Proteinases Secreted by Fasciola hepatica Degrade Extracelullar Matrix and Basement Membrane Components

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# PROTEINASES SECRETED BY *FASCIOLA HEPATICA* DEGRADE EXTRACELULLAR MATRIX AND BASEMENT MEMBRANE COMPONENTS

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ABSTRACT: The invasive stages of the parasitic trematode *Fasciola hepatica* release proteinases into the medium in which they are mantained. In this study, we investigated the interaction of *F. hepatica* excretory/secretory (E/S) products and 2 cysteine proteinases (CL1 and CL2) purified from these products with extracellular matrix and basement membrane macromolecules. *Fasciola hepatica* E/S products contained collagenolytic activity on fibrillar types I and III collagen as well as basement membrane type IV collagen. CL1 and CL2 were capable of degrading acid-soluble type III and type IV collagen but not insoluble type I collagen. In contrast, neither the E/S products nor the purified CL1 and CL2 showed elastinolytic activity. Fibronectin and laminin were degraded by E/S products and by CL1 and CL2. Sequence analysis of fibronectin degradation products showed that the fragments obtained corresponded to complete biologically active domains. These results indicate that the cysteine proteinases secreted by *F. hepatica* may be involved in the process of tissue invasion by the parasite.

*Fasciola hepatica* is an important trematode parasite of grazing animals and humans. Following ingestion of contaminated vegetation, newly excysted juveniles (NEJ) penetrate the intestinal wall and migrate to the liver where they spend up to 14 wk feeding on the host tissue. They then move into the bile ducts where they complete their maturation.

*Fasciola hepatica* secrete a number of proteinases, the most prominent of which are cysteinyl endopeptidases (Dalton and Heffernan, 1989). One of these enzymes, a cathepsin L proteinase (CL1), is secreted by all stages of the developing parasite; it is capable of cleaving host immunoglobulins and can prevent in vitro attachment of eosinophils to NEJs (Smith, Dowd, Heffernan et al., 1993; Smith, Dowd, McGonicle et al., 1993; Carmona et al., 1993). More recently, a second cysteinyl proteinase (CL2) was isolated from the medium in which *F. hepatica* was cultured. It was shown to differ from CL1 in its specificity for synthetic fluorogenic substrates, pH optimum, inhibition profile, and N-terminal sequence (Dowd et al., 1994). Because CL2 can cleave fibrinogen in a manner that produces a fibrin clot, it was postulated that it could prevent excessive bleeding at feeding points of the bile ducts (Dalton et al., 1994).

It has also been suggested that these CL1 and CL2 proteinases may act together in aiding parasite penetration and degradation of host tissue. Therefore, in the present study we examined the effects of *F. hepatica* E/S products and cathepsin L proteinases on the extracelullar matrix and basement membrane proteins collagen, elastin, laminin, and fibronectin.

## MATERIALS AND METHODS

### Preparation of *F. hepatica* in vitro released (excretory/secretory, E/S) products

Mature flukes were removed from the bile ducts of bovine livers obtained at a local abbattoir, washed 6 times in 0.01 M phosphate-buffered saline (PBS), pH 7.3, and maintained (1 mature fluke per ml) in RPMI-1640, pH 7.3, containing 2% glucose, 30 mM Hepes, and 25 mg $L^{-1}$ gentamycin for 6 hr (Sigma, St. Louis, Missouri). The medium (E/S products) was then removed and centrifuged at 15,000 rpm at 4 C. The supernatant was filtered through Whatman no. 1 filter paper and stored at −20 C until used.

### Enzymatic assays with fluorogenic substrates

Proteinase activity was measured fluorometrically using as substrate peptides coupled to 7-amino-4-methylcoumarin (NHMec). Each substrate was stored as a 1-mg/100 μl stock solution in dimethylformamide. Assays were conducted using a final concentration of 10 μM substrate in a volume of 1 ml. The mixtures were incubated at 37 C for 60 min before stopping the reaction by the addition of 200 μl of 1.7 M acetic acid. The amount of NHMec released was measured using a Sequoia Turner fluorometer with excitation at 370 nm and emission at 440 nm. One unit of enzymatic activity was defined as the amount that catalyzes the release of 1 μmol of NHMec per min at 37 C.

### Purification of cathepsin L proteinases

Cathepsins L1 and L2 were purified to homogeneity from E/S products by a combination of ultrafiltration on YM3 membrane (Amicon, Beverly, Massachussets), Sephacryl S200HR, and QAE-Sephadex (Pharmacia, Uppsala, Sweden) chromatography as previously described (Smith, Dowd, McGonicle et al., 1993; Dowd et al., 1994).

### Protein concentration

Protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, Illinois) in microtiter plates according to the method of Redinbaugh and Turley (1986). Bovine serum albumin was used as a protein standard. To avoid the interference of glucose and red phenol present in the RPMI from where the E/S products were obtained, 1 ml of E/S products was mixed with 100 μl of a 1% sodium deoxycholate solution in 0.1 M NaOH for 5 min on ice. Then, 200 μl of 50% (w/v) of trichloroacetic acid in water were added and after a further 15-min incubation, the sample was centrifuged at 15,000 rpm for 5 min and the pellet dissolved in distilled deionized water. The dissolved pellet was then used for protein determination.

### Collagen degradation assays

Lyophilized cathepsin L proteinases were dissolved in distilled water to a final concentration of 0.25–0.30 μg/μl and stored at −80 C until used. For the collagen degradation experiments, the following reagents were purchased from Sigma: Insoluble type I collagen from bovine Achilles tendon, acid-soluble type III collagen from calf skin, acid-soluble type IV collagen from Englebreth-Holm-Swarm mouse sarcoma, L-3-carboxy-2,3-trans-epoxypropionyl-leucylamido (4-guanidino) butane (E-64), phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), L-cysteine, dithiothreitol (DTT), *o*-phenanthroline, and the synthetic fluorogenic peptides. Collagen type III was dissolved in 5 mM acetic acid, pH 4.5, and adjusted with 0.1 M NaOH to pH 5.5. Acid-soluble collagen type IV solution (0.75 μg/ml) was adjusted with 0.1 M NaOH to pH 5.5 and stored at −20 C until used.

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Types I, III, and IV collagens (0.1–1.0 mg) were mixed in RPMI with *F. hepatica* E/S products (6 μg) or with purified cathepsin L1 (1–2 μg), cathepsin L2 (1–2 μg), or a mixture of both enzymes in a final volume of 100 μl. The mixtures were incubated at 37 C for 24 hr with continuous shaking. Reactions were stopped by boiling the mixture for 5 min. After centrifugation at 10,000 *g* for 5 min, aliquots were mixed with reducing sample buffer and analyzed on 8.5–12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli and Favre, 1973).

The effects of E-64 (10 μM), PMSF (1 mM), *o*-phenanthroline (10 mM) (protease inhibitors), and L-cysteine (10 mM) (an enhancer of cysteine proteinases) were assessed by adding these reagents to the enzyme preparations 20 min prior to the addition of the protein substrate. For digestion controls, collagens were incubated in RPMI, or RPMI with protease inhibitors, or L-cysteine, but in the absence of E/S products or the purified cathepsins.

The fluorogenic peptide Succ-Gly-Pro-Leu-Gly-Pro-NHMec cleavage (Nagai et al., 1960) was also used to assay E/S or cathepsin L proteinases for collagenolytic activity in the following buffers: 100 mM sodium acetate buffer, pH 4.4; 50 mM Tricine buffer containing 0.4 M NaCl and 10 mM $CaCl_2$, pH 7.5; 50 mM Tris-HCl buffer containing 5 mM $CaCl_2$, pH 7.4. The effects of the reducing agent DTT (10 mM) and cysteine proteinase inhibitor E-64 (10 μM) on enzyme activity were examined. All assays were performed at 37 C for 1 hr under continuous shaking. Proteins were stained with Coomassie brilliant blue R-250 (CBB-R250).

### Degradation assay of laminin and fibronectin

Thirty micrograms of bovine plasma fibronectin or laminin solution (1 mg/ml) from Englebreth-Holm-Swarm mouse sarcoma (Sigma) were incubated with 0.9 μg of CL1, CL2, or 1 μg of E/S products, with or without 10 mM DTT, at pH 5.5 in 0.1 M acetate buffer or pH 7.2 in PBS for 3 and 24 hr at 37 C. Protease inhibitors were used as described above. Products were resolved on a 5%–stacking/10%–separating SDS-PAGE gel for fibronectin, and both a 4%/5% gel, as well as a 4%/10% SDS-PAGE gel for laminin in order to detect both undegraded laminin and any degradation product from 10 to 400 kDa. Proteins were stained with CBB-R250.

### Amino acid sequence analysis of bovine fibronectin fragments

Digested bovine plasma fibronectin with either E/S products, CL1, and CL2 was run on a 12.5% SDS-PAGE, electrotransferred onto a polyvinylidenedifluoride (PVDF) membrane (Millipore, Bedford, Massachusets), and stained with 0.5% CBB-R250 in 40% methanol–5% acetic acid (sequanal grade) (Towbin et al., 1979). Bands were excised and sequenced. Automated amino acid sequence was carried out on a 477 A microsequencer (Applied Biosystems, Foster City, California), and the resulting phenylthiohydantoin (PTH) derivatives were identified using the on-line 120A PTH Analyzer (Applied Biosystems).

### Elastinolytic assays

Elastin degradation was measured by 2 different methods as follows. In the first, 10 ml of 1% (w/v) agarose, prepared in 20 mM sodium phosphate, pH 7.4, 0.15 M NaCl, 2 mM cysteine, 5 mM EDTA, containing suspended bovine ligamentum nuchae elastin (Sigma) (3 mg/ml), were poured onto glass plates (6 × 10 cm) and 3-mm wells were cut. Cathepsin L proteinases (1.5 μg) or E/S products (3 μg) were then dispensed into the wells. The plates were incubated at 25 C and 37 C in a humidified chamber. After 48 hr, zones of elastin digestion were measured (Senlor et al., 1971). In the second method, E/S products and cathepsin L were mixed with elastin–congo red (Sigma) at 37 C for 12 hr in 0.1 M citrate/phosphate buffer, pH 6.0, or 0.1 M glycine, pH 9.0. Following centrifugation, the optical density (OD) of the supernatant was measured at 550 nm (Naughton and Sanger, 1961). Porcine pancreatic elastase was used as a control.

## RESULTS

### Collagen degradation assays

*Fasciola hepatica* E/S products degraded native type I collagen as judged by a marked decrease in the amount of the crosslinked α and β chains and the appearance of new lower molecular-sized bands during an overnight incubation (Fig. 1, lanes 1 and 2). Collagenolytic activity of E/S products was enhanced by the addition of cysteine (Fig. 1, lane 3) and mostly inhibited by the addition of E-64 (Fig. 1, lane 4), indicating that cysteine proteinases are responsible for this activity. CL1 and CL2 did not degrade type I collagen to any appreciable extent, even in the presence of cysteine (Fig. 1, lanes 5, 6, 7, and 8); however, CL1 and CL2 did appear to have very weak collagenolytic activity but only in the presence of cysteine.

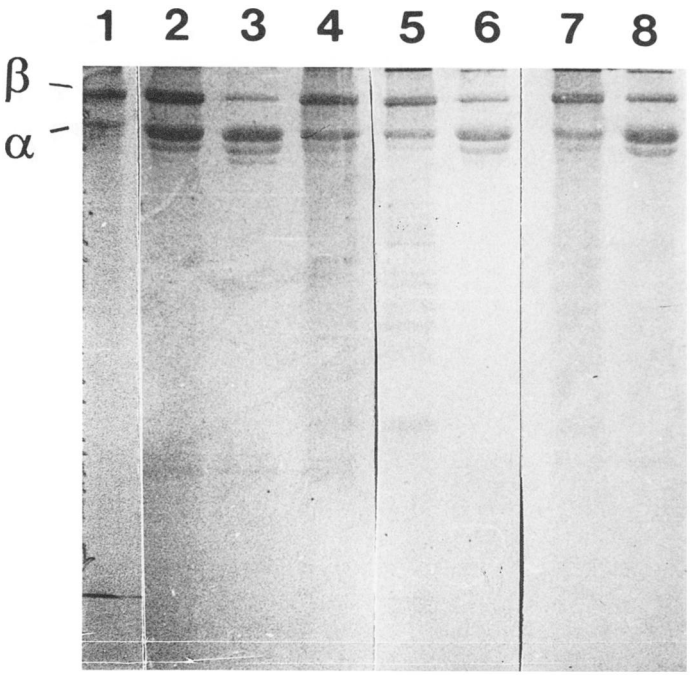


FIGURE 1. Type I collagen degradation by secreted proteinases of *Fasciola hepatica*. Aliquots of the following reaction mixtures were analyzed by 12.5% SDS-PAGE: (1) Type I collagen control (α and β chains are arrowed); (2) collagen and E/S products; (3) collagen, E/S products and cysteine; (4) collagen, E/S products and E-64; (5) collagen and CL1; (6) collagen, CL1 and cysteine; (7) collagen and CL2; and (8) collagen, CL2 and cysteine. All the reactions were performed at pH 6.0.

Type III collagen was completely degraded by the E/S products, CL1 and CL2, producing multiple degradation fragments noticeable in SDS-PAGE (Fig. 2). This cleaving activity in E/S products was thiol dependent and inhibited by E-64. The CL1 and CL2 enzymes produced a degradation pattern similar to that of the E/S products (Fig. 2).

Type IV collagen was completely degraded by the E/S products (Fig. 3, lane 2). It was partially degraded by the mixture of CL1 and CL2 in the presence of cysteine (Fig. 3, lane 10) and CL2 alone in the presence of cysteine (Fig. 3, lane 8), but it resisted degradation by CL1 or CL2 alone in the absence of cysteine. This enzymatic activity observed in the E/S products was also inhibited by E-64 and enhanced by cysteine (Fig. 3, lanes 3 and 4).

Collagenolytic activity was detected in the E/S products with the fluorogenic substrate Succ-Gly-Pro-Leu-Gly-Pro-NHMec but only when 10 mM DTT was added to the assay mixture with acetate buffer pH 4.4 (specific activity, μM AMC/min/mg

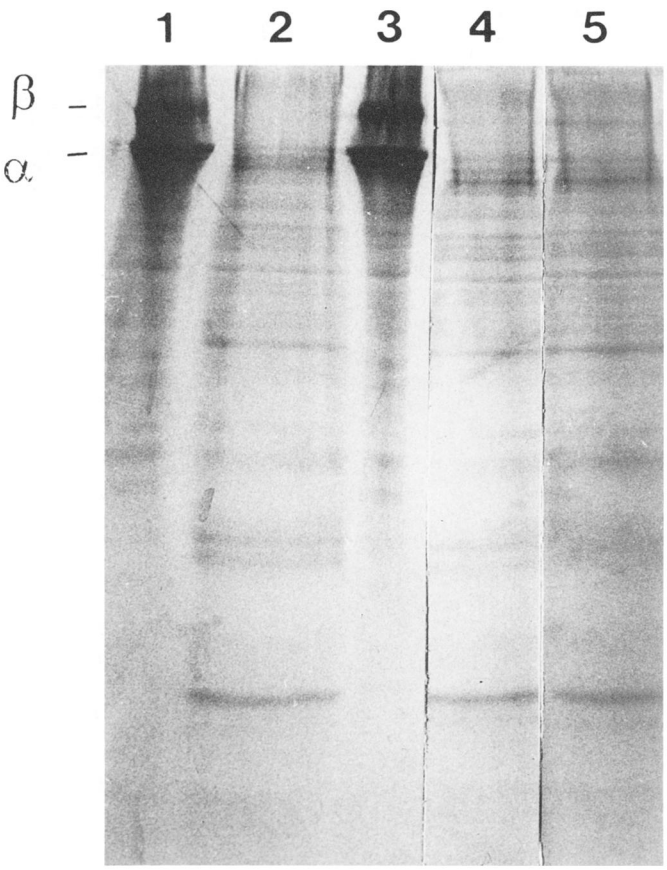


FIGURE 2. Proteolytic activity of *Fasciola hepatica*-secreted proteinases on type III collagen. Aliquots of the following mixtures incubated at pH 5.5 were analyzed by 12.5% SDS-PAGE: (1) acid-soluble type III collagen control (α and β chains are indicated by arrows); (2) collagen and E/S products; (3) collagen, E/S products and E-64; (4) collagen and CL1; and (5) collagen and CL2.

0.57) (Table I). CL1 and CL2 showed no activity on this substrate.

### Elastinolytic assays

Neither E/S products nor cathepsin L proteinases showed enzymatic activity against elastin by the elastin plate method or the elastin–congo red colorimetric method (data not shown).

### Degradation of laminin and fibronectin

E/S products, CL1 and CL2 hydrolyzed laminin to low molecular-sized products (Fig. 4). E-64 prevented the degradation of these molecules by E/S (Fig. 4, lane 4). Furthermore, the laminin-degrading activity of both CL1 and CL2 products was enhanced by the addition of 10 mM cysteine to the reaction mixture (Fig. 4, lanes 4–7).

Fibronectin was degraded by CL1 and CL2 without the addition of cysteine, mainly to fragments of 20–60 kDa, although many partial digestion products between 200 and 60 kDa were observed. CL1, CL2, and the combination of both enzymes, produced a similar digestion pattern. However, this pattern differed markedly to that produced by the E/S products where many more fragments were observed (Fig. 5).

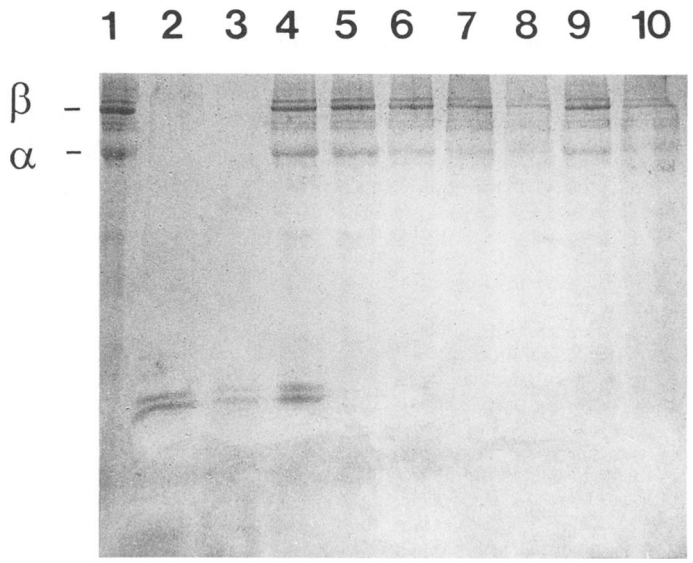


FIGURE 3. Cleaving activity of *Fasciola hepatica* secreted proteinases on type IV collagen. Products of the reactions of the following mixtures incubated at pH 5.5 were analyzed by 12.5% SDS-PAGE: (1) Type IV collagen control (α and β chains are arrowed); (2) collagen, and E/S products; (3) collagen, E/S products, and cysteine; (4) collagen, E/S products, and E-64; (5) collagen, and CL1; (6) collagen, CL1, and cysteine; (7) collagen, and CL2; (8) collagen, CL2, and cysteine; (9) collagen, CL1, and CL2; and (10) collagen, CL1, CL2, and cysteine.

### Amino acid sequence of fibronectin fragments

Analysis of the N-terminal sequence of the predominant 20-kDa digestion fragment (arrowed in Fig. 5B) revealed 2 sequences, Gln-Pro-Gln-Pro-(Cys)-Pro-Gln-Pro, and Gly-Pro-Pro-Pro-Tyr-Gly, which correspond to sequences beginning at residues 270 and 264, respectively, in fibronectin. A second band of approximately 29 kDa (Fig. 5A, arrowed), obtained by running CL1- or CL2-digested fibronectin on reducing SDS-PAGE, was analyzed and gave 2 amino-terminal sequences, Val-Ser-Gln-Ser-Lys-Pro-Gly-(Cys)-Tyr-Asp, and Gln-Ser-Lys-Pro-Gly-(Cys)-Tyr-Asp-Asn-Gly-Lys, which correspond to sequences beginning at residues 14 and 16, respectively, on the bovine fibronectin (Skorstengaard et al., 1986).

TABLE I. Specific activities of *Fasciola hepatica* E/S products and cathepsin L proteinases against the collagenase fluorogenic substrate Succ-Gly-Pro- Leu-Gly-Pro-NHMec

| | Cleaving Activity* | | | |
|---|---|---|---|---|
| Buffer | E/S products | CL1 | CL2 | CL1+CL2 |
| Acetate buffer pH 4.4 | 90† | 0 | 0 | 0 |
| Acetate buffer pH 4.4 + DTT | 570 | 0 | 0 | 0 |
| Tricine pH 7.5‡ | 0 | 0 | 0 | 0 |
| Tris-HCI pH 7.4‡ | 0 | 0 | 0 | 0 |

* Activity is expressed in nMNHMec/min/mg.
† Values were determined after subtracting background substrate (intrinsic fluorogenicity).
‡ Measurements were obtained with and without DTT in the corresponding mixture. Each value represents the mean of triplicate assays.

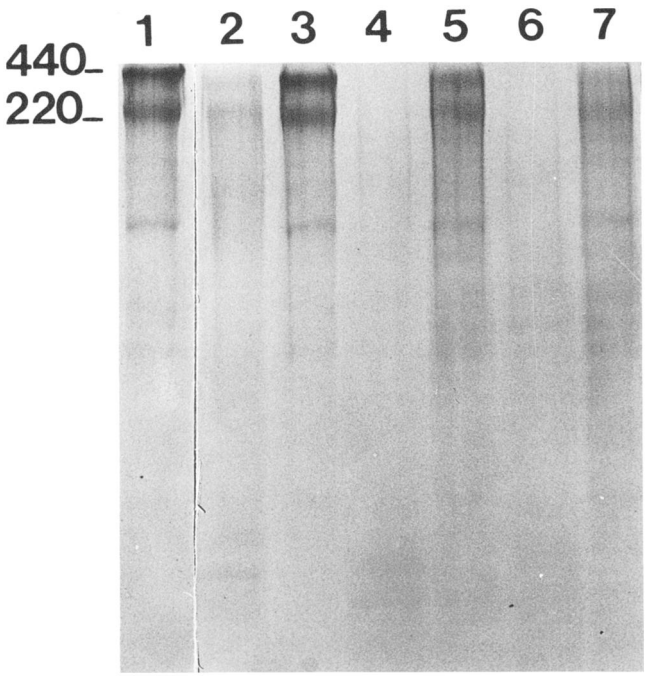


FIGURE 4. Laminin degradation by CL1, CL2, and *Fasciola hepatica* E/S products. Ten microliters of a 1-mg/ml laminin solution (lane 1) from basement membrane of Engelbreth-Holm-Swarn mouse sarcoma were incubated for 3 hr at 37 C at pH 5.5 with the following reactants: 5 μl E/S products (lane 2), 5 μl E/S products and E-64 (lane 3), 2 μl CL1 and cysteine (lane 4), 2 μl CL1 alone (lane 5), 2 μl CL2 and cysteine (lane 6), and 2 μl CL2 alone (lane 7). The resulting products were resolved on a 4%/10% reduced gel and proteins were stained by CBB-R250.

## DISCUSSION

In this study, we show that proteinases present in the E/S products of *F. hepatica* are capable of degrading fibrillar collagen types I and III, nonfibrillar collagen type IV, fibronectin, and laminin. The activities on these substrates were inhibited by E-64, indicating an involvement of cysteine proteinases. Types I and III collagen and fibronectin are constitutive components of the liver stroma and capsule, whereas type IV collagen and laminin are integral components of basement membranes (Martínez-Hernandez, 1984). Therefore, *F. hepatica* secretes the appropiate enzymes for degrading these molecules; thus, these enzymes may play a role in aiding the parasite in the penetration and migration through the intestinal wall and liver mass.

CL1 and CL2, 2 major cysteine endoproteinases present in E/S products, also degraded types III and IV collagens, fibronectin, and laminin and, therefore, appear to be responsible for part of the activity observed in the E/S products. However, these cathepsin Ls, either alone or in combination, were incapable of degrading native type I collagen. In addition, whereas we detected activity against the fluorogenic peptide Succ-Gly-Pro-Leu-Gly-Pro-NHMec in the E/S products, neither CL1 nor CL2 cleaved this substrate. These data indicate the presence of enzymes in the E/S products other than cathepsin Ls. We have detected a dipeptidylpeptidase in *F. hepatica* E/S products that can cleave dipeptides terminating in Gly-Pro (Carmona et al., 1994). This enzyme may act together with the cathepsin Ls in degrading type I collagen and Suc-Gly-Pro-Leu-Gly-Pro-NHMec.

Fibronectin is an important structural component of extracellular matrices because of its interactions with collagen, lam-

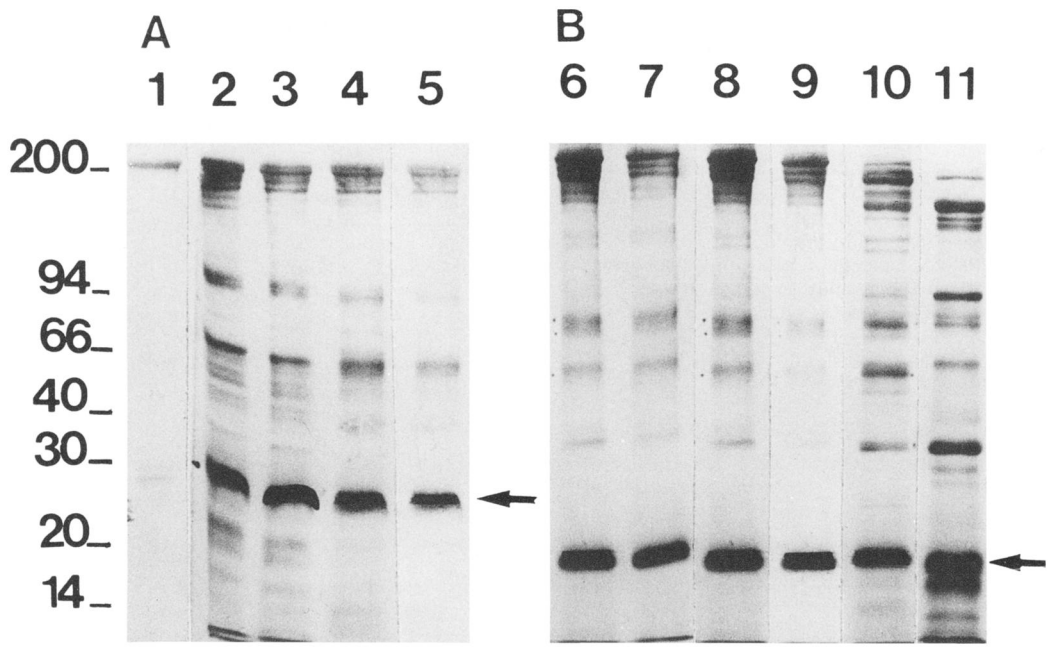


FIGURE 5. Fibronectin degradation by secreted proteinases of *Fasciola hepatica.* Products of reactions incubated in the presence of 10 mM (A) or without DTT (B) were analyzed by 12.5% SDS-PAGE. Reaction mixtures contained: (1) fibronectin only; (2) fibronectin and CL1, pH 5.5; (3) fibronectin and CL1, pH 7.2; (4) fibronectin and CL2, pH 5.5; (5) fibronectin and CL2, pH 7.2; (6) fibronectin and CL1, pH 5.5; (7) fibronectin, CL2, pH 5.5; (8) fibronectin, CL1, and CL2, pH 7.2; (9) fibronectin, CL1, and CL2, pH 5.5; (10) fibronectin and E/S products, pH 7.2; and (11) fibronectin and E/S products, pH 5.5. Arrows indicate the sequenced bands.

inin, hyaluronic acid, and cells (Yamada, 1991). The 29-kDa fragment produced by the degradation of fibronectin by *F. hepatica* E/S products, and which begins at positions 14 and 16, would contain the fibrin- and heparin-binding domain (FBR) and the 4 fibronectin type I repeats. The 20-kDa fragment beginning at positions 264 and 270 would contain most of the collagen-binding domain (CBR), 3 of the type I repeats, and 2 of the type II repeats. We did not locate a fragment that included the cell attachment (RGD) motif; however, only the predominant fragments were sequenced. Therefore, the *F. hepatica* proteinases seem to cleave fibronectin at sites that separate the biologically active domains. In this way, the parasite may "unlock" the extracellular matrix leaving it more susceptible to further digestion.

Collagenolytic activity has been described in several nematode parasites, e.g., *Strongyloides* spp. (Rege and Dresden, 1987), *Onchocerca volvulus* (Petralanda et al., 1986), and *Toxocara canis* (Robertson, et al., 1989). Rege and Dresden (1987) have shown that the collagenolytic activity from invasive larvae of *Stronglyloides ratti* was due to a cathepsin L proteinase with maximum activity at pH 4.5. The collagenolytic activity in the *F. hepatica* E/S products is similar to the *S. ratti* activity in terms of the class of proteinases involved, pH required for activity, and substrate degradation properties.

In summary, proteinases present in *F. hepatica* E/S products and, more specifically CL1 and CL2, are capable of degrading extracelullar matrix and basement membrane molecules. Thus, the inactivation of these secreted enzymes, either by immunoprophylaxis or chemotherapy may impair the migration and survival of the parasite in the mammalian host.

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