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De novo pathway is an active metabolic pathway of cysteine synthesis in *Haemonchus contortus*

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ABSTRACT

Haemonchus contortus, commonly known as Barber's pole worm, is an economically important gastrointestinal nematode of sheep and goats especially in tropical and sub-tropical regions of the world. Cysteine synthesis is a very important metabolic pathway for the parasite, however the functional aspects of cysteine synthesis in parasite are largely unknown. The key question which we have investigated in the study is; whether the parasite uses a *de novo* pathway of cysteine synthesis, which is unknown in multicellular organisms of the animal kingdom and known to be absent in mammals. Directional cloning of the cysteine synthase (CS) gene was done in pET303 champion vector using restriction sites XbaI and XhoI. The CS gene of the *H.contortus* was closely related to CS-A protein of *Oesophagostomum dentatum* and a hypothetical protein of *Ancylostoma ceylanicum*. Recombinant protein of the *H contortus* CS (rHC-CS) gene was expressed using pET303 vector in pLysS BL21 strain of *E.coli* and subsequently purified by Ni-NTA affinity chromatography. Western blot using anti-His tag antibody confirmed the presence of rHC-CS. Biochemical assay, FTIR and enzyme kinetics studies revealed that rHC-CS used O-acetyl serine as substrate to produce cysteine using *de novo* pathway and CS activity was also confirmed with the homogenate of *H.contortus*. Upregulation of CS transcripts in the adult and its downregulation in the L3 larval stage suggests that *de novo* pathway contributes to the cysteine requirement of mature *H.contortus*. It is concluded that *de novo* pathway is an active metabolic pathway in *H.contortus*.

1. Introduction

Haemonchus contortus is one of the most pathogenic, economically important, blood-sucking gastrointestinal nematode of sheep and goats worldwide [1]. The haemotophagous nematode sucks blood from the mucosal linings of the abomasum, the predilection site. *H. contortus* thrives well in the tropical regions, where hot and humid environments favour the hatching and perpetuation of the species. Grazing animals usually get infected with L3 stage infective larvae of *H. contortus*. The larger L5 and adult parasites may suck up to 30 μL blood per day, which results in the rapid development of anaemia, jowl edema, unthriftiness, debility and subsequent death even before the animal crosses the pre-patent period [2]. The life cycle of *H contortus* starts with the hatching of L1 larva from the eggs that eventually develop into infective L3 stage larva. L3 stage larva is picked up by the host while grazing and exsheaths in the rumen and moults to L4, which develops into L5 stage and adult parasite in the abomasum. Due to significant production and reproduction losses, infection with *H contortus* causes huge economic loss to farmers and the small ruminant industry encountered

Abbreviations: CS, Cysteine synthase; GIT, Gastrointestinal tract; E/S, Excretory/secretory; RTS, Reverse-transulphuration (RTS); CBS, Cystathionine beta-synthase; CGL, Cystathionine γ-lyase; SAT, Serine acetyltransferase; OAS, O-acetyl serine; rHC-CS, Recombinant *H.contortus* Cysteine synthase enzyme; FTIR, Fourier-transform infrared spectroscopy; PLP, Pyridoxal 5′-phosphate.

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annually across the globe. Though anthelminthic drugs are the mainstay tool used prophylactically and metaphylactically, the emergence of resistance to different classes of deworming drugs such as benzimidazole [3], organophosphates [4], salicylanilides [5], macrocyclic lactones [6], amino-acetonitrile derivatives [7,8] is an emerging concern. There is a dire need to develop suitable drugs or efficient vaccine candidates for the prevention of infestation in animals against this deadly parasite [9].

Understanding the metabolism biochemistry of parasite could provide vital information on important biochemical targets for rational drug development. The metabolism of cysteine has been studied enormously in protozoa, but there are limited information and studies on helminths. L-cysteine is a very important amino acid, and thus, it is essential for the survival of all organisms, ranging from bacteria to complex multicellular eukaryotes. Cysteine is responsible for maintaining the stability, structure, regulation of catalytic activity, and post-translational modification of various proteins [10] and is a basic structural constituent of all thiol antioxidants [11]. Cysteine becomes further important for *H.contortus* as its survival depends upon the evasion of the immune response, which it does by secreting many excretory/secretory proteins (E/S Proteins), most of which are cysteine proteases.

De novo and reverse-transsulfuration (RTS) pathways are the two primary pathways of cysteine biosynthesis. Reverse-transsulfuration (RTS) pathway has been demonstrated in fungi and mammals and is catalyzed by Cystathionine beta-synthase (CBS) (catalyzing the conversion of homocysteine and serine to cystathionine) and Cystathionine γ-lyase (CGL) (formation of cysteine from cystathionine) [12]. In *de novo* pathway, the first step is the conversion of serine to O-acetyl serine (OAS) by serine acetyltransferase (SAT) followed by reaction of OAS with sulphide to produce cysteine by cysteine synthases (CS) [13]. Several important studies have been undertaken for understanding the pathway of cysteine synthesis in various pathogenic organisms like *Trypanosoma* sp. [14–17], *Leishmania* sp. [18–21], *Acanthamoeba* sp [22]. and *Entamoeba* sp [23–25]. Walker and Barrett [26] studied very comprehensively the activities of enzymes of RTS pathway (CBS and CGL activities) in the homogenates of various parasites, including *H. contortus* and demonstrated CBS activity in the extracts of *H.contortus*.

In this study, we first produced recombinant CS of *H contortus* (rHC-CS) and demonstrated the presence of *de novo* biosynthetic pathway of cysteine in *H contortus*, which is not known in multicellular organisms of animal kingdom and is absent in mammals.

2. Material and methods

2.1. Collection of adult H. contortus samples

Live *H. contortus worms were* recovered from the abomasum of sheep after exsanguination from the slaughter house of CSWRI, Avikanagar. Immediately after collection, the sample was sorted to remove debris and other Strongylidae family worms, followed by its washing with PBS (pH-7.4) containing antibiotic/antimycotic solution (10,000 units penicillin, 10 mg streptomycin, and 25 μg amphotericin-B/mL) to avoid any contamination. Neat samples were stored in RNAlater at −80 °C for future use.

2.2. Collection and culture of stage (L1) and stage larvae (L3) of Haemonchus contortus

To obtain L1 and L3 larvae of *H. contortus*, a fresh faecal sample was collected per rectum from ewes experimentally infected with *H. contortus* and maintained in an experimental pen (under stall-fed condition) at Animal Health division of CSWRI. The detailed protocol is provided in the supplementary file.

2.3. Isolation of H. contortus RNA and cDNA synthesis

The fresh sample of *H. contortus* was processed for total RNA isolation using Trizol reagent (Invitrogen). The parasites (about 50 mg) were placed into pre-chilled pestle and mortar in which one mL of Trizol reagent was added. The cells were lysed with the help of pestle and the mixture was incubated at 25 °C for 5 min for the complete dissociation of nucleoprotein complexes. Then, 200 μL chloroform was added and the mixture was centrifuged at 12,000×*g* for 10 min at 4 °C. The aqueous phase was transferred into a fresh tube and RNA was precipitated by adding 0.5 mL isopropyl alcohol. The mixture was incubated at 25 °C for 10 min and again centrifuged at 12,000×*g* for 10 min at 4 °C. The RNA pellet was washed in 75% ethanol (prepared in DEPC-water), air-dried and dissolved in 20 μL DEPC treated nuclease-free water. The integrity of RNA was checked by agarose gel electrophoresis and quantified by NANOQUANT (Tecan Infinite M200, Switzerland) spectrophotometer. The extracted total RNA was used for cDNA synthesis. The cDNA was synthesized by using RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, USA) as per manufacturer's protocol.

2.4. Characterization of CS gene and its cloning in pET303 vector

The cDNA served as template for the amplification of the complete sequence of CS gene using specific primers. The primers for amplification of CS gene of *H. contortus* were designed using Primer3 software. The primer sequences are presented in Table .1. The PCR amplification of mRNA sequence of CS gene was performed using thermocycler PCR (MJ Research, USA) as per the following condition: initial denaturation at 95 °C for 5 min (1 cycle), denaturation at 95 °C for 1 min (35 cycles), annealing at 60 °C for 30 s (35 cycles), extension at 72 °C for 1 min (35 cycles) and a final extension at 72 °C for 10 min (1 cycles). The PCR product was purified by GeneJET Gel Extraction Kit (Thermo Fisher Scientific, USA) and ligated into pJET1.2 blunt-end cloning vector of CloneJET Kit (Thermo Fisher Scientific, USA) as per manufacture protocol. Then, the ligated product was transformed into $DH_5\alpha$ strain of *E. coli* (heat shock at 42 °C for 90 s). The positive clones were selected by colony PCR and plasmid PCR and confirmation was done by sequencing. The sequencing data were compared with the GenBank sequence by pairwise alignment to check the gaps and insertion and the annotated sequence was submitted to the NCBI database.

The sequence similarity and identity of CS gene with the CBS gene of humans was assessed by pairwise alignment tool (Clustal W). The sequence similarity of recombinant *H.contortus* CS (rHC-CS) enzyme was also checked by local alignment search tools, BLASTp and BLASTx used for protein to protein and DNA to protein alignment, respectively. The phylogenetic tree of rHC-CS was constructed by aligning the amino acid sequences using multiple sequences alignment tool of the Neighbor-Joining method of NCBI.

Two sets of primers were designed for the amplification and directional cloning of CS gene in pET303 vector (Table 1). One set of primer leads to incorporation of the gene with a C-terminal His tag (IS-1 for Insert-1) using the frame of the vector, while the other set was designed to include an N-terminal as well as C-terminal His tag (IS-2 for Insert-2). XbaI and XhoI were included as 5' overhangs on the forward and reverse primers, respectively for directional cloning of the genes in pET303 vector. A sequence overhang to include the Hexa His tag was included in the forward primer of IS-2 in addition to restriction site (XbaI) and reverse primer remained the same with XhoI restriction site.

The PCR amplification of the CS gene was done as described

Table 1
Sequence of the primers.

S.No	Oligo Name	Sequence	Description
1.	CS-F	ATGTCTCGTGATACTATGCTCCTCTC	CS gene amplification
2.	CS-R	GTACGCTTTTGCTCTGGAGATTGAT	
3.	IS-1F	ACATCTAGAATGTCTCGTGATACTATGCTCCTCTC	Expression primers for CS gene expression with C-terminal Hexa-His
4.	IS-1R	TTTCTCGAGGTACGCTTTTGCTCTGGAGATTGAT	Tag
5.	IS-2F	ACATCTAGAATGCATCACCATCACCATCACATGTCTCGTGATACTATGCTCCTCTC	Expression primers for CS gene expression with C-terminal and N-
6.	IS-2R (same as IS-1R)	TTTCTCGAGGTACGCTTTTGCTCTGGAGATTGAT	terminal Hexa-His Tag
7	CS (RT)F	GCGCTATTGAAAGGGCCCAGGAAC	Real-time quantification of CS gene
8	CS (RT)R	CCCGATCCAACTCCGAAACACACA	
9	$G6P_f$	ACGAGACCTACAATGCAGCC	Endogenous reference gene (Glucose 6 phosphate dehydrogenase) for
10	$G6P_r$	GCGAGACAGTTGGTGGTACA	relative quantification

earlier. We did not alter the sequence of the earlier designed primers, only the overhangs were modified suitably to complement the design of the cloning strategy. Amplified CS gene was cloned into a pET-303 expression vector with Xba1 and Xho1 restriction sites and confirmed by plasmid PCR and double restriction digestion.

2.5. Quantitative expression of CS gene at various developmental stages of H contortus

Real-time PCR (qPCR; StepOne plus, Applied Biosystems, Foster City, CA, USA) was used to elucidate the differential expression of CS gene. Specific real-time primers were designed using the complete sequence information of the CS gene. Glucose 6 phosphate dehydrogenase gene of *H.contortus* served as an endogenous reference to calculate delta Ct. Primers of both genes are illustrated in Table 1. The PCR reaction was done in a final reaction volume of 20 μL using Power SYBR Green (Applied Biosystems, Foster City, CA, USA), according to manufacturer's recommendations. All the reactions were placed in duplicates. Total RNA (4 ng) was used as the template for relative quantification. Reverse transcriptase control was used to avoid any gDNA contamination, and no template control was used as negative control. For PCR, the samples were activated and denaturation was performed at 95 °C for 10 min. Amplification was performed for 40 cycles at 95 °C for 15s, 60 °C for 30 s and 72 °C for 30s. A single peak on melt curve analysis (65 °C–95 °C, with increments of 0.3 °C/s) confirmed the specificity of the PCR products. The gene expression data have been normalized using relative quantification $2^{-\Delta\Delta Ct}$ method [27], and fold change was estimated against the L1 stage larval samples.

2.6. Expression and purification of rHC-CS

The rHC-CS was expressed after transforming the vector cassette containing the targeted gene (CS + pET303) in pLysS (BL21) strain of *E. coli*. The recombinant cells were bulk cultured in Luria-Bertani (LB) media containing ampicillin (100 μg/mL) and chloramphenicol (25 μg/mL) at 37 °C and induced by isopropyl-β D-thiogalactosidase (IPTG) at a final concentration of 1 mM for 4 h. The culture was pelleted at 4 °C and was dissolved in lysis buffer (200 mM sodium phosphate and 300 mM sodium chloride, pH 8.0) with 1 mg/mL lysozyme and 1 mM PMSF. The mixture was incubated on ice for 30 min and the cells were lysed by cold shock by brief repeated exposure to liquid N_2.. Then, they were placed on ice for 5 min and centrifuged at 12,000×*g* for 30 min at 4 °C. The supernatant was passed through 0.22 μm filter and the histidine-tagged fusion protein was purified from the supernatant by using Ni-NTA agarose column (Thermo Fisher Scientific, USA). The bound proteins were eluted with 100 mM and 250 mM imidazole concentration for IS-1 version of CS protein. CS protein (IS-2) was eluted with 250 mM and 500 mM imidazole concentration in the elution buffer. The fractions of different concentration were stored at −80 °C for further use. The purity and concentration rHC-CS were analyzed by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie brilliant blue (R250) staining. The concentration was determined by taking the absorbance of rHC-CS at 280 nm in a spectrophotometer and calculated based on the molar extinction coefficient of the protein.

2.7. Identification of tagged protein by western blotting

The purified protein of cysteine synthase rHC-CS was resolved on 10% SDS-PAGE gel. The membrane was presoaked in 50% ethanol for 30 s followed by two washes with distilled water and western blot transfer buffer, each for 5 min. Immunoblotting was performed using a slight modification of protocol standardized in the lab [28]. Briefly, the protein was transferred from gel to membrane by placing the gel at −ve side and membrane at + ve side in transfer buffer at 110 Vt for 90 min in ice. After that, the membrane was incubated in blocking buffer [2% BSA in Tris saline buffer (TBST)] overnight at 4 °C and washed thrice with TBST for 5 min. Then, the membrane was transferred in 10 mL of cocktail anti-his antibody solution (1:1000 dilution in blocking buffer) for 1.5 h on shaker, followed by its washing 4–5 times with TBST for 5 min. Then, the 10 mL of anti-mouse IgG secondary antibody conjugated with horseradish peroxidase (HRP) (1:3000 dilutions in blocking buffer) was added and incubated on a shaker for 1.5 h. Finally, the membrane was washed 4–5 times with TBST and the chromogenic reaction was done with 3,3′Diaminobenzidine (DAB)+ H_2O_2 staining in substrate buffer.

2.8. Cysteine synthase activity (CS) assay

The CS activity of the recombinant protein was assessed by following a slightly modified protocol of Gaitonde et al. [29]. The method is specific for the quantitative assessment of cysteine in the presence of other amino-acids and is based on the principle that in the presence of HCl at very low pH, cysteine reacts with ninhydrin to give an intense pink colour, which is not given by proline, cystine, ornithine, homocysteine, glutathione and all other naturally occurring amino acids.

The 100 μL reaction mixture [200 mM potassium phosphate buffer, 10 mM DTT, 1 mM PLP, 6.5 mM O-acetyl serine (OAS)] was added to protein (5 μg/mL). Protein control was included for each of the time points of the reaction. A control for OAS was also included. The mixture was incubated at 37 °C for 5 min. Then, 5 mM sodium sulfide was added and incubated at 37 °C for 0, 5, 10, 15, 20 and 25 min. After that, 15 μL of 20% TCA was added into the tubes to stop the reaction followed by its centrifugation at 12000×*g* for 5 min. Then, 100 μL of supernatant was added in 100 μL of acid ninhydrin

Reagent-2, which was freshly prepared. The reaction mixtures were boiled at 100 °C for 10 min and cooled for 5 min on ice. Then, the absorbance was recorded at 560 nm and analyzed with respect to control. L-cysteine (0–50 μmol) dissolved in acid-ninhydrin reagent served as standard and treated similarly as described earlier to quantify the amount of cysteine formed. Enzymatic activity of rHC-CS (μmol/min) was measured based on the regression line interpolated from the measured data points. The O-phosphoserine was also tested as a replacement of OAS in the above protocol to evaluate whether enzyme could use it as a substrate.

Further, we tested the CS activity in the crude lysate of *H contortus* that was prepared by sonicating 50 mg of worm pellets resuspended in one volume of 0.1 M phosphate-buffered saline (PBS), containing protease inhibitor cocktails (P8465, Sigma–Aldrich). Crude extracts were centrifuged for 1 h at 4 °C at 15000×*g*, and the supernatant containing 50 μg of total protein was used for the determination of CS activity as described earlier after calculating the total protein concentration using Pierce™ BCA Protein Assay Kit (ThermoFischer Scientific,USA).

2.9. FTIR analysis for qualitative determination and further confirmation of CS activity

The reaction was conducted using 5 μg/mL protein concentration for 5 different time points (A1-0 min, A2-5 min, A3-10 min, A4-15 min, A5-20 min). Protein control (PC) was also included and incubated for 25 min. After that, 15 μL of 20% Trichloro acetic acid was added into the tubes to stop the reaction followed by its centrifugation at 12000×*g* for 5 min. The aqueous supernatant was characterised using attenuated total reflection (ATR)-Fourier transform infrared (FTIR) spectrophotometer (Alpha-T, Bruker,USA) equipped with the OPUS software to determine various functional groups in the scanned sample. The FTIR spectra were obtained at a resolution of 2 cm^{-1} from 4000 to 800 cm^{-1}. Each FTIR spectrum was obtained after 32 numbers of scans.

2.10. Enzyme kinetic analysis of rHC-CS

Kinetic analysis of the rHC-CS sulphydrylation reaction was done at 37 °C with the assay mixture and conditions described above. The concentrations of OAS were changed from 0 mM to 18 mM at a fixed sodium sulphide concentration of 5 mM. Similarly, for measuring the kinetics of Na_2S utilization, the concentration of Na_2S varied from 0 to 10.5 mM at a fixed OAS concentration of 7 mM. The enzyme concentration used for doing the kinetic assays were 0.1 μg and 0.4 μg for Na_2S and OAS respectively. A 100 μL portion of the reaction mixture was prepared separately and was used for measuring cysteine production after different intervals of incubation. The produced L-cysteine was determined as described above. The initial velocity of the enzyme-catalyzed reaction was determined at each of the respective substrate concentration and kinetic parameters of reaction were determined by plotting the Michaelis-Menten equation using the non-linear regression function of GraphPad. Turnover number (kcat) was manually calculated based on the following formula ($kcat = V_{Max}/[ET]$).

2.11. Statistical analysis

Experiments from 2.9 to 2.10 were performed in triplicates and repeated thrice. Results are presented as mean and standard deviation of the mean. Real time PCR studies were performed in duplicates with three repeations and results are presented as means at 95% confidence interval. Normalized Data were analyzed and graphs were plotted using the Graphpad Prism 8.

3. Results

3.1. Sequence characterization of CS gene of the H.contortus and its expression in pLysS BL21strain of E.coli

PCR amplification of the CS gene with the self-designed primers was standardized at an annealing temperature of 60 °C and a 1002 bp PCR product was amplified. The purified PCR product was successfully cloned in pJET1.2 blunt cloning vector and sequence characterization was done by sequencing. A sequence of CS gene was annotated and submitted to NCBI for which an accession number was granted (GenBank MN733957). The study of the phylogram suggests that the CS gene of the *H.contortus* is more closely related to CS-A protein of *Oesophagostomum dentatum* and a hypothetical protein of *Ancylostoma ceylanicum* and distantly related to CS protein of *Caenorhabditis elegans* (Fig. 1).

Successful directional cloning of the CS gene was done in pET303 champion vector using restriction sites XbaI and XhoI. The confirmation of clones was done by doing a plasmid PCR as well as the successful release of the insert from the cloned plasmid.

We made futile attempts to express CS protein with N-terminal His tag which might be due to sequestration of the tag within the folded protein. Thus, we designed primers to produce CS protein with a C-terminal His tag (IS-1) and one with a His-tag at both C-terminal and N-terminal end (IS-2). Both the versions of CS gene (IS-1 and IS-2) were induced to express in pLysS BL21 strain of *E.coli*. Expression of CS gene could be distinctly observed in an induced version of the lysates compared to un-induced lysates (Fig. 2a as part of supplementary material).

Ni-NTA affinity chromatography was successfully done to purify the protein (Fig. 2). Purification of IS-1 and IS-2 versions of rHC-CS yielded 35 ng/μL and 42 ng/μL respectively, from 100 mL culture. The C-terminal hexahistidine tag was able to bind to the Ni-NTA agarose beads in both IS-1 and IS-2 versions of the rHC-CS. Hexahistidine tag at both N-terminal and C-terminal ends improved the binding of IS-2 to Ni-NTA agarose beads; for this reason, elution buffer with 500 mM imidazole was used to elute the IS-2 version of CS protein. The protein size on SDS PAGE was corresponding to the expected band size of 38 kDa and 40 kDa for IS-1 and IS-2, respectively. The purity was evident by the presence of a single band in dialysed rHC-CS (Fig. 3a). Western blot using anti-His tag antibody could confirm the presence of recombinant proteins rHC-CS proteins (Fig. 3b).

3.2. Culture of L1 and L3 stages of the H.contortus

Larval stages of *H.contortus* L1 and L3 were generated using a in-house coproculture technique (Fig. 4a and b). Adult stage was identified based on the distinct feature of a fully developed copulatory bursa and a characteristic Y-shaped dorsal ray (Fig. 4c).

3.3. Relative expression of CS gene transcripts at different developmental stages of H contortus

The designed real-time primers could successfully amplify the 169 bp product and were standardized for their annealing at 60 °C. The CS gene was upregulated about 2.5 fold in the adult as compared to the stage L1 larva of *Haemonchus contortus*. Stage L3 larvae had the lowest expression of CS gene having a nearly 7 fold downregulation as compared to stage L1 larva (Fig. 5). We estimated that the CS gene expression was found to be the highest in adults and it was significantly down-regulated in L3 larva. Thus, it was observed that the *de novo* pathway of cysteine synthesis is happening at all the stages of parasite's developmental cycle.

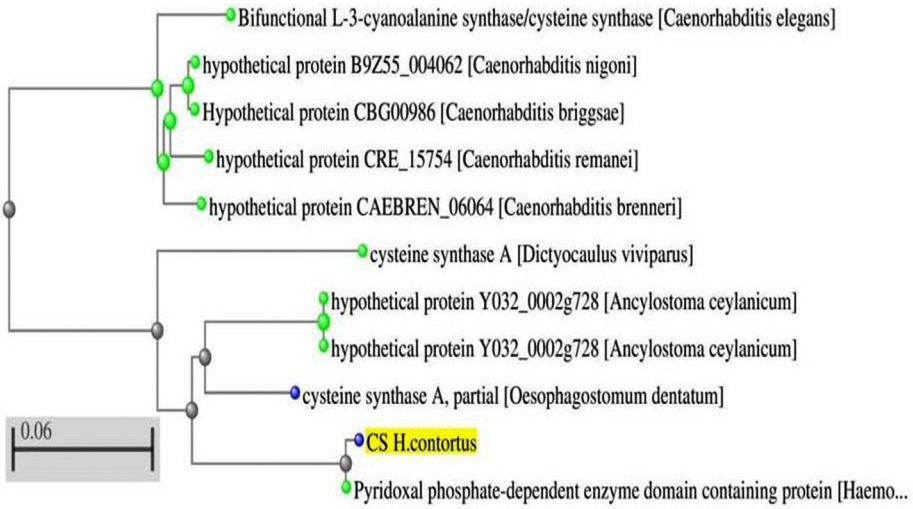

Fig. 1. Phylogram of *cysteine synthase* gene of *H. contortus*: *Phylogram analysis of H. contortus's CS gene with C.elegans, D. viviparus, O. dentatum and A. ceylanium. Phylogram depicts H. contortus, CS is distantly related to C.elegans, C.nigoni (B9Z55_004062), C.briggsae (CBG00986), C.remanei (CRE_15754), C.brenneri (CAEBREN_06064), closely related to D. viviparus, A. ceylanicum (Y032_0002g728), but more closely related to O. dentatum cysteine synthase A and Pyridoxal phosphate-dependent enzyme domain containing protein of H.contortus based on root and nodes length difference.*

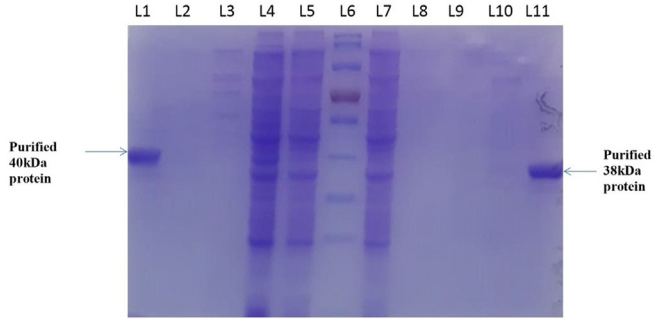

Fig. 2. SDS-PAGE analysis of expression and purification of IS-1 and IS-2 version of Cysteine synthase protein induced in pLysS BL21 strain of E.coli. Lane 1: purified protein of Insert2 at 500 mM concentration of imidazole, Lane 2: washing with 100 mM imidazole, Lane 3: washing with 50 mM imidazole, Lane 4: induced lysate of E.coli lysate containing IS-2, Lane 5: uninduced lysate of E.coli containing IS-2; Lane 6 contains Pageruler prestained protein ladder (Thermofischer Scientific, 26616), However, IS-1 protein of around 38 kDa was purified at 250 mM Lane 7: induced lysate of E.coli lysate containing IS-1, Lane 8:washing with 20 mM imidazole, Lane 9: washing with 50 mM imidazole, Lane10: washing with 100 mM imidazole concentration. Lane 11: purified protein of Insert1 at 250 mM concentration of imidazole.

3.4. Enzymatic activity of the rHC-CS in vitro

Enzymatic activity of the rHC-CS was determined *in vitro* by activating the enzyme using sodium sulphide by incubating it in the presence of PLP and O-acetyl serine as substrate. Both IS-1 and IS-2 version of rHC-CS were functional. The enzymatic production of cysteine at different time intervals was assayed for IS-1 version of rHC-CS as it contains only a single hexa-His tag. Time-dependent increase in the production of cysteine over 25 min provides proof of enzymatic activity of rHC-CS as compared to the protein control (Fig. 6). The protein control did not show any change in absorbance. Incubation of rHC-CS with 1:10 M ratio of CS enzyme to pyridoxal 5′-phosphate did not significantly increase the enzyme activity (data not shown), suggesting that the purified enzyme already had a full complement of PLP in it. The rHC-CS was unable to use O-phosphoserine as substrate. The crude lysate of *H contortus* converted O-acetyl serine into cysteine, indicating the presence of CS enzyme in the nematode [43 (±1.8) nmol min^{-1} mg $protein^{-1}$].

Kinetic behavior of the *rHC-CS* was studied for both the substrates at pH 8 and 37 °C and the kinetic constants are presented in Table 2. Michaelis-Menten plots and kinetic traces (Fig S_1 and Fig. S_2) with their linear equations are available as supplemenatary data. The V_{Max} and K_M values of the *rHC-CS* enzyme for OAS and Na_2S were quite different from each other. The apparent k_{cat} values of rHC-CS for OAS and Na_2S were 1044 and 1216 s^{-1}, respectively.

3.5. FTIR analysis for qualitative determination and confirmation of CS activity

FTIR spectra was successfully obtained for protein control (PC) and enzyme reaction incubated at various time points (A1-A5) over 4000–800 cm^{-1} (Fig. 7a). The peak at 1637 is ascribed to amide I, which is mainly associated with C=O. The broad peak at 3336 represents –OH group as well as amide A. The peak at 1336 cm^{-1} is associated with Amide III. The S–O–S vibration of protein is at 1074 cm^{-1} [30,31]. It has been reported that the cysteine shows characteristic peaks at 2357 cm^{-1} representative of S–H group, 1325 cm^{-1} due to C–N cm^{-1} stretching, 2920-2850 cm^{-1} indicates C–H stretching vibration, 1628 cm^{-1} belongs to N–H bending vibration of free amino groups, 1630 cm^{-1} ascribed to C=O stretching, 1439 cm^{-1} due to C–O stretching vibration, 873 cm^{-1} indicates C–S vibration, 3419 cm^{-1} is because of OH stretching and 2826 cm^{-1} denotes N–H asymmetric stretching [32,33].

As we intended to probe on cysteine production, the FTIR spectra over the range of 2500–2000 cm^{-1} were investigated. It can be seen that the protein control, had no presence of cysteine, whereas the spectra of A1 to A5 showed a peak at 2357 (Fig. 7b), confirming the presence of cysteine. It should be noted that the FTIR spectra are normalized at the transmittance value of 2357 cm^{-1} wavenumber. Thus, with an increase in time (From A1 to A5), the intensity and sharpness of the cysteine peak at 2357 cm^{-1} increased. This confirmed that the cysteine was produced in the reaction by the enzyme using Na_2S and OAS as substrates.

7(a) Normalized FTIR spectra was obtained for the reaction product of protein control (PC) and reaction carried over for A1 = 0 min, A2 = 5 min, A3 = 10 min, A4 = 15 min, A5 = 20 min by rHC-CS over 4000–800 $cm^{-1}$and (b) Normalized FTIR spectra of same samples depicted over the range to 2500–2000 cm^{-1}

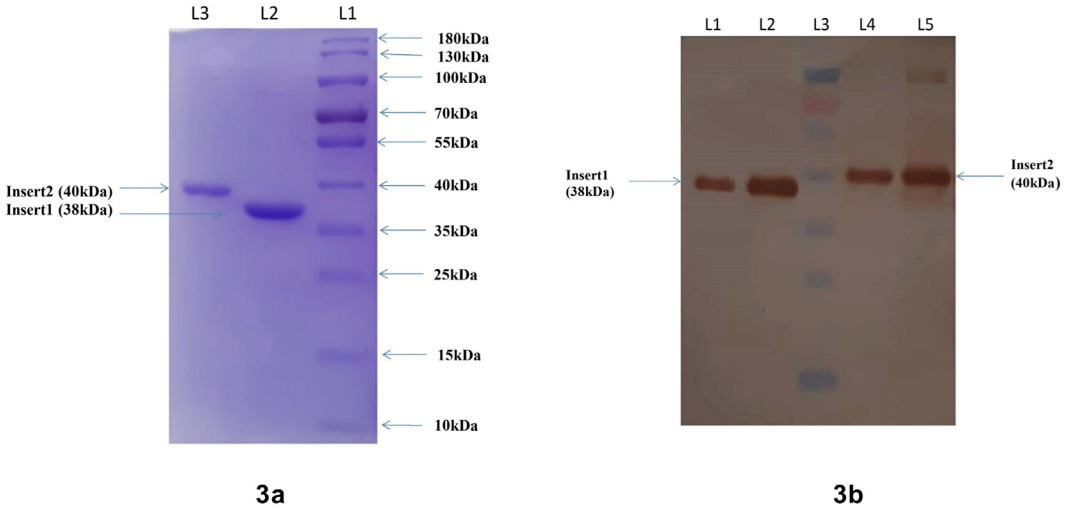

Fig. 3. a SDS-PAGE analysis of purified Cysteine synthase protein after performing dialysis. Lane 1 contains Pageruler prestained protein ladder (26616), Purified Insert1 CS protein of around 38 kDa in lane 2, Purified Insert 2 CS protein of about 40 kDa in lane 3. Fig. 3b Validation of purified Cysteine synthase by western blotting. Lane 1 and 2 represent purified fragments of Insert-1 (IS-1) CS whereas lane 4 and 5 indicate Insert-2 (IS-2) CS. Lane 3 contains Pageruler prestained protein ladder (26616).

4. Discussion

4.1. Characterization of CS gene and expression of rHC-CS protein

Cysteine is a key amino-acid for *H. contortus* adults because of haematophagous nutrition. Adult parasites utilize haemoglobin as an important source of nutrition. Cathepsin-B like cysteine proteases have their role in the digestion of proteins for nutrient acquisition. Knox et al. [34] showed that the cysteine proteases of *H.contortus* are capable of hydrolyzing haemoglobin, fibrinogen and albumin, thus helping in nutrient digestion [35]. Thus, as the parasite shifts to an adult haematophagous state, it will need to pump up its expression of these cysteine proteases [36], and it will have an increased requirement of cysteine. Enzymes of cysteine synthesis pathways and other PLP dependent enzymes have been studied to be potential drug targets in protozoans [37]. Williams et al. [18] using the in-silico genome mining approach, demonstrated that both RTS and *de novo* pathways are active in *Leishmania* spp. Romero et al., 2014 [15] could find only the RTS pathways to be an active synthetic pathway of cysteine in *Trypanosoma rangeli*; although the parasite had a coding gene for CS enzyme, however, it lacked two of the important Lys (Lys26 and Lys184) essentially required for the activity. Thus, different parasites adopts a selective approach to cysteine synthesis based on their microenvironments. Cysteine synthesis pathways and genes encoding them in *H.contortus* have not been characterized.

We characterized CS gene of the *H.contortus*, which is an important parasite of sheep and goat. The designed primers could successfully amplify the CS gene from the cDNA prepared from the isolated RNA of *H.contortus*. Phylogram suggested that the gene shows high sequence similarity with the cysteine synthase A protein of *Oesophagostomum dentatum* and a hypothetical protein of *Ancylostoma ceylanicum*, thus indicating the confirmation of gene as CS gene of *H. contortus* as the parasite belongs to the same phylum, i.e. Nematoda. The sequence of recombinant CS (rHC-CS) demonstrated only 38% identification with human Cystathionine beta-synthase (CBS). On performing blastp with human Cystathionine beta-synthase (CBS) against sequences of *H.contortus*, another hypothetical protein Pyridoxal phosphate-dependent enzyme domain-containing protein *Haemonchus contortus* (Sequence ID: CDJ82417.1) was demonstrating 61% sequence identity, which may indicate that the parasite may have other proteins in parallel for cysteine synthesis by RTS pathway as Walker and Barrett [26] could demonstrate some sort of cystathionine beta-synthase (CBS) and cystathionine γ-lyase (CGL) in the parasite. The CS gene, which translates to 334 amino-acids protein sequences, also demonstrated the presence of conserved CBS domain (11–305 amino acids), which is present on cystathionine beta-synthase (CβS) and cysteine synthase proteins with an e-value of 1.51e-16. In our earlier attempt, we failed to successfully purify the protein using an N-terminal His tag; therefore, the two versions of cysteine synthase protein were expressed, one with a C-terminal His Tag (IS-1/38 kDa) and one with His tag at both N-terminal and C-terminal (IS-2/40 kDa). The structural conformation of the rHC-CS protein consisted primarily of alpha-helical (45%) and βeta-sheet conformation. Ni-NTA affinity chromatography could very well purify the protein owing to very good binding with the Hexa-His tag. The western blotting with anti-His antibodies could detect and confirm the expression of both the recombinant versions of rHC-CS protein.

4.2. Relative expression of CS gene transcripts at different developmental stages of H contortus

Real-time primers were designed to amplify 169 bp product in the exonic region of CS gene, and they could successfully amplify the 169 bp product. The CS gene was found to have a nearly 2.5 fold greater expression in adults than the stage L1 *Haemonchus contortus* larva. Stage L3 larvas had the lowest CS gene expression having a nearly 7 fold down-regulation compared to stage L1 larva. Upregulation of CS gene transcripts in the adult stages compared to L1 and L3 larval stages could be due to the high cysteine requirement for producing adequate cysteine proteases to digest the blood meal [38]. Adult *H. contortus* demonstrate far greater expression of cathepsin-B protease genes (63 genes) than related free-living nematodes, representing 80% of all cathepsin cysteine protease genes in the genome [38]. Another very interesting finding is the significant down-regulation of the transcripts of CS enzyme in the L3 stage of *H.contortus*'s life cycle. It is the exsheathment process in L3 which marks the change from the free-living (L3) to the parasitic (xL3) larval stage, and then only *H. contortus* begins to grow, develop and starts feeding on the blood of the host [39]. It has been

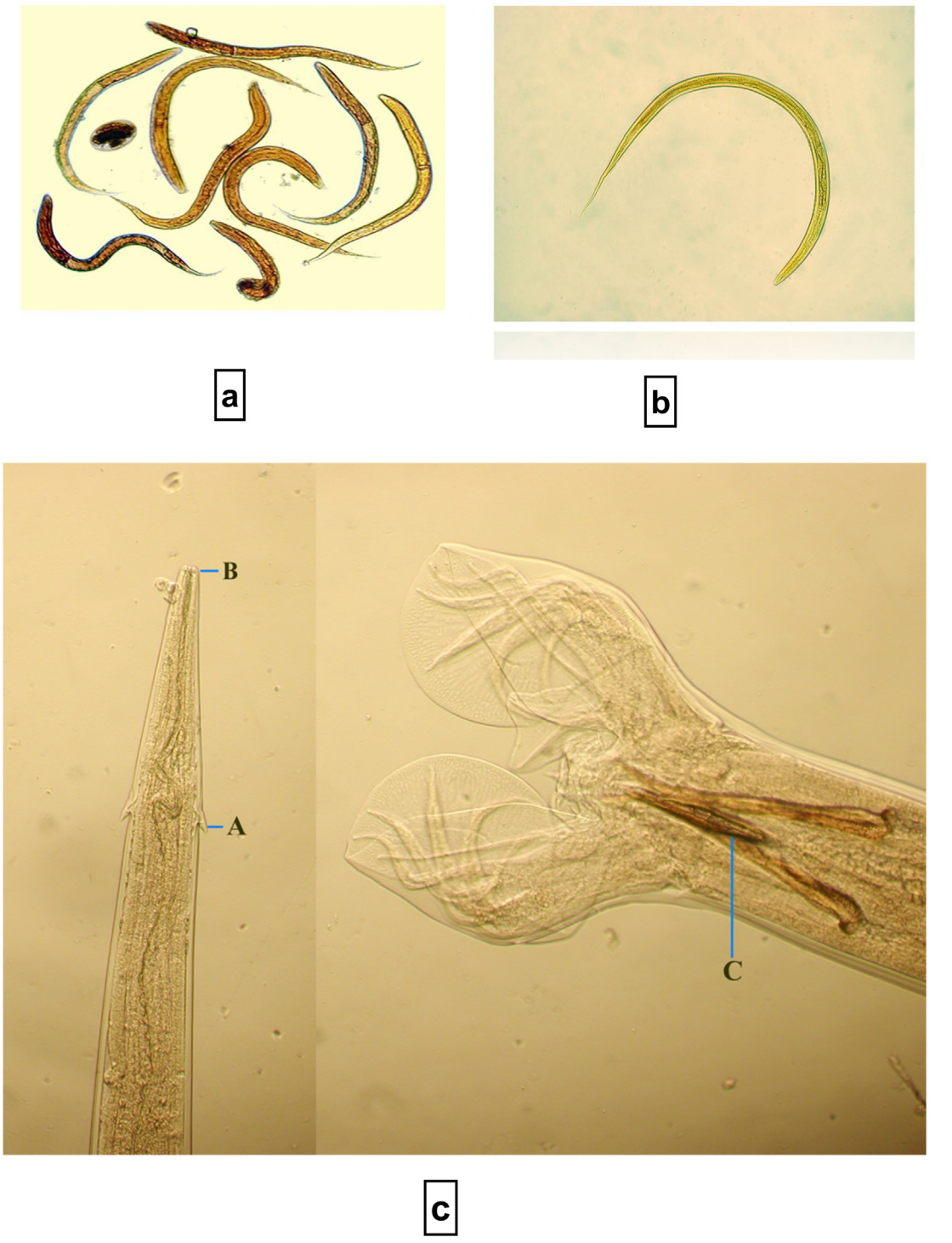

Fig. 4. Different developmental stages of H. contortus (a) L1 stage larvae obtained from the hatching of eggs of H.contortus (b) Characteristic L3 stage larvae obtained after the process of coproculture (c) Anterior and posterior end of adult male H. contortus. A: Cervical papillae, B: Buccal lancet, C: 'Y' shape spicules (100X).

very well known now that a dauer like development arrest happens to be at the L3 stage in the environment mediated through DA/DAF12 signalling system. This dauer like developmental stage is prominently characterized by developmental arrest, stress resistance and a significant extension of lifespan. Once conditions become favourable, which is generally sensed by the presence of bile hormones like Dafachronic acid [40], the L3-dauers exit the developmental diapause and rapidly progress into the L4 stage by sequential metabolic and developmental changes tightly regulated by a coordinated transcriptional network. Thus, the coprocultured L3 larvas were in the state of this developmental metabolic arrest, which is reflected as the decrease in the cysteine synthesis pathway gene at the L3 stage. Cathepsin-L like cysteine proteases are very active in L1 larva due to the very active process of embryogenesis, which may justify the higher expression of CS gene in L1 larva. It is also important to note that expression of CS gene, coding for an

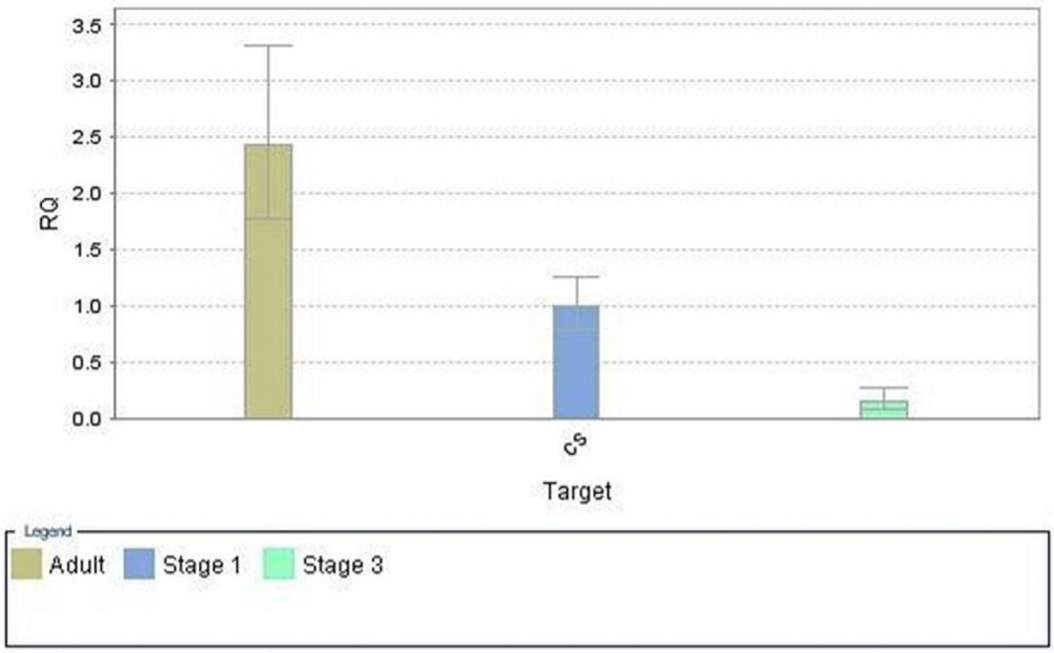

Fig. 5. Relative quantification of cysteine synthase (CS) gene by qPCR at different developmental stages of H. contortus.

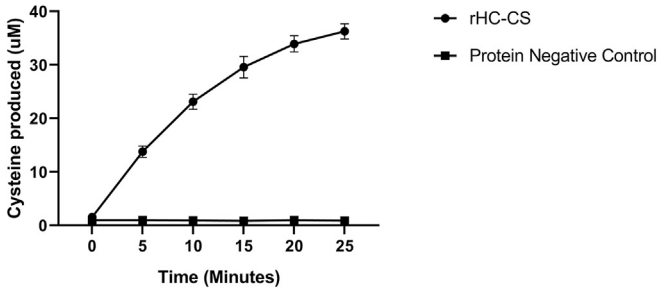

Fig. 6. Spectrophotometric assay for the quantification of cysteine production by rHC-CS. Cysteine synthesis activity was measured both for rHC-CS and protein control at six time points (t = 0, 5, 10, 15, 20 and 25 min). Protein negative control did not show any change with time.

Table 2
Kinetic properties of recombinant cysteine synthase of Haemonchus contortus

Kinetic constants of rHC-CS	OAS	Na_2S	Unit
V_{Max}	6.6 ± 0.85	1.92 ± 0.11	μM/min
K_M	9.3 ± 2.4	0.84 ± 0.21	mM
k_{cat}	1044	1216	s^{-1}
k_{cat}/K_M	1.1×10^6	1.45×10^6	$M^{-1}S^{-1}$

important enzyme of cysteine synthesis by *de novo* pathway, is happening at all the stages of the parasite's developmental cycle; however, its expression is modulated in close adjustment to the microenvironment of the parasite. It could be interesting to study the effect of modulation of cysteine expression on the growth, survival and development of *H.contortus* for future studies.

4.3. Haemonchus contortus does utilize de novo pathway for cysteine synthesis

The expressed 38 kDa IS-1 version of the rHC-CS protein was assayed for catalyzing the reaction of O-acetyl-L-serine (OAS) and bisulfide to produce L-cysteine and acetate. For testing cysteine production, one very important consideration for the assay should be that it should not react with the O-acetyl-L-serine in the reaction. The method of Gaitonde et al. [29] has been tested, and we found that O-acetyl-L-serine does not produce any colour on reaction with acid ninhydrin reagent-2, while cysteine produces bright pink colour. The same method was very specifically used by Fuzishima et al. [41] to assay the enzymatic CysM activity and some of its mutants in *E.coli*. We have ruled out any interconversion of cysteine to cystine by incorporating DTT in our reaction mixture, and in addition to that, cystine too is not detected and thus, does not interfere with reading at absorption maxima of 560 nm using acid ninhydrin. The time-course increase in cysteine production with a fixed amount of substrate confirms the in-vitro enzymatic activity of the enzyme.

We further confirmed by FTIR that the cysteine is getting produced in the reaction. Cysteine is an amino acid with a sulfhydryl group, and it has been reported that the cysteine shows a characteristic peak at 2357 cm-1, representative of the S–H group in FTIR [31,32]. We could further confirm cysteine production by the enzyme by a time-bound increase in the intensity and sharpness of the cysteine peak at 2357cm-1. It further confirms that the rHC-CS protein was catalyzing the production of cysteine.

Some of the O-acetyl serine sulphadrylases (OASS) of prokaryotic organisms could utilize O-phosphoserine (OPS) as an alternative substrate instead of OAS. rHC-CS did not show any activity with OPS. Kinetics of the enzyme was also studied to determine the V_{Max} and K_M of the OAS sulphadrylation reaction, which was slightly higher than *E.coli*'s OASS [42]. In alignment to our studies, most of the investigators have observed Michaelis-Menten kinetics of O-acetyl serine sulphadrylases [43], but positive cooperativity has been noted in some plant enzymes [43,44] and OASS isolated from *M. thermophile* [45], *Salmonella* serovar Typhimurium [46] and *Phaseolus* sp [47]. Inhibition of enzymatic activity has been reported for the OASS from *Salmonella* serovar Typhimurium at substrate concentrations above 7.5 mM O-acetyl serine [48] and in *Phaseolus* OASS at concentrations above 10 mM [47]. We did not

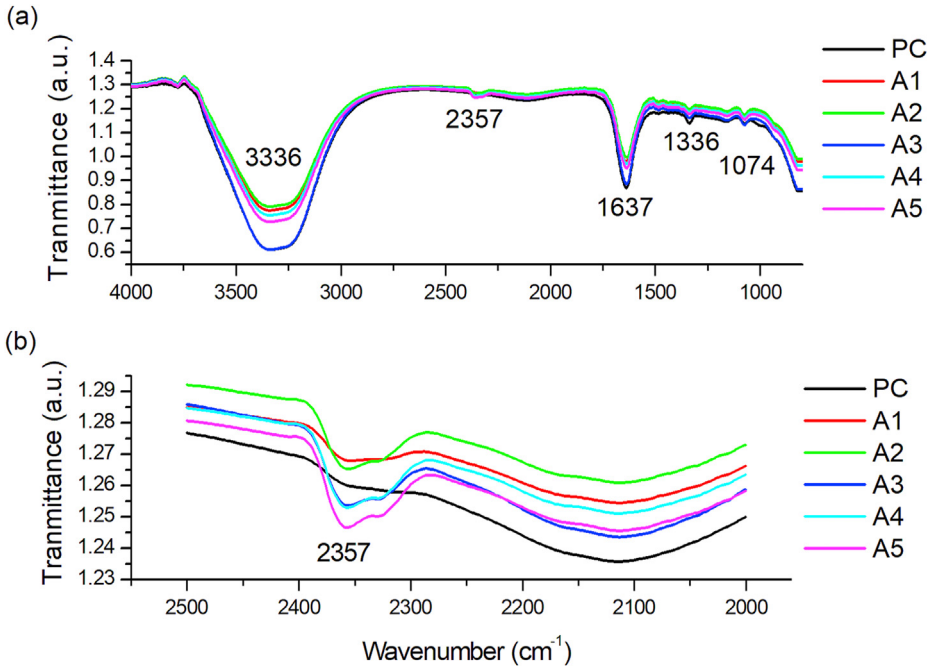

Fig. 7. FTIR examination of catalytic activity of rHC-CS.

observe enzymatic activity inhibition even at a concentration of 18 mM, which was the highest concentration of OAS studied in the current kinetic experiment. The observed K_M of sodium sulphide was slightly higher than prokaryotic OASS but was significantly lower to CBS from *Trypanosoma cruzi* [17] and *S. cerevisiae* CBS [49], which are also known to perform OAS sulphahydrylation. The cysteine synthase activity in *H.contortus* could be a mechanism to eliminate excess sulphide to avoid toxic effects, as the adult worms survive in the abomasum. This part of the compound stomach of ruminants is particularly important for protein digestion, and thus sulphide could be rich in its microenvironment. In addition to it, there is a strong presence of sulphur reducing bacteria (SRB) in the rumen (fermentative part of the compound stomach) of sheep, which reduce dietary sulphur to sulphide [50]. Thus, the sulphide content in rumen and abomasum could go very rich, particularly when animals are grazed on sulphate rich fodder sources [51]. The physiological significance of the discovered enzyme in context with host-pathogen interaction could be an important subject for further studies.

O-acetylserine sulfhydrylase A (CysK) is the pyridoxal 5′-phosphate-dependent enzyme which is important for catalyzing the final reaction of cysteine synthesis in bacteria. Because mammals could synthesize cysteine from methionine using RTS pathway and lack OASS, the enzyme is a potential target for antimicrobials. Isoenzyme specific inhibitors [52], fluoroalanine derivative inhibitors [53], thiazolidine inhibitors [54] and other inhibitors of the enzyme [55] have been identified to act as potential antimicrobials. There has been extensive research to elucidate potential newer activities and functions of the OASS enzyme [56,57].

The crude lysate of the *H.contortus* demonstrated very good cysteine synthase activity, which further corroborated our finding that the *de novo* pathway is very much active for cysteine synthesis in *H.contortus*. This discovery of a functional protein of *de novo* pathway in a multicellular organism opens up potential possibilities to further research the additional role and interactions of this enzyme with the parasite's biochemical milieu.

5. Conclusion

Cysteine synthase enzyme demonstrated the classical CS activity confirming that the *de novo* pathway is an active metabolic pathway of cysteine synthesis in *H.contortus.* The expression of CS gene was found to be highly up-regulated in adult worms and was found to be lowest in the L3 larval stage of *H.contortus*'s life cycle. It may, therefore, make sense to explore the importance of CS and its other functional role as well as interactions in pathogenic nematodes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2021.05.014.

Author credit role

Vijay Saxena – Investigation, Conceptualization; Methodology, Validation, Data Curation, Writing Original Draft, Project Adminstration, Formal Analysis, Review & Editing:Vedamurthy GV– Investigation, Conceptualization, Methodology, Validation, Funding Acquistion, Project Adminstration, Review & Editing: C.P.Swarnkar-Investigation, Methodology, Visualization; Vinod Kadam-

Investigation, Methodology, Review & Editing: Suneel Kumar Onteru-Formal analysis; Validation; Review & Editing; Haseen Ahmad- Investigation, Methodology; Raghvendar Singh -Formal analysis; Supervision; Resources.

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