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Short communication

Lack of protective efficacy in buffaloes vaccinated with *Fasciola gigantica* leucine aminopeptidase and peroxiredoxin recombinant proteins

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ABSTRACT

Gene coding for leucine aminopeptidase (LAP), a metalloprotease, was identified in the tropical liver fluke, *Fasciola gigantica*; that on sequence analysis showed a close homology (98.6%) with leucine aminopeptidase of the temperate liver fluke, *Fasciola hepatica*. The recombinant leucine aminopeptidase protein was expressed in *Escherichia coli*. *F. gigantica* peroxiredoxin, a hydrogen peroxide scavenger and an immunomodulating protein, was also cloned and expressed in *E. coli*. A vaccination trial in buffaloes was conducted with these two recombinant proteins, with 150 and 300 μg of leucine aminopeptidase and a cocktail of 150 μg each of recombinant leucine aminopeptidase and peroxiredoxin in three groups, respectively. Both Th1- and Th2-associated humoral immune responses were elicited to immunization with these antigens. A challenge study with 400 metacercariae did not show a significant protection in terms of reduction in the worm burden (8.4%) or anti-fecundity/embryonation effect in the immunized groups, as to the non-immunized control animals. Our observations in this buffalo vaccination trial are contrary to the earlier promise shown by leucine aminopeptidase of *F. hepatica* as a leading candidate vaccine molecule. Identification of leucine aminopeptidase gene and evaluation of the protein for its protective efficacy in buffaloes is the first scientific report on this protein in *F. gigantica*.

1. Introduction

Fasciola gigantica (tropical liver fluke) inflicts substantial productivity losses on the livestock industry in the tropical countries and adversely affects the health, weight gain, feed conversion efficiency and reproduction of buffaloes (FAO, 1994; Mehra et al., 1999). The pathogenesis of tropical fasciolosis in buffaloes has been remarkably complex, involving a liver migratory phase of the parasite causing traumatic hepatitis and a phase of maturation and establishment of the adult parasite in the bile ducts, causing hyperplastic obstructive cholangitis (Radostits et al., 1994; Aiello and Mays, 1998). India, being an agrarian society, has the largest buffalo population in the world, which constitutes a very significant component of the Indian livestock sector (Agricultural Research Data Book, 2002). There are endemic pockets of fasciolosis in India (Sanyal, 2001), a major constraint in buffalo productivity. Despite intensive research efforts, progress in the development of effective vaccine for *Fasciola hepatica* and *F. gigantica* control has been relatively slow (Mendes et al., 2010). Several parasite molecules have been identified that have the potential to induce a protective immune response in the laboratory and large animals but the protection conferred to the host has been shown to vary.

Leucyl aminopeptidases (LAPs), members of the M1 or M17 peptidase families, constitute a group of diverse and ubiquitous Zn-dependent metallopeptidases and play a crucial role in the parasite biology of protozoa and helminths. LAPs present broader amidolytic activity beyond leucine hydrolysis and participate in processing/maturation/activation or degradation of substrates (Rawlings et al., 2006; Matsui et al., 2006). The homo-hexameric M17 LAPs are being revealed as novel drug targets and vaccine candidates against parasitic infections. In helminths, LAPs have been poorly characterized although there is evidence that support their participation in vital processes in the parasite life cycle. LAP has been detected, purified and characterized in *Ascaris suum*, *Schistosoma mansoni*, *Schistosoma japonicum* and *Haemonchus contortus* (Rogers and Brooks, 1978; Rhoads and Fetterer, 1998; McCarthy et al., 2004; Abouel-Nour et al., 2005). The *F. hepatica* leucine aminopeptidase was identified, characterized (Acosta et al., 1998, 2008) and evaluated in a single vaccination trial in sheep, where the protein was shown as a relevant candidate for vaccine development (Piacenza et al., 1999). In *F. gigantica*, the pathogenic species of the tropical countries, the protein has not been identified.

The *F. hepatica* peroxiredoxin (Prx) plays an important role in the parasite's defense against host generated oxidants (McGonigle et al., 1997, 1998). The protein also acts as an immunomodulator by

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inducing the recruitment of alternatively activated macrophages to the peritoneum (Donnelly et al., 2005, 2008). Alternatively activated macrophages are key to promoting Th2 responses and suppression of Th1 inflammatory pathology during helminth infections. Using murine models for the infections caused by *F. hepatica* and *S. mansoni*, it has been shown that parasite peroxiredoxin induces alternative activation of macrophages by way of expression of surface markers, Ym1, Fizz1, arginase on macrophages, a key to promoting Th2 response in the host (Donnelly et al., 2005, 2008).

In the present study, gene coding for leucine aminopeptidase was identified in *F. gigantica* and recombinant protein expressed in *Escherichia coli*. Another protein of the parasite, peroxiredoxin, was also cloned and expressed. The two recombinant proteins were evaluated for vaccine potential in buffaloes.

2. Materials and methods

2.1. Parasite collection

F. gigantica flukes were collected from a local buffalo abattoir, Bareilly, U.P., India, in physiological saline and transported to the laboratory in chilled saline.

2.2. Cloning and expression of recombinant leucine aminopeptidase protein

The flukes were washed extensively in normal saline and total RNA was isolated from 30 to 50 mg of the parasite tissue, using Trizol reagent (Invitrogen, USA) following manufacturer's protocol. The RNA was used in the synthesis of cDNA using oligo-dT primer and M-MLV reverse transcriptase enzyme (MBI Fermentas, USA). The cDNA was subjected to polymerase chain reaction (PCR) using primers specific to the temperate liver fluke *F. hepatica* leucine amino-peptidase (GenBank accession no: AY644459), with sequences forward primer 5′-atggcggcgttggctgtgggcgt-3′ and reverse primer 5′-ctatttgaatcccagtcgtgg-3′. The full length *F. gigantica* leucine aminopeptidase open reading frame was amplified, cloned in a TA cloning vector (pDRIVE, Qiagen, Germany) and sequenced. The gene was sub-cloned in a prokaryotic expression vector (pPROEXHT-b, Life Technologies, USA) and the recombinant leucine aminopeptidase expressed in the *E. coli* DH5α at 28 °C for 6–8 h post-induction with 1 mM IPTG. The recombinant protein was purified by Ni–NTA affinity chromatography in its native conformation. Briefly, the bacterial cells were exposed to 3–4 cycles of alternate freezing and thawing at −40 °C and 37 °C. Bacterial cells were sonicated with 5 bursts of 30 s each at 10 amplitude (Soniprep, USA) and cell lysate centrifuged at 10,000 × *g*. Soluble recombinant leucine aminopeptidase was purified from the bacterial lysate by binding to Ni–NTA resin (Qiagen, GmbH, Germany) in tris-phosphate buffer (10 mM Tris, 100 mM NaH_2PO_4, pH 8.0; supplemented with 20 mM imidazole). A step elution protocol was developed to remove the contaminating bacterial proteins by washing the recombinant protein bound resin with 10 ml each of tris-phosphate buffer (pH 8.0), supplemented with imidazole concentrations of 25, 50 and 80 mM, respectively. The recombinant protein was eluted in the same buffer, containing 250 mM imidazole. The tris-phosphate buffer used in the above purification protocol was supplemented with 300 mM NaCl.

2.3. Expression of peroxiredoxin protein

F. gigantica cDNA was used for the PCR amplification of peroxiredoxin gene using primers specific to *F. gigantica* peroxiredoxin (Prx) gene (GenBank accession no: GQ 845012), with primer sequences forward 5′-atgttgcagcctaatatgccc-3′and reverse 5′-ctagttggctgaggagaaata-3′. The amplified PCR product was cloned in a TA cloning vector (pDRIVE, Qiagen, Germany) and sequence information determined. Peroxiredoxin gene was sub-cloned in a prokaryotic expression vector (pPROEXHT-b, Life Technologies, USA) and protein expressed in *E. coli* DH5α. Expression and purification protocol for the recombinant peroxiredoxin protein was identical to the one followed for recombinant leucine aminopeptidase, except for the resin washing being carried out in wash buffer, supplemented with 25, 35 and 50 mM imidazole, respectively. The recombinant protein was eluted at 250 mM imidazole concentration in the wash buffer.

2.4. Immunization protocol

Twenty nine buffalo calves, 8–10 months old were purchased through a local animal contractor from *F. gigantica* non-endemic areas of North India. Serological and faecal screening was carried out to rule out previous exposure to *F. gigantica*. Calves were maintained under normal husbandry conditions on a concrete floor in the experimental shed of the Institute. Experimental procedures were carried out as per the guidelines of the Institute Animal Ethics Committee. These calves were randomly distributed in Gr.I and Gr.II with 7 calves each, Gr.III with 6 calves and Gr.IV with 9 calves as non-immunized control group. Animals in Gr.I and II were immunized with 150 and 300 μg recombinant leucine aminopeptidase, respectively, and Gr.III calves received a cocktail of leucine aminopeptidase and peroxiredoxin proteins at 150 μg of each protein. Both the antigens were delivered to the host with montanide M-70 VG (Seppic, France) at 70:30 adjuvant–antigen ratios. The antigen–adjuvant emulsion was prepared in a glass homogenizer with a Teflon coated piston at a speed of 4000 rpm on ice for initial 2 min, followed by another 2 min homogenization at 4000 rpm on ice after a 2 min resting period. Antigen emulsion was injected to the calves via intramuscular route in the neck and glutial muscles. Three immunizations were given at 3 week interval. A gap of one month was maintained after the last immunizing dose before challenging these animals along with the un-immunized control calves. Animals were challenged *per os* with a dose of 400 viable metacercariae prepared in a wheat flour bolus. The metacercariae were harvested from *F. gigantica* infected *Lymnaea auricularia*, collected from the natural water bodies (ponds). Snails positive for *F. gigantica* metacercariae were maintained in the laboratory. Metacercariae were harvested on polyethylene strips and stored in distilled water at 4 °C before infection of the experimental animals. Metacercariae were *in vitro* hatched to newly excysted juveniles and confirmed for *F. gigantica* species by PCR amplification and sequencing of ITS-2 sequence of the ribosomal DNA in the juveniles. Buffalo calves were infected within one month of the harvesting of metacercariae. Experimental animals were euthanized by intravenous injection of 50–100 ml of saturated magnesium sulphate at 18–20 weeks post-challenge infection. Liver was cut into slices approximately 1 cm thick and squeezed in warm saline for fluke recovery.

2.5. Enzyme linked immunosorbent assay

Serum samples collected periodically from each animal under experiment were probed by ELISA (Wijffels et al., 1994). Checkerboard titration was initially performed to determine the optimum quantum of antigen, sera dilution and antiglobulin enzyme conjugate concentration. Polystyrine microtitre plates (Nunc, Denmark) were sensitized with 100 μl of 0.05 M carbonate–bicarbonate buffer, pH 9.6, containing 2 μg/ml of rLAP or peroxiredoxin antigen, followed by overnight incubation at 4 °C. The wells were washed with PBS containing 0.05% Tween-20 (PBS-T), thrice for 5 min each. Subsequently, blocking was performed using 3% skimmed milk in