Fasciola hepatica (L., 1758):
Studies on Protease and Protease Inhibitor Activity

Éva HAJDÚ — Dr. István MATSKÁSI — Dr. Sándor JUHÁSZ
Veterinary Medical Research Institute,
Hungarian Academy of Sciences, Budapest

"Fasciola hepatica (L., 1758): Studies on protease and protease inhibitor activity" -

ABSTRACT. Proteolytic enzyme and protease inhibitor activities were demonstrated
in somatic extracts and maintenance media of liver flukes (Fasciola hepatica).
Against previous observations the extract and the medium reveals BAEE- and BTEE-
splitting activities also at alkaline pH (8.0). The somatic extract inactivates the es-
terolytic effect of bovine trypsin and chymotrypsin. In vitro maintained live flukes,
too, were able to decrease the activity of bovine proteases added to the medium,
even after sealing of their oral sucker with the tissue cement Histoacryl.

The liver fluke (Fasciola hepatica) derives its nutrients from the liver tissue and
blood (HALTON, 1967). The muscular oral sucker and oesophageal suction play a decisive
role in the mechanism of nutrient intake, but it has been postulated that proteolytic enzymes
released by the parasite may also collaborate in the digestion of host tissue. The involvement
of parasite proteases in the nutrition process has been implied from light and electron micro-
scopic observations (THORSELL and BJÖRKMANN, 1965; HALTON, 1963, 1967; HOWELL,
1973).

PENNOIT de COOMAN and van GREMBERGEN (1942) were the first to detect pro-
teolytic enzyme activity against a natural protein (casein) in extracts prepared from liver
fluke homogenates; they designated the parasite protease tentatively as cathepsin C. In more
detailed studies LOCATELLI and BERETTA (1969) established and acid pH optimum (range:
1.8 - 3.0) of the enzyme activity on haemoglobin substrate, and inactivity of the protease at
a neutral pH.

An in vitro release of protease by the liver fluke was verified by THORSELL and
BJÖRKMANN (1965), and later by LOCATELLI and BERETTA (1969) on the basis of gelatinolysis.
The flakes placed in a gelatine-containing maintenance medium produced a lysis of
gelatin, signs of which were most conspicuous around the oral region. Flukes with a ligated
oral part failed to produce gelatinolysis.

Histological examinations suggested that the proteolytic enzymes arise by synthesis
in the parasite gut rather than by release from digested host tissues (Thorssell and Björkman,

The protease inhibitors produced and released by the parasite play an important role
in host-parasite interaction. Certain authors (PAPPAS and READ, 1972a; JUHÁSZ, 1979) be
lieve that such inhibitors protect the parasite itself, or its more sensitive organs, against
the proteolytic enzymes (trypsin, chymotrypsin) of the host. Evidence of the release of pro-
tease inhibitors has up to now been obtained in several Nematode (Ascaris suum, Ascaridia
galli, Nippostrongylus brasiliensis) and tapeworm species (Ligula intestinalis, Proteocephala-
PAPPAS and READ, 1972a, b; REICHENBACH-KLINKE and REICHENBACH-KLINKE, 1970;
MATSKÁSI and JUHÁSZ, 1977).

Only a single attempt has been reported at the detection of protease inhibitors in
fluke extracts (Fasciola hepatica and F. gigantica), but this failed altogether (KLIMENKO and KENINA, 1971).

Studies on the proteolytic enzymes of the liver fluke, with special regard to neutral proteases, and investigations into its supposed protease inhibitor activities are reported in this paper.

Material and Methods

Adult specimens of liver fluke were collected from naturally infected cattle in the Budapest abattoir. The parasites were washed in three changes of sterile physiological saline, and were processed further as follows:

Table 1

<table>
<thead>
<tr>
<th>pH</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAEE-splitting activity U/g</td>
<td>0</td>
<td>0.2</td>
<td>0.42</td>
<td>0.05</td>
<td>0</td>
<td>0</td>
<td>0.25</td>
<td>0.05</td>
</tr>
</tbody>
</table>

I. Preparation of liver fluke extracts

The washed parasites were homogenized in fivefold volume of distilled water related to their weight. The homogenate was centrifuged at 19,000 g for 60 min. at +5°C. The supernatant was collected for enzyme assays.

Table 2

<table>
<thead>
<tr>
<th>pH</th>
<th>1.5</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTEE-splitting activity U/g</td>
<td>0</td>
<td>0</td>
<td>11.15</td>
<td>19.9</td>
<td>2.7</td>
<td>1.52</td>
<td>1.0</td>
<td>1.2</td>
<td>0.45</td>
</tr>
</tbody>
</table>

II. Incubation in maintenance medium

Group 1

Single washed parasites were incubated either in 1 ml sterile Tyrode solution (pH 7.5), containing 0.3 M TRIS-HCl buffer, for 4 hours at 37°C, or in TC 135 (Difco) medium, containing 2000 IU/ml penicillin, 1 µg/ml Streptomycin and 100 µg/ml Nystatin, for 24 hours.

Group 2

Washed flukes were incubated in Tyrode medium as above, except that their oral sucker was closed with Histoacryl (Braun, GFR) tissue cement. Sealing was performed under a SM XX binocular stereomicroscope, and was rechecked for tightness at the conclusion of the experiment.
Group 3
Bovine trypsin (BDH) or bovine chymotrypsin (REANAL) of known activity was added to flukes incubated in TC 135 medium. Medium samples similarly incubated, and containing the same level of enzyme, but no fluke, were used as control. All media were assayed for protease activity after 24 hours incubation.

Table 3
BTEE-splitting activity in 0.2M Na-phosphate and Na-phosphate-HCl buffer

<table>
<thead>
<tr>
<th>pH</th>
<th>3.5</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTEE-splitting activity U/g</td>
<td>13</td>
<td>25</td>
<td>2.4</td>
<td>0.5</td>
<td>0.8</td>
<td>1.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Group 4
Flukes were incubated in Tyrode medium, containing 0.3 M TRIS-HCl buffer and bovine proteases of known activity, on the following schedules:

a. 1 ml Tyrode + 0.1 ml, 0.1% trypsin + fluke
b. same as in "a", with oral sucker of fluke closed
c. 1 ml Tyrode + 0.1 ml, 0.1% chymotrypsin + fluke
d. 1 ml Tyrode + 0.1 ml, 0.1% chymotrypsin (control)
e. same as in "d", with oral sucker of fluke closed
f. 1 ml Tyrode + 0.1 ml, 0.1% chymotrypsin (control).

Table 4
Haemoglobin degrading activity of the liver fluke somatic extract
(Pepsin and cathepsin-like enzyme determination)

<table>
<thead>
<tr>
<th>Buffer</th>
<th>0.3N HCl (pH 1.5)</th>
<th>0.04M Na-citrate (pH 2.8) §</th>
<th>1.35M CH₃ COOH 0.02M (NH₄)₂SO₄ (pH 3.5) §</th>
<th>0.4M citric acid-0.3M NaOH (pH 5.0) §</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔE₂₈₀</td>
<td>0.28</td>
<td>0.52</td>
<td>0.42</td>
<td>0.50</td>
</tr>
<tr>
<td>+ 0.07M cysteine+HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔE₂₈₀</td>
<td>0.94</td>
<td>0.96</td>
<td>0.97</td>
<td></td>
</tr>
</tbody>
</table>


Incubation lasted 4 hours at 37°C in all experimental and control series.
Protease activity was determined on BAEE (N-α- benzoyl-L-arginine-ethylester) (SCHWERT and TAKENAKA, 1955) or BTEE (N-benzoyl-L- tyrosine-ethylester) substrate (HUMMEL, 1959), using a Unicam SP 1000 Ultraviolet spectrophotometer, with thermostated cuvettes and an AR-25 Linear Recorder. All assays were carried out at 30°C, after 5-min
preincubation, in a final volume of 3 ml. One unit of protease activity has been defined as the amount of enzyme causing an increase of absorbance at 253 and 256 nm of 1.000 per minute upon hydrolysis of BAEE or BTEE as substrate, respectively.

Inhibitor activity was assessed by measuring the residual enzyme activity of trypsin and of chymotrypsin in an assay medium of known enzyme activity after addition of test material. The inhibitor unit was defined as the amount of inhibitor required to depress the activity of trypsin or chymotrypsin so as to cause a reduction of BAEE and BTEE hydrolysis by 1.000 O.D. per minute.

Table 5
Effect of protease inhibitors on the BTEE-splitting activity of the liver fluke extracts (0.15M Tris-HCl buffer, containing 8.3mM CaCl₂; pH 7.8)

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>BTEE-splitting activity U/g</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>extract</td>
<td>extract+inhibitor</td>
</tr>
<tr>
<td>soybean trypsin inhibitor 0.001%</td>
<td>3.25</td>
<td>1.85</td>
</tr>
<tr>
<td>phenylmethyl sulphonyl fluoride 1mM</td>
<td>8.00</td>
<td>2.50</td>
</tr>
<tr>
<td>tosylphenyl-alanil-chloromethane 1mM</td>
<td>8.00</td>
<td>1.50</td>
</tr>
</tbody>
</table>

Acid protease activity was also determined on 2.5% haemoglobin substrate. The proteolytic unit was expressed as the increase of optical density over the substrate-free control at 280 nm (ΔE280) (MYCEx, 1970).

The variation coefficient was assessed as 20% for 20 routine measurements in each test system.

In the inhibitor-activity assays, the test system, of 3 ml final volume, contained 2.3 ml 0.15 M TRIS-HCl buffer (pH 7.8), containing 8.3 mM CaCl₂, 0.1 ml incubation medium, 0.1 ml trypsin or chymotrypsin solution of known activity, and 0.5 ml substrate. The activities of trypsin and chymotrypsin were measured after 5-min and 15-min preincubation of enzyme plus sample, respectively.

Results

I. Protease activity of fluke extracts

a) BAEE-splitting activity

The BAEE-splitting activity of extracts was 0.138 U/g in 0.15 M TRIS-HCl buffer (pH 7.8), containing 8.3 mM CaCl₂. The pH optima for BAEE-hydrolyzing enzyme activity are shown in Table 1.

b) BTEE-splitting activity

This proved to be 1.53 U/g under the above conditions. Tables 2 and 3 show the pH-optimum of BTEE-splitting activity in different buffer systems.
c) Haemoglobin splitting activity

The proteolytic activity of liver fluke extracts was also examined on 2.5% haemoglobin substrate, in different buffer systems generally employed for the assay of cathepsin-like and pepsin-like proteases, in acid medium, at pH optima characteristic of certain cathepsines (MYCEK, 1970; RYLE, 1970). All measurements were additionally carried out in a cystein-activated system. The results are shown in Table 4.

Table 6
Effect of inhibitors on the BAEE-splitting activity of the liver fluke extracts (0.15M Tris-HCl buffer, containing 8.3mM CaCl₂; pH 7.8)

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>BAEE-splitting activity U/g</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean trypsin inhibitor 0.001 %</td>
<td>0.150</td>
<td>0.00</td>
</tr>
<tr>
<td>Phenylmethyl-sulphonyl-fluoride 1 mM</td>
<td>0.150</td>
<td>0.062</td>
</tr>
<tr>
<td>Tosyl-L-lysyl-chloromethane 1 mM</td>
<td>0.150</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Table 7
A comparison between the protease activities measured in media of flukes with open and closed oral suckers (Student's "t"-test)

<table>
<thead>
<tr>
<th>BAEE-splitting activity (U/ml)</th>
<th>( \bar{x} )</th>
<th>S</th>
<th>n</th>
<th>P%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral sucker open</td>
<td>0.278</td>
<td>±0.258</td>
<td>7</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Oral sucker closed</td>
<td>0.050</td>
<td>±0.050</td>
<td>7</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>BTEE-splitting activity (U/ml)</td>
<td>( \bar{x} )</td>
<td>S</td>
<td>n</td>
<td>P%</td>
</tr>
<tr>
<td>Oral sucker open</td>
<td>1.071</td>
<td>±0.396</td>
<td>7</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Oral sucker closed</td>
<td>0.507</td>
<td>±0.117</td>
<td>7</td>
<td>&lt; 5</td>
</tr>
</tbody>
</table>
Table 8

Effect of culture media incubated with flukes on the bovine trypsin and chymotrypsin activity

<table>
<thead>
<tr>
<th>BAEE-splitting activity mU/ml</th>
<th>inhibition in per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>bovine trypsin (control)</td>
<td>bovine trypsin + sample</td>
</tr>
<tr>
<td>110</td>
<td>61</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BTEE-splitting activity mU/ml</th>
<th>inhibition in per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>bovine chymotrypsin (control)</td>
<td>bovine trypsin + sample</td>
</tr>
<tr>
<td>110</td>
<td>116</td>
</tr>
</tbody>
</table>

* The values of the proteolytic activity released in the maintenance medium by the flukes and determined in earlier experiments were subtracted from the total activity of the bovine enzyme containing preincubated medium.

Table 9

Effect of intact flukes on the protease activity of the bovine enzyme containing culture media

<table>
<thead>
<tr>
<th>BAEE-splitting activity mU/ml</th>
<th>inhibition in per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>medium</td>
<td>secreted by the worms*</td>
</tr>
<tr>
<td>without worms (control)</td>
<td>incubated with worms</td>
</tr>
<tr>
<td>132</td>
<td>50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BTEE-splitting activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>medium</td>
</tr>
<tr>
<td>without worms (control)</td>
</tr>
<tr>
<td>32</td>
</tr>
</tbody>
</table>

* The values of the proteolytic activity released by the flukes and determined in other experiments were subtracted from the total protease activity of the bovine enzyme containing incubation media.
d) Effect of protease inhibitors on the proteolytic activity of liver fluke extracts

This was examined to obtain more information on the nature of the liver fluke's own proteases (Tables 5 and 6).

II. Protease activity of the maintenance medium

In the first experimental series the flukes were incubated in Tyrode solution for 4 hrs at 37°C. After removal of the flukes the BAEE and BTEE splitting activities of the medium were assessed as 0.278 and 1.071 U/ml, respectively.

Table 10

<table>
<thead>
<tr>
<th></th>
<th>BAEE-splitting activity</th>
<th>BTEE-splitting activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \bar{x} ) S n</td>
<td>( \bar{x} ) S n</td>
</tr>
<tr>
<td>without worms (control)</td>
<td>0.750 ( \pm ) 0.224 7</td>
<td>2.970 ( \pm ) 0.298 7</td>
</tr>
<tr>
<td>oral sucker closed</td>
<td>0.535 ( \pm ) 0.089 7</td>
<td>2.228 ( \pm ) 0.335 7</td>
</tr>
<tr>
<td>oral sucker open</td>
<td>0.471 ( \pm ) 0.138 7</td>
<td>2.042 ( \pm ) 0.356 7</td>
</tr>
</tbody>
</table>

* The activity of bovine trypsin.
** The activity of bovine chymotrypsin.

In the second experiment the flukes were incubated under the same conditions as above, except that their oral sucker was sealed with Histoacryl tissue cement. At the end of incubation the maintenance medium showed BAEE and BTEE splitting activities of 0.050 and 0.507 U/ml, respectively. Both enzyme activities differed significantly between the two groups (Table 7).

III. Results of fluke-inhibitor studies

a) Inhibitors released into the maintenance medium

Samples of TC 135 incubation medium were examined for influence on bovine pancreatic trypsin and chymotrypsin. The results are shown in Table 8. The samples of medium depress the trypsin and chymotrypsin activity.

b) Inactivation of host proteases in maintenance medium

The test system employed in this series contained 2.4 ml 0.15 M Ca-containing TRIS-HCl buffer (pH 7.8), 0.1 ml incubation medium, 0.5 ml substrate. The results are shown in Table 9.

It was established earlier that the flukes did themselves release proteases into the maintenance medium. As the applied methods did not make possible the differentiation in the
same sample of the fluke-protease from added bovine protease activity, the latter was determined by subtraction of the fluke enzyme activity measured in enzyme-free system from the total (bovine protease + proteases released by the worm) activity. The difference of the residual activity so established from the control was regarded as the measure of inactivation by the parasite-released protease inhibitors.

c) Location of the sites of inhibitor release

The foregoing experimental observations have unequivocally indicated that the liver flukes are able to inactivate host trypsin or chymotrypsin. Persistence of the inactivating effect in the maintenance medium after removal of the flukes suggested that the parasites synthesize protease inhibitors, and release these into their environment. No information was, however, emerging from these studies on the site(s) of inhibitor release. In order to obtain more information flukes with sealed oral sucker (group 4) were exposed to host enzymes of known activity (Table 10) in maintenance medium as previously described.

The incubation media of the parasites showed lower BAEE- and BTEE-splitting activity than the parasite-free control systems, but both substrate-splitting activities were higher in the media of flukes with closed oral sucker than of not sealed flukes, indicating a lower inhibition by the former. The data of the supposed inhibition are shown in Table 11.

The sealing of the oral part did not account for a notable depression of inactivation.

Table 11

<table>
<thead>
<tr>
<th></th>
<th>Activity of bovine + released fluke enzym (see the Table 7) U/ml</th>
<th>Inhibition (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>oral sucker</td>
<td></td>
</tr>
<tr>
<td></td>
<td>open</td>
<td>closed</td>
</tr>
<tr>
<td>BAEE-splitting activity</td>
<td>1.028</td>
<td>0.800</td>
</tr>
<tr>
<td>BTEE-splitting activity</td>
<td>4.041</td>
<td>3.477</td>
</tr>
</tbody>
</table>

Discussion

Earlier studies on liver flukes were centered on the detection of acid - cathepsin-like - protease production by the parasite. We succeeded in demonstrating proteolytic activity in fluke extracts and maintenance media also at slightly alkaline pHs. On the basis of their substrate specificity the parasite enzyme(s) appeared to be trypsin- and chymotryptic-like in nature. Experiments with protease inhibitors also indicated the presence of two - trypsinic and chymotryptic - types of protease activity.

Liver fluke extracts displayed a measurable BAEE- and BTEE-splitting activity also at low pHs. The BTEE-splitting activity showed two peaks in Na-phosphate buffer, an optimal one at pH 4.0, and a considerably lower peak at pH 8.0. BAEE-splitting also showed two maxima at pHs 4 and 8, respectively, in the same buffer system. This has suggested that the somatic extract of liver flukes probably contains several BAEE- or BTEE-splitting enzymes.
Measurements on a natural substrate (haemoglobin) at an acid pH, under activation with cysteine, have substantiated the earlier implication that the liver fluke also possesses proteases functioning in acid conditions (pH 1.5, 2.8, 3.5, 5.0). This accords well with the pertinent findings of PENNOIT de COOMAN and van GREMBERGEN (1942), HALTON (1967) and LOCATELLI and BERETTA (1969). The experimental observation that addition of cysteine-HCl accounted for an about twofold activity increase of the proteases at pHs 2.8, 3.5 and 5.0 supported their cathepsin-like nature.

Our experiments on flukes with sealed oral sucker have substantiated the observation of others (THORSELL and BJÖRKMAN, 1965; LOCATELLI and BERETTA, 1969) that the flukes release the digestive enzymes through their oral opening. Extra-corporeal digestion seems to play a substantial role in the nutrition of the liver fluke. The released proteases account for lysis of the surrounding host tissue and for partial digestion of the blood before its complete breakdown in the digestive tract.

The production of protease inhibitors by parasites has long been known (von BRAND, 1973). It has been extensively studied in Nematodes, and recently it has been demonstrated also in Cestodes (MATSÁSI and JUHÁSZ, 1977). The present experiments have substantiated the presence of protease inhibitors in the liver fluke.

Their biological role was much disputed, (von BRAND, 1973) recently the original explanation that they protect the parasite from the digestive enzymes of the host has gained ground again (PAPPAS and READ, 1972, b; JUHÁSZ, 1979). Flukes incubated in maintenance medium inactivated added bovine trypsin and chymotrypsin.

The incubation medium did in itself depress the activity of host proteases after removal of the flukes. Sealing of the oral part prevented intake of maintenance medium, and strongly depressed the oral release of enzymes as well. The flukes with closed oral sucker nevertheless inactivated the added host proteases of similar degree to non-sealed ones. Heat-inactivated (80°C for 15 min) parasites and incubation media also were able to depress bovine protease activity. The experimental observations support the conclusion that inactivation of host proteases by the liver fluke is due primarily to protease inhibitors produced and released by the parasite. It appears that the oral opening of the liver fluke plays no notable role in inhibitor release.

References


Received: 20 January, 1979

HAJDÚ, É.
Dr. MATSKÁSI, I.
Dr. JUHÁSZ, S.
Veterinary Medical Research Institute, Hungarian Academy of Sciences
H-1143 Budapest, Ungária krt. 21.