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Molecular and biochemical characterisation of abomasal nematode parasites *Teladorsagia circumcincta* and *Haemonchus contortus* phosphofructokinases and their recognition by the immune host

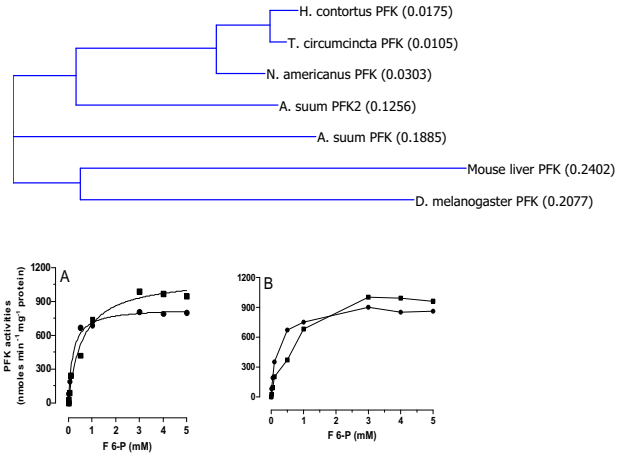
S. Umair [a,*], E. Dagnicourt [b], J.S. Knight [a], H.V. Simpson [c], A. Pernthaner [a]

[a] AgResearch Ltd, Private Bag 11-008, Palmerston North, New Zealand
[b] AgroSup Dijon, 26 Boulevard Petitjean, BP 87999 21079-Dijon cedex, France
[c] Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Private Bag 11-222, Palmerston North, New Zealand

HIGHLIGHTS

- Full length cDNAs encoding PFK were cloned from *T. circumcincta* and *H. contortus*.
- *Tc*PFK (2361 bp) and *Hc*PFK (2367) cDNA encoded 787 and 789 amino acid proteins.
- The predicted amino acid sequences showed 98% similarity with each other.
- The recombinant *Tc*PFK and *Hc*PFK had very similar kinetic properties.
- Antibodies from nematode-exposed sheep recognised recombinant PFK.

GRAPHICAL ABSTRACT



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ABSTRACT

Full length cDNAs encoding phosphofructokinase (PFK) were cloned from *Teladorsagia circumcincta* (*Tc*PFK) and *Haemonchus contortus* (*Hc*PFK). *Tc*PFK (2361 bp) and *Hc*PFK (2367 bp) cDNA encoded 787 and 789 amino acid proteins respectively. The predicted amino acid sequences showed 98% similarity with each other and 70% with a *Caenorhabditis elegans* PFK. Substrate binding sites were completely conserved in both proteins. Soluble N-terminal His-tagged PFK proteins were expressed in *Escherichia coli* strain BL21, purified and characterised. The recombinant *Tc*PFK and *Hc*PFK had very similar kinetic properties: the pH optima were pH 7.0, K_m for fructose 6-phosphate was 0.50 ± 0.01 and 0.55 ± 0.01 mM respectively when higher (inhibiting concentration, 0.3 mM) ATP concentration was used and the curve was sigmoidal. The V_{max} for *Tc*PFK and *Hc*PFK were 1110 ± 16 and 910 ± 10 nM min^{-1} mg^{-1} protein respectively. Lower ATP concentration (non-inhibiting, 0.01 mM) did not change the V_{max} for *Tc*PFK and *Hc*PFK (890 ± 10 and 860 ± 12 nM min^{-1} mg^{-1} protein) but the substrate affinity doubled and K_m for fructose 6-phosphate were 0.20 ± 0.05 and 0.25 ± 0.01 mM respectively. Recognition of *Tc*PFK and *Hc*PFK by mucosal and serum antibodies in

* Corresponding author. Fax: +64 6 353 7853.
E-mail address: saleh.umair@agresearch.co.nz (S. Umair).

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nematode exposed animals demonstrates antigenicity and suggests involvement in the host response to nematode infection.

1. Introduction

Parasite excretory/secretory (ES) products released into the host include enzymes, structural molecules, proteases and immunomodulators, some of which are recognised immunologically by the host (Harnett, 2014). These parasite chemicals are likely to be responsible for initiating pathological and inflammatory responses of host tissues to the presence of the luminal stages of nematode parasites. Analysis of ES products produced *in vitro* in *Ascaris suum* has shown that more than 200 proteins can be present (Wang et al., 2013) including the glycolytic enzyme phosphofructokinase-1 (PFK-1) (E.C. 2.7.1.11).

Glycolysis has a central role in the active energy metabolism required by rapidly growing nematode larvae and profuse egg-laying adult worms. PFK-1 irreversibly catalyses the phosphorylation of fructose 6-phosphate (F6P) to fructose 1,6-bisphosphate (F1,6BP) and is a regulatory point in glucose metabolism. A distinct enzyme PFK-2 phosphorylates F6P to fructose 2,6-bisphosphate (F2,6BP), a regulator of glycolysis. Mammalian PFK has three isoforms located in the liver, brain and muscle (Martinez-Costa et al., 2004), but only one was isolated in *Ascaris suum* (Starling et al., 1982). The enzyme properties and allosteric regulation of PFK-1 of *A. suum* (Hofer et al., 1982a, 1982b; Rao et al., 1987, 1991a, 1991b) and in homogenates of *Teladorsagia circumcincta* (Walker et al., 2012) are generally similar to those of mammalian PFK-1. Gene sequences are known for PFK-1 of *A. suum* (Kulkarni et al., 2005), *Haemonchus contortus* (Klein et al., 1991) *Caenorhabditis elegans* (Miersch and Doring, 2012) and *Schistosoma mansoni* (Ding et al., 1994), however, there appear to be no studies of the properties of recombinant nematode PFK-1.

In this study, the cDNAs encoding *T. circumcincta* and *H. contortus* PFK were cloned, expressed in *Escherichia coli* and the recombinant proteins were purified and their kinetic properties were determined. Enzyme-linked immunosorbent assays (ELISAs) were performed to determine if the enzymes were recognised by saliva and serum from sheep previously exposed to nematode parasites.

2. Materials and methods

All chemicals were purchased from Sigma Chemical Co. (Mo, USA) unless stated otherwise. Use of experimental animals for culturing and harvesting adult worms for RNA extraction has been approved by the Massey University Animal Ethics Committee (09/11).

2.1. Parasites

Pure cultures of *T. circumcincta* and *H. contortus* were maintained in the laboratory. Adult worms were recovered from the abomasa of infected sheep as described previously (Umair et al., 2013a). Briefly, abomasal contents were mixed 2:1 with 3% agar and the solidified agar blocks incubated at 37 °C in a saline bath. Clumps of parasites were collected from the saline soon after emergence.

2.2. cDNA isolation

Total RNA was extracted using Trizol (Invitrogen), from adult *T. circumcincta* and *H. contortus* according to Umair et al. (2013b). First strand cDNA was synthesised using the iScript Select cDNA Synthesis Kit (Bio-Rad).

A partial *T. circumcincta* phosphofructokinase (*Tc*PFK) cDNA was amplified by the PCR from plasmid DNA extracted from a cDNA library prepared from adult *T. circumcincta* RNA (Custom adult cDNA Library in pCMV.SPORT6.1, Invitrogen) using the degenerate primers PFK forward (5′-AGGGAGAACHATCATYGG-3′) and PFK reverse (5′-CGACCCATSACCTCAA-3′). Primers were based on an alignment of the predicted PFK protein sequences from *Caenorhabditis elegans* (GenBank Accession No. NP741738), *H. contortus* (AAA29181), *Ascaris suum* (AAR16088), *Caenorhabditis remanei* (XP003109499) and *Pristionchus pacificus* (Wormbase Accession No. PPA18824). An extended *Tc* PFK cDNA was amplified from *T. circumcincta* cDNA library plasmid DNA using the PCR and *T. circumcincta* PFK-specific primers, based on the partial *Tc*PFK cDNA, in conjunction with library vector-specific primers. The 3′ and 5′ ends of the *Tc*PFK cDNA were obtained by RACE (SMARTer RACE cDNA Amplification Kit, Clontech) using *T. circumcincta* adult RNA and primers based on this extended *Tc*PFK cDNA, as outlined by the manufacturer. Full length *Tc*PFK and *H. contortus* phosphofructokinase (*Hc*PFK; GenBank Accession No. M59805) cDNAs were amplified by the PCR from cDNA prepared from adult *T. circumcincta* or adult *H. contortus* RNA, using the primers TcPFK-NdeI-fwd (5′-ATCGCATATGAGCCTCAAAAGAAATATAC-3′) and TcPFK-XbaI-rev (5′-GATCTCTAGAGCTAATCATAATCTTCAG-3′) for *T. circumcincta* or *Hc*PFK-NdeI-fwd (5′-ATCGCATATGAGCCTCAAAAGGAATATAC-3′) and *Hc*PFK-XbaI-rev (5′-GATCTCTAGACACGGGCCTAATCGTAATC-3′) for *H. contortus*. The full length *Tc*PFK and *Hc*PFK cDNAs were cloned, using standard protocols, into the pCL476 vector using the restriction enzymes NdeI and XbaI (engineered into the forward and reverse primers, underlined in primer sequences) to allow the production of N-terminal His tagged recombinant proteins under the control of the heat-inducible tandem λP_R and P_L promoters (Love et al., 1996).

2.3. Expression and purification

E. coli strain BL21 (DE3), transformed either with pCL476 *Tc*PFK or *Hc*PFK, was grown in 10 ml Luria Broth (LB) containing ampicillin (100 μg/ml) for 16 h at 37 °C. The cultures were diluted to 500 ml with LB containing antibiotic and incubated at 32 °C and 250 rpm until the $OD600_{nm}$ was 1.4. The culture was brought rapidly to 42 °C in a 75 °C water bath and then incubated at 42 °C for 4 h at 250 rpm. Bacteria were harvested by centrifugation and the soluble extract was obtained by treating the resuspended cells with lysozyme (1 mg/ml), repeated freeze–thawing and sonication. The recombinant proteins were purified by Ni–NTA agarose (Qiagen) affinity purification using the manufacturer's standard protocol. Protein concentration was determined by the method of Bradford (1976).

2.4. Electrophoresis

SDS–PAGE was performed using NuPAGE Novex 4–12% Bis–Tris gels according to the instructions of the manufacturer (Invitrogen). Gels were stained with Coomassie Blue (Invitrogen). A western blot was also performed on the protein using a monoclonal anti-poly histidine-peroxidase antibody (Sigma). Gels were incubated overnight in 1:2000 antibody in buffer (4% skim milk powder in Tris-buffered saline and 0.1% Tween-20) at room temperature and developed to detect His-tagged recombinant protein.

2.5. Enzyme assays

PFK activities of recombinant *Tc*PFK and *Hc*PFK were determined by measuring the rate of NADH formation from NAD^+ in a