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FASCIOLA HEPATICA: RAPID IDENTIFICATION OF NEWLY EXCYSTED JUVENILE PROTEINS

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SUMMARY: The sensitivity of N-terminal sequencing has been used to identify proteins expressed by the newly excysted juvenile stage of the parasite *Fasciola hepatica.* Of the seven proteins identified, a number have significant sequence homology to the cysteine proteases: cathepsin B, cathepsin L and asparaginyl endoproteinase. Proteolytic acitvity was demonstrated using gelatin substrate sodium dodecyl sulphate polyacrylamide gel electrophoresis. In addition, a number of novel proteins were identified which shared no significant sequence homology to proteins in the databases. The availability of such N-terminal sequence information allows rapid identification of major proteins from scarce developmental stages and provides the basis for further molecular studies.

It has been established that antigens derived from the immature stages of the trematode parasite *Fasciola hepatica* induce the most significant protective immunity to challenge infection (1-3). In rats, it has been shown that newly excysted juveniles (NEJ) injected into immune rats are killed within 4 hours of infection (4). Despite their obvious importance, little is known about the molecular composition of NEJ liver flukes. This is mainly due to the minute amount of material that can be obtained from NEJ, making classical protein purification and analysis extremely difficult. In order to obtain more information on the proteins expressed by the early infective stage of *F. hepatica,* somatic proteins from *in vitro* derived NEJ were submitted to non-reducing SDS-PAGE, electroblotted onto a suitable sequencing support and stained with Coomassie blue. Individual bands were excised, pooled and their N-terminal sequence determined by Edman-degradation.

MATERIALS AND METHODS

N-terminal sequencing. *F. hepatica* metacercariae (Baldwin Aquatics, Monmouth, Oregon) were gassed with 40% CO_2 /10% O_2 /50% N_2 and incubated at 37°C for 1

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h. Taurocholic acid and L-cysteine (Sigma, St Louis, Mo) were then added to 0.45% w/v and 0.36% w/v, respectively, the medium gassed again and incubated for 2 h at 37°C. NEJs were separated from empty shells using a 100 μm membrane, extensively washed in PBS then sonicated in the presence of PBS containing protease inhibitors (1mM phenyl methylsulfonyl fluoride, 10mM iodoacetamide (Sigma) and 1mM EDTA). Samples were boiled and solubilized in SDS non-reducing sample buffer and electrophoresed on a 10% SDS-PAGE gel (5), then electroblotted onto Problott (Applied Biosystems, Inc.) membranes using transfer buffer containing 10mM 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 11 (Sigma), 15% methanol. Blotted proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma) and appropriate regions were excised and subjected to Edman degradation sequencing using an Applied Biosystems, Inc. Model 473A sequencer.

Gelatin substrate SDS-PAGE. NEJ somatic proteins were electrophoresed on a 10% gelatin substrate SDS-PAGE gel (6) in the presence and absence of 100μM of the irreversible cysteine protease inhibitor trans-epoxysuccinyl L-leucylamido (4-guanidino)butane (E64) (Sigma). Following electrophoresis, individual lanes were processed with the addition of 1 mM DTT (BioRad) in all wash and incubation buffers or the addition of both DTT and 10μM E64 to all buffers. After SDS removal, all lanes were incubated in 0.1 M Tris HCl, pH 7.5 at 37°C, overnight. Following staining with Coomassie R-250, all lanes were destained in 7% v/v acetic acid, 12.5% v/v methanol, to visualize zones of proteolytic activity.

RESULTS AND DISCUSSION

Sequence data was obtained from 7 distinct protein bands (Fig.1). N-terminal sequencing was repeated at least three times to confirm the validity of each sequence obtained. Although the proteins were separated using one dimensional SDS-PAGE only, single N- terminal sequences were obtained suggesting that they were not significantly contaminated with other proteins. The peptide sequences

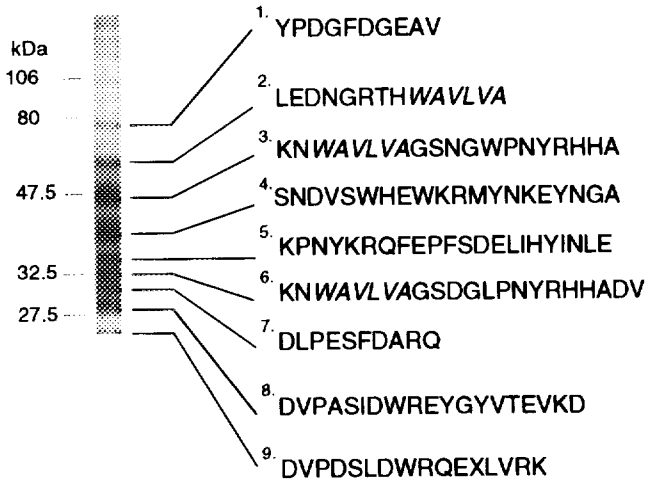

Fig. 1.
Non-reduced SDS-PAGE profile of NEJ proteins and derived N-terminal sequences.

obtained were compared with the GenBank, EMBL and Swiss Prot databases using the fasta algorithm (7).

One protein of approximately 75 kDa (Fig.1, band 1) was found to have 71% identity in a 7 amino acid overlap to rat phosphoenolpyruvate carboxykinase (PEPCK). In mammals, this enzyme is involved in gluconeogenesis, converting oxaloacetate to phosphoenolpyruvate. The enzyme in nematodes has been shown to be both functionally and kinetically distinct from the mammalian equivalent, carboxylating phosphoenolpyruvate to oxaloacetate (8) and is therefore seen as a potential target for anthelmintic drugs (9-10). The cDNA for PEPCK has been cloned from the nematodes *Hemonchus contortus* and *Ascaris suum,* and encodes 68.7 and 71.4 kDa proteins, respectively. To date, the enzyme has only been biochemically characterized in *F. hepatica* (11) and the related trematode, *Schistosoma mansoni* (12).

The 14 amino acid N-terminal sequence of somatic protein 2 (Fig.1) has no significant homology to any of the proteins found in the databases searched. It does however, feature the 6 amino acid hydrophobic sequence, WAVLVA, found on three of the NEJ somatic proteins sequenced here. Database searches revealed such a sequence is found only in the schistosome hemoglobinase, however, the remaining residues show no homology to this protein.

Somatic proteins 3 and 6 (Fig.1) both feature the WAVLVA sequence in the derived N-terminal sequence and share 82% identity with one another. They also show significant homology to the hemoglobinase of *Schistosoma japonicum* (76% identity) and *S. mansoni* (77% identity). The *S. mansoni* hemoglobinase has been shown to have a 50 kDa primary translation product which is reduced to a 31 kDa mature hemoglobinase following cleavage at both the N and C termini (13).These molecular masses compare favourably with the somatic proteins 3 and 6 sequenced in the present study (46 and 32 kDa, respectively). As the two NEJ proteins both share significant N-terminal homology with one another, it appears that in *F. hepatica*, cleavage might only occur at the C-terminus. Alternatively, the lower molecular mass protein sequenced here may be a break down product of the higher molecular mass species.

Recently, the schistosome hemoglobinase has been shown to share 75% absolute amino acid homology to legumain, an asparaginyl endopeptidase from *Canavalia ensiformis*, the jack bean plant (14). In addition, legumain shares 75 and 73% absolute homology with the N-terminal sequences of NEJ proteins 3 and 6 derived in the present study. The asparaginyl endoproteinases are members of a novel family of cysteine proteinases as their sequence, substrate specificity and inhibitor susceptibility is not comparable to the papain superfamily of cysteine proteases (14).

Somatic proteins 4 and 9 (Fig.1) show no significant homology to any proteins in the data bases searched. As analyzed by one-dimensional electrophoresis, protein 4 appears to be a dominant protein expressed by the NEJ and by later

developmental stages collected from the peritoneal cavity and liver mass of infected mice during a ten day infection period (Tkalcevic *et al.*, in preparation).

Proteins 5 and 7 (34 and 31 kDa approximately, respectively) (Fig.1) both share significant sequence homology with cathepsin B. The higher molecular weight protein shares 64% identity in a 14 amino acid overlap to *S. mansoni* cathepsin B (15). It also shares a 56% identity in an 18 amino acid overlap to the murine cathepsin B precursor while the lower molecular weight species shares 80% identity to the mature cathepsin B of both the mouse and rat. Recently, Heussler and co-workers (16) using degenerate oligonucleotide primers derived from conserved cysteine protease sequences, generated cDNA clones from adult fluke transcripts showing homology to both cathepsins B and L. The present study is the first to demonstrate expression of cathepsin B protein by the immature NEJ parasite. In contrast to *F. hepatica*, the schistosome cathepsin B has been widely characterised (15, 17). Klinkert and co-workers (15) demonstrated that the *S. mansoni* cathepsin B cDNA coded for a protein of 38.5 kDa but the native protein of 31 kDa, comparable to the molecular mass observed here, was detected in Western blots, suggesting a precursor protein processed into a smaller polypeptide. It is now believed that the 31 kDa cathepsin B like cysteine protease found in the gut of *S.mansoni* is the major enzyme involved in hemoglobin degradation (17).

NEJ protein 8 (Fig.1) shares 63% homology with human cathepsin L. Interestingly, NEJ protein 8 shares only 75% absolute N-terminal amino acid homology with the cathepsin L cDNA (Fhcat-1) derived from adult fluke (18). In addition, it only shows 69% and 56% amino acid homology with N-term 1 and N-term 2, respectively, the two cathepsin L sequences derived from adult excretory-secretory (ES) products in the same study. NEJ protein 8 also only shares 68% absolute homology with the secreted adult fluke cathepsin L proteins described by Smith (19) and Dowd (20). It has been suggested, that the two adult fluke proteinases described by Smith and Dowd represent two distinct subclasses of cathepsin L (20). It is possible that the NEJ cathepsin L may be different to its adult counterpart and so constitute a third subclass.

As with cathepsin B, the mature cathepsin L is also cleaved from a preproenzyme form. However, this precursor form was not identified in the present study. All cathepsins L described are proteins of approximately 27 kDa. A number of workers have shown that cathepsin L activity is found in liver fluke ES products (18-22) released from the NEJ, immature and adult stages of the parasite. Immunolocalization studies have demonstrated that the proteinases are packaged and secreted from vesicles within the adult gut epithelial cells (19). Cathepsin L has been implicated in host immune evasion mechanisms. It is thought that cathepsin L cleaves host immunoglobulin releasing the Fc portion from IgG and therefore prevents antibody mediated immune effector cell attachment (21).

Given that several somatic proteins identified in the present study appeared to be proteases, GS SDS-PAGE was used to demonstrate proteolytic activity. Acidic,

neutral and alkaline buffers were used (data not shown) and optimal activity was observed at pH 7.5 (Fig.2, lane a), with activity being further enhanced in the presence of the reducing agent, dithiothreitol (DTT) (Fig.2, lane b), suggesting the presence of cysteine proteases. Addition of the irreversible cysteine protease inhibitor trans-epoxysuccinyl L-leucylamido (4-guanidino) butane (E-64) to the sample and all buffers post-electrophoresis, substantially inhibited protease activity (Fig.2, lane c), confirming that the proteolytic activity observed in the two lower molecular mass bands was due to cysteine proteases. The position of the two cysteine protease bands correspond to the cathepsin B and cathepsin L proteases identified by N-terminal sequencing. Proteolytic activity was also observed in high molecular mass regions of the gel. These high molecular mass proteases could not be identified in this study by N-terminal sequencing due to the limiting amounts present in somatic NEJ preparations.

N-terminal sequencing rapidly revealed the identity of 6 major proteins expressed by the NEJ stage of *F. hepatica*. In addition, 3 novel NEJ proteins were identified which show no significant homology to any proteins in the data bases. Interestingly, while the N-terminal sequence could be easily obtained from the *in vitro* excysted NEJ stage, similar attempts to sequence proteins from later fluke stages recovered from the liver of infected mice were unsuccessful. This suggest that proteins expressed *in vivo* after infection of the host are somehow modified resulting in blocking of the N-terminus. This may be a way the parasite protects itself from the hostile environment of the host which includes the presence of amino peptidases. The deduced N-terminal amino acid sequences obtained can now be used to generate degenerate oligonucleotide probes for screening genomic or cDNA libraries or in PCR procedures, as well as for the production of peptide antibodies, and so form the basis for further characterization of NEJ proteins.

The combination of N-terminal sequencing and gelatin substrate gel electrophoresis revealed a predominance of proteins showing protease activity. It

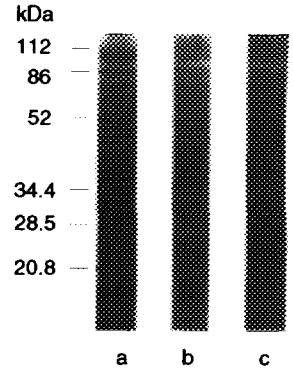

Fig. 2.
Gelatin substrate SDS-PAGE of NEJ proteins. Lane a: no DTT, no E-64; Lane b: with DTT; Lane c: with DTT and E-64.

has been demonstrated that in parasites, proteases are involved in the invasion of host tissues, the evasion of immune attack mechanisms and to provide nutrients for parasite survival. As they provide essential functions, they may serve as useful target sites for both chemotherapeutic and immunological control mechanisms.

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