Leucine Aminopeptidase Is an Immunodominant Antigen of Fasciola hepatica Excretory and Secretory Products in Human Infections

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The liver fluke Fasciola hepatica parasitizes humans and ruminant livestock worldwide, and it is now being considered a reemerging zoonotic disease, especially in areas in which it is endemic, such as South America. This study investigates the immune response to excretory and secretory products produced by F. hepatica in a group of patients from the Peruvian Altiplano, where the disease is highly endemic. Using a proteomic approach and immunoblotting techniques, we have identified the enzymes leucine aminopeptidase (LAP) and phosphoenolpyruvate carboxykinase as immunodominant antigens recognized by sera from fasciolosis patients. An indirect enzyme-linked immunosorbent assay using recombinant LAP as the antigen was developed to check sera from individuals of this region. Our results demonstrate that LAP produces a specific and strong reaction, suggesting its potential use in the serologic diagnosis of F. hepatica infections in humans.

Fasciolosis is an important disease caused by the liver flukes Fasciola hepatica and F. gigantica, infecting several mammalian species, including cattle and sheep, and consequently leading to significant global economical losses, valued at $3 billion annually (59). In addition, F. hepatica infestation recently has been recognized as an emerging/reemerging zoonotic disease, with an estimated prevalence of up to 17 million people infected and 180 million at risk for infection worldwide (30, 41). Those health problems are encountered especially in areas in which the disease is endemic, like the Andean region in Peru, where well-known human areas of hyperendemicity include the Puno Altiplano (18), the Cajamarca valley (26, 27), and the Mantaro valley (4, 47, 60).

Sensitive and specific diagnostic tools are necessary in order to treat patients early and to avoid the major clinical complications caused by the parasite. In this context, immunological probes are replacing direct diagnosis by detecting eggs in feces (20), although there is still a lack of consensus regarding the choice of the immunologic analysis for human fasciolosis. Among the immunological methods, enzyme-linked immunosorbent assay (ELISA) and immunoblotting are well known, while other new systems are being evaluated (35). Most of these tests use F. hepatica molecules present in excretory and secretory products (ESP), since they produce a more intense response from the host immune system than somatic antigens (7, 10, 16, 17, 50). The identification of such molecules could represent specific markers for the detection of F. hepatica. In this context, several molecules present in ESP have been characterized, including the cysteine proteases (11), Cu/Zn superoxide dismutase (25, 43), enolase (5, 24), fatty acid binding proteins (21), glutathione S-transferases (9, 64), leucine aminopeptidase (LAP) (1), saposin-like molecules (15, 19, 48), and thioredoxin-associated proteins (23, 24, 29, 32, 36, 51, 52, 56, 57).

The exopeptidase LAP (EC 3.4.11.1) originally was identified in detergent-soluble extracts of F. hepatica, with a very low level of activity detected in ESP (1). This peptidase has been shown to induce protection against fasciolosis in sheep (44), similar to that observed with endopeptidases like cathepsins (11, 33).

In this study, we have identified the enzymes LAP and phosphoenolpyruvate carboxykinase (PEPCK) as the reactive antigens present in F. hepatica ESP by using a combination of immunoblotting with human antisera and mass spectrometry (MS). Recombinant F. hepatica LAP (rLAP) also was recognized by individual positive serum samples by ELISA, adding a putative diagnostic value in addition to their protective properties.

MATERIALS AND METHODS

Preparation of parasite ESP. Livers from naturally infected sheep were collected from a local slaughterhouse, and parasites were removed immediately and washed extensively with prewarmed phosphate-buffered saline, pH 7.4 (PBS). Flukes were incubated at 37°C for 12 h with slow agitation in PBS containing 100 μg/ml streptomycin (Sigma), using 1 ml per fluke (14). Thereafter, the flukes were removed and the culture medium was centrifuged at 15,000 × g for 15 min at 4°C. The supernatant was precipitated using equal volumes of ice-cold 20% trichloroacetic acid and solubilized in loading buffer for gel electrophoresis. The...
protein content was measured using a modified Bradford assay (Bio-Rad) and adjusted to 10 mg/ml using an ultrafiltration membrane (YM-3; Millipore).

Production of rLAP\textsubscript{9} and anti-rLAP\textsubscript{9} sera. Based on published cDNA sequences of LAPs, degenerate primers were synthesized corresponding to conserved amino acid regions for metal binding (VGKK) and for the active site (NFTDAEGRL). Using adult fluke cDNA as a template, a 280-bp fragment was generated by PCR with these primers. Purified PCR products were subcloned into pCRTOPUSH (Invitrogen) and sequenced. New gene-specific primers were synthesized and used in rapid amplification of cDNA ends (RACE)-PCR. Products of 1.2 (5'RACE) and 0.7 kb (3'RACE) were obtained, subcloned, and sequenced. A full-length construct was generated by mixing gel-extracted 5'RACE and 3'RACE aliquots in a 1:100 PCR that contained no primers. The full-length fragment was obtained by extension from the overlapping region. After five cycles, new primers designed from both ends of the LAP\textsubscript{9} were added and used for an additional 25 cycles of amplification. These primers included BamHI and SalI restriction sites to facilitate subcloning in the expression vectors. The expected full-length cDNA product was obtained, subcloned, and sequenced (accession number AY64459). The full-length cDNA product was cloned in frame in BamHI and BgIII sites of linearized pTHiC Erichescheria coli expression vector (Invitrogen), fused downstream of the E. coli thioredoxin (txx) gene, and transformed into E. coli Top10 cells. Fused rLAP\textsubscript{9} was affinity purified using a beestatin-agarose column.

A New Zealand rabbit was immunized subcutaneously four times at 3-week intervals with purified rLAP\textsubscript{9} (50 μg) in Freund's complete or incomplete adjuvant. Anti-rLAP\textsubscript{9} serum was obtained 10 days after the final immunization.

Source of human sera and ELISA. Human sera were obtained from school children between 5 and 14 years of age from Cajamarca (Peru), Positive sera used in this study were purified based on a second antibody reaction as the reactivity in ELISA assays against ESP by following established protocols (18, 22). Negative sera corresponded to individuals from the same area with no presence of F. hepatica eggs in feces and also negative by ELISA assays using ESP. ELISA tests using rLAP\textsubscript{9} were performed essentially as described previously (61). An aliquot of 0.5 μg of the recombinant L. major protein in coating buffer (5 μg/ml) was added to the wells (100 μl) of flat-bottomed 96-well microtiter plates (NUNC). After incubation overnight at 4°C, the plates were blocked with PBS containing 4% nonfat dry milk for 1 h at 37°C. Serum samples diluted 1:5000 in PBS containing 4% nonfat dry milk and 0.05% Tween 20 were added to the wells (100 μl/well) and were incubated for 45 min at 37°C. After the samples were washed, peroxidase activity was detected by incubation with secondary antibodies coupled to HRP (1:10,000 in TBS plus 1% BSA). The antibody was detected with horseradish peroxidase conjugated goat anti-human IgG (Bio-Rad) in 1/500 in PBS containing 4% nonfat dry milk for 1 h at 37°C. After the samples were washed, peroxidase activity was detected by incubation with secondary antibodies coupled to HRP (1:10,000 in TBS plus 1% BSA).

**RESULTS**

Identification of F. hepatica immunogenic proteins in ESP detected by human sera from an area in which fascioliasis is endemic. To investigate the antigenicity of the ESP proteins, we used sera from patients diagnosed with fascioliasis from Cajamarca (Peru), an area where the disease is highly endemic (63). To identify immunogenic spots, a pool of positive sera (from patients eliminating parasite eggs in feces) were used to blot ESP subjected to 2D gels in the pH range of 5 to 8. Five major reactive spots were detected by Western blotting, ranging from a pI of 5.0 to 7.6 and having molecular masses between 35 and 120 kDa (Fig. 1A). After the filters were stripped and reprobed with sera obtained from healthy individuals from the same area in which the disease is endemic, two spots, previously immunodetected by positive sera, were detected (Fig. 1A and B). No other signal was obtained when negative sera were used, indicating the specificity of the detection (Fig. 1B). Matching immunoblots with their corresponding Coomassie-stained gels (Fig. 1C) allowed for the excision of the corresponding protein spots. The proteins contained in these spots were subjected to trypsin digestion and MS analysis. As shown in Table 1, spot 1 was identified as F. hepatica LAP (EC 3.4.11.1; NCBI accession no. AAV59016), with the
peptides identified by PMF covering 45% of the molecule. Spots 2 and 3 corresponded to two peptides each, which were assigned to PEPCK (EC 4.1.1.32) with 100% homology to the peptides from the Ascaris suum molecule (accession no. Q05893) (Table 1). Spots 4 and 5, immunodetected by both positive and negative sera (Fig. 1A and B), also corresponded to F. hepatica phosphopyruvate hydratase (enolase) (EC 4.2.1.11; accession no. A53665), with coverages of 69 and 43%, respectively (Table 1).

Confirmation of the identity of LAP was achieved by Western blotting using rabbit serum raised against the recombinant protein (rLAP$_{Fh}$) produced in E. coli. As shown in Fig. 2, these antibodies strongly reacted with the native LAP, detecting various spots of about 70 kDa ranging from pH 7.0 to 8.0. The recombinant LAP$_{Fh}$ fused to the His patch-thioredoxin (used as a control) was detected with lower mobility by the rabbit sera (Fig. 2).

**Immunological detection of rLAP$_{Fh}$ by ELISA.** To evaluate the possible diagnostic potential of the rLAP$_{Fh}$ protein, we next evaluated human serum samples individually for their reactivity against this antigen, establishing an indirect ELISA. Preliminary assays were performed to ascertain both the proper amount of rLAP$_{Fh}$ protein (ranging from 0.05 to 1.0 μg) to cover the ELISA plates (determined as 0.5 μg per well) and the dilution of sera employed (from 1/100 to 1/2,000; optimal results were obtained with the 1/500 dilution). The results from the negative sera ($n = 6$) indicated that the cutoff point for differentiating negative from positive sera was an OD value of 0.22. All of the confirmed F. hepatica-infected serum samples ($n = 4$) exhibited positive values greater than 0.398 (Fig. 3).

**DISCUSSION**

The control of fasciolosis historically has been limited by the lack of accurate and practical tests for the early diagnosis of F. hepatica infection in humans and other animals. Microscopic demonstration of parasite eggs in feces, the traditional diagnostic technique still widely used, is highly specific but has limitations in sensitivity. This limitation is due to the fact that it takes at least 4 to 10 weeks after infection for flukes to mature sexually and produce eggs (20). In addition, difficulties in obtaining and manipulating stool samples in the field, intermittence in egg shedding, and false positives by ingestion of infected raw liver contribute to the inaccuracy of the coprological diagnosis (20).

Alternatively, immunological assays can detect the infection in the initiation of egg release by analyzing antigens present in blood or released in stools (13, 34). In contrast to antigen detection, the investigation of circulating antibodies can be carried out easily, allowing earlier detection of infection and revealing current and past infections. Different indirect and capture ELISAs that are based on ESP (35, 53) or individual molecules from ESP, like Fas (10, 16) and cathepsins (8, 39), have been described. However, there is still a need for the identification of new specific markers for the detection of F. hepatica antibodies in serum.

The present study constitutes the first report of the proteomic identification of F. hepatica immunogens in humans from an area where the disease is highly endemic. A recent report has shown a preliminary antigenic characterization of adult F. hepatica vomit, detecting several proteins ranging from 8 to 85 kDa by using 1D electrophoresis and immunoblotting of those antigens with infected human sera (12). Here, we...
identify two specific antigens in the ESP: the enzymes LAP and PEPCK. In addition, we identified some enolase spots that also were reactive against negative sera (Fig. 2). Using a similar proteomic approach, Morphew et al. (36) have very recently described cathepsin L proteases as the most abundant and immunogenic molecules in infected sheep bile (36). In this context, previous studies have shown the potential of cathepsin L1 (either purified or produced as a recombinant protein in yeast or bacteria) as a diagnostic marker for human infections by techniques like ELISA and immunoblotting (8, 39, 40).

Interestingly, here we show that even though cathepsin L proteases seem to be abundant in *F. hepatica* ESP (58) (Fig. 1C), the human sera used in this study do not seem to react strongly against them (Fig. 1A). In recent studies with infected goats, no protein spots could be detected in the low-molecular-mass range (where cathepsin L proteases appear), and no reaction was observed by immunoblotting detecting major immunoreactive areas between pI 5 to 8.5 with infected goat antisera (38).

Enolase appears to be a major antigenic protein in several organisms, including parasitic protozoa like *Eimeria tenella* and *Plasmodium falciparum* (28, 54), yeasts like *Candida albicans* (45, 46), nematodes like *Trichinella spiralis* (37), and also trematodes like *Schistosoma bovis* (42), but its immunogenicity is controversial. A recent study was unable to detect anti-enolase IgE in sera from human patients infected with *Anisakis simplex*, suggesting an insufficient antigen presentation to induce anti-enolase antibodies in natural *Anisakis* infections (49). Moreover, Morphew et al. (36) have observed that enolase accumulates in the ESP of *F. hepatica* during the incubation process, even when the trematode is dead (36). The reactivity of negative sera against two spots corresponding to enolase, which presents at least seven isoforms when anti-enolase antibodies are used (5), could be due either to cross-reacting antibodies against the molecule from other pathogens present in those patients or to nonspecific detection by the human sera studied.

In contrast to enolase, we observed clear and specific recognition of LAP and PEPCK by the Peruvian sera studied (Fig. 1A). By following a similar proteomic approach, a recent report has identified PEPCK and enolase as immunogenic proteins in the related trematode *S. bovis* (42). PEPCK previously was identified as a major immunogen protein in eggs of *S. mansoni* and was identified as being responsible for T-cell responses (3).

LAP originally was isolated from an *F. hepatica* detergent-soluble extract, and histochemical methods, detecting a very low level of activity in ESP, showed that LAP activity was associated mainly with the epithelial cells that line the digestive tract of the parasite (1). Here, we clearly demonstrate the presence of LAP in ESP of *F. hepatica* by immunoblotting, either with human sera or with rabbit sera against the recombinant protein. Our results suggest the existence of distinct

![Image](http://cvi.asm.org/)

**FIG. 2.** Immunodetection of LAP in *F. hepatica* ESP using rabbit antisera raised against rLAP<sub>Fh</sub>. Proteins were subjected to IEF in a pH range of 5 to 8, followed by SDS-PAGE in 10% acrylamide gels. Following 2D electrophoresis, proteins were transferred to nitrocellulose and incubated with rabbit sera raised against the recombinant LAP<sub>Fh</sub>. The recombinant protein (rLAP) was included in the second dimension as a control.

![Image](http://cvi.asm.org/)

**FIG. 3.** Individual serum ELISA detection using rLAP<sub>es</sub> as the antigen. Median values from individual negative human sera (closed circles) and positive human sera (open circles) are shown. Values were defined as the mean OD values at 490 nm ± three times the standard deviations. Vertical bars represent the standard deviations.
enzyme isoforms, with only one of them being detected by human sera (Fig. 1A and 2). Since this enzyme most likely functions in the final stages of the catabolism of peptides that are generated by the degradation of host tissue by endoproteinas, such as the cathepsin L proteases, and are absorbed by the epithelial cells (1), it is possible that the molecule loses activity when secreted. This enzyme also has been detected in the tegument of S. mansoni (31, 62), and its presence in the ESP could be related to turnover processes.

The efficiency of LAP as an immunogen has been well documented, rendering protection in animals and confirming its recognition by the host immune system (44). Our assays use dilutions of human sera (1/500) in accordance with what has previously been published for the F. hepatica antigen Fas-2 (10, 17), being less concentrated than the samples used in other assays for either procathespins or cathespin molecules, which required dilutions of 1/200 (8, 39).

In the present study, we further suggest LAP as a promising diagnostic marker in immunological tests, since it is specific and strongly detected by sera of infected individuals. Future studies with a larger number of samples will address the confirmation of its diagnostic potential.

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