metal sensitivity nor in the modulation of the protein activity [16]. A survey of available helminth genomes (GeneDB at the Sanger Institute (genedb.org), NEMBASE4 (nematodes.org), The Genome Institute (nematode.net) and NCBI) found PCS genes to be widely present (Fig. 1). Single copy PCS genes were identified in other Clade V nematodes including parasitic (*Haemonchus contortus*, *Heligmosomoides polygyrus* and *Necator americanus*) as well as free-living members. *Necator americanus* is the other major species of human hookworm. PCS genes were also present in Clade III nematodes including the pig parasite *Ascaris suum* (a close relative of the human ascarid, *A. lumbricoides* causative agent of the important STN disease ascariasis) and the human filarial parasites *Loa loa* (loiasis, African eye worm), *Brugia malayi* and *Wuchereria bancrofti* (lymphatic filariasis, elephantiasis), and *Onchocerca volvulus* (onchocerciasis, river blindness). PCS genes appear to be absent from the genomes of Clade I, IVa, and IVb nematodes. These clades include the important human parasites *Strongyloides stercoralis*, *Trichuris trichiura* and *Trichinella spiralis*.

In addition, parasitic flukes (phylum Platyhelminthes, class Trematoda), such as *Schistosoma mansoni*, *S. japonicum*, *S. haematobium*, and *Clonorchis sinensis*, and tapeworms (phylum Platyhelminthes, class Cestoda), such as *Echinococcus granulosus*, *E. multilocularis*, and *Taenia solium*, each possess a single PCS gene [8, 29]. Human helminth parasites are often co-endemic and many individuals are infected with multiple helminth species. Therefore, since PCS genes are present as single copy genes encoding highly conserved proteins in a wide variety of parasitic helminths across widely divergent phyla, it may be possible to develop a broad-acting, PCS-targeted chemotherapy targeting multiple worms simultaneously. Given the financial limitations for programs to control NTDs, such a broad-spectrum therapy could greatly facilitate their efforts.

3.2. Expression analysis of AcePCS

Expression of the AcePCS gene was evaluated by measuring mRNA abundance in different stages of the worm life cycle using qPCR (Fig. 2). AcePCS mRNAs were detected in all investigated life stages of the parasite (eggs/L1, L3 stages and adults) and AcePCS transcript levels were found to be significantly higher in adult male worms compared to eggs/L1, L3 larvae and female adult worms (5-fold; $p < 0.01$, $p < 0.01$, $p = 0.0107$, respectively). This suggests that the enzyme may play a crucial role *in vivo* for adult males at the host-parasite interface during bloodfeeding or in the developmental or reproductive biology of male *A. ceylanicum* hookworms in the host small intestine. PCS expression in filarial nematodes, causative agents of lymphatic filariasis, can be explored *in silico*. Analysis of *B. malayi* expression profiles generated by deep sequencing mRNAs [30] shows that PCS mRNA is present in L3, L4, adult male, adult female, egg/embryo, and microfilarial life cycle stages with 4442, 681, 673, 1070, 970, 320 RPKMs (Reads Per Kilobase of exon model per Million mapped reads), respectively. PCS expression in adult filarial worms suggests that compounds targeting PCS may be active against this stage, which is not targeted by current therapies.

3.3. AcePCS activity

Recombinant AcePCS was successfully expressed in *E. coli* and purified to homogeneity by Ni^{2+} -affinity chromatography. Recombinant AcePCS was found to form homodimers by gel filtration chromatography (data not shown). AcePCS was active in phytochelatin synthesis (Fig. 3). In the first 5 min of the reaction, AcePCS mainly synthesizes PC_2 . Significant amount of PC_3 and PC_4 appear after 10 min incubation. After 20 min, the synthesis of PC_2 , PC_3 and PC_4 slow down. Higher molecular weight species of phytochelatin (PC_5 , PC_6 , and PC_7) were detected at 90 min (data not shown). During the time course analysis, no accumulation of γ -Glu-Cys was detected. Taken together, these data suggest that once cleaved from GSH, γ -Glu-Cys is immediately consumed for the synthesis of phytochelatins and that once synthesized, the phytochelatin species serve as substrate for the synthesis of higher molecular weight species. Hence, these data suggest that *in vitro*, AcePCS synthesizes phytochelatins by the same mechanism as other PCS proteins [11, 16].

To investigate the influence of metals and thiols on AcePCS activation, we followed PC_2 synthesis with either GSH or *S*-methyl-GSH, with or without cadmium, and with or without the reducing agent β -Me (Fig. 4). The use of *S*-methyl-GSH as sole substrate for the enzyme allows us to evaluate the enzyme activity without any reducing agent (GSH through its thiol group can play the role of reducing agent). We found that AcePCS was capable of synthesizing PC_2 from GSH in all conditions tested, with no significant difference of activity between each condition. However, when *S*-methyl-GSH is used as substrate and β -Me is not added, a significant inhibition of the enzyme occurs ($35\% \pm 10\%$ residual activity). Interestingly, the addition of metal in the absence of reducing agent almost completely abolishes enzyme activity ($3\% \pm 3\%$ residual activity). Therefore, the presence of a reducing

agent is necessary for efficient AcePCS transpeptidase activity. The rate of PC₂ synthesis by AcePCS in the presence of cadmium and -Me (1.1 ± 0.1 SH $\mu\text{mol mg}^{-1}$ protein min^{-1}) was found to be identical to the rate of SmPCS [12] and close to the rate of the Nostoc PCS [9]. It is however, less active than the *Arabidopsis thaliana* PCS, whose activity is 14 $\mu\text{mol mg}^{-1}$ protein min^{-1} [32].

We further investigated whether the enzyme was able to act as a peptidase on GSH *S*-conjugates. Monobromobimane (bimane), a compound that labels thiols, was used to prepare the GSH *S*-conjugate GS-bimane, and was tested as substrate for AcePCS. The reactions were analyzed by LC-MS for the appearance of the product -Glu-Cys-*S*-bimane after 30 minutes of incubation. We found that AcePCS cleaved the glycine from the GS-bimane conjugate to give the corresponding -Glu-Cys-*S*-conjugate (Fig. 4). As for the transpeptidase activity, we compared the hydrolytic activity of the enzyme in different conditions, with 100% enzyme activity being attributed to the activity measured in the condition with metal (0.1 mM CdCl₂) and the reducing agent (10 mM -Me). We found that metal did not significantly influence the activity of the AcePCS enzyme when the reducing agent is present ($77.5\% \pm 25\%$ without metal). However, in the absence of the reducing agent (no -Me) we measured an important inhibition of AcePCS; activity was found to be only $0.6\% \pm 0.3\%$ (with or without metal) (Fig. 4). Therefore, AcePCS catalysis must occur in a reducing environment for efficient hydrolytic activity with GS-bimane.

AcePCS does not display the metal dependency that most PCS enzymes display. This is the second description of a helminth PCS with no metal requirement for its activity with GSH [16]. Instead, the presence of thiols in the PC synthase reaction mixture was found to be essential for PCS activation, as previously described for other PCS enzymes [11, 16, 27]. It is thus unlikely that AcePCS is activated by direct interaction between the metal and the enzyme as previously suggested [27, 31]. In addition, the requirement for blocked thiols and particularly GS-metal complexes to serve as substrates [32] does not apply to AcePCS. The characterization of this new PCS protein highlights once again the complexity of this family of enzymes and provides a better understanding of PCS function in parasitic helminths.

4. Conclusions

We present here the identification of the gene encoding the PCS protein of *A. ceylanicum* and characterization of the recombinant protein. *In vitro*, recombinant AcePCS is capable of both transpeptidase and hydrolytic activities, through a thiol-dependant but metal-independent mechanism. This is the second description of a PCS from a parasitic helminth with no metal co-factor dependence for activation. Although the role of the enzyme in *A. ceylanicum* biology remains to be explored in more detail, the non-metal dependent activation and the low enzyme rate suggests a role for AcePCS in a pathway not necessarily related to direct metal detoxification. Rather, AcePCS may be responsible for xenobiotic and/or oxidative stress elimination, as it has been suggested for the PCS of *S. mansoni* [16]. Taken together, the ubiquitous expression of PCS genes throughout the life cycle of *A. ceylanicum* and other parasitic nematodes and the enhanced expression of AcePCS observed in mRNA populations of mature, bloodfeeding male hookworms suggests a major *in vivo* role for the enzyme in parasite development and biology. Finally, PCS is absent from the human genome; it raises great interest as a parasite-specific target for new chemotherapeutic interventions.

Acknowledgments

We thank Dr. Michelle L. Michalski for analysis of PCS expression in the *B. pahangi* deep sequencing database and Dr. Julian Nomme for performing the gel filtration chromatographic analysis.

Funding

These studies were supported in part by National Institutes of Health Health-National Institute of Allergy and Infectious Diseases (NIH-NIAID) awards R21AI097529 and R21AI081107 (DLW) and NIH Career Development Award K22 A08476 (JJV). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abbreviations

-ME	2-mercaptoethanol
AcePCS	<i>Ancylostoma ceylanicum</i> phytochelatin synthase
EST	expressed sequence tag
GSH	glutathione
NTD	neglected tropical disease
PCS	phytochelatin synthase
qPCR	real-time quantitative PCR
STNs	soil-transmitted nematodes

References

1. Vivares D, Arnoux P, Pignol D. A papain-like enzyme at work: native and acyl-enzyme intermediate structures in phytochelatin synthesis. *Proc Natl Acad Sci U S A*. 2005; 102:18848–53. [PubMed: 16339904]
2. Brautigam A, Schaumloffel D, Preud'homme H, Thondorf I, Wesenberg D. Physiological characterization of cadmium-exposed *Chlamydomonas reinhardtii*. *Plant Cell Environ*. 2011; 34:2071–82. [PubMed: 21819413]
3. Brulle F, Cocquerelle C, Wamalah AN, Morgan AJ, Kille P, Lepretre A, et al. cDNA cloning and expression analysis of *Eisenia fetida* (Annelida: Oligochaeta) phytochelatin synthase under cadmium exposure. *Ecotoxicol Environ Saf*. 2008; 71:47–55. [PubMed: 18083232]
4. Clemens S, Schroeder JI, Degenkolb T. *Caenorhabditis elegans* expresses a functional phytochelatin synthase. *Eur J Biochem*. 2001; 268:3640–3. [PubMed: 11432729]
5. Cobbett C, Goldsbrough P. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annu Rev Plant Biol*. 2002; 53:159–82. [PubMed: 12221971]
6. Ha SB, Smith AP, Howden R, Dietrich WM, Bugg S, O'Connell MJ, et al. Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. *Plant Cell*. 1999; 11:1153–64. [PubMed: 10368185]
7. Pal R, Rai JP. Phytochelatins: peptides involved in heavy metal detoxification. *Appl Biochem Biotechnol*. 2010; 160:945–63. [PubMed: 19224399]
8. Ray D, Williams DL. Characterization of the phytochelatin synthase of *Schistosoma mansoni*. *PLoS Negl Trop Dis*. 2011; 5:e1168. [PubMed: 21629724]
9. Rea PA, Vatamaniuk OK, Rigden DJ. Weeds, worms, and more. Papain's long-lost cousin, phytochelatin synthase. *Plant Physiol*. 2004; 136:2463–74. [PubMed: 15375203]
10. Tsuji N, Hirayanagi N, Okada M, Miyasaka H, Hirata K, Zenk MH, et al. Enhancement of tolerance to heavy metals and oxidative stress in *Dunaliella tertiolecta* by Zn-induced phytochelatin synthesis. *Biochem Biophys Res Commun*. 2002; 293:653–9. [PubMed: 12054653]
11. Tsuji N, Nishikori S, Iwabe O, Shiraki K, Miyasaka H, Takagi M, et al. Characterization of phytochelatin synthase-like protein encoded by alr0975 from a prokaryote, *Nostoc* sp. PCC 7120. *Biochem Biophys Res Commun*. 2004; 315:751–5. [PubMed: 14975765]
12. Vatamaniuk OK, Bucher EA, Ward JT, Rea PA. A new pathway for heavy metal detoxification in animals. Phytochelatin synthase is required for cadmium tolerance in *Caenorhabditis elegans*. *J Biol Chem*. 2001; 276:20817–20. [PubMed: 11313333]

13. Zenk MH. Heavy metal detoxification in higher plants--a review. *Gene*. 1996; 179:21–30. [PubMed: 8955625]
14. Blum R, Beck A, Korte A, Stengel A, Letzel T, Lendzian K, et al. Function of phytochelatase synthase in catabolism of glutathione-conjugates. *The Plant Journal : for cell and molecular biology*. 2007; 49:740–9. [PubMed: 17253989]
15. Wunschmann J, Krajewski M, Letzel T, Huber EM, Ehrmann A, Grill E, et al. Dissection of glutathione conjugate turnover in yeast. *Phytochemistry*. 2009
16. Rigouin C, Nylin E, Cogswell AA, Schaumlöffel D, Dobritsch D, Williams DL. Towards an understanding of the function of the phytochelatase synthase of *Schistosoma mansoni*. *PLoS Negl Trop Dis*. 2013; 7:e2037. [PubMed: 23383357]
17. Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. Helminth infections: the great neglected tropical diseases. *J Clin Invest*. 2008; 118:1311–21. [PubMed: 18382743]
18. Hotez PJ, Molyneux DH, Fenwick A, Ottesen E, Ehrlich Sachs S, Sachs JD. Incorporating a rapid-impact package for neglected tropical diseases with programs for HIV/AIDS, tuberculosis, and malaria. *PLoS Med*. 2006; 3:e102. [PubMed: 16435908]
19. Hotez PJD, E.; Heffernan, MJ.; Bottazzi, ME. Innovation for the ‘Bottom 100 Million’: Eliminating Neglected Tropical Diseases in the Americas. In: Curtis, NF.; Finn, A.; Pollard, AJ., editors. *Hot Topics in Infection and Immunity in Children IX*. Springer; 2013.
20. Fenwick A. The global burden of neglected tropical diseases. *Public health*. 2012; 126:233–6. [PubMed: 22325616]
21. Hotez PJ, Bethony JM, Diemert DJ, Pearson M, Loukas A. Developing vaccines to combat hookworm infection and intestinal schistosomiasis. *Nat Rev Microbiol*. 2010; 8:814–26. [PubMed: 20948553]
22. Keiser J, Utzinger J. The drugs we have and the drugs we need against major helminth infections. *Adv Parasitol*. 2010; 73:197–230. [PubMed: 20627144]
23. Denning DW, Hope WW. Therapy for fungal diseases: opportunities and priorities. *Trends Microbiol*. 2010; 18:195–204. [PubMed: 20207544]
24. Trivedi S, Arasu P. Evaluation of endogenous reference genes for real-time PCR quantification of gene expression in *Ancylostoma caninum*. *Mol Biochem Parasitol*. 2005; 143:241–4. [PubMed: 15993958]
25. Bungiro RD Jr, Greene J, Kruglov E, Cappello M. Mitigation of hookworm disease by immunization with soluble extracts of *Ancylostoma ceylanicum*. *J Infect Dis*. 2001; 183:1380–7. [PubMed: 11294670]
26. Schefe JH, Lehmann KE, Buschmann IR, Unger T, Funke-Kaiser H. Quantitative real-time RT-PCR data analysis: current concepts and the novel “gene expression’s CT difference” formula. *J Mol Med (Berl)*. 2006; 84:901–10. [PubMed: 16972087]
27. Oven M, Page JE, Zenk MH, Kutchan TM. Molecular characterization of the homo-phytochelatase synthase of soybean *Glycine max*: relation to phytochelatase synthase. *J Biol Chem*. 2002; 277:4747–54. [PubMed: 11706029]
28. Romanyuk ND, Rigden DJ, Vatamaniuk OK, Lang A, Cahoon RE, Jez JM, et al. Mutagenic definition of a papain-like catalytic triad, sufficiency of the N-terminal domain for single-site core catalytic enzyme acylation, and C-terminal domain for augmentative metal activation of a eukaryotic phytochelatase synthase. *Plant Physiol*. 2006; 141:858–69. [PubMed: 16714405]
29. Tsai IJ, Zarowiecki M, Holroyd N, Garcarrubio A, Sanchez-Flores A, Brooks KL, et al. The genomes of four tapeworm species reveal adaptations to parasitism. *Nature*. 2013; 496:57–63. [PubMed: 23485966]
30. Choi YJ, Ghedin E, Berriman M, McQuillan J, Holroyd N, Mayhew GF, et al. A deep sequencing approach to comparatively analyze the transcriptome of lifecycle stages of the filarial worm, *Brugia malayi*. *PLoS Negl Trop Dis*. 2011; 5:e1409. [PubMed: 22180794]
31. Ogawa S, Yoshidomi T, Yoshimura E. Cadmium (II)-stimulated enzyme activation of *Arabidopsis thaliana* phytochelatase 1. *J Inorg Biochem*. 2011; 105:111–7. [PubMed: 21134609]
32. Vatamaniuk OK, Mari S, Lu YP, Rea PA. Mechanism of heavy metal ion activation of phytochelatase (PC) synthase: blocked thiols are sufficient for PC synthase-catalyzed

transpeptidation of glutathione and related thiol peptides. J Biol Chem. 2000; 275:31451–9.
[PubMed: 10807919]

Highlights

- *Ancylostoma ceylanicum* possesses a gene encoding a phytochelatin synthase (AcePCS)
- High sequence identity and conservation of the catalytic triad is found in AcePCS
- *A. ceylanicum* PCS mRNA present in all life stages with highest level in adult males
- AcePCS is capable of synthesizing phytochelatins and hydrolyzing GSH *S*-conjugates
- AcePCS activities use a thiol-dependant and metal-independent mechanism

Figure 1. *Ancylostoma ceylanicum*

phytochelatin synthase (PCS) displays high amino acid sequence similarity to phytochelatin synthases from other parasitic helminths. An alignment of the N-terminal domain containing the conserved active sites of PCS proteins from parasitic helminths was created using ClustalW and produced with Boxshade. PCS amino acid sequences included in the alignment are Ace, *Ancylostoma ceylanicum* (KC914882); Bma, *Brugia malayi* (XP_001902100); Llo, *Loa loa* (XP_003139039); Asu, *Ascaris suum* (CDS_Asu|GS_24101 from Nematode net plus ADY48387); Sma, *Schistosoma mansoni* (XP_002569764); and Egr, *Echinococcus granulosus* (EgrG_000255400 from GeneDB at The Sanger Institute). The predicted active site Cys, His, and Asp (Glu in Egr) residues are highlighted with circles (•). Conserved residues are shown in reverse font; conservative changes are shown in grey. The C-terminal regions of these proteins are less well conserved and were not shown in the alignment.

Figure 2. *Ancylostoma ceylanicum*

phytochelatin synthase (AcePCS) mRNA is expressed throughout the hookworm life cycle and at significantly higher levels in adult male hookworms. AcePCS mRNA transcript levels were quantified in cDNA samples obtained from mixed populations of *A. ceylanicum* eggs and first stage larvae (Eggs/L1), infectious third stage larvae (L3), and adult male and female worms by qPCR. The 60S RNA gene from *A. ceylanicum* was used as a control transcript. Fold differences in AcePCS mRNA abundance were calculated using the 2^{-Ct} method for the purposes of graphical representation. Normalized qPCR data was statistically analyzed using a Student's t-Test.

Figure 3. *Ancylostoma ceylanicum*

PCS (AcePCS) is capable of synthesizing PC₂, PC₃, and PC₄ *in vitro*. The kinetics of phytochelatin polymer (PC₂, PC₃, and PC₄) synthesis was measured *in vitro* as described in Materials and methods. The reaction mixture contained 200 mM Tris-HCl (pH 8), 10 mM - Me, 10 mM GSH, 0.1 mM CdCl₂, and 4 μg of purified AcePCS. AcePCS-mediated synthesis of PC₂ (○), PC₃ (□), and PC₄ (△) are expressed in μmol per mg enzyme, as quantified by LC-MS.

Figure 4. *Ancylostoma ceylanicum*

PCS (AcePCS) activity is metal independent. PC₂ synthesis (black bars), *S*-methyl-PC₂ (grey bars), and glutathione *S*-bimane hydrolysis (open bars). The incubation mixture contained 200 mM Tris-HCl (pH 8), 10 mM GSH, 10 mM *S*-methyl-glutathione, or 1 mM glutathione *S*-bimane, and 3 μg of purified AcePCS, with 0.1 mM CdCl₂ and 10 mM -mercaptoethanol (-Me) as specified. PC₂ and *S*-methyl-PC₂ synthesis and glutathione *S*-bimane hydrolysis were quantified by LC-MS. Mean value of three separate assays expressed as activity as a percentage of the reaction with 100 μM CdCl₂ and 10 mM -Me added ± SE.