Original Article

Cloning and characterization of a selenium-independent glutathione peroxidase (HC29) from adult *Haemonchus contortus*

Wei Sun, Xiaokai Song, Ruofeng Yan, Lixin Xu, Xiangrui Li*

College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, Jiangsu, China

The complete coding sequence of *Haemonchus* (*H.*) *contortus* HC29 cDNA was generated by rapid amplification of cDNA ends in combination with PCR using primers targeting the 5′- and 3′-ends of the partial mRNA sequence. The cloned HC29 cDNA was shown to be 1,113 bp in size with an open reading frame of 507 bp, encoding a protein of 168 amino acid with a calculated molecular mass of 18.9 kDa. Amino acid sequence analysis revealed that the cloned HC29 cDNA contained the conserved catalytic triad and dimer interface of selenium-independent glutathione peroxidase (GPX). Alignment of the predicted amino acid sequences demonstrated that the protein shared 44.7∼80.4% similarity with GPX homologues in the thioredoxin-like family. Phylogenetic analysis revealed close evolutionary proximity of the GPX sequence to the counterpart sequences. These results suggest that HC29 cDNA is a GPX, a member of the thioredoxin-like family. Alignment of the nucleic acid and amino acid sequences of HC29 with those of the reported selenium-independent GPX of *H. contortus* showed that HC29 contained different types of spliced leader sequences as well as dimer interface sites, although the active sites of both were identical. Enzymatic analysis of recombinant prokaryotic HC29 protein showed activity for the hydrolysis of H_2O_2. These findings indicate that HC29 is a selenium-independent GPX of *H. contortus*.

Keywords: glutathione peroxidase, *Haemonchus contortus*, HC29 cDNA

Introduction

The most pressing issue in the control of parasites using anthelmintics and vaccines is rating the attractiveness of particular enzymes and metabolic pathways with respect to the likelihood of identifying specific and therapeutically useful inhibitors or potential antigen candidates. In mammals, reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radical act as important defensive factors against parasites. Such factors damage parasite lipids, proteins, and nucleic acids, eventually causing death of the parasite. However, parasites do produce some antioxidant enzymes that cleave ROS, constituting a defense mechanism. Glutathione peroxidase (GPX) is one of the essential antioxidant enzymes in parasites [15,26]. In particular, GPXs in parasites differ from those in mammals, which contain both selenium-dependent and selenium-independent forms. Selenium-dependent GPXs play a fundamental role in protecting against ROS, whereas selenium-independent GPXs function as a backup system [15]. In contrast, the selenium-independent GPX family is the main one in parasites, with selenium-dependent GPXs basically absent [5,26]. In addition to its breaking down ROS from hosts, parasite GPXs also play roles in protecting cells against the deleterious effects of reactive oxidants generated during aerobic metabolism [9,27]. Therefore, based on their critical functions in parasite survival and metabolism, selenium-independent GPXs could be considered as potential chemotherapeutic targets and vaccine candidates for parasite control [12].

Haemonchus (*H.*) *contortus* is a blood-ingesting nematode affecting ruminants that causes major losses to the global agricultural industry every year [16,24]. Control of *H. contortus* has thus far been carried out using anthelmintics and grazing management. However, the excessive and uncontrolled application of chemical drugs has resulted in the emergence of anthelmintic-resistant strains of the parasite, toxic residues in the human food chain, and environmental pollution [10,25]. These undesirable effects have further led to attempts to better understand the biology of *H. contortus*, with the eventual goal of developing

*Corresponding author: Tel: +86-25-84399000; Fax: +86-25-84398669; E-mail: lixiangrui@njau.edu.cn

alternate or supplementary means of control, including the development of molecular vaccines. Several enzymatic antioxidants have been considered as vaccine candidates in other parasites, including superoxide dismutase (SOD) for *Schistosoma* (*S.*) *mansoni*, glutathione S-transferase for *S. mansoni*, and peroxidoxin for *Fasciola hepatica* [6,21]. In *H. contortus*, a combination of cytosolic and extracellular SODs has been tested for its potential to protect lambs from *H. contortus* infection, demonstrating a slight reduction in worm burdens [20].

HC29 was originally identified in a study on differential gene regulation during *H. contortus* development using RNA arbitrarily-primed PCR [14]. *In situ* analysis of adult parasites revealed expression of HC29 in all examined organs of *H. contortus*, particularly in the intestinal microvilli and muscle cells lining the cuticle. The previously published partial sequence of HC29 EST in *H. contortus* (380 bp, accession No. AF305967) possesses significant similarity with *Caenorhabditis elegans* GPX R03G5.5 (accession No. U51994), namely 72% identity (85% similarity) at the amino acid level over 48 residues, and therefore could be a GPX molecule. Further, another GPX of *H. contortus* (accession No. AY603337) was identified previously. However, protein sequence analysis indicated that it is disparated from HC29 EST [2]. Until now, the full sequence and protein characteristics of *H. contortus* HC29 have not been reported.

In this research, the full-length cDNA sequence of *H. contortus* HC29 along with the enzyme activity of the recombinant protein were evaluated.

Materials and Methods

Parasite material and RNA preparation

Adult *H. contortus* worms were collected from goat abomasa as previously described [23]. Total RNA was prepared from pooled parasite samples by a single step protocol [7] and stored at −20°C until use.

3′-rapid amplification of cDNA ends (3′-RACE) and 5′-RACE

The 3′-end of the cDNA was amplified by a 3′-full RACE kit (TaKaRa Bio, Japan) using the gene-specific primers 3 outer primer (OUP) and 3 inner primer (INP) (Table 1), which were designed based on *H. contortus* EST (GenBank accession No. AF305967) in combination with the 3′OUP and 3′INP in the kit (Table 1).

The 5-end of the cDNA was amplified by 5′-RACE PCR using a 5′-full RACE kit (TaKaRa Bio, Japan). Primary PCR was performed using the primers 5OUP (Table 1) and 5′OUP, followed by a second PCR using 5INP (Table 1) and 5′INP.

Products from both of the second-round PCRs were recovered using an agarose gel DNA purification kit (ver.

Table 1. All of the primers used in this experiment

Name	Sequences (5′→3′)
3OUP	ATTCGGACCCACAACAGAGC
3INP	GAACGCTCTTCTCGTCTCCACTTT
3′OUP	TACCGTCGTTCCACTAGTGATTT
3′INP	GCGGATCCTCCACTAGTGATTTCACTATAGG
5OUP	ACGAGAAGAGCGTTCAGCGAG
5INP	TTCACAACATTCCCTTCTCTGTCCACC
5′OUP	CATGGCTACATGCTGACAGCCTA
5′INP	CGCGGATCCACAGCCTACTGATGATCAGTCGATG
QCS	GAAAGGTTTAATTACCCAAG
QCA	AAACAAGTTAGATTCATTTTGCT
ORFS	AGTGAATTCATGGCAGCAACTAACG (*Eco*RI)
ORFA	TTTCTCGAGCTAAAGCTTGGTGGTG (*Xho*I)

OUP: outer primer, INP: inner primer. Initiation and termination codons are shadowed.

2.0; TaKaRa Bio, Japan) according to the manufacturer's instructions and then ligated into pMD-18T cloning vector (TaKaRa Bio, Japan). Clones containing inserts of the expected size were identified by *Xba*I and *Hind*III digestion and then sequenced by Invitrogen, USA. The complete sequence of the cDNA was deduced from the overlapping sequences of both amplification products using BioEdit (ver. 7.0.1; North Carolina State University, USA).

Synthesis of complete HC29 cDNA and open reading frame

The full-length cDNA and open reading frame (ORF) of HC29 cDNA were generated by RT-PCR using the primers QCS and QCA for the full-length cDNA and ORFS and ORFA for ORF (Table 1). The purified PCR products were ligated into pMD-18T cloning vector (TaKaRa Bio, Japan), transformed, and then sub-cultured. Randomly selected clones containing inserts of the expected size were then identified by *Xba*I and *Hind*III digestion. The sequences of the clones were also verified by Invitrogen (USA). All oligonucleotides used in this study (Table 1) were synthesized by Invitrogen (USA).

Sequence analysis

BLASTP and BLASTX were used for sequence similarity searches between HC29 and reported GPX [1]. The sequences of both HC29 and the reported GPX protein were aligned using Clustal W 1.82 [33]. Protein motifs, glycosylation sites, and secondary structures were predicted using programs accessible on the Internet, including Motifscan [11] and PSIpred [22]. Phylogenetic tree analysis was generated using Clustal X 2.0 [19] and MEGA 4.1 [30].

Construction of expression vector

Expression vector of recombinant HC29 protein was constructed by inserting full-length HC29 ORF into pET-28a plasmid (TaKaRa Bio, Japan). Briefly, recombinant pMD-18T containing HC29 ORF was digested with *Eco*RI and *Xho*I, after which the HC29 ORF fragment was inserted into pET-28a vector (TaKaRa Bio, Japan) that was previously linearized with similar enzymes to give pET-28a/HC29 expression vector. The recombinant vector was then transformed into *Escherichia* (*E.*) *coli* DE3 stain competent cells, and positive clones were verified by enzyme digestion.

Expression of recombinant HC29 protein

Transformed *E. coli* harboring pET-28a/HC29 was sub-cultured in Luria Bertani media supplemented with kanamycin (100 μg/mL) and incubated at 37°C until an OD$_{600}$ of 0.4~0.6. Expression was induced with isopropyl-β-D-thiogalactopyranoside (Sigma, USA) to a final concentration of 1 mM. After 5 h of incubation at 37°C, bacteria cells were harvested and expression assayed by SDS-PAGE.

Purification of recombinant HC29 proteins

Following induction, bacterial pellets were collected, after which recombinant proteins were harvested by centrifugation, lysed for 30 min on ice in PBS buffer, and sonicated. After centrifugation at 10,000 × g, the supernatant was loaded onto a Ni^{2+}-nitrilotriacetic acid column (GE Healthcare, USA) and purified according to the manufacturer's instructions. An elution buffer (40 mM NaH$_2$PO$_4$, 300 mM NaCl, pH 8.0) containing various amounts of imidazole was then evaluated for optimal release of the His-tagged proteins from the nickel resin. Purity of the proteins was estimated by 12% SDS-PAGE, and protein concentration was determined by Bradford assay [4] using bovine serum albumin as a standard.

Generation of polyclonal antibodies

To prepare polyclonal antibodies, about 0.3 mg of the purified HC29 protein was mixed with Freund's complete adjuvant, which was injected subcutaneously into SD rats (Experimental Animal Center of Jiangsu, China) at multiple places. Rats were then boosted four times at 2-week intervals after the first injection, and bleeding was carried out 10 days after. The serum containing specific anti-HC29 polyclonal antibodies was then collected, sampled, and stored at −20°C. Goat sera used in this research were collected from naturally infected goats.

Western blot analysis

Western blotting was performed after 12% SDS-PAGE. Separated protein bands, whole protein of adult worms, and purified recombinant protein were then electro-phoretically transferred from the gel slab to a nitrocellulose filter (Sigma, USA) using a Trans-Blot system (Bio-Rad, USA). This was followed by incubation for 1 h in blocking solution containing 5% (w/v) skimmed dry milk, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.01% (w/v) NaN$_3$, and 0.05% (v/v) Tween-20, followed by incubation with primary antibodies (rat sera and naturally infected goat sera) for 1 h at 37°C (rat sera diluted 1 : 1,000 in 5% skimmed dry milk powder/TBS and goat sera diluted 1 : 100). Secondary antibody (HRP-conjugated goat anti-rat IgG, HRP-conjugated rabbit anti-goat IgG; Sigma-Aldrich, USA) diluted 1 : 5,000 in 5% dry milk powder/ TBST (TBS in 0.05% Tween-20) was incubated through a nitrocellulose filter for 1 h at 37°C one more time. The immunoreaction was visualized using freshly prepared DAB (Sigma-Aldrich, USA) as a chromogenic substrate after 2~5 min.

Determination of recombinant HC29 protein enzymatic activity

Enzymatic activity was investigated by DTNB assay as previously described [13]. In the DTNB assay, these enzymes catalyzed the reduction of H$_2$O$_2$ by oxidizing glutathione (GSH), and GPX activity was confirmed by the deduction of GSH. The enzyme tubes were incubated at 37°C containing 1 mL of 2 mM GSH, 1 mL of 0.4 M sodium phosphate buffer (pH 7.0) with 4×10^{-4} M EDTA, 0.5 mL of 0.01 M NaN$_3$ (to inhibit catalase), 100 μg of recombinant HC29 protein, and water to a total volume of 4 mL. After 5 min of preincubation, 1 mL of 1.25 mM H$_2$O$_2$ (prewarmed to 37°C) was added. Following this, at 3 min intervals, 1 mL aliquots of the incubation mixture were added to 4 mL of metaphosphoric acid precipitation solution as the filtrate. GSH concentration was then determined by mixing 2 mL of filtrate with 2 mL of 0.4 M Na$_2$HPO$_4$ and 1 mL of DTNB reagent. A$_{412}$ was recorded within 2 min after mixing. An enzyme-free tube (with H$_2$O substituted for enzyme) acting as a negative control was established simultaneously. HC29 group and enzyme-free group were carried out with five replications per group.

Results

Characteristics of HC29 cDNA sequence

The product of 5′-RACE-PCR was a fragment of 523 bp. A 22 bp nematode-specific spliced leader sequence type 1 (SL1) was identified from 5 to 26 bp at the 5′-end of this fragment. The 3′-RACE-PCR product contained a polyadenylation tail at position 498 bp downstream of stop codon TAG. HC29 transcript was 1,113 bp long and obtained by splicing both the 3′- and 5′-RACE fragments. Its nucleotide and inferred amino acid sequences were submitted to GenBank under accession No. GQ927327. The sequence AATGAA at position 18 bp upstream of the

gaa agg **ttt aat tac cca agt ttg agt** tac tta ctt ctc gcc gtt ttc ttc aac tct caa gtg tcg ca

1	ATG GCA GCA ACT AAC GTC TAT CAG TTC AAG GTG AAG GAC GCC GAC GAG AAG GAG GTT TCA TTG
1	M A A T N V Y Q F K V K D A D E K E V S L
64	GAT AAG TAC AAG GGA AAA GTT CTC ATC ATT GTC AAC GTC GCA TCT CAA TGT GGT CTG ACA AAC
22	D K Y K G K V L I I V N V A S Q C G L T N
127	TCA AAT TAC ACA CAA TTC AAA GAG CTT CTT GAC AAA TAC AAG TCG CAA GGA TTG GAA GTA GCT
43	S N Y T Q F K E L L D K Y K S Q G L E V A
190	GCG TTT CCG TGT AAT CAG TTC GGC GGA CAG GAA CCA GCC TGC GAA ATT GAT ATC AAG AAT TTC
64	A F P C N Q F G G Q E P A C E I D I K N F
253	GTA GCA AAC AAA TTC AAC TTT GAG CCT GAT CTC TAC GCC AAA GTC AAT GTG AAC GGT GAC AAC
85	V A N K F N F E P D L Y A K V N V N G D N
316	GCG GAT CCT CTA TTC AAG TAT TTG AAG AAA GAG CAA GGA GGG ACA ATG TTC GAC GCC ATT AAA
106	A D P L F K Y L K K E Q G G T M F D A I K
379	TGG AAC CTC ACA AAG TTT TTG GTG GAC AGA GAA GGG AAT GTT GTG AAG AGA TTC GGA CCC ACA
127	W N L T K F L V D R E G N V V K R F G P T
442	ACA GAG CCA AAA GAT ATG GTA AAG GAC ATT GAG AAA CTT CTT GCA AGT GGC ACC ACC AAG CTT
148	T E P K D M V K D I E K L L A S G T T K L
505	TAG
	*

agt ttt aaa ctt tac tac aca tct ctg ctt ctg tat att ctt cta acc tta gta tac ttt gct aag ttg cta cgt tgt tct aca aaa aaa aca cat ctc ctt

gga tta ttt ttg aaa aat gct ttt ccg aag cct cgc tga acg ctc ttc tcg tct cca ctt taa ctc cta cgt gcc ggg ttc ttc cgc gtg aaa agt gta

gat atc tac ctc gtg tat tta acc ttg ctt cta gtc tat tcc ata ata act gac tgt gta gac tag ctg aac tgt agg att tga cca ata taa acg aga

cca gta ttt tcg ttt ggc atg ttt gta atg taa tca tat taa acc tta gct atc tgt ata gtc aat aca tac tgt atc aaa tta ttt ttc ttg tag cgt ttc att

tac gtt ttg cga tat gtc agc cag tgc tgt aat tga aga ttt ctt tgg ttg aat gtt tta aaa cgt ttt agc aaa **atg aat** cta act tgt tta aaa aaa aaa

aaa aaa aaa aaa a

Fig. 1. Alignment of nucleotide and predicted amino acid sequences of HC29 cDNA. Nucleotides consisting of the non-coding regions are in lowercase letters while nucleotides of the presumed coding region are in uppercase letters. The initiation ATG and stop TAG codons are shaded. Distal polyadenylation signal sequence AATGAA is shaded. The nucleotides and amino acids are numbered along the left margins. Conserved residues of the sequence are shown in boxes. The catalytic triad active sites are shaded. The spliced leader sequence is indicated in shadow.

polyadenylation site was identified as a distal polyadenylation stop signal of the HC29 cDNA gene. The ORF from 65 to 571 bp was 507 bp in size (Fig. 1) and encoded a polypeptide of 168 amino acid.

Characteristics of amino acid sequence of HC29

The predicted protein of HC29 consisted of 58 hydrophobic amino acids, 43 hydropolar amino acids, 24 strongly basic amino acids, and 23 strongly acidic amino acids. A search of the protein databases using the Basic Local Alignment Search Tool network service (National Center for Biotechnology Information, USA) indicated that the encoded protein had 44.7 to 80.4% similarity with a number of GPXs in the thioredoxin-like family (Table 2). The phylogenetic tree generated from the protein sequences (Fig. 2) revealed a close relationship between HC29 and GPX of *Caenorhabditis elegans*.

Amino acid sequence alignment between HC29 and the GPXs of other species revealed that HC29 protein possessed the catalytic triad active site residues C^{38}, Q^{73}, and W^{127} (Fig. 3). The dimer interface sites G^{72}, P^{75}, C^{77}, D^{80}, N^{83}, and N^{87} were also predicted in the GPXs, indicating a tetrameric structure for HC29. A GPX (GKvLIIvNVaSqCGlT) was identified in the putative amino acid sequence at position 26 ~ 41 by the ScanProsite database tool. Two N-glycosylation sites (^{44}NYTQ^{47}, ^{128}NLTK^{131}) were predicted by the CBS web server (Center for Biological Sequence Analysis, Denmark).

Alignment of HC29 and previous GPX from *H. contortus*

DNA sequence alignment between HC29 and *H. contortus* GPX showed that both sequences possessed different types of SL sequences (Fig. 4). HC29 had a SL1 type sequence (^{5}GGTTTAATTACCCAAGTTTGAG^{26}), whereas GPX had a SL2 type sequence (^{1}GGTTTTAACCCAGTTACTCAAG^{22}), as reported by Bagnall and Kotze [2].

Table 2. Analysis of identity and similarity between HC29 and glutathione peroxidase (GPX) homologues of the thioredoxin-like family

GPX of other species	Identity to HC29 (%)	Similarity to HC29 (%)
*Caenorhabditis briggsae*AF16 (XP_002631686)	67.9	80.4
Caenorhabditis elegans (NP_492598)	67.9	79.2
Globodera rostochiensis (CAD38524)	55.2	69.6
Trypanosoma brucei (CAC83347)	35.1	48.5
Plasmodium falciparum (NP_701484)	56.0	70.2
Bovine gpx1 (X13684)	33.0	44.7
Canis lupusfamiliaris GPX 1(NM_001115119)	33.7	44.9
Anopheles gambiae (EAA08523)	30.1	46.2
Mus musculus glutaredoxin 2 (NM_001038592)	38.5	54.1
Venturia canescens (AAK09374)	35.1	48.5
Pig GPX (L12743)	34.6	48.4
Toxoplasma gondii (AAT47115)	46.2	62.1
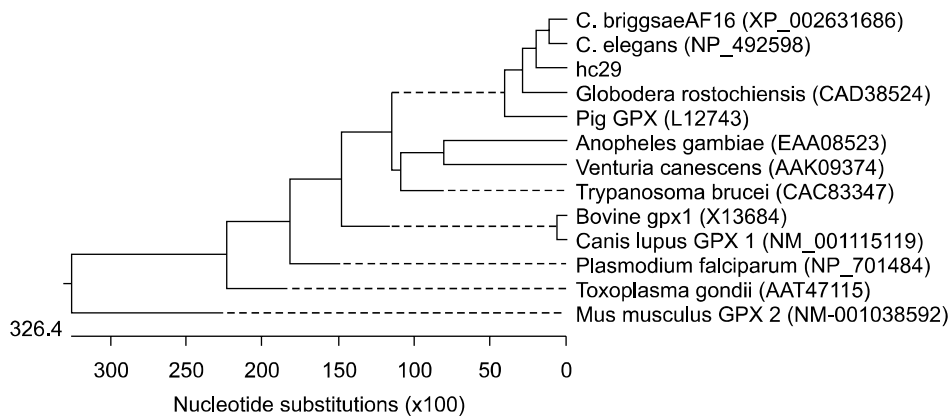
Fig. 2. Phylogenetic tree based on the predicted amino acid sequences of selected glutathione peroxidases (GPXs) against HC29 protein. Sequences were derived from GenBank or EMBL databases.

Alignment of the amino acid sequence of HC29 with that of reported GPX revealed 30.8% identity and 48.6% similarity at the amino acid level (Fig. 5). A cysteine residue coded by UGU located in the active site is a specific structure of selenium-independent GPXs, whereas selenium-dependent GPXs possess a seleno-cysteine residue coded by UGA. Both HC29 and the reported GPX protein contained a cysteine residue coded by UGU in their active sites (position 38 for HC29; position 50 for GPX). The amino acid sequence of HC29 possessed the same catalytic triad active site residues C^{38}, Q^{73}, and W^{127} as those of the reported GPX. However, the predicted dimer interface site residues of HC29 were different. Specifically, the dimer interface site residues of HC29 consisted of G^{72}, P^{75}, C^{77}, D^{80}, N^{83}, and N^{87}, whereas those of the reported GPX consisted of L^{83}, P^{86}, E^{88}, E^{91}, N^{94}, and Y^{98}. Furthermore, HC29 contained a GPX motif sequence (GKvLIIvNVaSqCGlT) at amino acid position 26~41, whereas the reported GPX had a GPX motif sequence (GQvLLIiNVaTfCAyT) at amino acid position 38~53.

Expression of recombinant HC29 protein

The expression product from *E. coli* migrated to an approximate molecular mass of 22 kDa, as visualized under reducing conditions (Fig. 6, Lanes 4~8). Both the *E. coli* transformed with pET-28a and the cells transformed with recombinant plasmid before induction did not produce expressive target bands (Fig. 6, Lanes 1~3).

Western blot assay

Native protein extracts of *H. contortus* probed with rat anti-recombinant HC29 serum displayed a single band with a size nearly the same as that of the recombinant protein (Fig. 6).

There was no detectable band in the Western blot analysis of recombinant HC29 protein, which was electro-
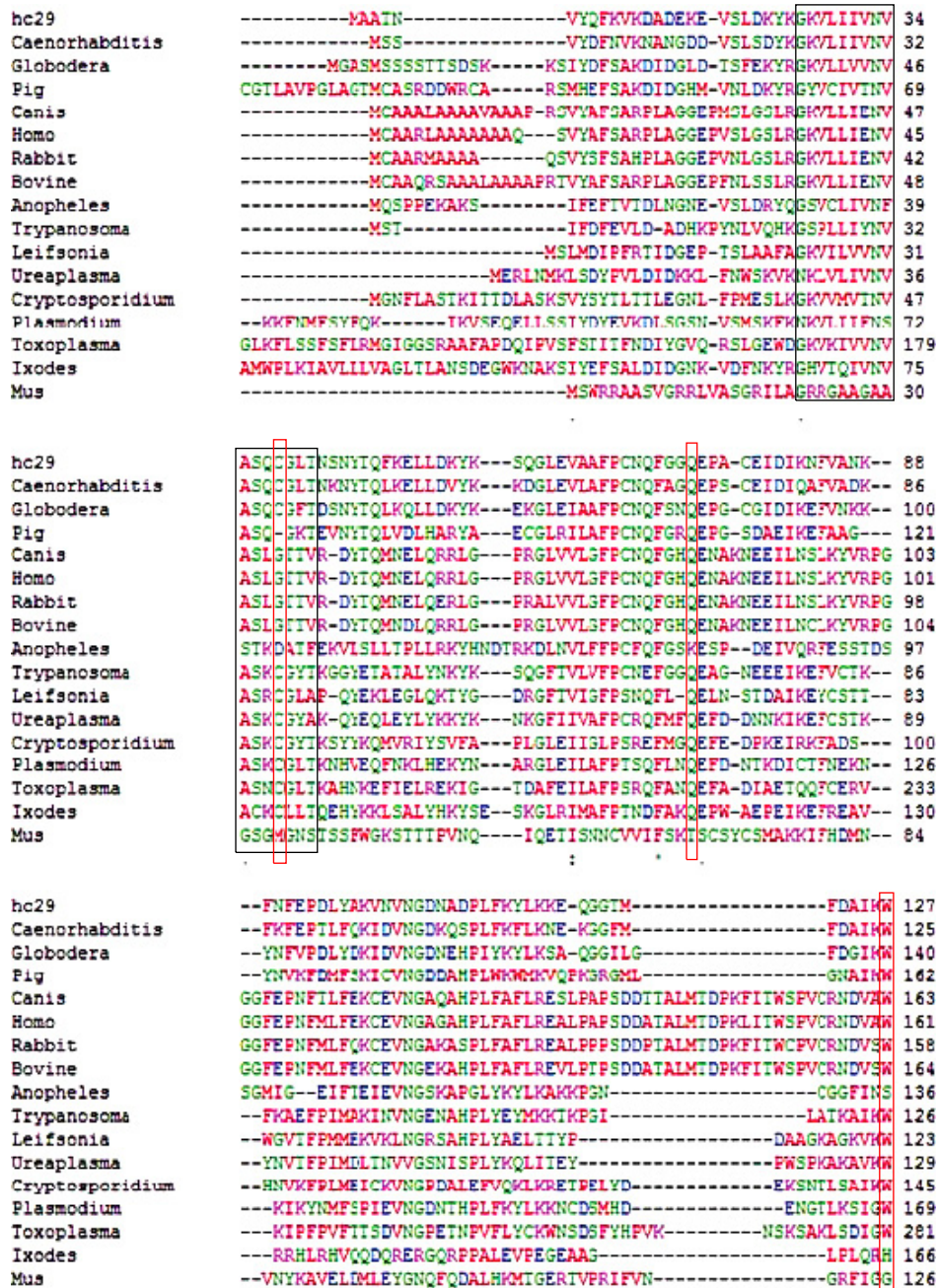
Fig. 3. Alignment of the critical GPX residues in the amino acid sequences of GPX homologues. The GPX motif and catalytic triad sites are boxed.

transferred to a nitrocellulose membrane and probed with serum from goats naturally infected with *H. contortus* parasites as the primary antibody (data not shown).

Determination of enzymatic activity

Low but significant GPX activity for decreasing H_2O_2 was demonstrated by recombinant HC29 protein. The absorbency of the substrate in the HC29 group (0.4384 ± 0.0015) decreased significantly compared to that in the

enzyme-free group (0.3960 ± 0.0066) ($p < 0.05$).
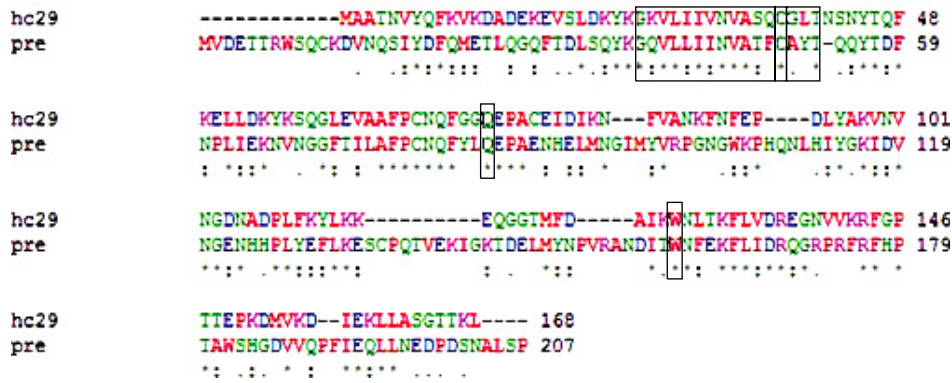
Discussion

A number of GPXs were isolated or recombined from a wide range of nematodes [15]. However, previous reports on GPX from *H. contortus* are quite limited in number. In the present investigation, the complete sequence of a *H. contortus* GPX cDNA termed HC29 was cloned and
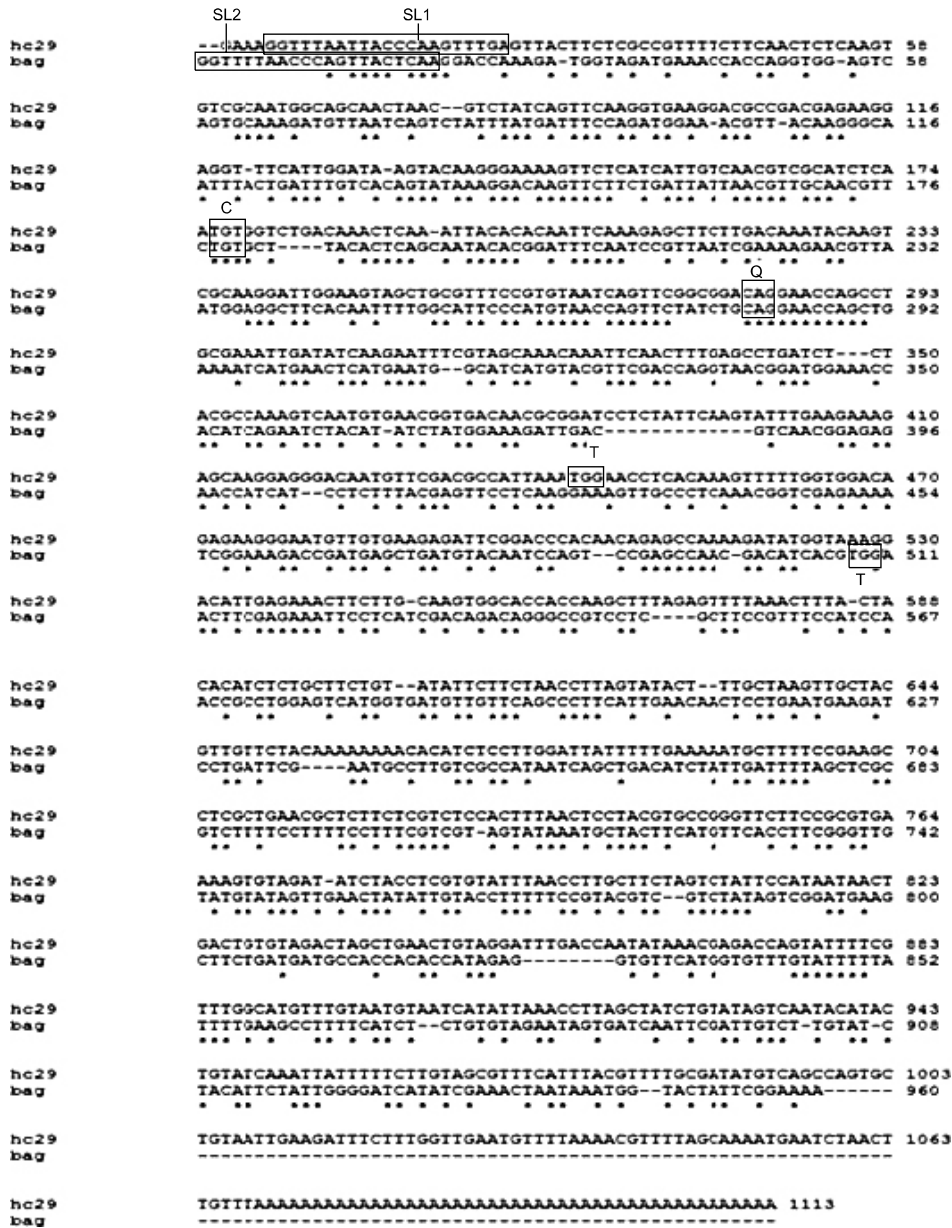
Fig. 4. Alignment of nucleic acid sequences of HC29 and GPX. The sliced leader sequences and codons of the catalytic triad sites are boxed. hc29: HC29, bag: reported GPX by Bagnall and Kotze [2].

characterized for the first time. In a comparison with the predicted amino acid sequences of other GPXs, HC29 demonstrated 44.7～80.4% similarity with GPX homologues in thioredoxin-like family. The conserved motif of the catalytic register along with the conserved catalytic triad active site residues C^{38}, Q^{73}, and W^{127} of thioredoxin-like family GPXs were identified in HC29 protein. Enzymatic activity assay of recombinant HC29 protein showed that it could catalyze H_2O_2 by oxidizing GSH to oxidized glutathione. All of the above results suggest that HC29 is a GPX of the thioredoxin-like family. GPXs in mammals include both selenium-dependent and selenium-independent

enzymes, the differences between which lie in the catalytic triad active sites and 'stem-loop'. In selenium-independent GPXs, the active sites consist of C-Q-W. However, in selenium-dependent ones, the cysteine is substituted by seleno-cysteine. On the other hand, the 'stem-loop' is a specific RNA structure present only in the 3′-untranslated region (UTR) of selenium-dependent GPXs and functions in recognizing UGA as a seleno-cysteine residue and not a stop codon [28]. In this research, we observed a cysteine residue at position 38 as well as the lack of a 'stem-loop' sequence in the 3′-UTR by using RNA draw software to analyze the full RNA sequence. Based on our results, we could conclude that

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hc29  ------------MAATNVYQFKVKDADEKEVSLDKYKGKVLIIVNVASQCGLINSNYTQF  48
pre   MVDETTRWSQCKDVNQSIYDFQMETLQGQFTDLSQYKGQVLLIINVATFCAYT-QQYTDF  59
                .:*:*::::  : :  ..*.:***:**:*:*:***:  *. : .:**:*

hc29  KELLDKYKSQGLEVAAFPCNQFGGQEPACEIDIKN---FVANKFNFEP----DLYAKVNV  101
pre   NPLIEKNVNGGFTILAFPCNQFYLQEPAENHELMNGIMYVRPGNGWKPHQNLHIYGKIDV  119
        : *::*  .  *: : ******  :*** : :: *   :*    .::*    .:*.*::*

hc29  NGDNADPLFKYLKK----------EQGGTMFD-----AIKWNLTKFLVDREGNVVKRFGP  146
pre   NGENHHPLYEFLKESCPQTVEKIGKTDELMYNFVRANDIIWNFEKFLIDRQGRPRFRFHP  179
        **:* .**::::**:     :. *::  * *:*: ***:***:*:.  ** *

hc29  TTEPKDMVKD--IEKLLASGTTKL----  168
pre   IAWSHGDVVQPFIEQLLNEDPDSNALSP  207
        *: .:. * :  **:** ... .
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Fig. 5. Alignment of amino acid sequences between HC29 and GPX. The GPX motifs and catalytic triad sites are boxed. hc29: HC29, pre: reported GPX by Bagnall and Kotze [2].
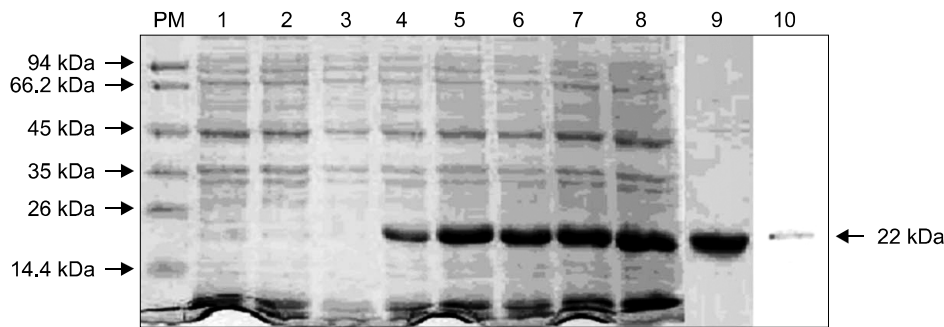
Fig. 6. Expression of HC29 protein and Western blotting. Gels were stained with Coomassie blue, and 15 μL samples were loaded per lane. Lane PM: Standard protein molecular weight marker, Lane 1: Extracts of *Escherichia* (*E*.) *coli* (BL21 strain) transformed with pET-28a empty expression vector before induction with isopropyl-β-D-thiogalactopyranoside (IPTG, negative control), Lane 2: Extracts of *E. coli* (BL21 strain) transformed with pET-28a empty expression vector after 5 h of induction with IPTG (negative control), Lane 3: Extracts of *E. coli* (BL21 strain) transformed with pET-28a/HC29 before induction with IPTG, Lanes 4～8: Extracts of *E. coli* (BL21 strain) transformed with pET-28a/HC29 after 1～5 h of induction with IPTG, Lane 9: Purified recombinant HC29 protein supernatants, Lane 10: HC29 natural protein in soluble proteins of *Haemonchus contortus* was identified using rat sera as a primary antibody.

HC29 is a selenium-independent enzyme.

In a DNA sequence comparison, HC29 was found to be different from the reported GPX of *H. contortus* (accession No. AY603337) [2]. DNA sequence comparison also revealed that the reported GPX possessed SL2, whereas HC29 possessed SL1. Amino acid sequence alignment between HC29 and the reported GPX revealed that these two shared 30.8% identity and 48.6% similarity, along with different dimer interface sites. Furthermore, HC29 displayed a GPX motif sequence (GKvLIIvNVaSqCGlT) at position 26～41, whereas the reported GPX had a different GPX motif sequence (GQvLLIiNVaTfCAyT) at position 38～53. These results indicate that the HC29 is a novel GPX of *H. contortus*.

Most nematode mRNAs possess a 5′-SL sequence. SL sequences in nematodes are generated by trans-splicing, which is vital to the maturation of pre-mRNAs. SL1 was the first SL ever reported in nematodes, and a series of SL sequences have been subsequently described [3]. SL1 plays main roles in regulating the optimal length of the 5′-UTR in mature mRNA as well as the formation of nucleotide construction benefit for gene translation [29, 35]. However, SL2 is closely linked (temporally or mechanistically) to 3′-end formation and polyadenylation of upstream genes [17]. Further, the sequence of SL2 is quite distinct from that of SL1. Genes containing SL2 all appear to be located within ～150 bases downstream of another gene containing SL1 on the same strand [18]. Therefore, the reported GPX was determined to be consecutively downstream of HC29 on the same strand of the primary transcript.

A dimer interface plays an important role in the formation of enzyme dimers. Existence of dimer interface sites in GPXs indicates that these enzymes possess a tetrameric structure, or a dimer of dimers. Both HC29 and the reported GPX of *H. contortus* were determined to possess dimer interface sites, which implies a polymeric structure.

Comparison with the amino acid sequence of the reported

GPX showed that HC29 also possessed the same catalytic triad active sites C-Q-W, suggesting that both GPXs belong to the GPX superfamily. Analysis of the GPX motif sequence using the ScanProsite database showed that HC29 had a GKvLIIvNVaSqCGlT motif, whereas the reported GPX had the sequence GQvLLIiNVaTfCAyT. Although these motif sequences are different, both are conserved in the GLUTATHIONE_PEROXID_1 (accession No. Ps00460, Prosite) family. Therefore, we could conclude that HC29 and the reported GPX of *H. contortus* belong to the GLUTATHIONE_ PEROXID_1 (accession No. Ps00460, Prosite) family.

The amino acid sequence comparison also showed that both HC29 and the reported GPX possessed the same cysteine residue in their active sites and both were selenium-independent GPXs. The cysteine residues were found to be highly conserved in the GPXs of *Brugia pahangi*, *Brugia malayi*, *Wuchereria bancrofti*, and *Dirofilaria immitis* [8,34]. Therefore, these findings suggest that the GPXs of nematode are selenium-independent.

It has been reported that, in all aerobic organisms, selenium-dependent GPXs are the main enzymes for scavenging H_2O_2 and preventing the further formation of ROS. In addition, selenium-independent GPXs have been found to adequately function as a back-up system for selenium-dependent GPXs in the absence of sufficient concentrations of selenium [15]. Surprisingly, these selenium-dependent enzymes are absent in parasitic nematodes, including *H. contortus* and filarial nematodes. This lack of enzymes capable of dealing with H_2O_2 is puzzling and should be further researched [5,26].

In this research, low but significant enzymatic activity was observed in recombinant HC29 protein by Hafeman assay [13]. Similarly, Tang *et al*. [31] previously found that a recombinant selenium-independent GPX of *Brugia phangi* expressed in insect cells (gp29) shows very low activity against H_2O_2. This indicated that the recombinant GPX of nematode has low enzyme activity under artificial conditions or that H_2O_2 is not the optimal substrate of this enzyme. To address this, Tang *et al*. [32] suggested that GPXs mainly play roles in repairing oxidatively damaged membranes rather than acting directly as antioxidant enzymes. This could explain the low activity of the recombinant GPX under artificial conditions. However, exactly why recombinant GPX of nematode displays low activity under artificial conditions should be further researched. In this study, the immunoblot assay results indicated that the native HC29 of *H. contortus* was 22 kDa in size, similar to that of the recombinant protein. This suggests that perhaps GPX does not undergo post-translational modification or only a small modification occurs that is undetectable by SDS-PAGE.

HC29 was expressed particularly in the intestinal microvilli and muscle cells lining the cuticle [14]. However, in this research, the recombinant protein was not recognized using naturally infected serum. This suggests that the native HC29 of *H. contortus* is neither secreted into its native host tissues under physiological conditions nor recognized by the host immune system. As this result runs contrary to the known location of HC29, more investigations are needed.

Acknowledgments

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