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CHARACTERIZATION AND PARTIAL PURIFICATION OF A LEUCINE AMINOPEPTIDASE FROM *FASCIOLA HEPATICA*

Daniel Acosta, Fernando Goñi*, and Carlos Carmona†
Unidad de Biología Parasitaria, Instituto de Higiene, Av. A. Navarro 3051 CP11600, Montevideo, Uruguay

ABSTRACT: An aminopeptidase activity capable of hydrolyzing different aminomethylcoumarin amino acids, but mainly leucine-7-amino-4-methylcoumarin (Leu-NHMc), was detected in deoxycholic soluble extracts from adult *Fasciola hepatica*. The enzyme (EC 3.4.11.1) was partially purified by gel filtration and EAH-Sepharose affinity chromatography using bestatin as a ligand. Results obtained by gel filtration, direct fluorogenic substrate analysis in polyacrylamide gel, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis suggest that in a native form the enzyme might be aggregated as a high molecular weight complex. By affinity chromatography on concanavalin A–Sepharose, the enzyme did not bond to the column showing that it lacks mannose residues. The *F. hepatica* aminopeptidase was characterized as a metalloproteinase based on its activation by Mn^{2+} and Mg^{2+}, and its inhibition by EDTA, 1,10-phenanthroline, and bestatin. It has an optimal activity at a pH between 8.0 and 8.5. Histochemical localization revealed strong leucine naphthylamidase activity at the cells lining the gut epithelium of the parasite.

Fasciola hepatica, the common liver fluke, has a worldwide distribution and can infect a wide range of mammalian hosts, including man. Following ingestion the parasite burrows through the host gut wall and migrates to the liver where it causes extensive damage before moving into the bile duct. It has been proposed that the parasite should possess mechanisms by which it can actively penetrate the host tissue simultaneously avoiding immune attack.

Fasciola hepatica secretes a number of proteinases, the most prominent being cysteinyl endopeptidases (Dalton and Hefferman, 1989). One of these enzymes, the cathepsin L proteinase (CL 1), is secreted by all stages of the developing parasite and it is capable of cleaving host immunoglobulins (Carmona et al., 1993; Smith et al., 1993). More recently, a second cysteinyl endopeptidase (CL 2) has been isolated from the medium in which *F. hepatica* was cultured (Dowd et al., 1994). In vitro studies demostrated that both cathepsins have collagenolytic activity and are capable of degrading extracellular and basement membrane macromolecules (Berasain et al., 1997).

On the other hand, much less work has been devoted in looking for fluke exopeptidases. Recently, a dipeptidylpeptidase was isolated and characterized from *F. hepatica* excretion/secretion (E/S) products and its role is thought to be related to the digestive system of the parasite (Carmona et al., 1994).

In other related parasites, e.g., *Schistosoma mansoni,* a different group of exoproteinases, the aminopeptidases, has been described and shown to be membrane associated (Xu et al., 1990). Aminopeptidases can catalyze the hydrolysis of amino acid residues at the amino terminus of peptide substrates and it has been suggested that, among other functions, it has involvement in protein maturation, the terminal degradation of proteins, and also plays a key role in the regulatory process of cellular metabolism (Bachmair et al., 1986).

In order to investigate the presence of aminopeptidase activity in *F. hepatica,* we have studied the E/S products and the somatic and membrane extracts of adult flukes. Here, we report the presence of aminopeptidase activity in *F. hepatica* detergent-soluble extract. We have partially purified, characterized, and histochemically localized a leucine aminopeptidase from adult flukes.

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* Cátedra de Inmunología, Facultad de Quimica, Instituto de Higiene, Av. A. Navarro 3051 CP11600, Montevideo, Uruguay.
† To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

Deoxycholic acid (DOC), 7-amino-4-methylcoumarin (NHMec) coupled to different amino acids and peptides as described below, α-D-mannopyranoside, EDTA, o-1–10 phenanthroline, cysteine, bestatin, dithiothreitol (DTT), 2β-mercaptoethanol, iodoacetamide, pepstatin, aprotinin, leupeptin, sodium borohydride, diprotin B, and leucine-4-methoxy β-naphthylamide were obtained from Sigma (St. Louis, Missouri). The Bio-beads SM-2 column was from Bio-Rad (Richmond, California). Superdex 200, EAH-Sepharose 4B, carbodiimide, concanavalin A (Con A)–Sepharose 4B, and protein standards for electrophoresis were purchased from Pharmacia (Piscataway, New Jersey). The BCA protein assay kit was from Pierce (Rockford, Illinois).

Preparation of membrane extract

Adult *F. hepatica* were collected from cattle livers at a local abattoir. The flukes were immediately carried to the laboratory in bile at 35 C and washed 6 times in 0.01 M phosphate pH 7.2/0.15 M NaCl (PBS) at 4 C. The flukes were killed by freezing for 30 min at −20 C, washed twice with PBS at 4 C, and drained. One gram wet weight of tissue was incubated in 10 ml of 1% DOC in 0.15 M glycine, pH 9.0, 0.5 M NaCl, for 60 min at room temperature, 30 min at 37 C, and then 30 min at 4 C. The DOC-extracted material was centrifuged at 20,000 *g* for 60 min and the supernatant stored at −80 C until used.

Somatic extracts

Live adult flukes were washed several times with PBS and then homogenized with PBS in a glass tissue grinder. The homogenate was ultrasonicated on an ice bath for 3 min, in 60-sec bursts (20% power) with 30-sec pauses, using an Ultrasonic Homogenizer 4710 (Cole-Palmer Instrument Co., Chicago, Illinois), then centrifuged at 48,000 *g* for 1 hr, and the supernatant stored at −80 C until used. For the obtention of somatic extract after detergent extraction, the same procedure was applied to the parasite structures after DOC extraction.

Excretion/secretion products (E/S)

Live adult flukes were carried to the laboratory as above and E/S products were obtained as described by Dalton and Hefferman (1989). Briefly, the flukes were washed 6 times in PBS pH 7.3 and then maintained in RPMI-1640, pH 7.3, containing 2% glucose, 30 mM Hepes, and 25 mg/L gentamicin overnight. The culture medium was removed, centrifuged at 12,000 *g*, and stored at −20 C until used.

Bovine liver extract

Bovine liver extract from a cow raised in a *Fasciola*-free area was obtained using the same procedure as for *F. hepatica* somatic extract. Briefly, liver tissue from each lobule was mixed in PBS, homogenized, ultrasonicated, and centrifuged to obtain a protein concentration of 10 mg/ml.

Enzymatic assays with synthetic fluorogenic substrates

Aminopeptidase activity was measured fluorometrically using NHMec, coupled to different amino acids as substrates. Leu-NHMec, Ser-NHMec, Phe-NHMec, Arg-NHMec, Ala-NHMec, Pro-NHMec, Gly-NHMec, Glu-NHMec, and Tyr-NHMec were stored as 10 mM stock solutions in dimethylformamide or distilled deionized water prior to use. Assays were carried out using a final concentration of 20 μM substrate, 50 μl of each extract, and 0.1 M glycine, pH 8.0, to a final volume of 1 ml. The mixture was incubated for 60 min at 37 C before stopping the reaction by the addition of 200 μl of 1.7 M acetic acid. In each assay, a standard calibration curve of NHMec from 0.1 μM to 1.0 μM was run, made in distilled deionized and under the same experimental conditions. The amount of NHMec released was measured in a Sequoia-Turner M 450 fluorometer (Sequoia-Turner, Tokyo, Japan), using excitation and emission wavelengths set at 360 nm and 430 nm, respectively. One arbitrary unit of enzymatic activity was defined as the amount that catalyzes the release of 1 nmol of NHMec per min at 37 C.

Aminopeptidase purification

Detergent from the membrane extract was removed on a Bio-beads SM-2 column equilibrated in 0.01 M K_2HPO_4, pH 7.4. The membrane extract fraction with aminopeptidase activity was applied onto a calibrated Superdex 200 HR 16/50 column equilibrated with 0.05 M Tris buffer, pH 8.5. Samples were eluted with 0.05 M Tris buffer, pH 8.5 at a flow rate of 3.5 ml/min and collected in 1 ml fractions. Protein elution was monitored as absorbance at 280 nm using a flow-through spectrophotometer (Pharmacia, Uppsala, Sweden). Leucine aminopeptidase activity was measured using the substrate Leu-NHMec. Fractions containing leucine aminopeptidase activity were pooled and applied to a bestatin affinity column.

The bestatin affinity column (5 × 1 cm) was prepared according to Kurauchi et al. (1986) as follows. Five milliliters of EAH-Sepharose 4B were suspended in 12 ml of water adjusted to pH 4.5 with HCl, and 5 ml of a 1-mg/ml bestatin solution in acid water was added to the suspension. Next, 5 ml of a 10-mg/ml solution of carbodiimide dissolved in acid water was added to the resin/bestatin mixture. The reaction mix was gently rolled for 5 hr at room temperature, maintaining the pH at pH 5.0 by the addition of 0.1 M HCl. The resin was then allowed to settle, the supernatant was removed, and the resin was sequentially washed with 20 volumes each of deionized water; 0.1 M sodium acetate, 0.5 M NaCl, pH 4.0; 0.1 M Tris, 0.5 M NaCl, pH 8.0, and finally deionized water. Prior to application of the sample, the bestatin affinity column was equilibrated with 0.05 M Tris buffer, pH 8.5. Superdex 200 pooled fractions with aminopeptidase activity were filtered through a 0.45-μm nylon membrane filter (Millipore, Bedford, Massachusetts) and applied to the column. The column was then washed with 10 bead volumes of the equilibration buffer at a flow rate of 0.3 ml/min. Elution was carried out stepwise with 0.05 M Tris-HCl buffer, pH 8.5, made to 0.1 M, 0.25 M, 0.5 M, and 1.0 M NaCl. Active fractions were pooled, dialyzed against 0.1 M glycine, pH 8.0, containing 0.02% sodium azide, and stored at 4 C until used.

To determine the molecular size of the leucine aminopeptidase, the gel-filtration column was calibrated with the following standards: thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158; albumin, 67 kDa; ovoalbumin, 43 kDa, chymotrypsinogen A, 25 kDa; ribonuclease A, 13.7 kDa.

Concanavalin A chromatography

Fractions with aminopeptidase activity obtained from the Superdex 200 column were applied to a Con A–Sepharose 4B column (5 × 1 cm). The column and sample were equilibrated with 0.02 M Tris, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.4. The column was washed with 10 bead volumes of equilibration buffer. Elution was carried out with equlilibration buffer containing 0.5 M α-D-mannopyranoside. One-milliliter fractions were collected and aminopeptidase activity was determined as described.

TABLE I. Specific activity of different *Fasciola hepatica* extracts on aminopeptidase fluorogenic substrates.*

	Membrane extract	Somatic extract	Somatic extract after DOC treatment	E/S products	Bovine liver extract
Leu	569.6	1,903	929.6	0.0	449.6
Arg	251.8	471.3	378.2	0.0	162.7
Phe	66.0	138.8	92.4	0.0	240.8
Tyr	22.3	66.5	65.5	0.0	94.8
Ala	22.3	219.8	75.6	0.0	212.6
Pro	13.6	69.4	50.4	0.0	34.6
Glu	12.6	20.2	43.7	0.0	69.1
Ser	9.7	2.9	23.5	0.0	10.4
Gly	3.9	0.0	21.8	0.0	3.8

* Each substrate was tested at 20 μM concentration and 100 μg of protein were used for each assay. Assays were carried out in glycine 0.1 M, pH 8.0. Each value represents the average of triplicate samples incubated separately. Values are expressed in U/mg.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

To analyze samples at different purification steps, 12.5% SDS-PAGE was performed under nonreducing conditions as described by Laemmli and Favre (1973). After electrophoresis, the gels were stained with Coomassie brilliant blue R-250. Phosphorylase B (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and lactoalbumin (14 kDa) were used as molecular weight markers.

Direct fluorogenic substrate analysis in polyacrylamide gels

Direct fluorogenic substrate analysis in 8% polyacrylamide gels was performed as described by Robertson et al. (1990) with the exception that SDS was not included in the gel or the running buffer . The samples were electrophoresed at 4 C, constant current set at 25 mA, for 1 hr. The gel was incubated at 37 C for 10 min in 0.1 M glycine pH 8.0, containing 1 mM $MgCl_2$ and 20 μM Leu-NHMec. After incubation the gel was gently soaked in distilled deionized water. Fluorescent bands locating the substrate were visualized by the use of a Funakoshi NTM-10 UV transilluminator (Funakoshi, Tokio, Japan) and recorded with a Polaroid camera fitted with a Kodak Wratten gelatin filter (model 2E).

Protein concentration

Protein concentration was measured using the BCA protein assay kit in microtiter plates according to the method of Redinbaugh and Turley (1986). Bovine serum albumin was used as a protein standard.

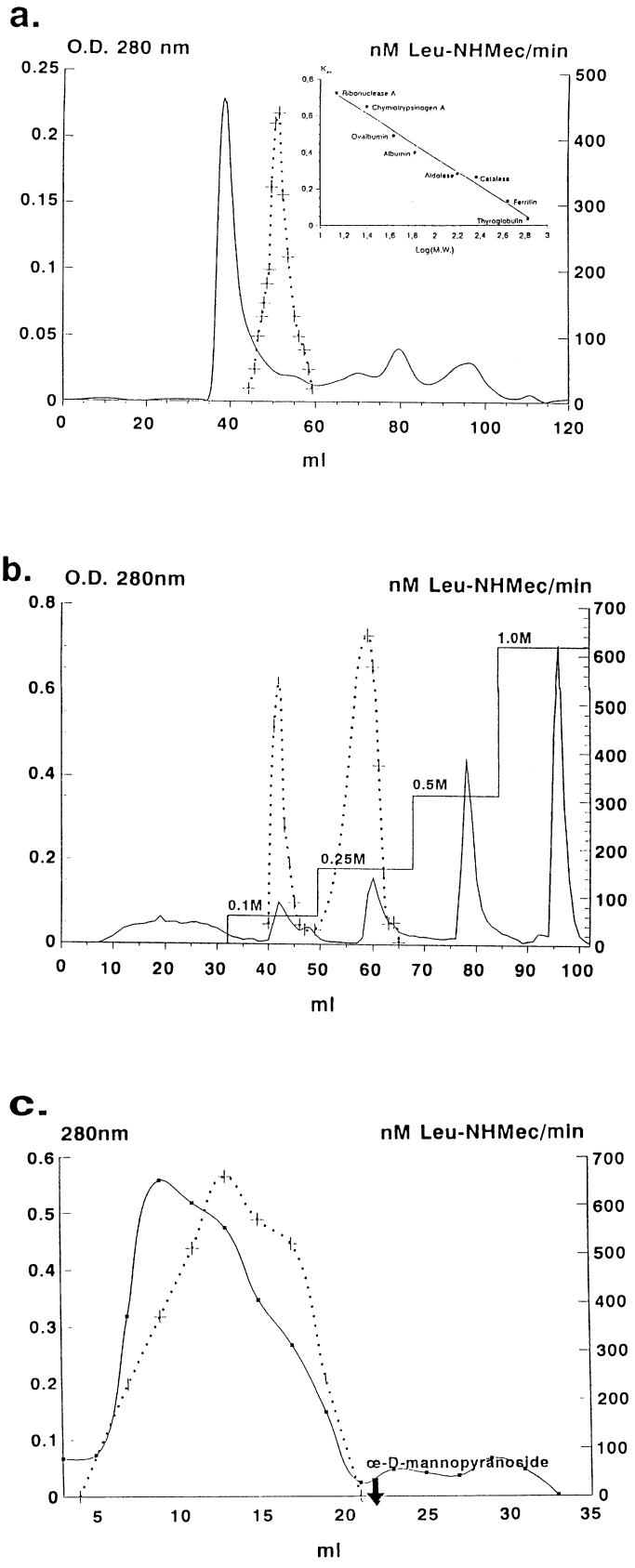
Aminopeptidase characterization

The optimum pH for enzymatic activity was determined by incubating the partially purified enzyme in 0.1 M glycine solutions brought to pH from 5.0 to 10.0.

The effect of metal ions on the enzymatic activity was assayed by incubating the partially purified enzyme in different concentrations of $MgCl_2$, $MnCl_2$, $ZnCl_2$, $ZnSO_4$, $PbCO_3$, $CaCl_2$, $Cd_2(SO_4)_3$, $HgCl_2$, $CoCl_2$, $CuSO_4$, and $FeSO_4$. A control in 0.1 M glycine, pH 8.0, containing no metal ions was measured simultaneously.

Inhibition assays

The partially purified enzyme was incubated for 1 hr in the presence of either: 10 mM EDTA; 1 mM *o*-1,10-phenanthroline; 10 mM cysteine; 50 μM, 10 μM and 1 μM bestatin; 5 mM DTT; 10 mM 2β-mercaptoethanol; 10 μM iodoacetamide; 1 mM pepstatin; 1–100 mM aprotinin;



5 μg/ml leupeptin, 10 mM sodium borohydride; or 10–100 μM diprotin B . Activity was measured using Leu-NHMec as substrate. Results are expressed as percentage of inhibition respect to a control without inhibitors.

Leucine aminopeptidase histochemistry

Leucine aminopeptidase activity was localized histochemically using the method described by Everson Pearse (1972). Adult *F. hepatica* recovered from abattoir were washed in 0.15 M NaCl and submerged in liquid nitrogen. Four-micrometer sections were cut at −20 C, then air dried, and incubated in leucine-4-methoxy β-naphthylamide. The chromogenic substrate (1 mM) was dissolved in 1 ml dimethylformamide to which 10 ml of 0.1 M acetate buffer pH 6.5, 0.15 M of NaCl, 1 ml of 20 mM KCN, and 10 mg of fast blue B salt were added sequentially. The sections were incubated at 37 C for 20 min, rinsed in 0.15 M NaCl for 3 min, immersed in 0.1 M cupric sulfate for 2 min, rinsed again, and preserved in glycerine gelatin. Control sections were incubated in a substrate-free medium and in complete medium with 10 mM EDTA as inhibitor for metal-dependent aminopeptidase activity.

RESULTS

Enzymatic assay with fluorogenic substrates

The comparative hydrolysis of NHMec-amino acids by *F. hepatica* membrane extract is shown in Table I. The highest specific activity corresponded to Leu-NHMec, but substantial activity was also found against Arg-NHMec, whereas the hydrolytic activity on Phe-NHMec was about 1/10 of that on Leu-NHMec and the rest of the NHMec-amino acids assayed was less than 5% of that on Leu-NHMec.

Leu-NHMec activity was found in membrane extract, somatic extract, and somatic extract after DOC treatment, whereas E/S products showed minimal activity only after a 10-fold concentration. On the other hand, the bovine liver extract showed

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FIGURE 1. Purification of liver fluke leucine aminopeptidase. **A.** Superdex 200 chromatography. The fraction with leucine aminopeptidase obtained from passage through a Bio-beads SM-2 column was concentrated and applied to a Superdex 200 gel-filtration column (1.6 × 50 cm). Elution was performed using a 0.05 M Tris, pH 8.5, mobile phase. Protein elution was monitored by absorbance at 280 nm (———). Leucine aminopeptidase activity in collected fractions was assayed using the fluorogenic substrate Leu-NHMec . All active samples were pooled (+). The inlet shows the calibration for the *F. hepatica* leucine aminopeptidase using the following molecular weight markers: thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; chymotrypsinogen A, 25 kDa and ribonuclease A, 13.7 kDa. **B.** Affinity chromatography. Pooled fractions with leucine aminopeptidase activity from Superdex 200 (25.8 mg) were applied to an EAH-Sepharose 4B affinity column using bestatin as ligand. Column and sample were equilibrated in 0.05 M Tris-HCl pH 8.5. The column was extensively washed with the same buffer at a flow rate of 0.3 ml/min and aminopeptidase then eluted with a nonlinear NaCl gradient. Protein eluting from the column was monitored by absorbance at 280 nm (———). Leucine aminopeptidase activity was detected with the substrate Leu-NHMec (+). All active samples eluted at 0.1 M and 0.25 M NaCl were pooled to obtain the partially purified leucine aminopeptidase. **C.** Concanavalin A affinity column. Leucine aminopeptidase pooled fractions obtained by gel filtration were applied to a Con A affinity column (5 × 1 cm) equilibrated in 0.02 M Tris, 0.5 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$, pH 7.4. Elution was carried out using 0.5 M α-D-mannopyranoside. Each fraction was assayed for leucine aminopeptidase activity using Leu-NHMec as substrate (+). Protein content was determined by absorbance at 280 nm (■). The arrow indicates addition of α-D-mannopyranoside.

TABLE II. Purification of *Fasciola hepatica* leucine aminopeptidase.*

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Membrane extract	79.8	44,448	557	1	100
Superdex 200	25.8	23,514	911	1.6	52.9
Bestatin affinity column	2.31	9,876	4,272	7.6	22.2

* Enzyme activity was measured using the substrate Leu-NHMec. The starting material corresponded to approximately 200 adult flukes.

substantial aminopeptidase activity for all the amino acids-NHMec assayed except Pro, Gly, and Ser-NHMec.

Purification of membrane-associated aminopeptidase

The membrane-associated aminopeptidase from adult *F. hepatica* was extracted with 1% of DOC and partially purified by gel-filtration and affinity chromatography.

The proteinase eluted from the gel-filtration column in a narrow peak with an apparent molecular weight of about 240 kDa (Fig. 1a). At pH 8.5, the enzyme bound to the inhibitor bestatin coupled to EAH Sepharose and was subsequently eluted in 2 steps at 0.1 M and 0.25 M NaCl (Fig. 1b). Aminopeptidase pooled fractions obtained by gel filtration did not bind to Con A (Fig. 1c).

The specific activity of the partially purified enzyme was 7.6-fold greater than the crude extract with a recovery of 22.2% of the total activity present in the original membrane extract. The proteinase isolated in this study had a final specific activity of 4,272 units/mg (Table II).

Determination of the optimum pH

The assay performed in various 0.1 M glycine buffers showed that maximum activity was obtained at pH between 8.0 and 8.5 (Fig. 2).

SDS-PAGE

Analysis of the partially purified fractions on nonreducing SDS-PAGE stained with Coomassie blue revealed protein bands in the range of 67 kDa to 20.1 kDa molecular weight. After bestatin affinity chromatography, there was an important enrichment of a band with an apparent molecular weight of 65 kDa and the concomitant present of 2 minor faster migrating bands (Fig. 3A).

Direct fluorogenic substrate analysis in polyacrylamide gels

The membrane extract, Superdex, and bestatin affinity chromatography active fractions were compared electrophoretically in native 8% polyacrylamide gels. Leucine aminopeptidase in membrane extract migrates as a single band, whereas Superdex and bestatin affinity chromatography samples showed the enrichment of this activity and the appearance of 2 minor bands (Fig. 3B).

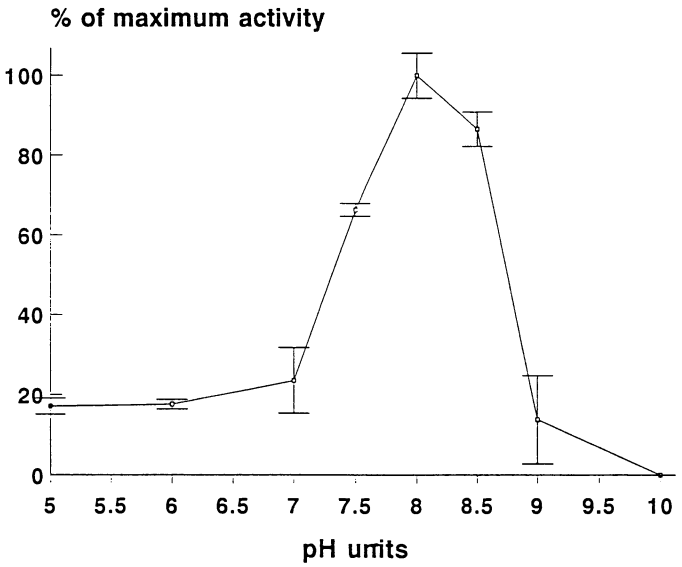

FIGURE 2. Optimum pH of *Fasciola hepatica* leucine aminopeptidase. Assays were carried out at 37 C for 1 hr. Each point represents the mean and SD of triplicate samples incubated separately.

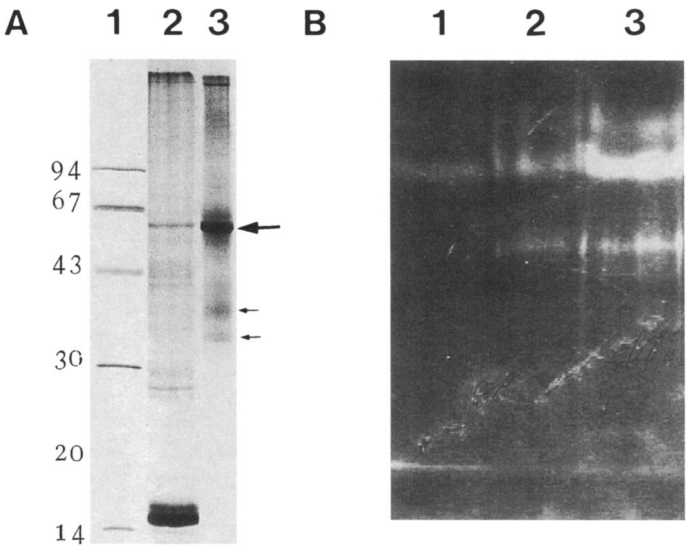

FIGURE 3. **A**. SDS-PAGE analysis of the purification steps of *Fasciola hepatica* leucine aminopeptidase. Thirty micrograms of protein was loaded in each lane. The gels were run and stained with Coomassie brilliant blue R-250 as described in the Materials and methods. The figure shows molecular weight markers (lane 1), membrane extract (lane 2), and bestatin affinity chromatography pooled fractions (lane 3) analyzed by 12.5% SDS-PAGE under nonreducing conditions. Arrows point to the enhanced 65-kDa band (big arrow) and the other 2 minor faster migrating bands (small arrows). **B.** Direct fluorogenic substrate analysis in polyacrylamide gels. Samples (50 μg) were electrophoretically separated under nondenaturing conditions by 8% polyacrylamide gel electrophoresis (without SDS) at 4 C during 1 hr. Following electrophoresis, the gel was incubated at 37 C with 0.1 M glycine, pH 8.0, containing 1 mM $MgCl_2$ and 20 μM Leu-NHMec. After 10 min the gel was removed, soaked gently in distilled deionized water, transilluminated, and photographed. Lane 1: membrane extract; lane 2: Superdex; lane 3: bestatin affinity chromatography.

TABLE III. Inhibition studies on *Fasciola hepatica* partially purified leucine aminopeptidase.*

Inhibitor	Concentration	Activity (U)	Remaining activity (%)
EDTA	10 mM	29.2 ± 1.1	4
O-phenanthroline	1 mM	38.6 ± 2.6	5.3
Bestatin	50 μM	53.2 ± 5.9	7.3
	10 μM	55.4 ± 3.9	7.6
	1 μM	89.7 ± 4.2	12.3
Cysteine	10 μM	53.2 ± 4.6	7.3
DTT	5 mM	60.2 ± 3.7	8.3
2-Mercaptoethanol	10 mM	104.3 ± 9.5	14.3
Iodoacetamide	10 μM	87.3 ± 8.0	12
E-64	1 μM	120.2 ± 14.1	16.5
Sodium borohydride	50 mM	343.1 ± 42.6	47
Pepstatin	1 mM	649.7 ± 39.0	89
Aprotinin	100 mM	703.7 ± 48.3	96.4
Leupeptin	5 μg/ml	824.9 ± 67.7	113
Diprotinin B	100 μM	893.0 ± 79.3	122.4
Control	—	730.0 ± 55.4	100

* Assays were carried out in 0.1 M glycine, pH 8.0. Activity was measured using Leu-NHMec as substrate. Results are expressed as total activity and percentage of remaining activity with respect to a control without inhibitor. Each value represents the average of triplicate assays. Two hundred micrograms of protein were used for each assay.

Inhibition assays

The effect of potential inhibitors on the enzymatic activity is presented in Table III. EDTA and *o*-1,10-phenanthroline, both chelating agents, decreased aminopeptidase activity by 96% and 94.7%, respectively. Bestatin, which specifically binds to metalloproteases of the leucine aminopeptidase type, inhibited 87.7% of the enzymatic activity at 1 μM, 92.4% at 10 μM, and 92.7% at 50 μM.

The thiol compounds, cysteine, DTT, and 2-mercaptoethanol inhibited enzymatic activity by 92.7%, 91.75%, and 85.7%, respectively. The cysteine protease inhibitors iodoacetamide and E-64 produced more than 83% inhibition and sodium borohydride reduced the activity to 53%.

No inhibition was observed when the aspartic protease inhibitor pepstatin, the serine protease inhibitors aprotinin, and leupeptin and the specific dipeptidyl aminopeptidase IV inhibitor diprotinin B were used.

Effect of metal ions on the aminopeptidase activity

The effect of different divalent metal ions on enzymatic activity is presented in Table IV. The basal aminopeptidase activity was progressively activated by $MnCl_2$ and $MgCl_2$ by more than 300%. The same increase of activity was observed with $CoCl_2$ in the range of 10 μM to 100 μM, whereas $ZnCl_2$ and $CaCl_2$ increased only moderately the activity in the range of 1 μM to 100 μM.

Higher concentrations of $MgCl_2$ did not inhibit the enzyme activity; $MnCl_2$ in the same range of concentrations was also stimulatory, whereas $ZnCl_2$, $CoCl_2$, and $CaCl_2$ inhibited the enzyme. The other metal ions assayed produced a decrease of the enzymatic activity in various degrees (data not shown).

Leucine aminopeptidase histochemical localization

Cryostat sections of *F. hepatica* incubated with leucine-4-methoxy β-naphthylamide revealed strong activity in the cells lining the gut epithelium (Fig. 4a). This intense and homogeneous orange reaction contrasted the positive but light staining observed in cells just under the basement membrane over the entire dorsal body surface (Fig. 4b). Leucine naphthylamidase activity was not seen in any other tegumentary structure; however, in vitelline glands, a nonspecific intense brown staining precluded the visualization of any possible aminopeptidase activity.

DISCUSSION

In the present study, we report the characterization, partial purification, and histochemical localization of a leucine aminopeptidase (EC 3.4.11.1) from adult *F. hepatica* flukes. Maximum activity was obtained at alkaline pH. The pattern of NHMec-amino acid hydrolysis obtained on membrane extract, somatic extract, and somatic extract after DOC treatment was very similar because only Leu-NHMec, Arg-NHMec, and Phe-NHMec of the 9 NHMec-amino acids were significantly cleaved. In contrast, the bovine liver extract showed a distinct and broader spectrum of substrate specificity. This result indicates that the aminopeptidase is of parasite origin.

By gel filtration, the enzyme was eluted at an apparent molecular weight of 240 kDa; however, the band pattern observed on SDS-PAGE suggests that the enzyme might be composed of a high molecular weight complex, a common feature among aminopeptidases (Taylor, 1993). The bestatin affinity chroma-

TABLE IV. Effects of metal ions on *Fasciola hepatica* partially purified leucine aminopeptidase activity.*

Metal	Concentration				
	1 μM	10 μM	100 μM	1 mM	10 mM
$MnCl_2$	1,626 ± 119	2,184 ± 175	3,112 ± 193	3,368 ± 186	2,123 ± 179
$MgCl_2$	818 ± 36	1,337 ± 101	1,929 ± 78	2,330 ± 220	2,867 ± 242
$CoCl_2$	1,237 ± 90	2,831 ± 212	2,612 ± 184	1,478 ± 87	211 ± 17
$ZnCl_2$	869 ± 48	1,319 ± 144	895 ± 53	650 ± 58	435 ± 28
$CaCl_2$	796 ± 51	1,125 ± 67	630 ± 31	630 ± 43	511 ± 55
Control	671 ± 30				

* A control in 0.1 M glycine, pH 8.0, containing no metal ions was measured simultaneously. Each value represent the mean ± SD of triplicate assays. Two hundred micrograms of protein were used for each assay.

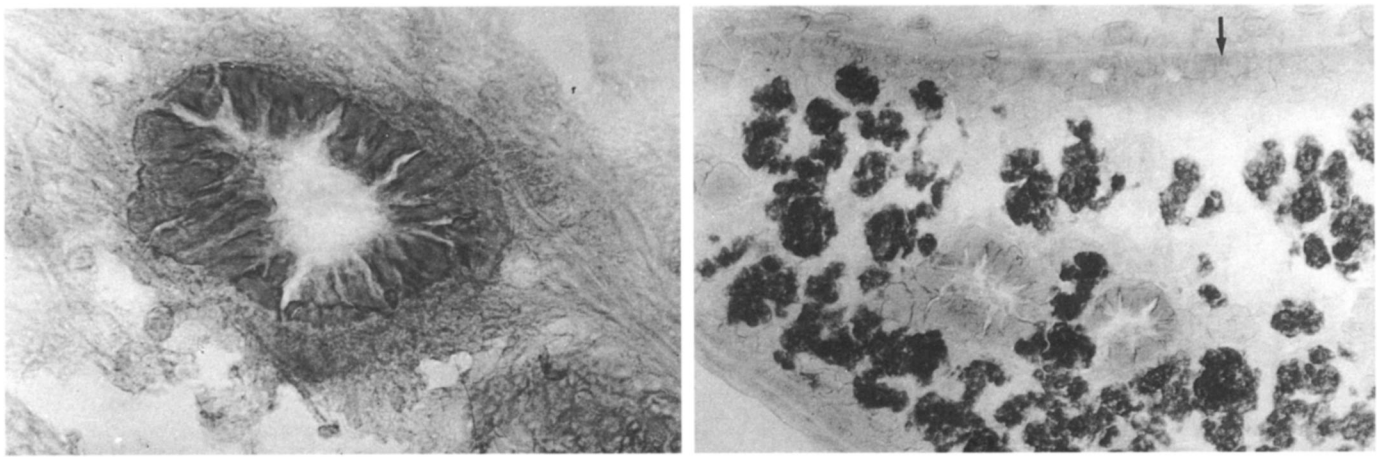

FIGURE 4. Histochemical localization of leucine aminopeptidase in adult flukes. Transversal 4-μm cross sections were incubated in leucine-4-methoxy β-naphthylamide as chromogenic substrate. **A.** The intense leucine naphthylamidase activity at the fluke gut epithelium (×250). **B.** Leucine naphthylamidase activity at the gut ephitelium. An arrow points to the light staining observed under the basement membrane. The intense staining seen in the vitelline glands precluded the visualization of any possible aminopeptidase activity (×100).

tography was an effective purification step because it separated the leucine aminopeptidase from most other detergent-solubilized proteins and increased the specific activity 7.6-fold. In addition, when partially purified samples were run on 8% native polyacrylamide gels, the enrichment of a major band of leucine aminopeptidase activity present in the original membrane extract was observed close to the other 2 minor bands. That could be due to the presence of different isoforms of the enzyme. The result obtained by affinity chromatography on Con A indicated that the enzyme lacks mannose residues. This finding contrasts with the results obtained with the membrane-associated leucine aminopeptidase purified from *S. mansoni* (Xu et al., 1990). In this sense, no other assay was performed to assess the presence of other carbohydrates with the molecule.

Aminopeptidases are a family of zinc-dependent enzymes that have 1 or 2 zinc ions per active site and are implicated in different biological processes (Chevrie et al., 1996). Because little is known about aminopeptidase catalytic mechanisms, classification generally has been based on the rate of cleavage of analog peptides and metal ion content.

The bovine lens leucine aminopeptidase was extensively studied and shown to be a homohexameric metalloenzyme containing 2 metal coordination sites in each subunit, usually occupied by Zn^{2+}. Whereas 1 Zn^{2+} ion is tightly bound and essential for the enzymatic activity, the second can be displaced by other metal ions. When in excess, Zn^{2+} can occupy both sites, thereby displacing other metal ions important for the catalytic reaction (Burley et al., 1992). Because Mn^{2+} and Mg^{2+} were the only metals that substantially enhanced hydrolysis of *F. hepatica* leucine aminopeptidase in all the ranges assayed, those ions could be good candidates for displacing the Zn^{2+} on the second coordination site as to obtain maximum activity.

Thus, the leucine aminopeptidase from *F. hepatica* is a metalloenzyme with properties similar to those from *Solanum tuberosum* L. and *Arabidopsis thaliana,* which also show enhanced activity with Mn^{2+} and Mg^{2+} and are inhibited by EDTA, *o*-1,10-phenanthroline, and bestatin (Bartling and Weiler, 1992; Herbers et al., 1994). The reduced activity obtained after incubation with E-64, cysteine, DTT, 2-mercaptoethanol, and iodoacetamide suggests that a cysteine residue or a free sulfhydryl group should be relevant to the active site.

Macromolecules, such as hemoglobin, are essential nutrients for trematodes. However, they must be enzymatically digested within the lumen of the intestinal tract to prepare them for absorption. Leucine aminopeptidase activity was found in adult *S. mansoni* crude extract (Cesari et al., 1983) and membrane extract in parasite eggs (Xu and Dresden, 1986). However, histochemical studies failed to show membrane-associated leucine aminopeptidase in the digestive tract of adult worms (Bogitsh and Dresden, 1983); thus, the final degradation of hemoglobin to diffusible peptides or amino acids in these organisms remains unclear (Dalton et al., 1995).

We have previously showed that cysteine endoproteinases of *F. hepatica* are able to degrade extracelullar matrix and basal membrane components to discrete polypeptides (Berasain et al., 1997). Furthermore, a dipeptidylpeptidase was detected in the E/S products of all developmental stages of the liver fluke and was shown to produce dipeptides that could be absorbed through the parasite digestive tract (Carmona et al., 1994).

In *F. hepatica,* the leucine aminopeptidase activity was located histochemically at the parasite gastrodermis whereas no aminopeptidase activity was found in E/S products. Thus, small polypeptides and dipeptides produced by both cathepsin Ls and the dipeptidylpeptidase could diffuse through the gastrodermis for the final breakdown to amino acids by aminopeptidase.

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