Identification of enolase as a plasminogen-binding protein in excretory–secretory products of *Fasciola hepatica*

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Abstract **We have followed a combined proteomic approach to identify proteins of *Fasciola hepatica* that could be involved in host–parasite interactions. Using two-dimensional gel electrophoresis, far Western immunoblot and mass spectrometry analyses, we have identified the enolase enzyme, present in the excretory/secretory materials of *F. hepatica*, as a human plasminogen-binding protein. This enzyme has an apparent molecular weight of 47 kDa with p*I* ranging from 6.2 to 7.2. These results suggest that enolase could act as a plasminogen receptor.**

Key words: Excretory–secretory; Enolase; Plasminogen binding; *Fasciola hepatica*

1. Introduction

Fascioliasis is a disease caused by digenetic trematodes of the genus *Fasciola*, of which *Fasciola hepatica* is the most common. This species is well known as a fluke of livestock causing significant economic losses with increasing importance in human health, where there have been reported cases in the five continents [1]. Humans are usually infected by the ingestion of aquatic plants that harbor the infectious metacercariae. The parasite excysts and migrates through the intestinal wall to the peritoneal cavity and penetrates in the liver parenchyma causing considerable damage including extensive hemorrhaging and perforations [2,3]. *F. hepatica* secretes proteases like cysteine proteases and cathepsins, which are believed to play important roles in tissue penetration, immune evasion and pathogenesis [3–7]. Other molecules identified in the excretion/secretion (E/S) materials of *F. hepatica* that could modulate the host response include detoxifying enzymes like protein disulfide isomerase [8] and glutathione-S-transferases (GSTs) [9].

Plasminogen is a 92 kDa plasma proenzyme of the serine protease plasmin which plays important roles in processes like fibrinolysis [10] and degradation of extracellular matrices [11]. A vast number of bacteria pathogens interact with plasminogen enhancing their proteolytic activity and tissue damage [12]. One of the best characterized cellular plasminogen receptors in mammals is the glycolytic enzyme enolase [13,14]. Enolase has been also localized on the surface of several pathogens like bacteria, fungi or protozoa [15], and very recently in helminths [16]. This enzyme has been implicated in autoimmune diseases, and its utilization by pathogens in invading host tissues is well documented [15]. Recent studies have indicated that enolase present in the cell walls of *Staphylococcus aureus* enhances the activation of plasminogen [17], favors *Streptococcus* adherence to human pharyngeal cells [18], and that, in *Candida albicans*, it promotes host endothelial invasion by binding to plasmin(ogen) [19].

The enolase gene from *F. hepatica* was previously cloned in a study aimed at identifying mRNA regions involved in trans-splicing [20]. Here we present evidence suggesting that enolase plays a role in the fibrinolytic system based on its identification as a specific human plasminogen interacting protein.

2. Materials and methods

2.1. E/S antigens of F. hepatica

Livers from naturally infected sheep were collected from a local slaughterhouse and parasites were immediately removed and washed exhaustively with pre-warmed phosphate buffered saline pH 7.4 (PBS). Flukes were then placed at 37°C for 12 h with slow agitation in PBS containing 100 μg/ml streptomycin (Sigma) at a ratio of 1 ml per adult [21]. Thereafter the flukes were removed and the culture media centrifuged at 15 000×*g* for 15 min at 4°C. E/S products were then precipitated using equal volumes of ice-cold 20% trichloroacetic acid (TCA) and solubilized in loading buffer for gel electrophoresis. The protein content was measured by the protein assay method (Bio-Rad) and adjusted to 10 mg/ml using an ultrafiltration membrane (YM-3, Millipore).

2.2. Two-dimensional (2D) electrophoresis

To carry out the 2D gel electrophoresis the protein samples were solubilized at room temperature (RT) with a buffer containing 7 M urea, 2 M thiourea, 4% 3[3-cholaminopropyl diethylammonio]-1-propane sulfonate (CHAPS) (w/v), 20 mM dithiothreitol (DTT) and 2% (v/v) Biolytes 3-10 and bromophenol blue (all chemicals from Bio-Rad), and were then applied onto a linear pH 6–8 ReadyStrip™ IPG strip (11 cm long, Bio-Rad). Isoelectric focusing was performed on a Bio-Rad PROTEAN® IEF cell at 20°C using the following program: (1) passive rehydration for 16 h; (2) 300 V for 1 h (step and hold); (3) 4000 V for 2 h (linear voltage ramping until reaching 4000 V); and (4) 4000 V for 6.5 h (step and hold). After electrofocusing, the strips were reduced (2% DTT) and then alkylated (2.5% iodoacetamide) in equilibration buffer containing 6 M urea, 0.375 M Tris pH 8.8, 2% sodium dodecyl sulfate (SDS) and 20% glycerol, and the second dimension was performed using 10% polyacrylamide gels. Proteins from SDS–polyacrylamide gel electrophoresis (PAGE) were stained with Coomassie blue or electroblotted onto a nitrocellulose membrane.

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2.3. Ligand blotting

For detecting plasminogen-binding activity, far Western blot assays were performed after 2D electrophoresis. Gels were transferred to nitrocellulose membranes in 20 mM Tris, 192 mM glycine, methanol 20% v/v, pH 8.3 as previously described [22]. Filters were stained with Ponceau S (Sigma) for 10 min, and blocked for 3 h in 10 mM Tris–HCl, pH 7.4, 150 mM NaCl (Tris buffered saline, TBS) containing 5% non-fat dry milk. After extensive washings with TBS containing 0.05% Tween-20 (TBST), blots were incubated overnight at 4°C with human plasminogen (Fluka) at 35 μg/ml in TBST containing 25 mM ethylenediamine tetraacetic acid (EDTA) plus 1% bovine serum albumin (BSA). After extensive washing in TBST, blots were then incubated in TBST with 1% BSA containing 1:200 (v/v) monoclonal anti-human plasminogen antibodies (Technoclone) for 2 h at RT. The bound antibody was detected by incubating blots 1 h at RT with goat anti-mouse IgG horseradish peroxidase (HRP) conjugated (Bio-Rad) at 1:10 000 (v/v) in TBST with 1% BSA. Immune complexes were visualized using Lumi-light Western blotting substrate (Roche) following the manufacturer's instructions, and exposed to X-OMAT film (Kodak).

2.4. Western blot analysis

Immunoblot analysis with anti-α-enolase (from *Streptococcus pneumoniae*) antibody [23] (1:500 in PBST with 5% fat-free milk) was performed after electroblotting a 2D gel onto a nitrocellulose membrane. Bound antibody was detected using an anti-rabbit HRP-conjugated secondary antibody at 1:5000 in PBST. Filters were processed as described above.

2.5. Mass spectrometry (MS) and identification

Each anti-plasminogen reactive spot (2D) was excised from the gel, digested with sequencing grade trypsin (porcine, Promega) and excised from the gel following the protocol of Shevchenko et al. [24] with some modifications. Samples were resuspended in 3 μl of 0.1% TFA (trifluoroacetic acid) in preparation for MS. For matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) MS, α-matrix (α-cyano-4-hydroxycinnamic acid, Bruker Daltonics, Bruker) was prepared at 0.3 g/l in ethanol:acetone 2:1 and DHB (2,5-dihydroxybenzoic acid, Bruker Daltonics, Bruker) at 5g/l in 33% acetonitrile, 0.1% TFA. The internal standards used were Peptid Calibration Standard (Bruker Daltonics, Bruker) at 10 pmol/μl. Matrix and standards were mixed in equal volumes and then added to an equal volume of sample before 0.5 μl of each was spotted onto the AnchorChip™ 600/384 T F target plate (Bruker Daltonics, Bruker) and allowed to dry. Samples were analyzed on a Reflex IV spectrometer (Bruker Daltonics, Bruker) with delayed extraction operated in reflection mode. Acquired spectra were analyzed directly using XMASS (Bruker Daltonics, Bruker).

Database searches using the non-isotopic peptide masses were performed using Biotools (Bruker Daltonics, Bruker) with MASCOT software (http://www.matrixscience.com). Peptide modifications included in the search were carbamidomethyl (C) (fixed) and oxidation (M) (variable). All searches used the MSDB20020219 database and were constrained to a peptide mass tolerance of 100 ppm.

3. Results

With the aim of characterizing proteins secreted by *F. hepatica* that could be involved in host–parasite interactions, we investigated the ability of *F. hepatica* E/S materials to bind human plasminogen. E/S material was subjected to 2D gel electrophoresis and transferred to nitrocellulose. Immobilized spots were analyzed for their ability to bind human plasminogen. Interestingly, protein spots corresponding to a molecular mass of 47 kDa clearly bound human plasminogen in vitro (Fig. 1A). These proteins were highly abundant in the *F. hepatica* E/S material, as shown by Coomassie staining of identical 2D gels (Fig. 1B). The binding was highly specific, since no other major protein spots exhibited plasminogen-binding activity. As expected, human plasminogen was detected as a 92 kDa band when using anti-plasminogen antibodies (data not shown).

These 47 kDa protein spots were excised from the polyacrylamide gel, subjected to MS and peptide mass fingerprinting, and subsequently identified as *F. hepatica* enolase (Protein database accession number A53665). This result indicated that enolase is present at different isoelectric points (p*I*) ranging from 6.2 to 7.2 (Fig. 1B). The peptide coverage of one of these enolase spots (p*I* 7.0) reached 62.6% (Fig. 2). One of the peptides identified by MS had a similar amino acid composition (55.5% identity, 70.3% similarity) to a putative plasminogen-binding motif recently reported for the *S. pneumoniae* enolase [25] (Fig. 2).

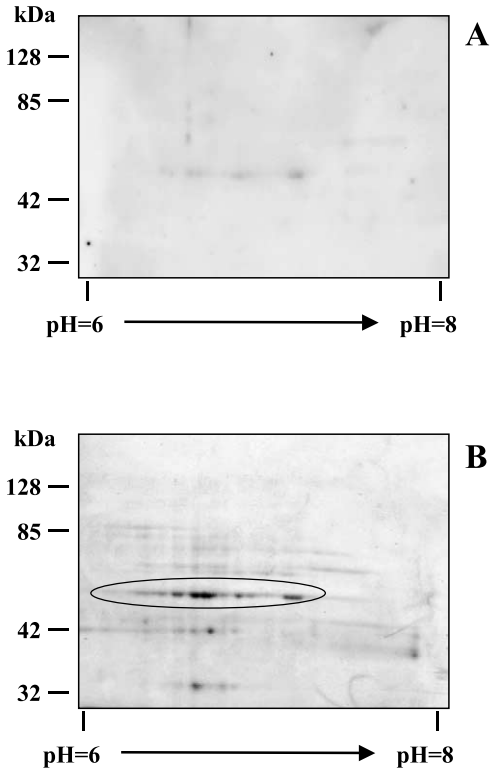

Fig. 1. *F. hepatica* enolase present in E/S material binds plasminogen in vitro. A: Far Western blot analysis after 2D gel electrophoresis of *F. hepatica* E/S proteins run on an 11 cm pH 6–8 IPG strip in the first dimension and then on gels of 10% SDS–PAGE in the second dimension. B: Coomassie blue staining of an identical gel as in A. The position of enolase is marked. Molecular weight markers in kDa are indicated on the left.

To further confirm that the identity of the protein that binds to human plasminogen is *F. hepatica* enolase, we next performed an immunoblot assay after 2D electrophoresis of *F. hepatica* E/S materials, using recombinant *S. pneumoniae* α-enolase (47 kDa) as a positive control [23]. As shown in Fig. 3, the same protein spots corresponding to a molecular mass of 47 kDa that bound human plasminogen in vitro, cross-reacted with antibodies directed against *S. pneumoniae* α-enolase [23]. These spots exhibited the same apparent molecular weight as the recombinant enolase control (r-Eno).

4. Discussion

Following a proteomic approach, we have clearly shown that *F. hepatica* E/S proteins bind human plasminogen in vitro. Furthermore, we have identified *F. hepatica* enolase as being responsible for this binding, suggesting that it may function as a plasminogen receptor. Enolase was identified using

MAIKAIHARQIFDSRGNPTVEVDVTTAKGLFRAAVPSGASTGVHEALELRDG

PPGYMGKGVLKAVANVNSQIAPNLIKSGINVTDQAAVDKFMLDLDGTPNKEK

LGANAILGVSLAXCKAGAAEKGLPLYKYIATLAGNKEVIMPVPSFNVINGGS

HAGNKLAMQEFMIMPTGASSFTEAMKIGSEVYHNLRAVIKSKYGLDACNVGD

EGGFAPSIQDNLEGLELLRTAIDKAGYTGKVXIAMDCAASEFY-KEGK-YDL

.* *..***.**** ** * **

DFKNPKSQASSWITSDAMADVYKKMMSTYPIVSIEDPFDQDDWPAWTKLTGE

*

CKIQIVGDDLTVTNPLRVQKAIDQKACNCLLLKVNQIGSVSESIKACKMAQE

AGWGVMVSHRSGETEDNFIADLVVGLRTGQIKTGAPCRSERLAKYNQLLRIE

EDLGGAAKYAGENFRRP

Fig. 2. Identification of *F. hepatica* enolase peptides (underlined) (Protein database accession number A53665) after trypsin digestion and peptide mass fingerprint from the 47 kDa band excised from a spot at p*I* 7.0 from 2D electrophoresis using Mascot searching software. A putative plasminogen-binding motif [25] is shadowed, indicating identical amino acids with an asterisk (*), and conserved amino acids with a dot (.). Dashes in the amino acid sequence indicate residues absent in the deduced *F. hepatica* enolase sequence.

2D gel electrophoresis followed by MS and peptide mass fingerprinting, and confirmed by Western blot analysis. Unfortunately, at present this approach cannot be fully exploited in *F. hepatica* because the sequence of the genome of this organism is not complete and therefore proteomic databases are very limited [26].

The *F. hepatica* enolase is represented by a group of isoforms ranging in p*I* from 6.2 to 7.2. A previous study identifying proteins present in E/S materials by 2D electrophoresis associated the enzyme phosphopyruvate hydratase (enolase) to a single spot of p*I* 6.56 and a theoretical molecular weight of 46.2 kDa [27]. Our study confirms this apparent molecular weight of 47 kDa, very similar to the theoretical molecular weight reported for the purified enzyme from *S. pneumoniae* that we used as a positive control [23,28]. Other enolases exhibit differences in weight, such as the recombinant Eno1 from *Pneumocystis carinii* that shows a molecular weight of 52 kDa [29]. We cannot discard different possibilities for the size variation, like post-translational modifications (i.e. glycosylation), or even pre-translational modifications like RNA splicing. In fact, the *F. hepatica* enolase gene was cloned when searching for repetitive regions of the *F. hepatica* mRNA involved in trans-splicing [20].

Recent studies have shown similar plasminogen-binding capacity for enolases from other pathogenic organisms, including bacteria like *S. aureus* [17], *Streptococcus* [23,28,30], fungi like *P. carinii* [29] and helminths like *Onchocerca volvulus* [16]. These studies suggest an important role of this association for the invasion of host tissues by the pathogenic agent [15]. Interestingly, recent reports have pointed to an enhanced activation of plasmin(ogen) upon interaction with the pathogen-derived enolase [12,19]. Furthermore, a change in the cellular localization of distinct enolase isoforms has been correlated with pathogenicity in the parasitic protozoan *Toxoplasma gondii* [31]. Moreover, *Streptococcus* G group surface enolase seems to act as a spreading factor during tissue invasion [18].

The amino acid sequence of *F. hepatica* enolase lacks the carboxy-terminal lysine residues of the plasminogen-binding motif [20] which is found in pathogens like *P. carinii* [29] and *Streptococcus* species [23,30,32]. But interestingly enough, it does contain a region of 28 amino acids (amino acids 235–253) (Fig. 2), which exhibits high homology with a novel plasminogen-binding domain recently identified in *S. pneumoniae* [25], suggesting this region may be responsible for the binding. Further studies should address these questions.

We still do not know whether other receptors for host fibrinolytic proteins are present in the E/S material of *F. hepatica*, but the evidence of plasminogen-binding molecules like enolase opens this possibility. Future studies will also address the potential immunogenicity of this and other E/S molecules.

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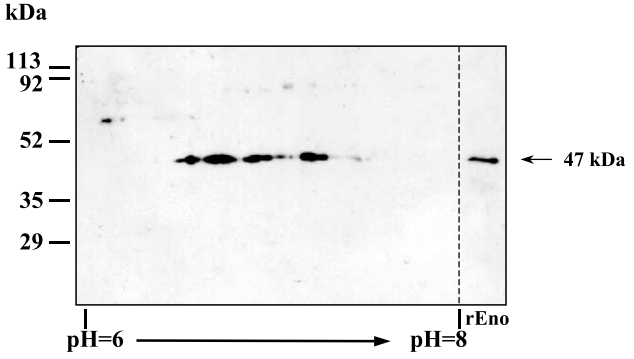

Fig. 3. *F. hepatica* enolase is recognized by antiserum against *S. pneumoniae* enolase. Western blot analysis after 2D gel electrophoresis of *F. hepatica* E/S proteins run on an 11 cm pH 6–8 IPG strip in the first dimension and then on gels of 10% SDS–PAGE in the second dimension, including 50 ng of recombinant α-enolase (r-Eno) from *S. pneumoniae* as an internal control [23]. The immunoblot was performed using rabbit polyclonal antibody raised against the α-enolase of *S. pneumoniae* [23]. The position of enolase is indicated at 47 kDa. Molecular weight markers in kDa are indicated on the left.

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