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Molecular expression and characterization of GCP7 gene of *Haemonchus contortus*

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ABSTRACT

Haemonchus contortus is a highly pathogenic and most economically important parasite of sheep and goats worldwide. The cysteine proteases from *H. contortus* are prime targets for vaccine development. In the present communication we report the molecular expression and characterization of cathepsin B-like cysteine protease, GCP7 gene of *H. contortus* to study its efficiency as target protein for immunoprophylaxis against haemonchosis in sheep and goats. The complete ORF of GCP7 gene, devoid of the signal sequence, was amplified by RT-PCR from mRNA isolated from *H. contortus* and was cloned initially into pTZ57R/T cloning vector and then sub-cloned in the pET32a(+) expression vector to produce GCP7 antigen. The nucleotide and deduced amino acid sequence of the GCP7 was aligned against the related sequences of *H. contortus* available in public domain for *in silico* analysis by DNA STAR and MEGA version 4.0 softwares. The nucleotide sequence revealed that the GCP7 gene of *H. contortus* (Indian isolate) encodes 324 amino acids (devoid of signal sequence) and its nucleotide sequence had 95.9% to 99.4% sequence homology with that of U.S.A. and previously published Indian isolates. A high level expression of recombinant (r) GCP7 protein was observed in the molecular range (Mr) of 55 kDa. The rGCP7 protein was confirmed by its specific immunoreactivity against known reference positive sheep sera.

Key words: Cloning, Cysteine proteinase, Expression, *Haemonchus contortus*, GCP7, Sheep

Haemonchus contortus is an economically important and highly pathogenic blood feeding nematode parasite with high biotic potential causing parasitic gastro-enteritis in small ruminants' in many parts of the world (Skuce *et al.* 1999). *Haemonchus contortus* infection can cause, particularly in young ones, anaemia and weight loss that in few cases lead to death of the animals (Bakker *et al.* 2004). Control of *H. contortus* is currently achieved by the regular use of anthelmintics and losses due to anthelmintic treatment against *H. contortus* in India alone have been estimated to be $103 million/year (McLeod 2004, Waller and Chandrawathani 2005). Further, this method of control leads to the inevitable development of anthelmintic resistance (Wolstenholme *et al.* 2004). The widespread emergence of multiple anthelmintic resistant *H. contortus* strains and increasing concern about drug residues in the food chain and the environment has dramatically accelerated the need to develop alternative, sustainable control measures to prevent the economic impact of this parasite (Coles 1998, Newton and Munn 1999, Wolstenholme *et al.* 2004). Several promising antigens have been identified as vaccine candidates for the development of a vaccine against *H. contortus* and significant among these are so-called 'hidden' antigens, such as H11 (Smith *et al*. 1997), cysteine proteinases (Knox *et al.* 1993), H-gal-GP (Smith *et al.* 1994) and natural antigens such as adult 15/24 kDa excretory/ secretory antigens (Schallig *et al*. 1994) and Hc-sL3 (Raleigh *et al.* 1996).

The cysteine proteases are considered as one of the prime targets for vaccine development against parasitic helminths (Loukas *et al.* 2004, Redmond and Knox 2004, Selzer *et al.* 1999). Cysteine proteases secreted from the gut of *H. contortus* are the most active proteases of the excretory-secretory (ES) products (Parkinson *et al.* 2004) and are likely to be involved in induction of protective immunity (Bakker *et al.* 2004). Many immunization trials in sheep against *H. contortus* have been carried out either with a cysteine proteinase-enriched fraction (TSBP) isolated from

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the gut of adult parasites or using recombinant cocktail of cathepsin B-like cysteine proteinases *viz.* hmcp1, 4 and 6 (Knox *et al.* 1999, 2005, Redmond and Knox 2004) with promising results.

However, apart from the vaccination trials on sheep with hmcp1, 4, 6 and AC-5, cysteine proteases, perusal of literature shows that there are no reports available on the expression and protection studies involving other cysteine proteases identified and characterized so far. The GCP7 is another cysteine protease of *H. contortus*, expressed in gut (Rehman and Jasmer 1998) and found in both intestinal and ES products (Shompole and Jasmer 2001, Yatsuda *et al.* 2006). The predicted mature GCP7 encodes for a 33 kDa protein comprising of 348 amino acids (Shompole and Jasmer 2001) and had a high degree of sequence identity with numerous cathepsin B-like cysteine protease sequences (CBLs) from parasitic and free living nematodes.

In the present communication we report molecular cloning, expression and preliminary characterization of GCP7 gene from the Indian (Bareilly region, Uttar Pradesh) isolate of *H. contortus*.

MATERIALS AND METHODS

Parasite collection: Adult nematode parasites were collected in PBS (pH 7.4) from abomasa of sheep and goats, slaughtered at local abattoir, Bareilly. Parasites were identified by using morphological keys (Soulsby 1982). Then the worms were used either for direct extraction of RNA or preserved in RNA Later and stored at -20^0 C for further use.

Isolation of total RNA and complementary DNA (cDNA) synthesis by reverse transcription: Total RNA was extracted from *H. contortus* using Trizol reagent (Life Technologies) as per manufacturer's recommendations. Complementary DNA (cDNA) was synthesized from the total RNA using oligo (dT) 18 primer following standard protocol (Sambrook and Russell 2001).

Oligonucleotide primers: A pair of PCR primers, specific for a 1051 bp coding sequence of GCP7 gene of *H. contortus* was designed from published sequence (Accession No: AF046229) and custom synthesized. The restriction sites for *Nco* I and *Sal* I were incorporated in the forward (GCP7F – 5' TTCCCATGG**CTCAAAAATTCACCAGATTAG AAGAG** 3') and the reverse primers (GCP7R – 5' GGGGTCGAC**TACAGCTAGTTACGAAAATCACAC** 3'), respectively.

Polymerase chain reaction (PCR) based amplification of GCP7 gene of H. contortus*:* The PCR assay for amplification of GCP7 coding sequence (devoid of signal sequence) from Bareilly isolates of *H. contortus* was laboratory standardized in 25 µl reaction volume containing 70 ng of cDNA, 10 pmol each of forward and reverse primers (GCP7F and GCP7R), 1.5 mM $MgCl_2$, 200 µM of each dNTPs, and 1U of Truestart Hotstart *Taq* DNA polymerase (MBI Fermentas, Lithuania). The reaction was carried out in a PTC 200 thermal cycler (MJ Research, USA) with a preheated lid. The cycling conditions were standardized as an initial denaturation of strands for 4 min at 95°C, followed by 35 cycles of denaturation at 95°C for 1 min, annealing of primers at 65°C for 1 min and extension of strands at 72°C for 1.5 min. A final extension of the synthesized strands was given at 72°C for 15 min. The PCR amplification was confirmed by running the product on an ethidium bromide stained 1.2% agarose gel and visualization of the amplicon on a transilluminator under UV light.

Molecular cloning and characterization of GCP7: The 1051 bp PCR amplicon was purified using gel extraction kit (Qiagen, Germany) and cloned in pTZ57R/T cloning vector (Fermentas, Lithuania) following standard protocol. The identification of the positive clones was based on blue white colony screening and colony PCR. The confirmation of the transformed colonies was further made by restriction analysis of the plasmid DNA isolated from the white colonies using *Nco* I and *Sal* I. The positive construct was named as pTZ57GCP7.

A subculture of positive clone harbouring the desired GCP7 gene was custom sequenced for nucleotides at Department of Biochemistry, Delhi University, New Delhi. The sequence alignment and analysis were done with the MegAlign component of the DNAStar programme (Version 5.0 DNASTAR, Madision, Wis.). Phylogenies were constructed by neighbour-joining using p-distance model using homogeneous pattern among lineages and tested by bootstrap with 1000 replicates, using MEGA version 6.0 (Tamura *et al.* 2013).

Expression and confirmation of rGCP7: Both the pTZ57GCP7 construct containing GCP7 gene and the pET-32a(+) expression vector (Novagen) was double digested with *NcoI* and *SalI* restriction enzymes following standard protocol for ligation of the product in the reading frame. *Escherichia coli* BL21(DE3) pLysS cells were transformed with the recombinant pET32a plasmid construct containing GCP7 gene insert and the positive clones were identified by colony PCR using the same pair of primers (Sambrook *et al*. 2001). Further confirmation of the transformed colonies was made by restriction enzyme digestion of the recombinant plasmid DNA using *Nco*I and *Sal*I carried out at 37ºC for 1 h.

The positive clones containing GCP7 gene insert were induced with 1 mM IPTG. The expressed recombinant protein was purified under denaturation condition by affinity chromatography using Ni-NTA agarose with certain modifications introduced in the purification parameter (Qiagen, Germany) and purity of the eluted protein was checked by SDS-PAGE using 12% gel under denaturing conditions at 100 V for 2–3 h. The immunoreactivity of the recombinant protein was evaluated by western blotting against infected sheep sera.

RESULTS AND DISCUSSION

Haemonchus contortus, a highly pathogenic GI nematode of sheep and goats and economically important parasite in small-ruminant farming (Sykes 1994). Due to

widespread emergence of *H. contortus* strains that are resistant to the anthelmintic drugs currently available, much effort has been directed towards development of a vaccine (Smith 1999). The cysteine proteases from parasitic helminths are prime targets for vaccine development and the most promising vaccine candidates identified in *H. contortus* so far (Knox 2005). Cysteine proteases secreted by *H. contortus* are probably involved in tissue penetration, feeding and defence against effector mechanisms of the host immune responses which have led to their purification and evaluation in protection trials (Knox 2001). Though, immunization trials in sheep against *H. contortus* have been carried out either with a cysteine proteinase-enriched fraction (TSBP) isolated from the gut of adult parasites or using recombinant cocktail of cathepsin B-like cysteine proteinases *viz.* hmcp1, 4 and 6 (Knox *et al.* 1999, 2005, Redmond and Knox 2004) with promising results, no reports are available on the expression and protection studies involving other cysteine proteases identified and characterized to date. Hence, in the present study, we cloned and expressed the GCP7 cysteine protease from the Indian (Bareilly region, U.P.) isolate *H. contortus* for charecterization and protection studies.

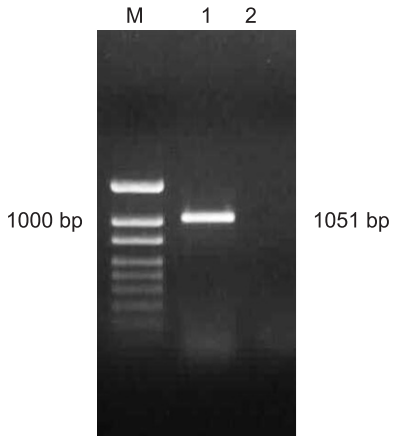


Fig. 1. Agarose (1.2%) gel showing the PCR amplification of GCP7 gene fragment (without signal sequence) specific for *Haemonchus contortus*. Lane M indicates 100 bp plus DNA marker. Lane 1 indicates GCP7 PCR product. Lane 2 indicates negative control with water.

Molecular cloning and characterization of GCP7 gene: The coding sequence of GCP7 gene (devoid of signal sequence) of *H. contortus* (Bareilly isolate) was PCR amplified using *de novo* primers with an annealing temperature of 65°C. The amplicon was resolved as a single band of 1051 bp (Fig. 1). The selection of positive colonies was done by colony PCR using the specific primers, which amplified 1051 bp product and the insert was released by *Nco*I and *Sal*I endonuclease digestion of the plasmids extracted from the transformed *E. coli* cells. The nucleotide sequence of GCP7 was deposited to GenBank database (accession number: KT726318). The sequence information revealed 975 bp nucleotides encoding 324 amino acids which form the primary structure of the GCP7 protein, with theoretical molecular weight and isoelectric point of 36.39 kDa and 8.08 respectively. The *in silico* analysis of GCP7 gene of *H. contortus* (Bareilly isolate) by GeneTool software showed the base composition of A: 292; T: 242; G: 249; C: 192 with AT density of 54.8%, and GC density of 45.2%.

The nucleotide and deduced amino acid sequences of GCP7 gene of *H. contortus* (KT726318) was aligned and analysed *in silico* using DNA STAR and MEGA version 6.0 softwares against the sequences from other isolates of *H. contortus* available in public domain. *Haemonchus contortus* Indian isolate used in the present study showed 99.4% homology (Fig. 2a) with the published sequences of *H. contortus* USA isolate (AF046229), 95.9% and 96.8% homology with the previously published Indian isolates (Button form (GQ 327962) and Linguiform (GQ 327963)) respectively. A total of six nucleotide substitutions were

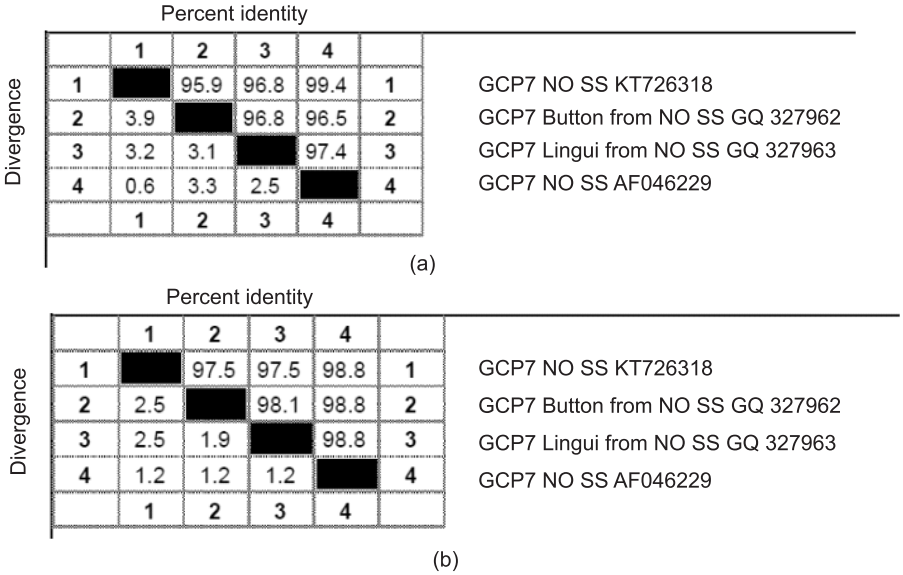


Fig. 2a. Sequence pair distances of GCP7 (without signal sequence) Clustal V method. b. Deduced amino acid pair distances of GCP7 (no signal sequence) Clustal V method.

found in Indian isolate used in the present study (KT726318) in comparison to USA isolate, however, there were 19 nucleotide substitutions when compared with the previously published Indian isolates (Button form GQ 327962 and Linguiform GQ 327963). Based on deduced amino acid sequence analysis, the Indian isolate (KT726318) used in the present study revealed 98.8% homology (Fig. 2b) with the USA isolate (AF046229) and 97.5% homology with the previously published Indian isolates. There were substitution of amino acids at 4 positions in the Indian isolate used in the present study when compared with USA isolates and 5 amino acid substitutions with previously published Indian isolates.

The GCP7 protein has one potential N-linked glycosylation site. Further, it contains pro region and occluding loop region (Rehman and Jasmer 1998). The occluding loop has been implicated in mechanism of autolytic cleavage of the pro region and enzyme stability (Rehman and Jasmer 1999). On amino acid sequence analysis, it was evident that the GCP7 gene of Indian isolate used in the present study also had one potential N-linked glycosylation site, pro region and occluding loop region .

A phylogenetic tree for nucleotides and amino acids of GCP7 gene of *H. contortus* (without signal sequence) Bareilly isolate was constructed by neighbour-joining using p-distance model using homogeneous pattern among lineages and tested by bootstrap with 1000 replicates, using MEGA version 6.0 to delineate its relationship with other referral isolates (Figs. 3a & 3b) and it clearly showed that the Indian isolate of *H. contortus* (KT726318) used in the present study is more closely related to USA isolate already available in public domain so far as the nucleotide and amino acid sequence homology is concerned.

Expression and immunoreactivity of rGCP7: High level expression of GCP7 was achieved in *E. coli* BL21(DE3) pLysS cells at 4 h of induction with 1 mM IPTG. The SDS-PAGE revealed the molecular mass of rGCP7 as approximately 55 kDa (Fig. 4a), which included the histidine, Trx and S tags. The specific recombinant GCP7 protein was purified to homogeneity using Ni-NTA agarose beads under denaturing conditions (Fig. 4b).

The identity of the recombinant protein was established by immunoblot assay using specific Ni-NTA HRP conjugate (Fig. 5). Further, the immunoreactivity of the expressed protein was confirmed by western blot analysis against known reference positive sheep sera (Fig. 6) with strong immunoprecipitation band at 55 kDa region.

This is the first report on molecular expression and characterization of GCP7 gene of *H. contortus* for its potential application for immunologically based control of haemonchosis in small ruminants. However, the expressed rGCP7 protein has to be evaluated for its immunization efficacy in small ruminants.

It is the first of its kind in molecular expression of GCP7 gene of *H. contortus* for application in immunoprophylaxis studies against *H. contortus* infection in sheep and goat in the world. Further works on the characterization of GCP7 protein and conduction of immunization trial with the recombinant GCP7 protein of *H. contortus* in sheep and goat is needed for its application and commercial exploitation as a vaccine candidate.

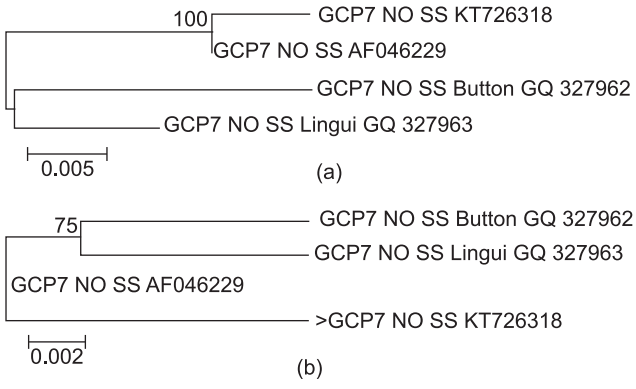


Fig. 3a. Phylogenetic analysis by neighbour-joining using p-distance on GCP7 sequence. Numbers at nodes represent percentage occurrence of clades in 1000 bootstrap replications of data. b. Phylogenetic analysis by neighbour-joining using p-distance on deduced amino acid GCP7 sequence. Numbers at nodes represent percentage occurrence of clades in 1000 bootstrap replications of data.

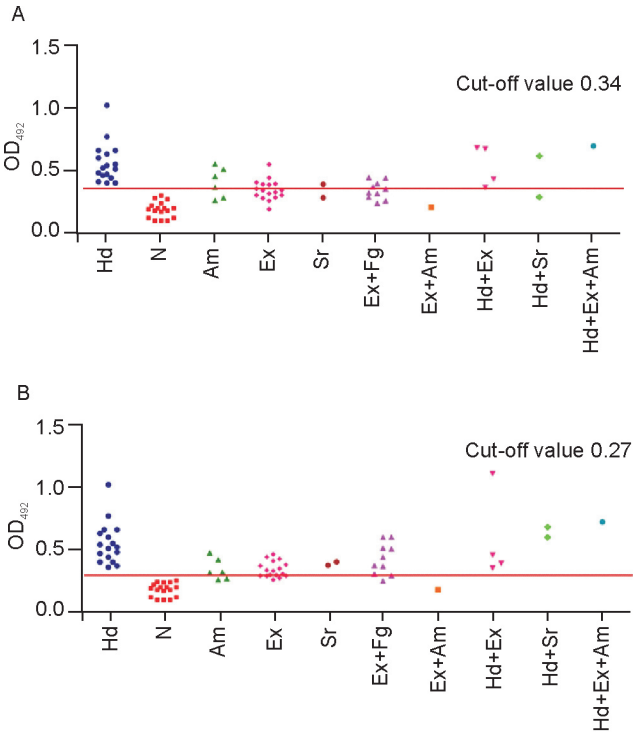
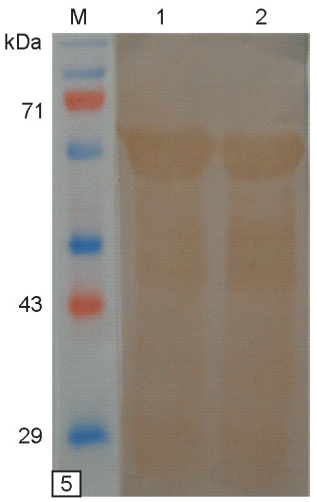
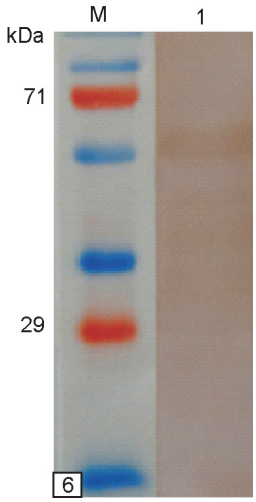


Fig. 4a. SDS-PAGE analysis of expression of GCP7 protein. Lane M indicates prestained protein molecular weight marker (Fermentas). Lane 1 indicates uninduced control (0 h culture) and Lanes 2–6 indicates induced cultures at 2, 4, 6, 8 h and over night respectively. b. SDS-PAGE analysis of the purified recombinant GCP7 protein. Lane M indicates protein molecular weight marker (Fermentas). Lane 1 indicates control before purification, Lane 2 indicates flow through. Lane 3 indicates wash through and Lanes 4&5 indicates purified rGCP7 protein.





Figs 5–6. **5.** Western blot analysis of purified rGCP7 using Ni-NTA HRPase conjugate. Lane M indicates pre-stained protein molecular weight marker (Fermentas) and Lanes 1&2 indicates purified rGCP7 protein. **6.** Western blot showing purified rGCP7 reactivity to *H. Contortus* infected sheep sera. Lane M indicates pre-stained protein molecular weight marker (Fermentas) and Lane 1 indicates purified rGCP7 protein.

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