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A Cysteine Proteinase from the Liver Fluke *Fasciola* spp.: Purification, Characterization, Localization and Application to Immunodiagnosis

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Abstract

A proteolytic enzyme has been purified 576-fold from the homogenate of adult *Fasciola* spp. by ammonium sulfate fractionation, Sephadex G-75 gel filtration, and activated thiol-Sepharose covalent chromatography. The purified enzyme had a molecular weight of 27000, and consisted of about 20% acidic, 10% basic, and 40% hydrophobic amino acids. Ten micromolars of leupeptin, antipain, chymostatin, and *p*-tosyl-L-lysine chloromethyl ketone (TLCK) inhibited the globinolytic activity by 90, 86, 84, and 87%, respectively, while 10 mM dithiothreitol enhanced the activity by 260% of the control. Pepstatin, phenylmethylsulfonyl fluoride, and ethylenediaminetetraacetic acid had no effect. These results indicate that the *Fasciola* enzyme is categorized as a cysteine proteinase. Monoclonal antibodies specific for the *Fasciola* proteinase were prepared in this study and utilized for immunohistochemistry of the enzyme. The *Fasciola* enzyme was found to be localized in the intestinal epithelial cells of the adult parasite, suggesting that it is secreted from the epithelial cells into the intestinal lumen to act as a digestive proteolytic enzyme. The purified *Fasciola* proteinase was verified to be valuable as a species-specific, sensitive antigen for enzyme-linked immunosorbent assay in human fascioliasis.

Key words: *Fasciola,* cysteine proteinase, monoclonal antibodies, localization, immunodiagnosis, fascioliasis

Introduction

The liver fluke *Fasciola hepatica* dwells in the bile ducts of host animals and thus depends on the nutrients present in the parasitic habitat in the hosts. There is, however, much argumentation about the feeding process of the parasite. Dawes (1963) reported that the fluke fed on bile and bile duct epithelia, while recent studies by Sukhdeo *et al.* (1988) showed that adult *F. hepatica* fed on hemorrhagic ulcers in the hyperplastic mucosa of the host bile ducts.

Some reports suggest that *F. hepatica* is hematophagic (Jennings *et al.*, 1956; Symons and Boray, 1968). Immediately after the parasite ingests the host blood, red cells are rapidly disrupted in the intestine of the parasite (Halton, 1967). In addition, the black pigmented contents in the cecum of the parasite have been chemically identified to be the degradation products of hemoglobin (Todd and Ross, 1966). Therefore, it is conceivable that the host hemoglobin, the major component of red blood cells, may be an important protein nutrient for this liver fluke.

Little is known about the catabolic process of host hemoglobin in *F. hepatica*. Concerning proteolytic enzymes which may initiate the degradation of hemoglobin and is designated as "hemoglobinase" in *Schistosoma mansoni* (Dresden and Deelder, 1977), Rupova and Keilová (1979) reported the presence in *F. hepatica* of an enzyme hydrolyzing bovine hemoglobin at an acidic pH and belonging to the class of aspartic acid proteinases. Simpkin *et al.* (1980) found a low molecular weight proteinase (12 kilo dalton,

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kDa), and Coles and Rubano (1988) showed a "hemoglobinase" with a molecular weight of 27 kDa in the gut exudates of *F. hepatica*. However, the present knowledge of the *Fasciola* proteinase is far from a full understanding of its biochemical and antigenic properties.

We report here the purification, biochemical characterization, and tissue localization of a *Fasciola* proteolytic enzyme and the usefulness of the enzyme as an antigen for immunodiagnosis in human fascioliasis. Monoclonal antibodies against the fluke proteinase were produced and utilized to determine immunohistochemically the localization of the enzyme in the parasite tissues.

Materials and Methods

Parasites: Adult worms of *Fasciola* spp. were collected from cattle livers at a slaughterhouse in Tokyo. The flukes were thoroughly washed with physiological saline solution, lyophilized, and stored at −20°C. Since the Japanese *Fasciola* species have not been completely described (Oshima, 1989), the parasite is designated as *Fasciola* spp.

Human sera: Sera from patients with schistosomiasis japonica were obtained in Leyte Is., the Philippines in 1975, and those with schistosomiasis mansoni were collected in Recife, Brazil in 1979. Human sera of fascioliasis were obtained from patients in the districts of Okayama, Hiroshima, and Hyogo, Japan from 1986 to 1988. Other human sera positive for other parasite infections were from our university hospitals. Normal sera were from the Central Clinical Laboratory, Juntendo University Hospital.

Enzyme assay: The *Fasciola* proteolytic activity was measured with [^{3}H]-labeled globin as a substrate, as described previously (Yamasaki *et al.*, 1987). The enzyme was incubated with the radioactive substrate (2500 dpm/300 μg globin) at 37°C for 20 min in the reaction mixture (pH 4.5, 200 μl) containing 1 mM ethylenediamine-tetraacetic acid (EDTA) and 10 mM dithiothreitol (DTT). The reaction was terminated by addition of dioxane (800 μl), and the radioactivity remaining in the supernatant was determined in a liquid scintillation spectrophotometer.

Enzyme purification: All steps of the purification procedure were carried out at 0–4°C unless otherwise stated. Lyophilized worms (40 g) were suspended in 50 mM sodium acetate buffer (pH 3.9, 70 ml) containing 0.15 M NaCl, 1 mM EDTA, and 10 mM DTT (Buffer A), minced with scissors, and homogenized in a Potter-type Teflon homogenizer. The homogenate was sonicated at 20 KHz and at 150 Watts for 15 sec 4 times in a sonicator (Tomy Seiko model UP-200P, Tokyo). After centrifugation of the homogenate at $16000 \times g$ for 30 min, the precipitate was homogenized in Buffer A (60 ml), and again centrifuged.

To the combined supernatant fraction (130 ml) was added solid ammonium sulfate to give 50% saturation and the mixture was stirred for 1 hr, followed by centrifugation at $16000 \times g$ for 15 min. The resulting pellet was dissolved in 50 mM sodium acetate buffer (pH 3.9, 26.5 ml) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (Buffer B), and dialyzed against Buffer B overnight. The precipitated materials were removed by centrifugation at $29000 \times g$ for 30 min.

A portion (8 ml) of this sample was applied on a column (3×56 cm) of Sephadex G-75 which had been previously equilibrated with Buffer B. Enzyme elution was done with Buffer B at a flow rate of 16.0 ml/hr and fractions of 3.6 ml were collected. The fractions containing globinolytic activities were pooled (126 ml) and concentrated by ultrafiltration on an Amicon PM-10 membrane filter. The concentrated enzyme solution (17.5 ml) was dialyzed against 0.1 M sodium acetate buffer, pH 5.0, containing 0.3 M NaCl and 1 mM EDTA (Buffer C).

The enzyme solution (1.5 ml) from the preceding step was mixed with 0.5 M DTT (30 μl) and incubated for 30 min at room temperature. The mixture was passed through a column (1.6×5.6 cm) of Sephadex G-25 equilibrated with Buffer C. Another batch of the enzyme (1.5 ml) was run parallel with this procedure of enzyme activation, and the enzyme from 2 columns was pooled (6.7 ml). The pooled sample was then loaded onto a column (2×7 cm) of activated thiol-Sepharose 4B (Pharmacia Fine

Chemicals) equilibrated previously with Buffer C. After a sufficient wash of the column with Buffer C, the *Fasciola* proteolytic enzyme was eluted with 5 mM L-cysteine in Buffer C. The effluent fractions were monitored at 280 nm and at 343 nm for 2-thiopyridone released from the column. Since the compound yielded a significant absorbance at 280 nm, net absorbance at 280 nm for protein was calculated on the basis of extinction coefficient of 2-thiopyridone, $\varepsilon_{343} = 8.08 \times 10^3 M^{-1} \cdot cm^{-1}$, and $\varepsilon_{280} = 7.70 \times 10^3 M^{-1} \cdot cm^{-1}$ (Stuchbury *et al.*, 1975).

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE): The purified *Fasciola* proteinase was estimated for purity and molecular weight by SDS-PAGE, essentially as described by Laemmli (1970).

Amino acid analysis: As described by Simpson *et al.* (1976), the purified *Fasciola* proteinase was hydrolyzed under the reduced pressure at 115°C for 22 hr in 4 N methanesulfonic acid (1.0 ml) containing 0.2% 3-(2-aminoethyl)indole. The hydrolysate was neutralized with 3.5 N NaOH (1.0 ml) and analyzed by a Hitachi model 835 amino acid analyzer.

Protein determination: Protein concentrations were determined by the method of Hartree (1972) using bovine serum albumin as standard.

Preparation of monoclonal antibodies specific for the Fasciola *proteinase*: Partially purified *Fasciola* proteinase (Sephadex G-75 fraction dialyzed against 0.85% NaCl, 100 μg) was mixed with an equal volume (0.25 ml) of Freund's complete adjuvant and injected subcutaneously into the female, 6-week-old BALB/c mouse. Two weeks after this immunization procedure, an intraperitoneal booster injection of the enzyme (100 μg) without adjuvant was conducted to the mouse. One week later, a second booster was given. Mouse spleen as the primed lymphocyte donor was taken 4 days after the last booster injection, and the cell suspension was prepared for fusion with the myeloma cell line P3-X63-Ag8-653 in 50% polyethyleneglycol (MW 1500, BDH). Cell fusion, HAT (hypoxanthine/aminopterin/thymidine) selection, and cloning of hybridomas were carried out as described by Oi and Herzenberg (1980).

Enzyme-linked immunosorbent assay (ELISA): The method of ELISA described by Matsuda *et al.* (1984) was applied to screen hybridomas secreting anti-*Fasciola* proteinase monoclonal antibodies. Partially purified enzyme (Sephadex G-75 fraction) dialyzed against 50 mM carbonate buffer, pH 9.6, was used as antigen to coat the 96-well microplates (Coster Co.) at the protein concentration of 5 μg/ml, and peroxidase-conjugated anti-mouse IgG (Miles Yeda Ltd.) was used as the secondary antibody. In the ELISA of sera from patients with parasitic diseases, peroxidase-labeled anti-human IgG (Miles Yeda Ltd.) was used.

Immunoblotting: SDS-PAGE of the *Fasciola* proteinase was done on a 12.5% gel containing 0.2% SDS, and then transblotted to nitrocellulose membrane at 80 V for 2 hr. The membrane was incubated with 3% bovine serum albumin solution at 4°C overnight, and subsequently with monoclonal antibodies. After the excess antibodies were washed off, the immune complexes of the enzyme and monoclonal antibodies on the membrane were visualized by incubating the membrane with the peroxidase-conjugated anti-mouse IgG; 3,3-diaminobenzidine (0.3 mg/ml) was used for the peroxidase color development.

Indirect fluorescent antibody technique: To examine the localization of the *Fasciola* proteinase in the parasite tissues, the indirect fluorescent antibody technique was employed. Fresh adult *Fasciola* worms were embedded in Tissue-TGK II (Miles Inc.) and immediately frozen in liquid nitrogen. Sections of 2-μm thickness were prepared in a cryostat, and incubated with monoclonal antibodies (10 μl/section) at 25°C for 1 hr. Then, the sections were washed 3 times with phosphate buffered saline (PBS) and incubated with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (Sevac) for 40 min at 25°C. After washing with PBS, the sections were mounted in glycerol and examined under a Zeiss fluorescence microscope.

Results

Purification of the Fasciola *proteinase.* The results of purification of the enzyme are sum-

Table 1. Purification of the *Fasciola* proteinase

Fraction	Total activity ($\times 10^{-4}$ dpm)	Protein (mg)	Specific activity ($\times 10^{-4}$ dpm/mg)	Purification (-fold)	Yield (%)
Homogenate	28763	21450	1.3	—	100
Supernatant	27182	8818	3.1	2.4	94.5
50% $(NH_4)_2SO_4$	15443	762	20.3	15.6	53.7
Sephadex G-75	11582	96	120.6	92.8	40.3
Thiol-Sepharose 4B	8240	11	749.1	576.2	28.6

The starting material was 40 g of the lyophilized parasites. See Materials and Methods for details of the purification procedure. Enzyme activities were measured with [^{3}H]-globin as substrate under the standard assay conditions.

marized in Table 1. Most of the globinolytic activity present in the homogenate was recovered in the supernatant fraction. Ammonium sulfate fractionation yielded the increase in the specific activity from 3.1×10^4 to 20.3×10^4 dpm/mg protein. The enzyme was further purified by Sephadex G-75 gel filtration, with 6-fold increased specific activity.

Figure 1 shows a typical elution profile of the enzyme on a column of activated thiol-Sepharose 4B. The activity demonstrated in the pass-through fraction is probably due to the enzyme, cysteine residue(s) of which might be oxidized before application to the column, since the column capacity was so high as to covalently bind the loaded enzyme. Addition of 5 mM L-cysteine to Buffer C entailed the elution of the greater part of globinolytic activities (Fig. 1). Consequently, the enzyme was purified 576-fold, with the highest specific activity of 749.1×10^4 dpm/mg protein (Table 1).

Physicochemical properties of the Fasciola *proteinase.* The purified enzyme migrated as a single band upon SDS-PAGE, with a molecular weight of 27 kDa (Fig. 2). Table 2 shows the amino acid composition of the purified enzyme. The enzyme consisted of about 20% acidic, 10% basic, and 40% hydrophobic amino acids; relatively higher contents of aspartate and glutamate may confer acidic nature on the enzyme though Asx and Glx include asparagine and glutamine, respectively. Of the 6 cysteine

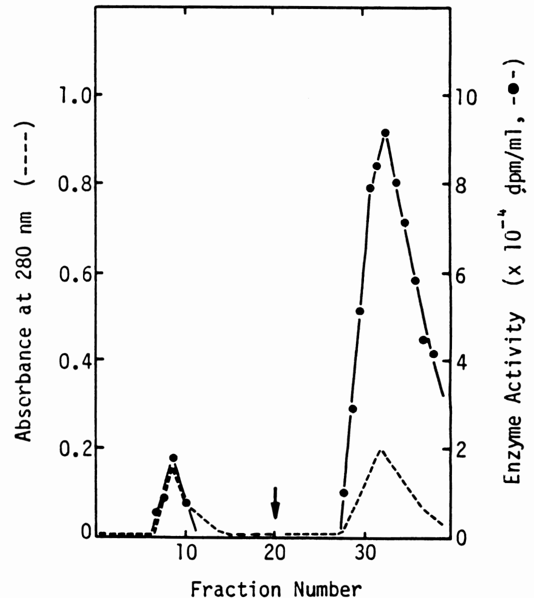

Fig. 1. Covalent chromatography of the *Fasciola* proteinase on a column of activated thiol-Sepharose 4B. Activated enzyme (6.7 ml, 4.9 mg protein) was applied on a column of thiol-Sepharose 4B, as described in Materials and Methods, and eluted with 5 mM L-cysteine in Buffer C (the arrow) at a flow rate of 10.8 ml/hr; fractions of 3.7 ml were collected. An aliquot (20 μl) of each fraction was assayed for the globinolytic activity (–•–). Net absorbance at 280 nm (–––) for protein was calculated as described in Materials and Methods.

residues (Table 2), one residue should be involved in the catalytic activity of the enzyme, as des-

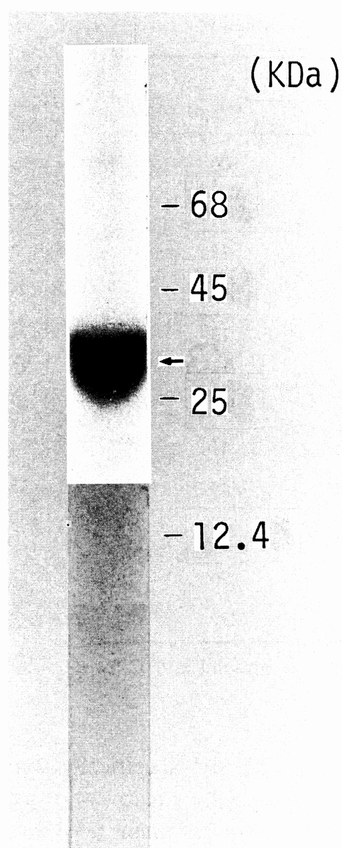

Fig. 2. SDS-polyacrylamide gel electrophoresis of the purified *Fasciola* proteinase. The enzyme (38 μg) reduced with 1% 2-mercaptoethanol was run on 15% polyacrylamide gel containing 0.2% SDS; the gel was stained with Coomassie Brilliant Blue and destained. The positions of the molecular weight standards, bovine serum albumin (68 kDa), ovalbumin (45 kDa), α-chymotrypsinogen A (25 kDa), and horse cytochrome c (12.4 kDa), are indicated. The arrow indicates the purified enzyme.

Table 2. Amino acid composition of the *Fasciola* proteinase*

Amino acid	Number of † residues per mole		% of total
Acidic	Asx ‡	18	19.5
	Glx	23	
Basic	Lys	8	10.0
	Arg	10	
	His	3	
Hydrophobic	Ala	14	40.5
	Val	16	
	Leu	11	
	Ile	6	
	Pro	7	
	Phe	5	
	Tyr	14	
	Trp	5	
	Met	7	
	Gly	27	30.0
	Ser	18	
	Thr	12	
	Cys	6	
Total		210	100.0

* Hydrolysis of the purified enzyme (2.0 mg) in 4 N methanesulfonic acid containing 3-(2-aminoethyl) indole was performed as described in Materials and Methods.

† Calculated on the basis of the molecular weight of 27000, determined upon SDS-PAGE as in Fig. 2.

‡ Asx represents L-aspartate plus L-asparagine, and L-glutamate plus L-glutamine for Glx.

cribed below.

Effects of proteinase inhibitors and activator on the purified Fasciola *proteinase.* The compounds listed in Table 3 were preincubated with the enzyme and the remaining activities were measured by the standard assay system. Peptide aldehydes, leupeptin, antipain, and chymostatin, and TLCK decreased the activity to 10, 14, 16, and 13% of the control, respectively. *p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) and *N*-ethylmaleimide were also inhibitory against the enzyme activity. High concentration of phenylmethylsulfonyl fluoride (PMSF) and EDTA, serine and metallo-proteinase inhibitors, respectively, did not yield significantly reduced activity. Pepstatin, a strong inhibitor specific for aspartic acid proteinase, had no effect. By contrast, 10 mM DTT enhanced the activity by 260% of the control. These results indicate that a cysteine residue is involved in the active site of the *Fasciola* proteolytic enzyme.

Monoclonal antibodies specific for the Fasciola *proteinase and localization of the enzyme in parasite tissues.* We have established

Table 3. Modulation by various effectors of the *Fasciola* proteolytic activity

Effector	Concentration	Activity (% of control)
None (control)	—	100
Leupeptin	10 μM	10
Antipain	10 μM	14
Chymostatin	10 μM	16
p-Tosyl-L-lysine chloromethyl ketone (TLCK)	10 μM	13
p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)	10 μM 250 μM	70 30
N-Ethylmaleimide	5 mM	41
Phenylmethylsulfonyl fluoride (PMSF)	5 mM	87
Ethylenediaminetetraacetic acid (EDTA)	5 mM	97
Pepstatin	10 μM	96
Dithiothreitol (DTT)	10 mM	259

The purified enzyme (1.3 μg) was preincubated with each compound at 0°C for 30 min and assayed as described in Materials and Methods.

3 different cell lines of hybridomas, which produce IgG_1 monoclonal antibodies, 3A2-H5-A5, 4D8-A3-A8, and 1H11-F11-E7. Upon immunoblotting analysis, these three monoclonal antibodies clearly recognized a single protein band of 27 kDa, corresponding to the molecular weight of the purified *Fasciola* proteinase.

To further clarify the specificity of these monoclonal antibodies, the partially purified *Fasciola* proteinase (Sephadex G-75 step) was incubated with each monoclonal antibody and then with anti-mouse IgG antibody; subsequently, the mixture was centrifuged to separate the precipitate and supernatant, followed by enzyme assays for both the precipitate and supernatant fractions (Fig. 3). 3A2-H5-A5 resulted in the lowest globinolytic activity remaining in the supernatant (3.7% of the control), while this monoclonal antibody yielded the highest proteinase activity (31.6% of the control) in the precipitates. Upon SDS-PAGE of the precipitate under the nonreducing condition, only IgG molecules and the enzyme with a molecular weight of 27 kDa were observed (data not shown). These results indicate that this monoclonal antibody specifically binds the *Fasciola* proteinase. Low recovery of activity in the precipitate plus supernatant (35.3%) suggests the strong binding of the enzyme with this antibody. 4D8-A3-A8 and 1H11-F11-E7 also bound the enzyme, demonstrating 30 and 23% activities in the precipitates, respectively, and 50% activities in the supernatant fractions; the precipitated materials were also composed of 27 kDa protein and IgG. Neither normal mouse IgG nor anti-*Trypanosoma cruzi* monoclonal antibody used as negative controls, precipitated the proteolytic activity, and the majority of the activity (83—110%) was found in the supernatant (Fig. 3).

Using a monoclonal antibody, 3A2-H5-A5, strong fluorescence was observed in the epithelial cells and microvilli of the intestine of the adult parasite (Fig. 4). The other two monoclonal antibodies gave similar results. The tegumental surface and parenchymal tissues had no fluorescence (Fig. 4). Control experiments using non-specific monoclonal antibodies did not result in any positive fluorescence. These observations may indicate that the *Fasciola* proteolytic enzyme is

released from the intestinal epithelial cells into the intestinal lumen to act as a digestive enzyme.

Species specificity and application of the Fasciola *proteinase as an antigen for ELISA*. Sera from patients with fascioliasis (13 cases), schistosomiasis japonica (13 cases), schistosomiasis mansoni (10 cases), paragonimiasis miyazakii (3 cases), angiostrongylosis, gnathostomiasis, sparganosis and amebiasis, and normal human sera (26 samples) were examined in ELISA, using the partially purified *Fasciola* proteolytic enzyme as the antigen (Fig. 5). The homologous combina-

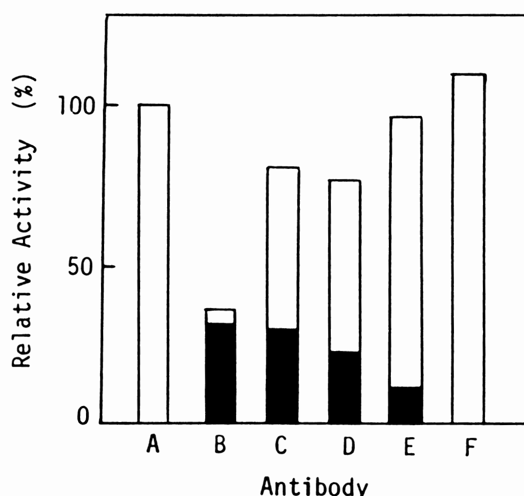

Fig. 3. Proteolytic activities of the *Fasciola* enzyme after binding with monoclonal antibodies. Monoclonal antibodies used here were purified from mouse ascites on a column of Affi-gel Protein A (Bio-Rad) according to the method of manufacturer's instruction. The partially purified enzyme (4 μg) was incubated with each monoclonal antibody (5 μg) at 37°C for 2 hr and then with anti-mouse IgG (20 μg) at 37°C for 2 hr. The mixture was centrifuged at 15000 rpm for 20 min at 4°C, and the enzyme activities in the supernatant (□) and precipitate (■) were measured as described in Materials and Methods. (A): Control assay without any monoclonal antibody. Monoclonal antibodies against the *Fasciola* proteinase: 3A2-H5-A5 (B), 4D8-A3-A8 (C), and 1H11-F11-E7 (D). Normal mouse IgG (E) and anti-*Trypanosoma cruzi* monoclonal antibody (F) were used as nonspecific antibodies.

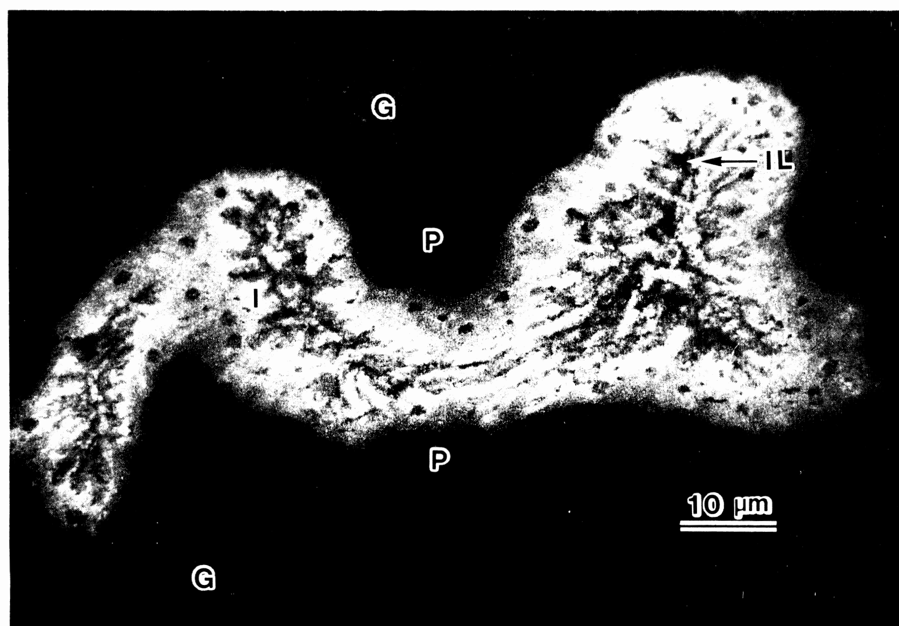

Fig. 4. Tissue localization of the *Fasciola* proteinase. A cryostat section of the adult parasite was incubated with 3A2-H5-A5 monoclonal antibody (10 μl) at 25°C for 1 hr and then with FITC-labeled anti-mouse IgG. Specific fluorescence is seen in the intestine (I). Genital glands (G) exhibit autofluorescence. IL, intestinal lumen; P, parenchymal tissue.

tion between the *Fasciola* enzyme and fascioliasis sera gave the highest average absorbance at 405 nm of 1.603±0.394 (mean±SD), with only one cross-reacted case of schistosomiasis japonica. No false negative fascioliasis was observed with the *Fasciola* enzyme. Heterologous combinations between the *Fasciola* proteinase and the sera of schistosomiasis japonica or schistosomiasis mansoni yielded the lower absorbance of 0.573± 0.168 or 0.511±0.278 (Fig. 5). The average absorbances between the *Fasciola* enzyme antigen and the sera of other parasitic diseases or normal humans were lowest, 0.268±0.053 (N=4) or 0.228±0.095. These absorbance values in the heterologous combinations are significantly different from the value in the homologous combination at the $p<0.001$ level.

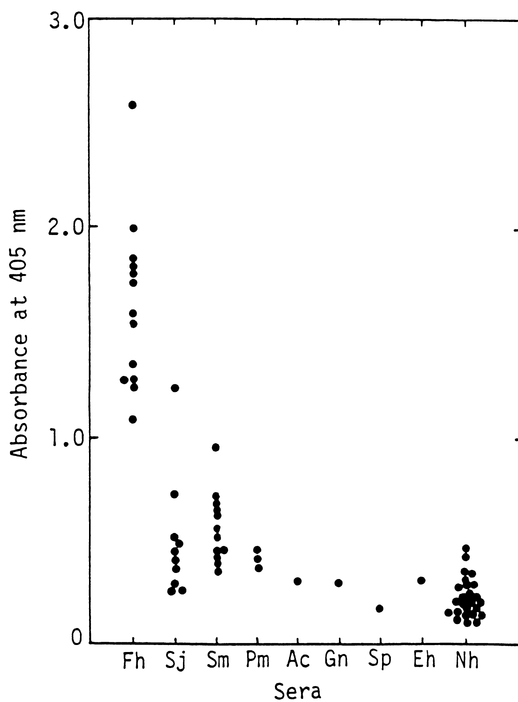

Fig. 5. ELISA for sera from patients with various parasite infections and for normal human sera, using the *Fasciola* proteinase as antigen. The assays were conducted as described in Materials and Methods. Test sera were obtained from patients with fascioliasis (Fh), schistosomiasis japonica (Sj), schistosomiasis mansoni (Sm), paragonimiasis miyazakii (Pm), angiostrongylosis cantonensis (Ac), gnathostomiasis (Gn), sparganosis (Sp), amebiasis (Eh), and from normal humans (Nh).

These results indicate that the *Fasciola* proteolytic enzyme, even at its partially purified state, has high species specificity and is valuable as a sensitive ELISA antigen for immunodiagnosis of human fascioliasis.

Discussion

In the present study, we have succeeded in purifying a *Fasciola* proteinase to the homogeneous state, and this enables us to further characterize the enzyme and to compare it with other *Fasciola* proteinases reported by different research groups. We have established a relatively simple, reproducible purification procedure of the enzyme, utilizing activated thiol-Sepharose 4B covalent chromatography at the final step of purification (Fig. 1). The purified *Fasciola* proteinase had a molecular weight of 27 kDa (Fig. 2). Of several reports, only two described the successful purification of the proteinases from adult *F. hepatica,* showing molecular weights of 12 kDa (Simpkin *et al.*, 1980) and 14.5 kDa (Rege *et al.*, 1989).

These differences in molecular weight are thought to be attributable to the different *Fasciola* strains or species, different methods of enzyme purification and of enzyme assays with use of different substrates. Simpkin *et al.* (1980) employed a medium in which the "British strain" of *F. hepatica* adult parasites was cultivated to obtain their gut contents as the starting material for purification of the 12 kDa enzyme. By contrast, Rege *et al.* (1989) purified the 14.5 kDa proteinase from the homogenate of "American strain" adult parasites through high speed centrifugation and high performance liquid chromatography. The "Japanese *Fasciola* spp." have been thought to be composed of coexisting *F. hepatica* and *F. gigantica,* but have not been clearly identified (Oshima, 1989).

Rege *et al.* (1989) noted that the repeated freezing and thawing of the 14.5 kDa proteinase resulted in the appearance of an additional 28 kDa band on SDS-PAGE analysis, and that 1 M urea caused the conversion from the 28 kDa band to the 14.5 kDa protein. In the presence of 7 M

urea in SDS-PAGE analysis, however, the highly purified *Fasciola* proteinase migrated as a single band with a molecular weight of 27 kDa (Yamasaki, unpublished data). Thus, our 27 kDa proteinase differ from 12 and 14.5 kDa enzymes in molecular structure. Coles and coworkers (Simpkin *et al.*, 1980; Coles and Rubano, 1988) reported that, in contrast with their 12 kDa proteinase in the intestinal regurgitation of the "British strain" flukes, the partially purified gut regurgitant proteinase of the "American strain" of *F. hepatica* were composed of two proteins with similar molecular weights of approximately 27 kDa. However, it was not clear whether either of these two proteins possessed the hemoglobinolytic activity.

The globinolytic activity of our purified *Fasciola* proteinase is decreased by typical cysteine proteinase inhibitors (leupeptin, antipain, chymostatin, TLCK, and *N*-ethylmaleimide), while the enzyme requires a reducing agent such as DTT for the maximal activity. These results indicate that the 27 kDa *Fasciola* proteolytic enzyme belongs to the class of cysteine proteinases. In general, cysteine proteinases are more sensitive to TLCK than to TPCK (Murachi, 1977), consistent with the present results obtained (Table 3). In addition to the inhibitory action of leupeptin on the *Fasciola* proteinase, the compound was thought to bring about an affinity labeling of the active site cysteine, yielding an irreversible inactivation of the enzyme (Aoki *et al.*, 1983).

It is very likely that the adult *Fasciola* possess different classes of proteolytic enzymes. Rupova and Keilová (1979) described the presence of an aspartic acid proteinase with hemoglobinolytic activity at pH 3. The 14.5 kDa enzyme is found to be a cysteine proteinase with hydrolytic activities against synthetic substrates and Azocoll (Rege *et al.*, 1989). Chapman and Mitchell (1982) reported a unique proteinase with properties common to the papain- or cathepsin B-like enzyme in the immature *F. hepatica*; the enzyme was highly active in hydrolyzing mammalian immunoglobulins *in vitro*, suggesting an evasion mechanism of the young parasites during migration in their mammalian hosts. Hemoglobin was also susceptible to this enzyme.

The 27 kDa cysteine proteinase had about 20% acidic and 10% basic amino acids (Table 2), consistent with acidic isoelectric points (Yamasaki *et al.*, 1982). Out of six cysteine residues per enzyme molecule (Table 2), one residue should be involved in the active site as described previously, and four residues could produce two -S-S- bonds, possibly leaving one free cysteine residue. The enzyme which carries amidase activities hydrolyzing α-*N*-benzoyl-L-arginine amide (BAA) and α-*N*-benzoyl-DL-arginine-β-naphthylamide (BANA) is most probably a glycoprotein, judging from the color development for the enzyme by periodic acid/Schiff (PAS) staining (Yamasaki *et al.*, 1982). These observations indicate that the 27 kDa *Fasciola* proteolytic enzyme shares common properties with lysosomal cysteine proteinases, cathepsin B, H, and L (Barrett, 1977).

Recent studies on nucleotide sequences for two genes encoding the *S. mansoni* proteolytic enzymes showed that the deduced amino acid sequence of one cysteine proteinase was highly homologous to that of mammalian lysosomal cathepsin B (Klinkert *et al.*, 1989), and that another cysteine proteinase called "hemoglobinase", sharing no homology with any other cysteine proteinase, was specifically present in this blood-dwelling trematode parasite (Davis *et al.*, 1987; Klinkert *et al.*, 1989). Whether the 27 kDa *Fasciola* proteinase resembles either of these *S. mansoni* enzymes remains to be elucidated. The determination of the primary structure of the *Fasciola* enzyme may deepen understanding of the phylogenetic relationship of these cathepsin-like enzymes or "hemoglobinases" in helminth parasites.

We have succeeded in the production of monoclonal antibodies specific for the *Fasciola* proteinase. The specificities of the three monoclonal antibodies were confirmed by two different criteria. The first criterion was that the antibodies recognized only the 27 kDa protein on the immunoblot analysis of the partially purified enzyme fraction used as the antigen. The second criterion was that the antibodies precipitated only the enzyme which actually hydrolyzed the

substrate [^{3}H]-globin and was proven to have the molecular weight of 27 kDa, although the affinity of each monoclonal antibody for the enzyme appeared to vary (Fig. 3). Of the three monoclonal antibodies, one having a markedly high affinity for the *Fasciola* proteinase resulted in a very low remaining activity in the supernatant obtained by centrifugation of the enzyme-antibody complexes. However, whether the differences in apparent binding potentials of these antibodies for the enzyme are due to the different epitope recognition or the same epitope recognition with completely different affinities, is an interesting question to be clarified.

The strictly evaluated specificity of monoclonal antibodies for the 27 kDa proteinase ensured the use of these antibodies to visualize immunohistochemically the localization of the enzyme in a tissue section of the adult *Fasciola* spp. Evidence that the 27 kDa proteinase is localized only in the intestinal epithelial cells and microvilli (Fig. 4), together with a different line of evidence that the "American strain" *F. hepatica* adults vomit a 27 kDa proteolytic enzyme (Coles and Rubano, 1988), leads us to consider the *Fasciola* proteinase to be synthesized in the intestinal epithelia, secreted into the lumen, and then vomited to the media. Monoclonal antibodies against the *S. mansoni* cysteine proteinase were also used to localize the enzyme in the gastrodermis of this adult parasite (Chappell and Dresden, 1987). Immunocytochemical analysis with use of the colloidal gold technique revealed that the gold particles accumulated in the gastrodermal syncytium and in the border of the gastrodermis and intestinal lumen (Chappell and Dresden, 1986); this may imply that the enzyme is synthesized in the gastrodermis and secreted into the intestinal lumen.

In the adult *S. mansoni,* host red blood cells injested into the digestive tract are degraded by the possible action of parasite-derived hemolytic factor(s), releasing hemoglobin (Kasschau and Dresden, 1986). Hemoglobin is then subjected to hydrolysis by the endopeptidase activities of the "hemoglobinase" and/or cathepsin-like cysteine proteinase, yielding fragments of hemoglobin. These cysteine proteinases are likely to be activated by a high concentration of glutathione derived from the red blood cells (Chappell and Dresden, 1986). Aminodipeptidyl peptidases I and II and other related hydrolases may possibly further decompose the fragmented hemoglobin to yield smaller polypeptides and amino acids which may be absorbed as nutrients by the parasite intestine (Bogitsh and Dresden, 1983).

On the other hand, the adult *Fasciola* worms may depend on the bile, epithelial cells and mucosa, and blood from the hemorrhagic ulcers of the host bile ducts as nutritional sources. The adult *Fasciola* seems to feed on these components, since residual materials or cellular debris have been observed in the gut lumen of the fluke (Yamasaki, unpublished data), and therefore it is thought that the hemoglobin released is extracellularly hydrolyzed by digestive proteinase(s) such as 27 kDa cysteine proteinase in the parasite intestinal lumen. Subsequently, the dipeptidyl peptidase I, previously called cathepsin C, which is active at pH 5.0 against *o*-acetyl 5-bromoindoxyl in the presence of cysteine (Halton, 1969), would be involved in the sequential process of hemoglobin digestion and uptake in *F. hepatica.*

The antigenicity of the partially purified *Fasciola* proteinase was examined by Aoki (1980), who showed that a radioallergosorbent test (RAST) using the *S. japonicum, S. mansoni,* and *Fasciola* proteinases as antigens could detect specific IgE antibodies, and that the species specificities of these enzyme fractions were quite high. On the other hand, Coles and Rubano (1988) reported the antigenicity of the two 27 kDa proteins of *F. hepatica* intestinal exudation in immunoblotting that demonstrated the cross reaction with antisera from mice infected with *S. mansoni.* We consider that the cross reactivity is most probably attributable to one of two 27 kDa protein (Coles and Rubano, 1988), since our partially purified *Fasciola* proteinase fraction containing the major protein of 27 kDa demonstrated very high species specificity in ELISA for sera from patients with various parasitic infections (Fig. 5). In addition, our ELISA method using the 27 kDa proteinase fraction is quite sensitive for human fascioliasis, showing

only one case of cross reaction with schistosomiasis japonica serum. Therefore, we expect to utilize this method for the prognosis of fascioliasis, after treatment with the effective antihelminth drugs such as bithionol.

There have been 62 reported cases of human fascioliasis in Japan (Matsuoka *et al.*, 1987). However, the diagnosis of *Fasciola* infections is not always easy since the stool examination is often ineffective particularly in the cases of heterotopic parasitism by the liver flukes. Therefore, our ELISA method with high sensitivity and species specificity could be utilized not only as a supplementary immunodiagnostic technique but also as a method of definite diagnosis.

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