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Cloning and Characterization of Aldolase from Parasitic Nematode *Haemonchus contortus*

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Abstract: Aldolase (ALD) was a glycolytic enzyme which catalyzes the cleavage of fructose 1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. In the present research, the *ALD* gene of *Haemonchus contortus* (HcALD) was first cloned and characterized. Specific primers for the Rapid Amplification of cDNA Ends (RACE) were designed based on the Expressed Sequence Tag (EST) to amplify the 3' and 5' ends of HcALD. The full length of HcALD cDNA was obtained by overlapping the sequences of 3' and 5' extremities. The Open Reading Frame (ORF) of HcALD was amplification by Reverse Transcription PCR (RT-PCR) and expressed in prokaryotic cell. Then, the biochemical activities of the recombinant HcALD protein were analyzed by assays of enzymatic activity, thermal and pH stabilities. The result showed that the full length cDNA of HcALD was 1235 bp, containing 27 bp of 5' Un-Transcript Region (5' UTR), 1098 bp of ORF and 110 bp of 3' UTR. The deduced amino acid sequence of HcALD was highly similarity to the ALDs from the nematodes *Caenorhabditis elegans*, *Brugia malayi* and *Onchocerca volvulus*. The biochemical assay showed that the recombinant HcALD exhibited enzymatic activity and the optimum temperature and pH for the reaction were 40°C and 7.5, respectively.

Key words: *Haemonchus contortus*, aldolase, RACE, enzyme activity assay, region

INTRODUCTION

Haemonchus contortus is one of the major gastrointestinal parasites of ruminants, causing great losses to the agricultural industry world wide (Knox *et al*., 1993; Newlands *et al*., 2001). Parasitism by this blood feeding nematode causes serious effects ranging from mild anemia to mortality in animals, primarily lambs and kids (Nikolaou and Gasser, 2006). The widespread emergence of *H. contortus* resistant strains to anthelmintic drugs currently available (Wolstenholme *et al*., 2004) has dramatically accelerated the need for alternative, sustainable control measures. Among them, the immunization of small ruminants against *H. contortus* has been tested over the last decades utilizing different immunizing preparations (Knox and Smith, 2001). And some of them have been proved for partial protective efficiency with reduced faecal egg output and worm burdens (Smith and Munn, 1990; Munn *et al*., 1993a, b; Redmond and Knox, 2004). However, for the difficulty to get enough native protein and the poor protection of recombinant proteins, no commercial vaccine against *H. contortus* was made up to date (Newton and Munn, 1999). More research should be done on discovering protective antigens of this parasite.

Recent research showed that DNA vaccine encoding cysteine protease, glutathione peroxidase, glyceraldehyde-3-phosphate dehydrogenase and aminopeptidase of *H. contortus* gave partial protection against the parasite infection with 28-38% reduction in worm burdens (Muleke *et al*., 2007; Sun *et al*., 2011; Han *et al*., 2012; Zhao *et al*., 2012). Parasitic nematodes utilize glucose for energy production and glycogen is the major substrate for energy conservation (Kohler, 1985). These may indicate that the enzymes involving in metabolizing of *H. contortus* could be the target for vaccine candidates.

Fructose-1, 6-bisphosphate Aldolase (ALD) is a glycolytic enzyme which catalyzes the conversion of Fructose-1, 6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Rutter, 1964). The enzyme is classified into two groups, class I and II. Class I aldolase forms a covalent Schiff-base intermediate with the substrate whereas class II aldolase requires divalent cations for the reaction (Rutter, 1964). There have been many reports of molecular cloning and structural analyses of class I aldolase genes for vertebrates including human (Rottmann *et al*., 1987), mouse (Stauffer *et al*., 1990), rat (Mukai *et al*., 1991), frog (Atsuchi *et al*., 1994), fish (Llewellyn *et al*., 1995) and

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chicken (Wang *et al*., 2007). The aldolase genes of parsites such as *Trypanosoma brucei* (Clayton, 1985), *Plasmodium falciparum* (Knapp *et al*., 1990), *Caenorhabditis elegans* (Inoue *et al*., 1997), *Onchocerca volvulus* (McCarthy *et al*., 2002), *Brugia malayi* (Ghedin *et al*., 2007) and *Schistosoma mansoni* (Marques *et al*., 2008) have also been cloned and analyzed. However, only an EST of *H. contortus* ALD has been published (GenBank Accession No. CB016007). In the current study, researchers report the complete coding sequence of the aldolase of this nematode (HcALD) and the enzymatic activity of its recombinant protein.

MATERIALS AND METHODS

Parasite preparation and mRNA extraction: Adult *H. contortus* worms were collected from the abomasums of the donor goats as described earlier (Muleke *et al*., 2006). The nematodes were washed three times with normal saline and stored in liquid nitrogen. Total RNA was prepared from pooled parasites utilizing the single step protocol (Chomczynski and Sacchi, 1987).

3' RACE and 5' RACE: Primers used for 3' and 5' RACE were listed in Table 1 which were designed based on *H. contortus* EST (GenBank accession No. CB016007) and the primers provided in the RACE kit (TaKaRa Biotech. Dalian, China). The oligonucleotides were synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China).

The 3' end of cDNA was amplified by 3' full RACE kit (TaKaRa Biotech., Dalian, China) using the forward gene specific primers Ald-3-F1 and Ald-3-F2. The primary PCR System (final volume, 25 μL) contained 2 μL of 3'-RACE-Ready cDNA, 1.0 U LA Taq® polymerase (TaKaRa Biotech, Dalian, China), 40 μM 1×cDNA Dilution Buffer II, 50 μM 10×LA PCR Buffer II (Mg^{2+} Free), 3.0 mM $MgCl_2$ and 400 nM of each primer. The primary PCR was performed using Ald-3-F1 and 3' outer primer followed by treatment at 94°C (3 min), 30 cycles at 94°C (30 sec), 55°C (30 sec) and 72°C (1 min) and a final extension at 72°C for 10 min. The product of the reaction was used as a template in a second PCR with primers Ald-3-F2 and 3' inner primer. This second PCR System contained the same ingredients as the first except that it lacked the 40 μM 1×cDNA Dilution Buffer II and contained 400 μM dNTP mixture. The parameters for the second PCR were the same as those in the first run. The HcALD 3' end fragment was then obtained.

The 5' end of the cDNA was amplified by 5' RACE PCR using the same method as the 3' RACE PCR. The primary PCR was performed using Ald-5-R1 and 5' outer primer with Ald-5-R2 and 5' inner primer in the second PCR.

Both of the products of the second PCRs were cloned into the pMD18-T vector (TaKaRa Biotech, Dalian, China) and sequenced by Invitrogen Biotech. Co., Ltd. (Shanghai, China). The complete sequence of the HcALD cDNA was deduced from the overlapping sequences of both amplification products using BioEdit Version 7.0.1 (T.A. Hall, North Carolina State University, USA).

Sequence analysis: Sequence similarity was studied using the BLASTP and BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi). ALD protein sequences were aligned using CLUSTALW1.82. The signal peptide, secondary structure and protein motifs were predicted using approaches accessible on the internet: SignalP (http://www.cbs.dtu.dk/services/SignalP/), PSIpred (http://bioinf4.cs.ucl.ac.uk:3000/psipred/), Motifscan (http://myhits.isb-sib.ch/cgi-bin/motif_scan), respectively. Phylogenetic analyses among ALDs were inferred by the Neighbor Joining (NJ) Method.

Expression and purification of recombinant HcALD: The complete *H. contortus* aldolase ORF was cloned using the sequence-specific primers Ald-F (5'-GGATCCATGG CATCGTACT-3') contained a BamH I site (underlined) and Ald-R (5'-CTCGAGTCAATAGGCATGATT-3') contained a Xho I site (underlined). The PCR reactions (final volume, 25 uL) contained 2 uL cDNA, 2.5 U Taq DNA polymerase (TaKaRa Biotech, Dalian, China),

Table 1: Primers used in 5' and 3' RACE

Primer name	Sequence (5'-3')	Length (bp)	Description
Ald-3-F1	TCCATAGATGCCAGAAGATTACC	23	Forward primer specific for 3' end of HcALD in primary PCR
Ald-3-F2	AGGGCACCAAGCCTTCTCACGAT	23	Forward primer specific for 3' end of HcALD in second PCR
3' outer primer	TACCGTCGTTCCACTAGTGATTT	23	Reverse primer for 3' end of HcALD in primary PCR (provided in 3' RACE kit)
3' inner primer	CGCGGATCCTCCACTAGTGATTTCACTATAGG	32	Reverse primer for 3' end of HcALD in second PCR (provided in 3' RACE kit)
Ald-5-R1	CAGTCTCGGTAATCTTCTGGCATCT	25	Reverse primer specific for 5' end of HcALD in primary PCR
Ald-5-R2	TGTGGGAAGGTGTGGTGGCGGAAATCT	27	Reverse primer specific for 5' end of HcALD in second PCR
5' outer primer	CATGGCTACATGCTGACAGCCTA	23	Forward primer for 5' end of HcALD in primary PCR (provided in 5' RACE kit)
5' inner primer	CGCGGATCCACAGCCTACTGATGATCAGTCGATG	34	Forward primer for 5' end of HcALD in second PCR (provided in 5' RACE kit)

1.5 mM $MgCl_2$, 400 uM dNTP mixture, 50 uM 10×PCR Buffer (Mg^{2+} Free) and 400 nM of each primer. Thermal cycling was carried out at 94°C for 3 min followed by 30 cycles of 94°C (45 sec), 55°C (45 sec), 72°C (75 sec) and a final extension at 72°C for 10 min.

The amplicon was cloned into the pMD18-T vector. Then, the recombinants were digested with BamH I and Xho I and the target gene was subcloned into the bacterial expression vector pET32a (+) (Novagen, USA) using the standard method. The recombinant plasmid was sequenced to confirm that the HcALD insert was in the proper reading frame and without PCR errors. The recombinant plasmid pET32a (+)/HcALD was transferred into competent *E. coli* BL21 (DE3) and the recombinant protein was induced and expressed by addition of 1 mM Isopropyl Thiogalactoside (IPTG; Sigma-aldrich, USA) to the cell culture after the OD600 of the culture reached 0.5 at 37°C. The cells were incubated at 37°C for 5 h after the addition of IPTG. The cell lysates were prepared by sonication and analyzed by SDS-PAGE using 12% gels to confirm the distribution of the expressed recombinant protein.

To purify the recombinant protein, induced *E. coli* cells were harvested by centrifugation and sonicated for 15 min on ice. After centrifugation at 10,000×g, the supernatant was added to a Ni^{2+}-Nitrilotriacetic Acid (Ni-NTA) column (GE Healthcare, USA) and purified according to the manufacturer's instructions. An elution buffer (300 mM NaCl, 40 mM NaH_2PO_4, pH 8.0) containing 400 mM of imidazole was used to wash the His-tagged proteins from the Ni-NTA column. Purity of the protein was detected by 12% SDS-PAGE and the concentration of purified protein was determined according to the Bradford procedure (Bradford, 1976) using Bovine Serum Albumin (BSA) as a standard. The protein was stored at -20°C in 50% glycerol for later use.

Western blot analysis: After electrophoresis in SDS-PAGE (12% w/v), Western blotting was performed. Recombinant proteins were electrophoretically transferred from the gel to a nitrocellulose filter (Sigma, USA). The blots were then incubated for 1 h in blocking solution containing 5% (w/v) fat-free dry milk, 0.1 M PBST (pH 8.0) and 0.05% (v/v) Tween-20 and incubated with the primary antibodies (goat sera, collected from native goats infected with *H. contortus*.) for 1 h at 37°C (dilutions 1:100 to goat sera in 5% (w/v) fat-free milk/PBST). The membrane was washed 4 times for 10 min each in PBST and then incubated with the second antibody (HRP-conjugated rabbit anti-goat IgG, Sigma Immuno-Chemicals) in 1:5000 dilution of 5% milk powder/PBST for 1 h at 37°C. After four rounds (10 min each round) of washing with PBST, the immuno-reaction was visualized using freshly prepared DAB (Sigma) as a chromogenic substrate after 2-5 min.

Determination of the recombinant HcALD activity and the optimal pH and temperature for the enzyme reaction: The ALD activity of the purified recombinant protein (HcALD) was measured according to the method of Rajkumar *et al.* (1966) with some modifications. About 1 mL reaction mixture contained 50 mM Triethanolamine-HCl buffer (pH 7.5), 0.2 mM NADH, 2 mM Fructose-1,6-bisphosphate (Sigma, USA), 100 ug BSA, 76 Unite of α-Glycero phosphate dehydrogenase (Sigma, USA), 480 U Triosephosphate isomerase (Sigma, USA) and 50 ng HcALD (purified recombinant protein)

To determine the apparent optimal temperature, reactions were carried out at temperatures of 20, 30, 40, 50, 60 and 70°C using a thermostated cuvette holder connected to a refrigerated bath circulator. To determine the optimal pH, 50 mM Triethanolamine-HCl buffer of pH 6.0, 6.5, 7.0, 7.5, 8.0 8.5 and 9.0 was used and the reaction was carried out for 3 min after the addition of 50 ng recombinant HcALD protein. The changes in absorbency were monitored at 340 nm before the addition of the recombinant protein and at the end of the reaction.

One unit of enzyme activity was defined as the amount of enzyme that catalyzed the reduction of 1 μmol of fructose-1,6-bisphosphate per min under the conditions used. Meanwhile, the protein extracted from *E. coli* transformed with pET 32a(+) (empty vector) was used as negative control.

RESULTS AND DISCUSSION

Cloning and sequence analysis of HcALD: By overlapping the 3' and 5' RACE fragments, a 1235 bp transcript was obtained. A 22 bp Spliced Leader sequence (SL1) was identified at the 5' end. The 27 bp 5' UTR was detected before the ATG initiation codon followed by a 1098 bp ORF terminated with the TGA stop codon. At the 3' end of the cDNA, 110 bp of 3' UTR with a poly-A tail (11 bp long) was found (Fig. 1). The ORF was found to encode a protein of 365 amino acids with a molecular mass of 39,697 Da, the deduced theoretical pI of the protein was 7.77. By analysis with the SignalP, no signal peptide was found in the predicted protein. Motifscan analysis showed that an enzymatic activity site of aldolase (^{224}LEGTLLKPNM^{233}) was present in this putative protein (Fig. 2).

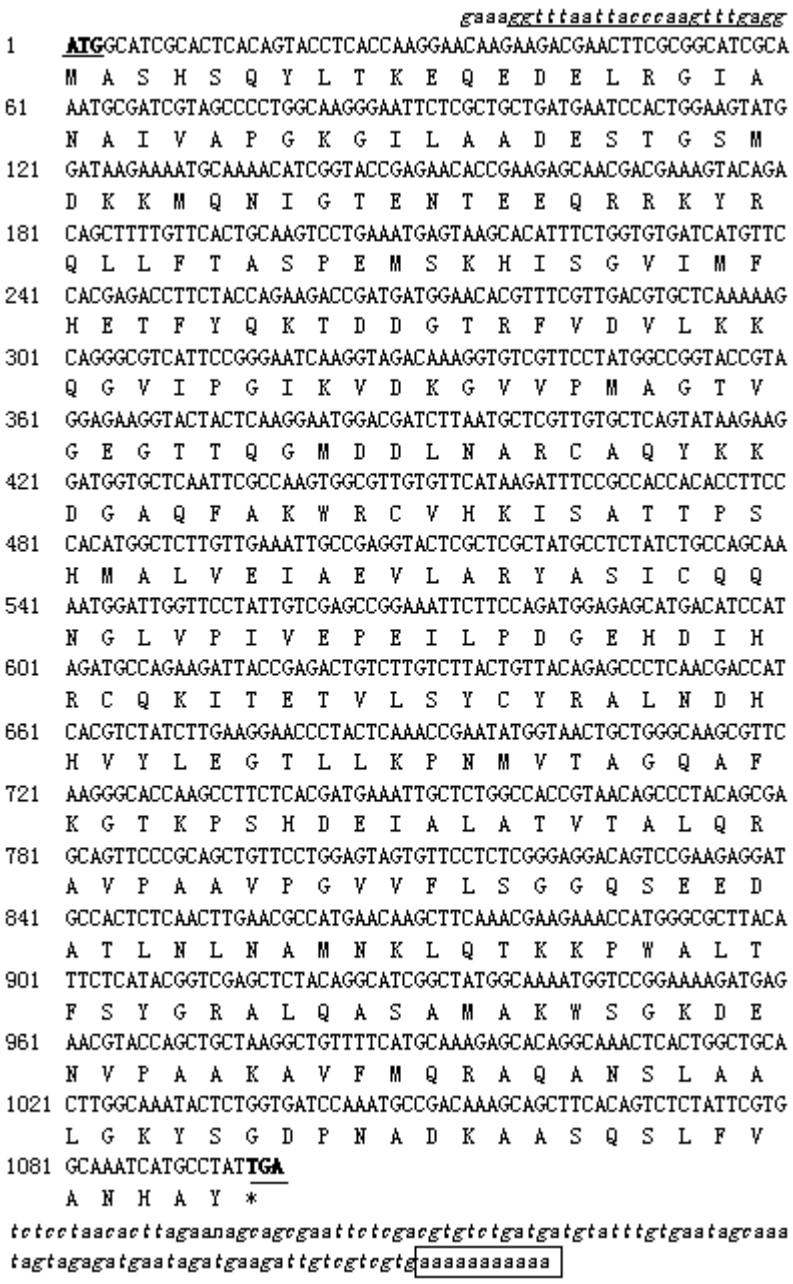

Fig. 1: Nucleotide sequence of the HcALD cDNA and its deduced amino acid (GenBank accession No. HQ529288). The coding region (ORF) was shown in capital letter, starts at nt 1 and ends at nt 1098. The ATG start codon and the TAA stop codon were bold and underlined. The 5' and 3' UTR were showed in small letters. SL1 sequence at the 5' end was underlined and the polyadenylation at the 3' end was boxed

Alignment of multiple ALD sequences: Alignment of the predicted amino acid sequence of *H. contortus* aldolase with these from other animals showed that this protein had a significant similarity (65-80% identical at the amino acid level) with ALDs in nematodes and mammals (Fig. 2). The similarity of this protein to aldolase of *C. elegans* was 80%. Phylogenetic analysis of the various ALD was performed by the Neighbor Joining (NJ) Method (Fig. 3). HcALD was phylogenetically close to the ALD from *C. elegans* and remotely related to that from clam.

Expression and purification of recombinant protein: SDS-PAGE showed that the recombinant protein was mostly found in the sonicated bacterial supernatant. After

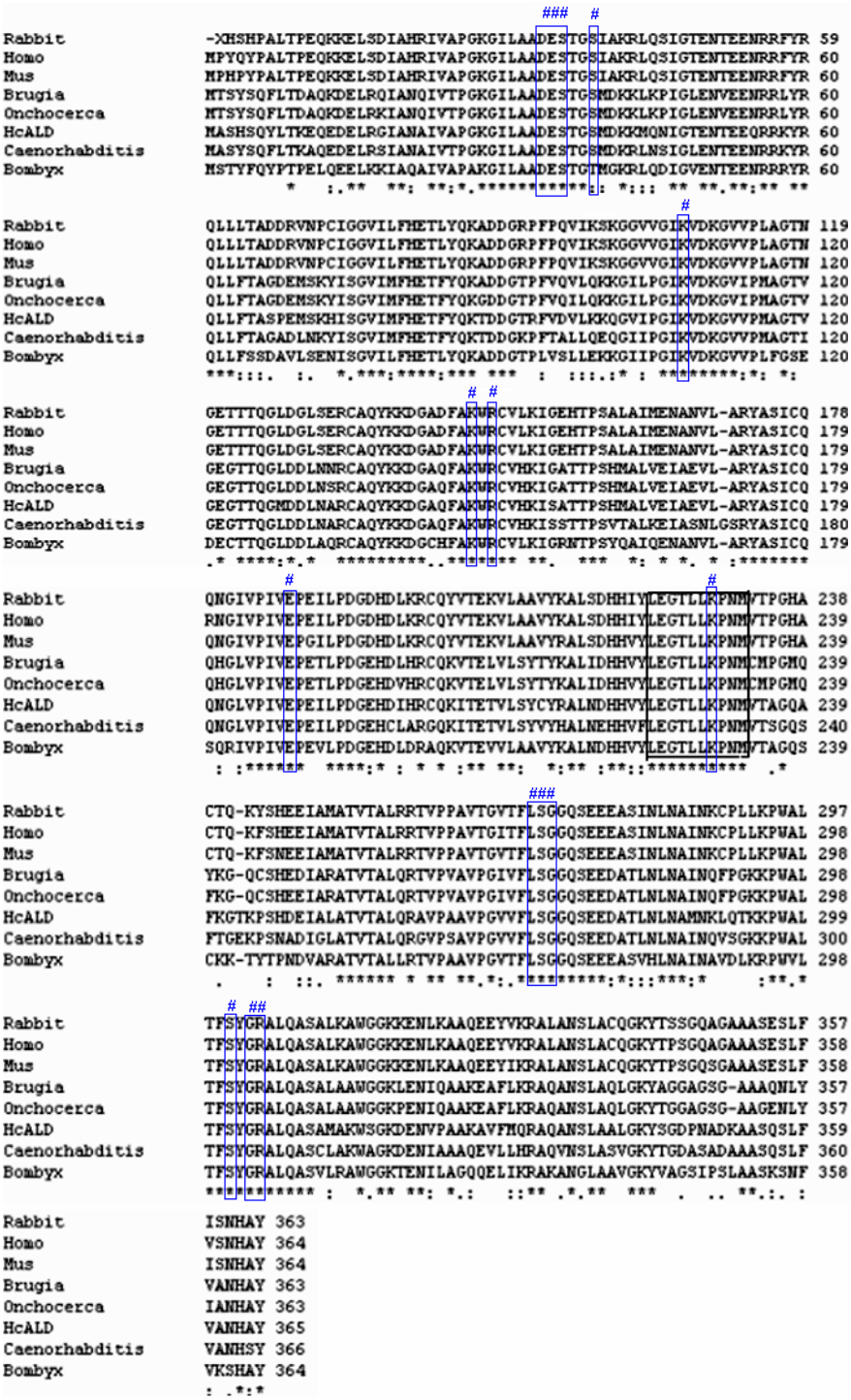

Fig. 2: CLUSTAL multiple sequence alignment of the deduced amino acid sequence of aldolase from *H. contortus* (HcALD), Rabbit (*Oryctolagus cuniculus*, 1ADO_A), Homo (*Homo sapiens*, CAG46678.1), Mus (*Mus musculus*, AAH66218.1), Brugia (*Brugia malayi*, XM_001894495.1), Onchocerca (*Onchocerca volvulus*, AF155220.1) Caenorhabditis (*Caenorhabditis elegans*, P54216.1) Bombyx (*Bombyx mori*, NP_001091766.1). Identical residues are marked with asterisks (*). The dashes (-) demonstrate gaps introduced between the sequences. Amino acid residues essential to the enzymatic activity of ALD are indicated in the black box. The conserved residues play roles in enzymatic activities were boxed blue and marked with hash marks (#) above the aligned sequences

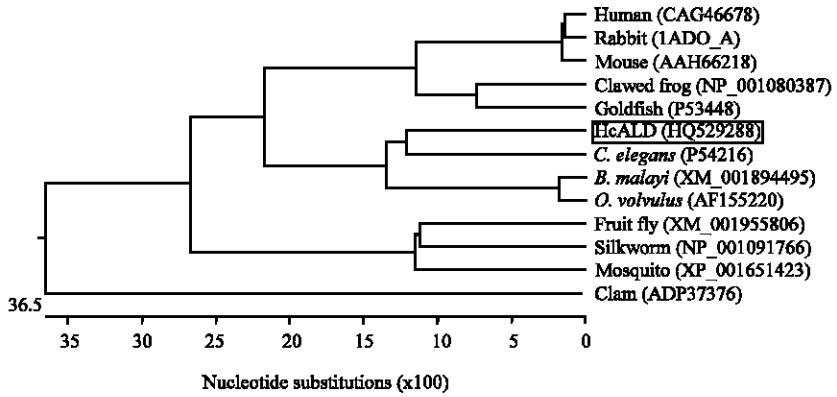

Fig. 3: Phylogenic trees inferred from homologies of ALD amino acid sequences utilizing the NJ Method. The scale bar represents one amino acid substitution/100 amino acid residues. The ALDs are represented by the name of their species. In parentheses, amino acid database accession numbers are listed. NJ analysis was carried out using the program CLUSTAL W Version 1.8

purification from the supernatant by chromatography on the Ni-NTA, the protein was seen as a single band with the molecular mass of 58 kDa on the SDS-PAGE gel (Fig. 4, lane 9). Because of the 18 kDa fused protein in the vector, the recombinant protein's molecular weight was more than the value of 39,697 Da calculated based on the deduced amino acid sequence.

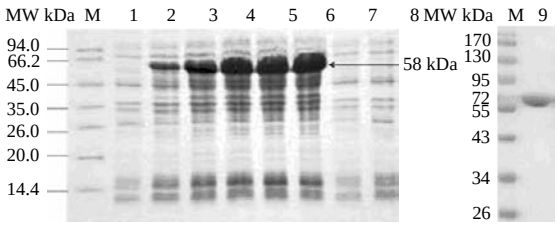

Fig. 4: Expression and purification of HcALD protein. Gels were stained with Coomassie blue and 15 μL samples were loaded per lane. Lane M: standard protein molecular weight marker, Lane1-6: *E. coli* (BL 21strain) transformed by recombinant plasmid pET32a (+)/HcALD was induced by IPTG for 0, 1, 2, 3, 4 and 5 h, respectively the fusion protein weighted about 58 kDa was marked with an arrowhead. Lane 7-8: *E. coli* (BL 21 strain) transformed by pET-32a empty expression vector (negative control) induction with IPTG for 0 and 5 h, respectively, Lane 9: Purified recombinant HcALD fractions after Ni-NTA affinity chromatography

Western blot: The immunoblotting results showed that the HcALD was recognized by sera from goat naturally infected with *H. contortus* (Fig. 5). This may indicated that HcALD could be excreted or secreted by the parasite during the infection.

Enzymatic activity assay: In the enzymatic activity assay, the purified protein yielded a specific activity of 13 U mg^{-1}. The assay showed that temperatures varying between 35 and 45°C did not greatly affect enzymatic activity. However, thermal inactivation occurred above 60°C and resulted in losing of the most activity at 70°C (Fig. 6a). Studies on the effect of temperature on enzymatic activity revealed an optimal value of about 40°C. The optimal pH assay indicated that this recombinant protein exhibited the highest aldolase activity at about pH 7.5 (Fig. 6b). When the pH was higher above 8.0, the HcALD displayed a sharp decrease in catalytic activity. However, the enzymatic activity decreased not so sharply when pH varied between 6 and 7.

A number of aldolases have been isolated from a wide range of nematodes (Inoue *et al*., 1997; Ghedin *et al*., 2007; McCarthy *et al*., 2002). However, there are only limited reports on *Haemonchus contortus* aldolase. For the first time in the present study, researchers reported the cloning, expression and characterization of a *H. contortus* cDNA sequence that encodes a 40 kDa protein.

Based on multiple sequence alignment of ALDs, the predicted amino acid sequence of the HcALD had an 80% identity with ALD of *Caenorhabditis elegans* and comparable similarity to other species: *Caenorhabditis briggsae* (79%), *Brugia malayi* (79%) and *Onchocerca volvulus* (78%). All of these indicated a high level of identity to members of the aldolase class I family. The primary sequence of HcALD revealed several

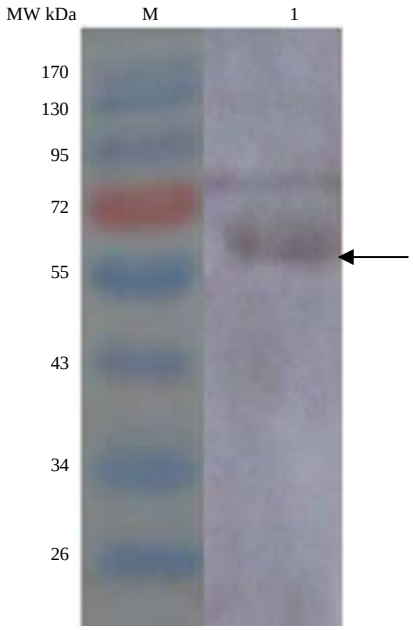

Fig. 5: Western blotting analysis of recombinant HcALD protein with sera from goat infected with *H. contortus* naturally (Lane 1, marked with an arrowhead). Lane M: standard protein molecular weight marker

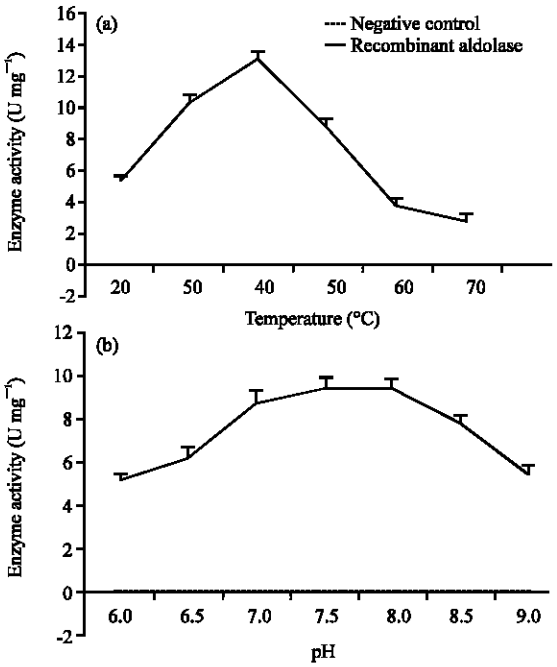

Fig. 6: Effect of temperature and pH variations on relative activity of purified *H. contortus* Aldolase (HcALD). a) Enzyme activity of purified HcALD was tested at a temperature range from 20-70°C, revealing the effects of the most suitable temperature; b) Relative enzymatic activity of purified aldolase was measured in the Triethanolamine-HCl buffer using pH range 6.0-9.0

structural features characteristic of ALDs including conserved amino acid residues Asp[34], Lys[147], Arg[149], Lys[230], Ser[302], Gly[304], Arg[305] and the terminal Tyr[365] which were thought to be involved in catalysis. The residues surrounding the active site residue Lys[230] namely ^{224}LEGTLLKPNM^{233}, exactly matched the consensus sequence that was conserved in both prokaryotic and eukaryotic ALDs. Finally, these results were further strengthened by the *in vitro* test showing that the recombinant HcALD protein had the enzymatic activity of Fructose-1, 6-bisphosphate aldolase. All of the above results suggested that it was a member of the aldolase family.

In this investigation, the mRNA coding for HcALD in *H. contortus* was shown to be preceded by a spliced leader. The 22 nucleotides at the 5'-end of mature mRNA of. HcALD were identical to the trans-spliced leader SL1 of *C. elegans*. In *C. elegans*, trans-splicing of mRNAs is a common phenomenon with at least 70% of mRNAs having an SL1 sequence (Zorio *et al*., 1994). Additionally, SL1 tends to be spliced very close to the translation start site. It plays major roles in the formation of the optimal length of 5' UTR in mature mRNA and in the formation of nucleotide constructs that benefit the translation of genes (Stover and Steele, 2001; Williams *et al*., 1999). In this study, researchers have shown that the presence of SL1 genes in *H. contortus* results in mRNA containing an SL1 sequence at its 5' end.

No signal sequence was found in this deduced protein indicating that it might be an intra-cellular protein. However, recombinant HcALD could be detected by the sera of infected goats in western blot assay. These observations appear to be contradictory. One possible explanation is that the HcALD enzyme could be released from the worm during the molting. In the earlier study, it was found that aldolase of *Onchocerca volvulus* was located in the body wall and the reproductive tract of adult females and the immunoelectron microscopy of *O. volvulus* L3 demonstrated binding in the region where the cuticle separates during molting in the channels connecting the esophagus to the cuticle and in the basal lamina surrounding the esophagus and the body cavity (McCarthy *et al*., 2002). Another possibility is that the HcALD from the dead adult parasite could be absorbed by the host and recognized by the host immune

system. The similar phenomenal was found in Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) from the same parasite (Han *et al*., 2011).

It was reported by Rhodes (1972) that aldolase could be the target of some anthelmintic drugs. Another similar study proved that the activities of enzymes participated in glycolytic cycles could be inhibited by tetramisole *in vitro* (Kaur and Sood, 1982). McCarthy *et al.* (2002) found that mouse immunized by recombinant aldolase of *O. volvulus* gave 50% of protection in reduction of survival larvae. These observations provided support for the further study of parasite enzyme as a vaccine candidate.

CONCLUSION

In this study, it was also found that the recombinant HcALD could be recognized by the serum from naturally infected goats. This indicated that HcALD may be excreted or secreted by the parasite during the infection.

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