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F I N A L R E P O R T

"PROTEOLYTIC ENZYMES OF *Fasciola hepatica* AS MARKERS OF HUMAN AND ANIMAL INFECTION IN CATTLE RAISING AREAS OF PERU"

PROJECT AID - PSTC 936-5542
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I. INTRODUCTION.

Animal and human levels of infection by *Fasciola hepatica* are very high in all cattle-raising areas of Perú and many other South American countries. The problem is serious for public health and economic reasons. Overwhelming infection of cattle, sheep, and other animals is not rare, and it leads to cachexia, anemia and edema. Valuable animal protein (including livers of infected animals) is lost for human consumption. In endemic areas of Perú parasitism by *Fasciola hepatica* in sheep and cattle is close to 100%. Infection rates as high as 50% have been reported in rural children, while such rates in cities of the same areas are between 5 and 11%. Human infection is related to use of watercress (*Nasturtium officinale*) in salads, both in rural areas and in cities, or to ingestion of larvae (metacercariae) in contaminated water.

The economic impact of fascioliasis for Perú is very great: losses arise from elimination of infected livers at abattoirs, decreased meat, milk and wool production by infected cattle and sheep, expenses for veterinary fasciolacidal drugs, and the need for medical attention of infected people. *Fasciola hepatica* infection in humans may cause an acute syndrome which includes acute pain, fever, eosinophilia and hepatomegaly, and may lead to hemorrhage, liver rupture, shock, and even death. The chronic stage of human fascioliasis has scant symptomatology, but complications such as bile duct obstruction, liver fibrosis, and extrahepatic localizations may occur. The degree of liver damage is proportional to the worm burden and the allergic reactivity of the patient.

Control of this zoonosis is a complex problem; accurate, sensitive diagnostic tests are an essential first step toward effective control. In particular, early diagnosis, now impossible in the first weeks of the disease, may aid significantly in efforts to keep cattle *Fasciola*-free in the critical periods of calfhood, pregnancy and lactation. Dosing with toxic drugs is specially undesirable during those periods. The advantage of early diagnosis in humans is obvious: if the disease is treated when the worms are at the larval stage, liver damage is minimized, as the extensive burrowing characteristic of the chronic stage is not reached.

The overall objectives of the project were:

a. The isolation and characterization of proteolytic enzymes of *Fasciola hepatica*, and

b. To study the specific antibodies to these enzymes in natural or experimental fascioliasis

The antigens obtained could be used for early diagnosis, estimation of worm load, ascertaining success of chemotherapy and epidemiological field studies.

We have isolated and characterized the proteolytic enzymes of the parasite. These enzymes show good antigenicity as they induce antibodies in experimental and natural infections in animals and humans. Initial tests performed with conventional techniques and the proteinase antigen isolated promise adequate performance once they are modified for mass screening and for individual followup of patients and cattle.

The specific experimental work achieved is described in the following pages.

II. MATERIALS

1. Adult worms (*Fasciola hepatica*) were obtained from the naturally infected cattle in abattoirs in Lima, Perú. The worms were washed in 0.9 % NaCl and homogenized in the same solution. Following centrifugation at 2,000 g, the separated pellet and supernatant fractions were lyophilized; these samples were studied in Lima and also transported to Baylor College of Medicine.
2. Adult worms obtained the same way were washed in saline solution and immediately lyophilized. This material was used both in Lima and at Baylor.
3. Adult worms (120 worms/50 ml) were resuspended in distilled cold water (4°C) in order to make them regurgitate excretion/secretion (E/S) products. The material was filtered through Whatman Nº 1 filter paper, lyophilized and stored until needed.
4. Juvenile forms were obtained from the life cycle of the parasites maintained experimentally in the laboratory.

III. METHODS

A. Life Cycle

The life cycle of the parasite was obtained through *Lymnaea viatrix*. It was necessary to have a room with aquaria. Snails were brought from the Mantaro valley, one of the most important cattle-raising areas of Perú as well as one with the highest prevalence of cattle and human infection with *Fasciola hepatica*. Eggs of the snails were used to begin rearing them in the

laboratory. Juvenile snails were infected with miracidia of *Fasciola hepatica* obtained from the eggs of the parasite. Eggs were incubated in the laboratory to reach the miracidium stage; their hatching was prevented by refrigeration (+10°C until use. Each snail was incubated with 4-5 miracidia during one hour. As it is known, the cycle in the snail lasts 1-2 months before the presence of cercariae; at that moment the snails were placed in small glass containers to induce the transformation of cercaria to metacercaria. These were collected to be used in enzymatic assays.

B. Fractionation:

The sample was resuspended in 0.2 M citrate buffer, pH 4.9 (1/20-1/100 w/v) and homogenized in cold in a glass-glass homogenizer or with a French press. The homogenate was centrifuged at 15,000 rpm for 10 minutes at 4° C; the supernatant (S_1) was separated and the pellet (P_1) was resuspended in the same buffer (Triton X-100 was initially included in this step, but was later eliminated) and centrifuged at 15,000 rpm for 60 minutes at 4° C, to yield S_2 and P_2. See Figure 1.

C. Proteinase assays

1. Low molecular weight substrates: Two fluorescent substrates were used (CBZ-Phe-Arg-AFC and CBZ-Arg-Arg-AFC). Assays were performed in 0.2 M citrate buffer, pH 4.9, unless otherwise stated. Thiols (5 mM) needed to be present in the system.
2. Azocoll assay: 5 mg/ml substrate in same buffer as above. Activity was measured at 520 nm.
3. Hemoglobin: Human hemoglobin at a final concentration of 20 ug/ml was incubated with enzyme extract in 0.2 M citrate buffer, containing 5 mM cysteine. The reaction was stopped at different intervals by adding an equal volume of 0.5 M Tris-HCl, pH 6.9 containing 2-mercaptoethanol, glycerol and sodium dodecyl sulphate (SDS). Samples taken at 0, 6, 12 and 24 hours after initiation of the reaction were subjected to electrophoresis in 15 % polyacrylamide gel under denaturing conditions.
4. Bovine Serum Albumin: The assay was performed the same way as for hemoglobin.

D. Modulators

The effect of different types of proteinase modulators on the activity of the enzyme was assayed, using hemoglobin and CBZ-Phe-Arg-AFC as substrates. Modulators used were phenyl-methyl-sulfonyl fluoride (PMSF), pepstatin, iodoacetate, EDTA, Ep-459, E-64, and calcium chloride.

E. Ammonium Sulphate Purification

Ammonium sulphate precipitations were performed as a first purification step. Salt concentrations used were 30, 50, 60, 70 and 90 % (w/v). Ammonium sulphate was slowly added at room temperature, and samples were left overnight at 4°C to let the precipitation go beyond completion. They were centrifuged at 10,000 rpm at 4 °C for 10 minutes and the supernatant was discarded. The pellet was dissolved in 0.2 M citrate buffer pH 4.9 and dialyzed against citrate buffer for 24 hours to eliminate the salt. Protein concentration and enzymatic activity were determined in each fraction.

F. Gel Filtration

Two different matrices were used: An AcA 54, an acrylamide-agarose-gel, was used at Baylor, and Sephadex G-100 was used in Lima. Both were properly calibrated using molecular weight standards (Blue Dextran, 2'000,000; Bovine Serum Albumin, 62,500; pepsin, 33,000; chymotrypsin, 22,600 and lisozyme, 13,900 daltons) and 0.2 M citrate, pH 4.9 as eluting buffer.
The Sephadex column was attached to an Isco UA-5 detection unit.

G. Cationic Exchange - High Performance Liquid Chromatography (CE-HPLC)

A polycationic exchanger (Polycat A, Custom LC Inc., Houston) was used in an LKB HPLC system. Samples and solutions were prefiltered through 0.2 um filters (Uniflo, Schleicher and Schuell). Samples were eluted with 1.0 M NaCl.

H. Hydrophobic Interaction Chromatography - High Performance Liquid Chromatography (HIC-HPLC)

A PolyPROPYL (C3) Custom LC, Houston 5um dialmeter column was used in an LKB HPLC system. Two buffers were used: Buffer A (0.9 M citrate, pH 5) for column equilibration and Buffer B (0.01 M citrate pH 5) for elution.

I. Chromatofocusing

A CX 10 ultrafiltration unit, with Polybuffer PBE 94 (Pharmacia Fine Chemicals) was used. A pH gradient from pH 4.0 to 7.0 was obtained with Polybuffer 74.

J. Glycoprotein Assay

1. Samples were subjected to electrophoresis and "Western blotted". The primary ligand was Concanavalin A (Con A) and

the secondary ligand a Peroxidase-conjugated anti Con-A antibody.

2. Lentil-lectin-Sepharose 4B was resuspended in proper buffer. 0.2 M citrate buffer as eluting buffer, and 0.1 M D-mannose as desorbing agent were used.

K. Serologic assays

1. Enzyme-Linked ImmunoSorbent Assay (ELISA)

Polyvinyl microtitulation (96-well) plates were coated with partially purified proteinase (1µg/ml) at 50 ul per well and stored at 4°C overnight. Each well was blocked with caseine 1% in Phosphate Buffered Saline - Tween 0.05% (PBS-Tween) overnight. The plates were rinsed 3 times with PBS-Tween 0.05 % before the addition of the first antibody, coming from immunized humans, animals or from the cloned hybridoma cells. The plates were incubated 1 hour at 37°C, rinsed as above, and the second antibody (50 ul of rabbit anti mouse whole IgG conjugated with horseradish peroxidase) was added. The plate was incubated 1 hour at 37°C, rinsed, and the substrate (ABTS plus H2O2) added, for development of the green color. The reaction was stopped with SDS (sodium dodecyl sulfate).

The ELISA was used to follow the rise in titer in immunized mice, to screen the supernatant of hybridoma clones for antibody *versus Fasciola hepatica*, isotyping monoclonal antibodies, and to determine the levels of antibody in patients.

2. Dot Blot

Different preparations of the enzyme were applied (200 ul) onto nitrocellulose paper and dried with the aid of an aspirator. The paper was cut into strips which were incubated with serum of immunized or infected animals, or supernatant of the positive antibody-producing hybridoma clones. Each strip was rinsed three times with PBS-0.05 % Tween. Nitrocellulose strips were incubated with primary antibody 1-2 hours at 37°C, rinsed as described above, and incubated with horseradish peroxidase-secondary Ab conjugate, for 1-3 hours; then rinsed and substrate (DAB/chloronaphtol) was added for 5 minutes, or until color was developed. The reaction was stopped with water.

3. Western Blot

Preparations of the enzyme were loaded onto SDS-15% PAGE and run at 30 mA. The proteins were transferred to nitrocellulose paper at 0.25 mA for 4 hours. After the transfer, proteins were stained with Ponceau S and blotted overnight with 1 % caseine in PBS . Nitrocellulose strips were incubated with the primary antibody and processed in the same way as in the dot blot technique.

4. Immunofluorescence

Whole worms were fixed with Rossman's fixative and slices in cross and longitudinal sections were incubated with the first antibody 1/100 (immunized mouse serum or infected mouse serum) for at least two hours. Sections were rinsed with PBS and incubated with fluoresceine isothiocyanate-conjugated second antibody. Sections were well rinsed before observation under the fluorescence microscope.

L. Polyacrylamide Gel Electrophoresis

Electrophoresis runs in 15 % polyacrylamide gels were performed in the presence of SDS and 2-mercaptoethanol. Average current used at Baylor was 20 mA for 2-3 hours. Average voltage used in Lima was 60 volts for 12 hours. Several molecular weight markers were used: Bovine Serum Albumin (62,500 d), Alcohol Dehydrogenase (42,000 d), Trypsin (21,500 d) Soybean Trypsin Inhibitor (21,000 d), Lysozyme (14,000 d), Cytochrome C, Aprotinin (6,500 d), and Insulin B chain (3,400 d).
Gels were developed either with Coomassie blue or silver stain.

M. Cellulose Acetate Electrophoresis

This technique separates molecules based on their electrical charge, and not on their molecular weight. Tris-Barbital (Ionic strength = 0.15 μ) was used as running buffer and 150 volt for 20 minutes were applied to the samples. The plates were stained with Ponceau red.

N. Hemolytic Activity

The assay was performed with washed human erythrocytes (RBC); 10 ml human bood samples were collected in 1 ml 3.8% sodium citrate and centrifuged at 1,000 rpm for 20 minutes. The supernatant fraction was discarded and the pellet washed three times in 10 volumes 0.95% NaCl, pH 7.3, and one in 10 volumes isotonic buffer pH 7.3 (0.1 M glycine in 0.6 % NaCl). For the hemolytic assay, samples contained 0.8 ml isotonic buffer, 0.1 ml washed RBCs and 0.1 ml of the *Fasciola* fraction to be examined. Samples were incubated at 37°C and the reaction was stopped by cooling the tubes at 4°C and centrifuging them at 1,000 rpm for 5 minutes. Hemolysis was determined by absorbance at 540 nm.

O. Immunization

Female BALB/c mice were immunized with the partially purified proteinase obtained through AcA 54 gel filtration. The animals were injected subcutaneously (multiple sites) with a total inoculum of 100 μg of antigen in 0.3 ml. From 6 mice, two were untreated, two inoculated with Ribi's adjuvant, and two with Freund's adjuvant. Complete Freund's adjuvant was used only in the first injection.

The mice were bled 48 hours after immunization and one week after each booster.

P. Fusion

Three days before fusion an intravenous booster was given (20 ug of partially purified proteinase in 100 µl) to the immunized animals. The antigen was partially purified through HIC and gel filtration.

Day 0: the immunized animals were bled just before the fusion, sacrificed by cervical fracture and the spleen was removed under sterile conditions. The spleen was homogenized in Dulbecco's modified Eagle Medium (DMEM) and the suspension of splenocytes was centrifuged (15,000 rpm for 5 minutes). The same procedure was followed with myeloma cells (non-Ig secreting murine P3 x 63 A G 8.653). Viability of cells was determined using Trypan Blue. Splenocytes and myeloma cells were mixed 1:1 (5 x 10^{-6} cells). Cells were mixed and centrifuged, and polyethyleneglycol (PEG) was added with constant stirring until 5 % final concentration was reached. DMEM without fetal calf serum was added in 1 minute; 7 ml more of medium were added in 2-3 minutes. The final suspension was centrifuged at 1,500 rpm x 10 minutes, and cells were resuspended in such a volume of DMEM (with added antibiotics, Streptomycin, Penicillin and 5-Flurocytosine, Fetal Bovine Serum and glutamine) as to obtain 10 cells/ml. This suspension was poured into 96-well culture plates, at 100 ul per well, and incubated at 37°C and 7 % CO_2.

Day 3: (After fusion): Cells were fed with DMEM plus Hypoxanthine, Aminopterin and Thymidine (HAT-containing medium).

Day 14: Cells were fed as needed with HAT-containing medium until day 25. Cells were then fed with complete DMEM.

Day 16: Supernatants of each well were screened for specific anti*Fasciola* antibody using ELISA.

Q. Selection of Clones

The cells contained in wells with ELISA positive reaction were split into 96-well microtiter plates, allowed to grow and screened again by ELISA. Positive wells were diluted using the technique of two limiting dilutions and screened again by ELISA. Production of monoclonal antibodies (mAb) in mouse ascitic fluid was also used to obtain mAb. The desired clone was injected intraperitoneally into BALB/c mice previously primed with Pristane brand of mineral oil, Aldrich Chemical Co.). Peritoneal fluid was obtained from mice 10 days later.

R. Endogenous inhibitor of *Fasciola hepatica* proteinases

To determine whether there was any endogenous inhibitor of proteinase activity, the partially purified enzyme (HIC pool) was assayed by itself mixed with equal volumes of buffer and also with fractions of the first peak of the HIC column. See Table 5.

III RESULTS

A. Proteolytic Activity

The proteinases from *Fasciola hepatica* were able to degrade most of the substrates available. Bovine Serum Albumin (BSA) was the only substrate upon which no action was observed. Most of the activity was found in the supernatant fraction (S1), using either whole homogenized worms or Excretion/Secretion (E/S) products. When the French press was used to disrupt the worms, almost 90 % of the activity was recovered in the S1 fraction, compared with 60 - 70 % recovery obtained when glass-glass homogenizer was used. (Table 1)

Activity was shown to be dependent on thiol sources (Table 2), and the activation depended on the step of purification at which the activity was determined. There was 5 fold stimulation in crude extracts, and a 20 fold activation in more purified fractions. Activity against Hemoglobin is shown in Figure 2. The proteinase activity was pH dependent, showing maximum activity at pH 4.9 using Phe-Arg or Azocoll as substrates (Figure 3).

Proteinase activity was shown in extracts from juvenile *Fasciola hepatica*, using Azocoll; the small amount of material obtained made it impossible to compare the properties of the adult and juvenile enzymes.

B. Modulators

Enzymatic activity was not inhibited by inhibitors specific for serine-proteinases (PMSF), metallo-proteinases (EDTA), or aspartil-proteinases (pepstatin), using CBZ-Phe-Arg-AFC as substrate, at the concentrations indicated in Table 3. Ep-459, E-64, and iodoacetate, specific cysteinyl proteinase inhibitors, completely inhibited enzyme activity using the same substrate. This same effect could be seen using hemoglobin as a substrate (Figure 4).

C. Ammonium Sulphate Precipitation

Results are shown in Table 4. As observed, the highest proteolytic activity using Azocoll as substrate was found at a 50 % ammonium sulphate concentration. Enzyme activity was also

assayed in the presence of 5 mM DTT, 5 mM cysteine or 5 mM calcium chloride (Table 4 and Figure 5)

D. Gel Filtration

Different samples were loaded on the AcA 54 column: Supernatant (Figure 6), Pellet, and S1 (Figure 7) from *Fasciola* extracts at different concentrations. All samples showed several protein peaks, and just one proteolytic peak at 28,000 to 32,000 daltons. The E/S products loaded on Sephadex G-100 showed several protein peaks, the largest of them located near the internal volume (Figure 8). Activity was also eluted as a single peak of the same molecular weight as above. Activity was also pH dependent.

E. Cationic Exchange-HPLC

Active fractions from AcA 54 were pooled and loaded onto the cationic exchange column. Results were not satisfactory and the procedure was discarded.

F. Hydrophobic Interaction Chromatography

This column separated proteins into two peaks. The larger of the two peaks, eluted with Buffer A, contained little proteinase activity, while the smaller peak, eluted with Buffer B, contained most of the proteinase; Figure 10 and Table 5. SDS-PAGE separates the proteinase activity into two components of different molecular weight; Figure 11.

G. HIC - Gel Filtration - HPLC

Whole worm homogenate (50 mg/ml) was prepared as usual, and loaded onto the HIC (C3) column. The peak of activity was pooled and filtered through PPK 125. Once resolved in a polyacrylamide gel, two major bands of proteins were observed (Figure 12).

H. Chromatofocusing

Fractions eluted from the AcA 54 column showing proteolytic activity were pooled and concentrated using a CX 10 ultrafiltration unit. The concentrated sample was dialyzed to reduce salt concentration and loaded into the column. Results are shown in Figure 13. There is only one important peak of proteolytic activity, with an isoelectric point at about pH 6.5. The same result was obtained using a Chromatofocusing-HPLC column.

I. Glycoprotein assay

The active fraction eluted from chromatofocusing was subjected to the technique described in II G. Previous reports on *Schistosoma mansoni* proteinase suggested the existence of carbohydrates in the structure of the enzyme. Moreover, it is known that *Fasciola hepatica* contains large amounts of glycoproteins in its surface. Con-A was used to determine the presence of D-mannopyranosyl, D-glucopyranosyl or sterically related residues in the *Fasciola* proteinase. No positive results were found in the immune transfer assay. Next a Lentil lectin-Sepharose 4B was used, and no specific binding was observed (Figure 14). The same results were obtained changing pH to 7.0 and 8.0.

J. Polyacrylamide Gel Electrophoresis

Supernatant from *Fasciola hepatica* as described in Methods (20 mg dry weight/ml) were electrophoresed and four major protein bands located near the running front, at 14 kd, 30 kd and 35 kd were obtained (Figures 15 and 16). Previous results in AcA 54 and Sephadex G-100 gel filtration showed a peak of activity at a molecular weight of 30 kd. Therefore, it was assumed that the third band in the PAGE (30 kd) has the proteolytic activity. In order to isolate the protein from the untreated gel, it was washed several times with distilled water to remove the SDS present. The portion of the gel that suposedly had the enzymatic activity was cut and triturated passing it through syringe needles. The resulting material was centrifuged al 3,000 rpm for 10 minutes, and azocollytic activity was determined in the supernatant and pellet fractions. No activity was found in the 30 kd band, nor in the remaining gel. This negative result could be explained in terms of the denaturalizing conditions in which the electrophoresis was performed, if they were so dramatic that the enzyme was not able to recover its activity.

Lyophilized parasite E/S products were electrophoresed and silver stained. Three major bands at molecular weights of 14 kd, 30 kd and near the sample application were found. Active fractions derived from the E/S gel filtration were pooled together and concentrated. Just two protein bands were obtained (no 14 kd band) (Figure 17).

K. Electrophoresis on Cellulose Acetate

Several bands were obtained with this technique. As it was run under non denaturalizing conditions, the protein bands were separated and assayed from enzymatic activity (Figure 18). No activity could be found in any of the fractions using Azocoll as substrate; this could be due to the low amount of sample loaded.

L. Hemolytic Activity

Worm homogenates and E/S products showed no hemolytic activity in the conditions described under Methods.

M. Western Blots

Human sera from patients with fascioliasis recognized two "bands", one at 28 kd and the second at 10 kd; Figure 19. Sera from immunized BALB/c mice recognized the same bands. One of the clones recognized the lower molecular weight band; Figure 20.

N. Immunofluorescence

Immunofluorescent assays with immunized animals showed a specific reaction against the protein synthesized in the *Fasciola* gut. The human sera from patients with fascioliasis recognized the same region even though the background was high. Figure 21 shows the reactions of mouse and patient sera against sections of *Fasciola*. It is also interesting that in the longitudinal sections there is a specific fluorescence along the integument. Monoclonal antibody against antigen from *Schistosoma mansoni* recognizes the brush border of the gut in *Fasciola*.

O. Selection of Clones

A fusion experiment yielded 300 hybridomas. Out of these, 24 produced antibodies measurable by ELISA, and 8 were selected for further cloning by limiting dilution. The 2 fastest growing, antibody-producing clones were finally selected.

P. Immunological Assays

1. ELISA: This technique was very sensitive, with antigen at 50 ng/50 µl/well. Sera from two patients gave positive results even when diluted 1:4096. The ELISA test showed both had predominantly IgM antiibody; Figure 22. The same procedure was adequate to detect antibodies in immunized animals. Ribi's adjuvant seemed to be better than Freund's adjuvant, with an area under the titer curve 1.9 times greater, and the added advantage of not producing a granuloma or hypersensitivity. In the case of hybridomas prepared for monoclonal antibody production, isotype analysis showed the clone studied secreted IgM antibody, kappa light chain specific; Figure 23.

2. Dot Blot: Sera from immunized, infected animals or supernatants of positive clones showed positive reactions with different enzyme preparations, although there was a high background. The *Fasciola* peak eluted from AcA 54 was

unreactive with *Schistosoma mansoni* infected mice sera and with normal mice sera as first antibodies.

Q. Endogenous Inhibitor of *Fasciola hepatica* Proteinases.

The assay for detection of inhibitor activity against partially purified proteinases in the first peak of the HIC column was positive; Table 6 shows the results obtained. This explains the greater proteinase activity found in purified proteinase as compared to total homogenates.

IV. COMMENTS

The results shown in this final report point out the accomplishment of the overall project objectives. The proteolytic enzymes of *Fasciola hepatica* seem to be good antigens, specially the light molecular weight enzyme (12 kd). It was recognized with sera of infected animals and humans; in addition the production of monoclonal antibodies was not difficult. There was no cross reaction when these antigens were tested with sera from mice immunized with *Schistosoma mansoni*, showing specificity exists.

We think it feasible, using an ELISA technique as shown, or a simpler method, to follow the course of the disease in humans, specially as related to therapeutic outcome, and to determine the prevalence of the infection in cattle raising areas.

The light proteolytic enzyme probably has an uncomplicated aminoacid structure, so that its synthesis may be possible through genetic engineering. This would allow large scale production of antigen for practical uses, with fermentation technology.

The development of this project has made it possible to bring together three research centers in three Universities, Baylor College of Medicine, Universidad Peruana Cayetano Heredia, and Universidad Nacional Mayor de San Marcos. This joint effort is resulting in further cooperative work, specially since there are now two well-trained Peruvian professionals, who have had an important experience in biochemistry laboratories at Baylor. They will be assuming progressively more important roles in the development of Peruvian science and technology.

TABLE 1

S A M P L E	Total Activity AFC* nM/hr	Protein mg	Specific Act. AFC nM/hr x mg prot
Homogenate	458.32	14.19	31.97
S_1	318.06	6.89	46.16
P_1	121.04	4.92	24.62
AcA 54	242.93	1.12	216.70
Chromat.	171.00	N.D.	---

* AFC: Aminofluorocoumarine

TABLE 2

Effect of Thiols and Ep-459 on Proteolytic activity (S1)

Control 2 hr.	%	DTT (5mM) 2 hr.	%	Ep 459 (1μM) 24 hr.	%
26.70	100	59.86	224	0	0

TABLE 3

Effect of Proteinase Modulators on AcA 54 Pool

Modulator	Concentration	% Control Activ.*
PMSF	1.0 mM	110
EDTA	5.0 mM	120
Pepstatin	100 ug/ml	86
	1.0 mM	111
$CaCl_2$	2.5 mM	113
	5.0 mM	119
Iodoacetate	1.0 uM	0
Ep 459	1.0 uM	0

* Activity assayed with CBZ-Phe-Arg-AFC.

TABLE 4

Effect of Modulators on Ammonium Sulphate Fractions

% $(NH_4)_2SO_4$	Proteolytic (Azocollytic) Activity*			
	Buffer	+DTT	+Cys	+$CaCl_2$
30	15.3	58.4	52.7	25.2
50	60.0	98.3	99.1	47.8
60	49.2	84.5	80.3	38.7
70	32.0	55.8	55.8	32.5
90	28.6	41.0	46.1	29.3

* Specific Activity: $A_{320\ nm} \times mg\ prot^{-1} \times min^{-1} \times 10^{-3}$

TABLE 5

Proteolytic Activity from HIC pool

SAMPLE	Protein mg/ml	Total Activity	Specific Activity
PC*	0.210	79.6	15.79
HIC	0.037	339.3	452.40

* Precolumn: activity of crude homogenate.

TABLE 6

Endogenous Inhibitor against *Fasciola hepatica* Proteinases

Fraction of "Inhibitor" (Tube Nº)	Percentage of Original Activity (%)	Percentage Inhibition (%)
12	38.1	61.9
13	38.3	61.7
14	45.8	54.2
15	52.4	47.6
16	59.1	40.9
17	62.0	38.0

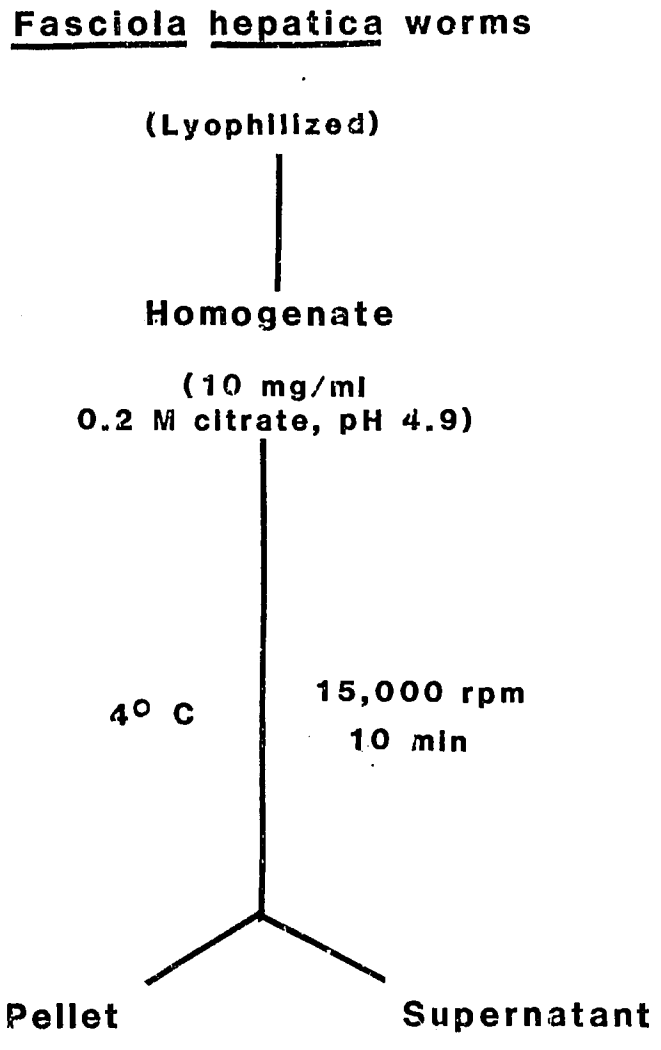


FIGURE 1

Fasciola hepatica Fractionation Scheme. Supernatant S_1 and Pellet P_1 .

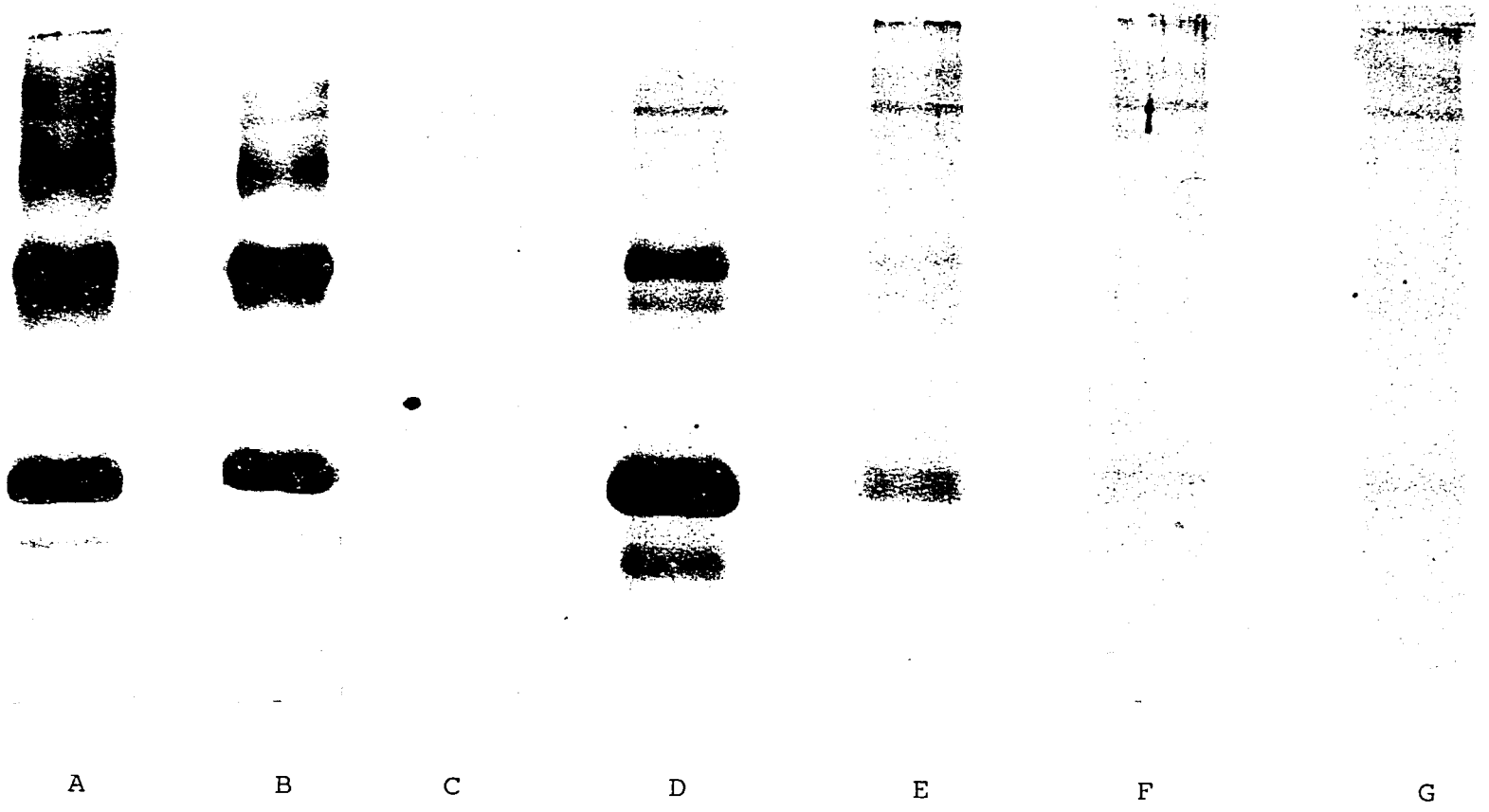


FIGURE 2

Hemoglobin digestion by ACA 54 pool: Hemoglobin control at 0 hr (A), and 24 hr (B); enzyme control (C); substrate digestion at 0 hr (D), 6 hr (E), 12 hr (F), and 24 hr (G).

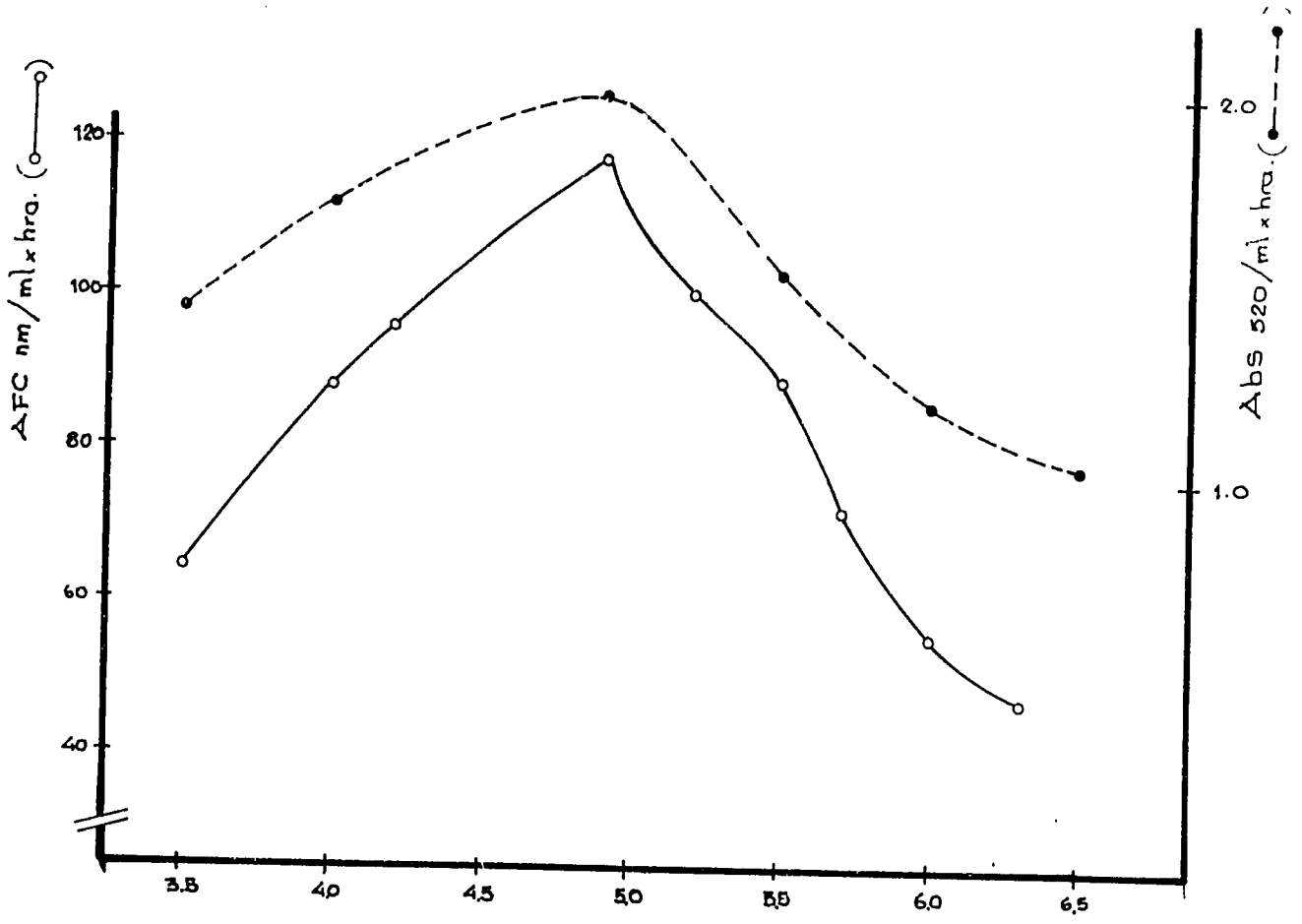


FIGURE 3

Effect of pH on enzymatic activity of Fasciola hepatica protease. (o——o): fluorescent substrate CBZ-Phe-Arg-AFC; (o-----o): Azocoll substrate. Both were measured under the same conditions: 0.2 M sodium citrate, 5 mM DTT.

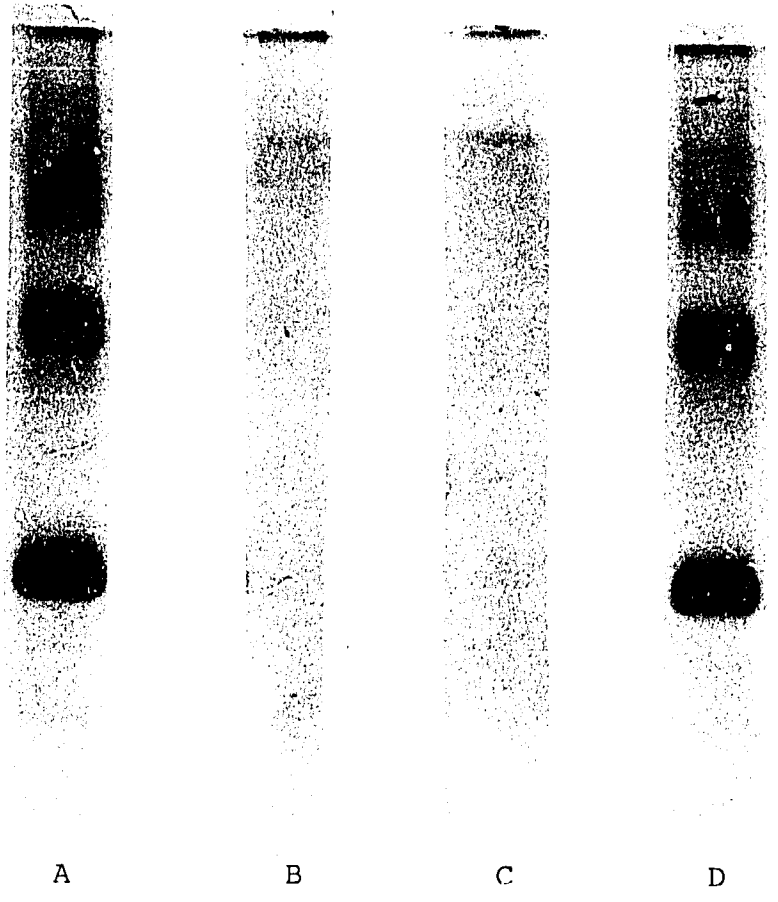


FIGURE 4

Effect of protease inhibitors on Fasciola hepatica protease. A: hemoglobin control (12 hrs); B: hemoglobin + protease (12 hrs); C: hemoglobin + protease + pepstatin (12 hrs); D: hemoglobin + protease + Ep-459 (24 hrs).

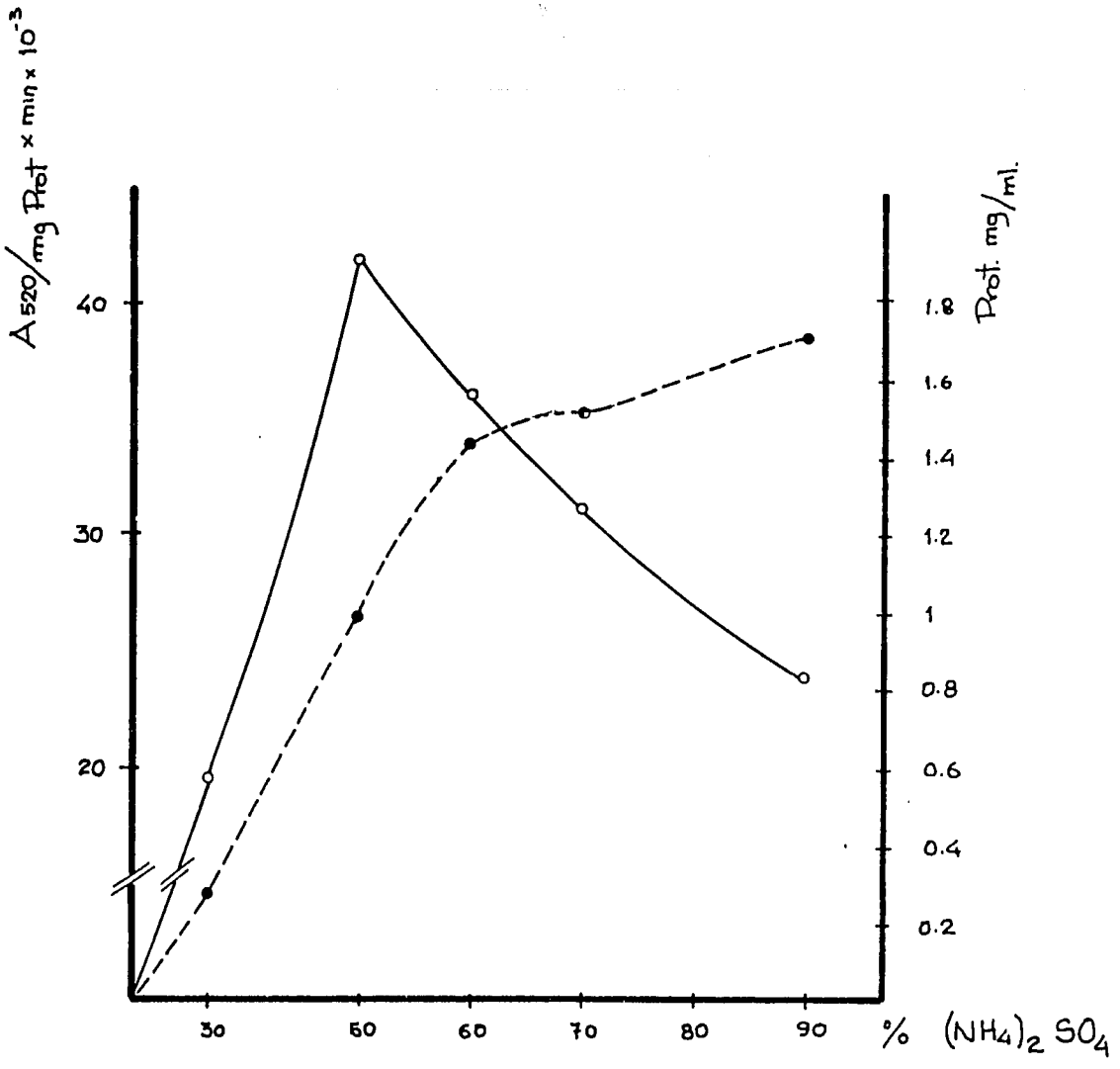


FIGURE 5A

Azocollitic activity in $(NH_4)_2SO_4$ fasciola fractions (o——o) and protein profile measured using Lowry method (●----●).

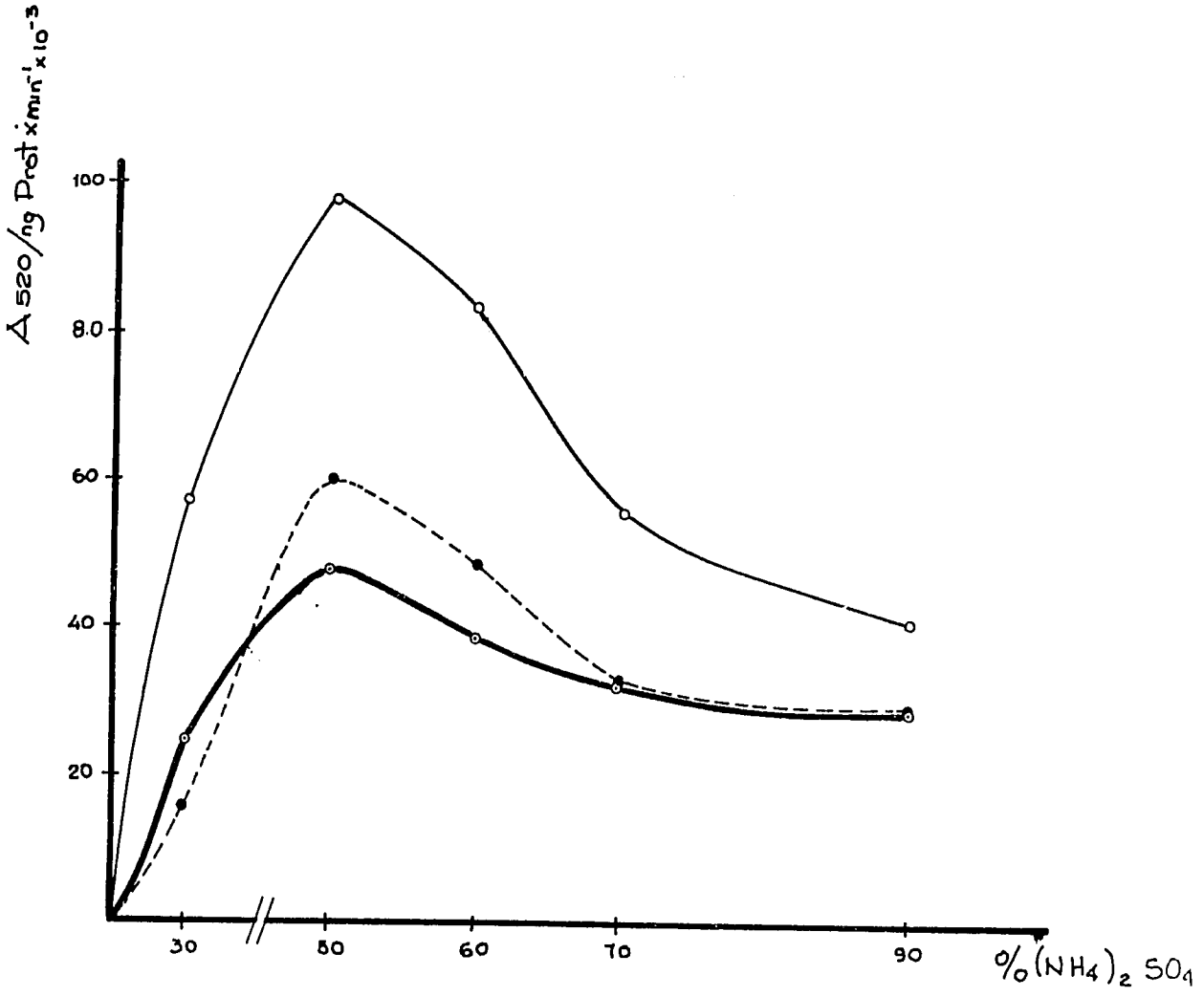


FIGURE 5 B

Effect of modulators on proteolytic activity of $(NH_4)_2SO_4$ *Fasciola hepatica* fractions. Activity was measured using Azocoll as substrate. (⊙——⊙): control; (●------●): + 5 mM $CaCl_2$; (o——o): + 5 mM DTT.

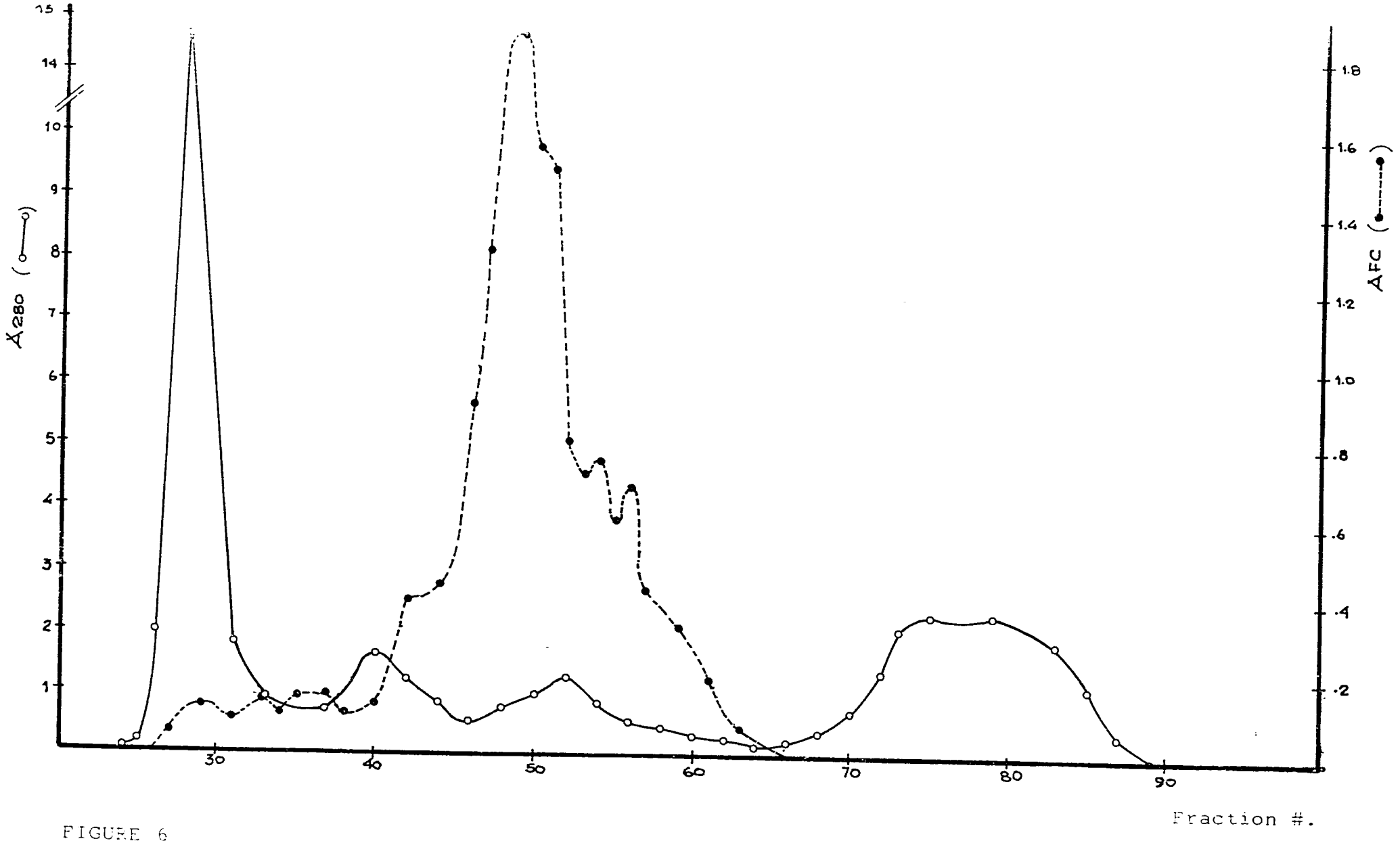


FIGURE 6

Fasciola hepatica supernatant was loaded on an AcA 54 column; eluting buffer, 0.2 M Sodium citrate, pH 4.9. Proteolytic activity was measured using CBZ-Phe-Arg-AFC as substrate; protein was determined as absorbance at 280 nm.

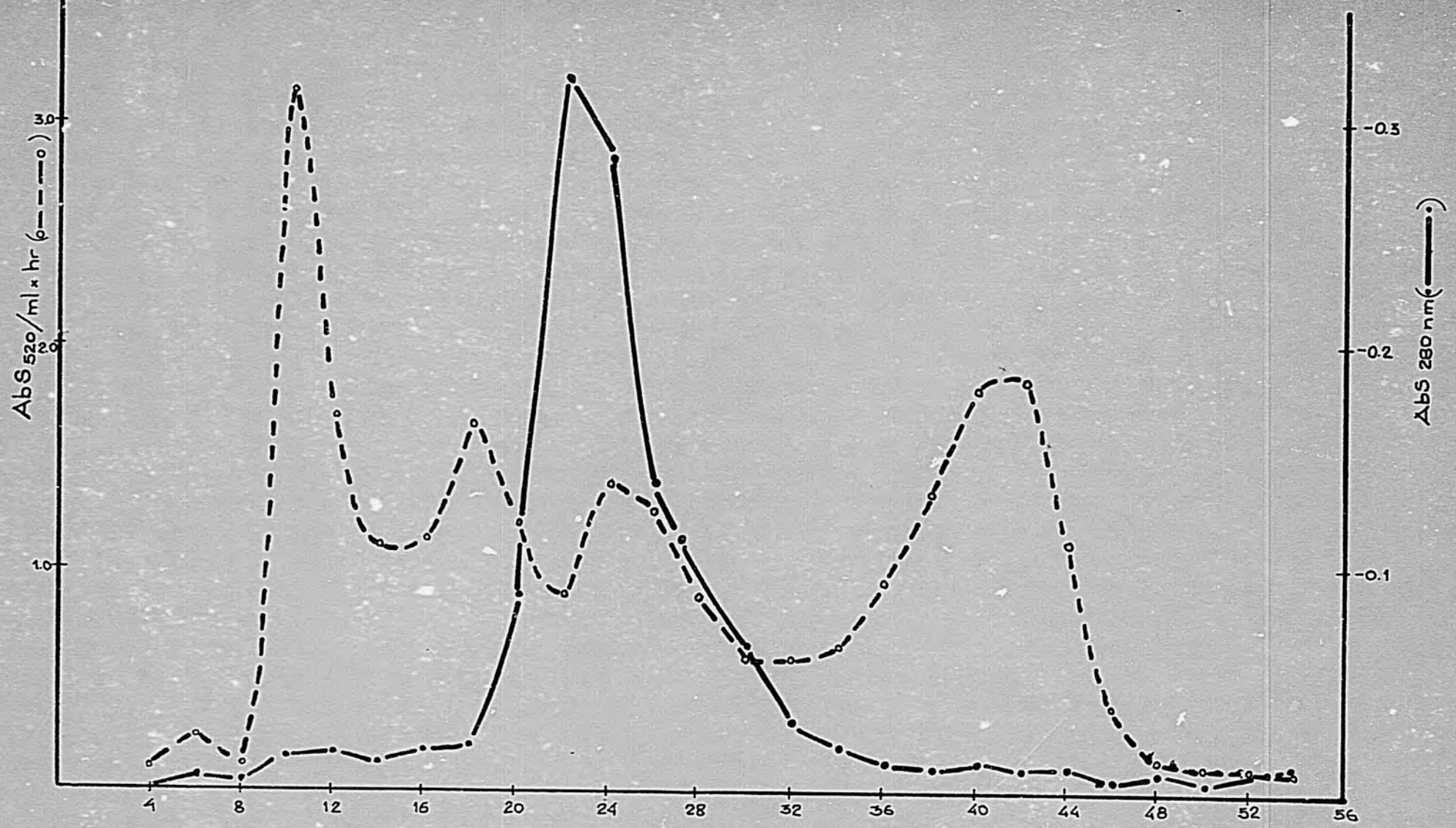


FIGURE 7

Fasciola hepatica S_1 fraction was loaded on an AcA 54 column; eluting buffer, 0.2 M Sodium citrate, pH 4.9. Proteolytic activity was measured using Azocoll as substrate; protein was determined as absorbance at 280 nm.

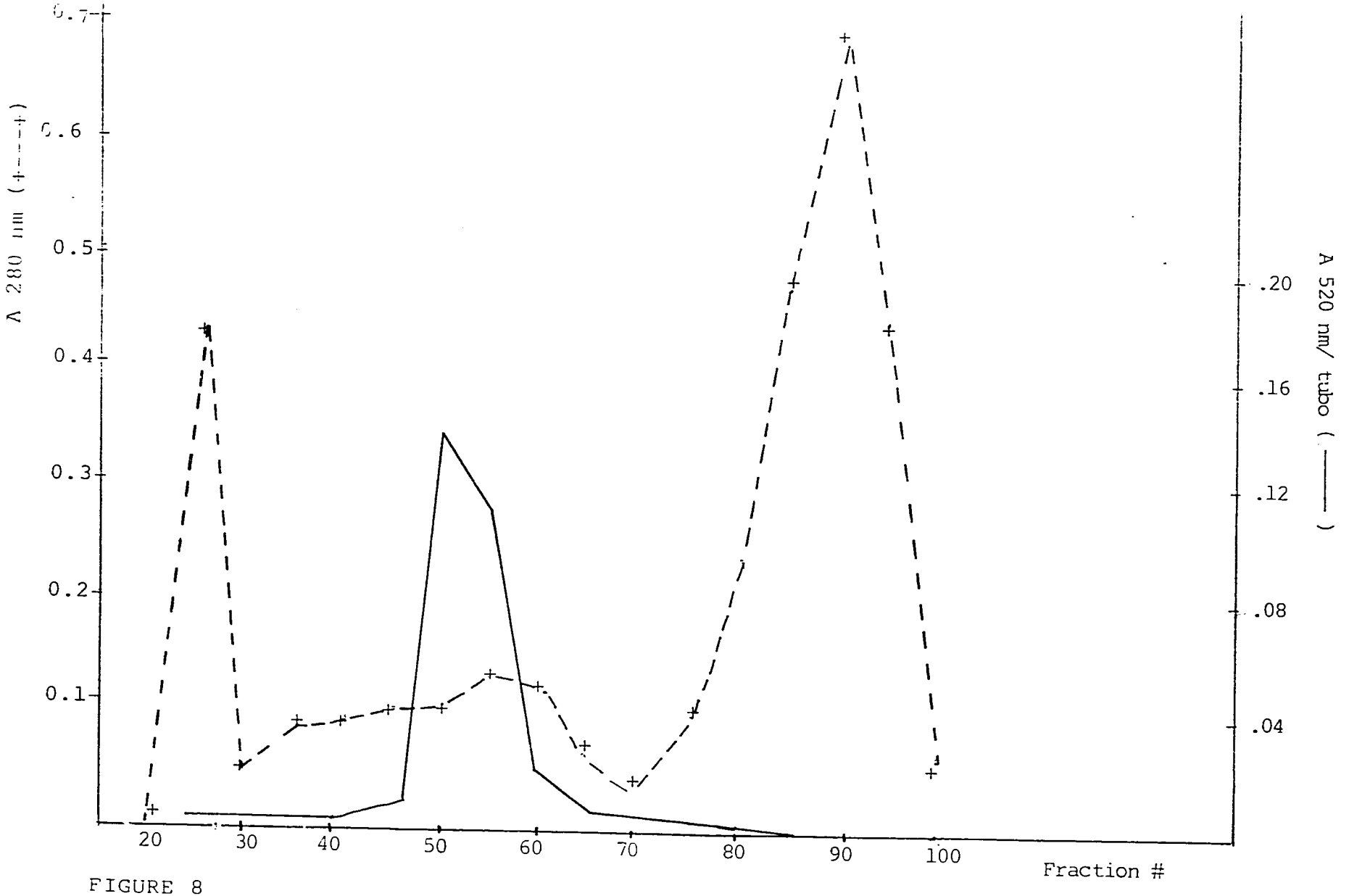
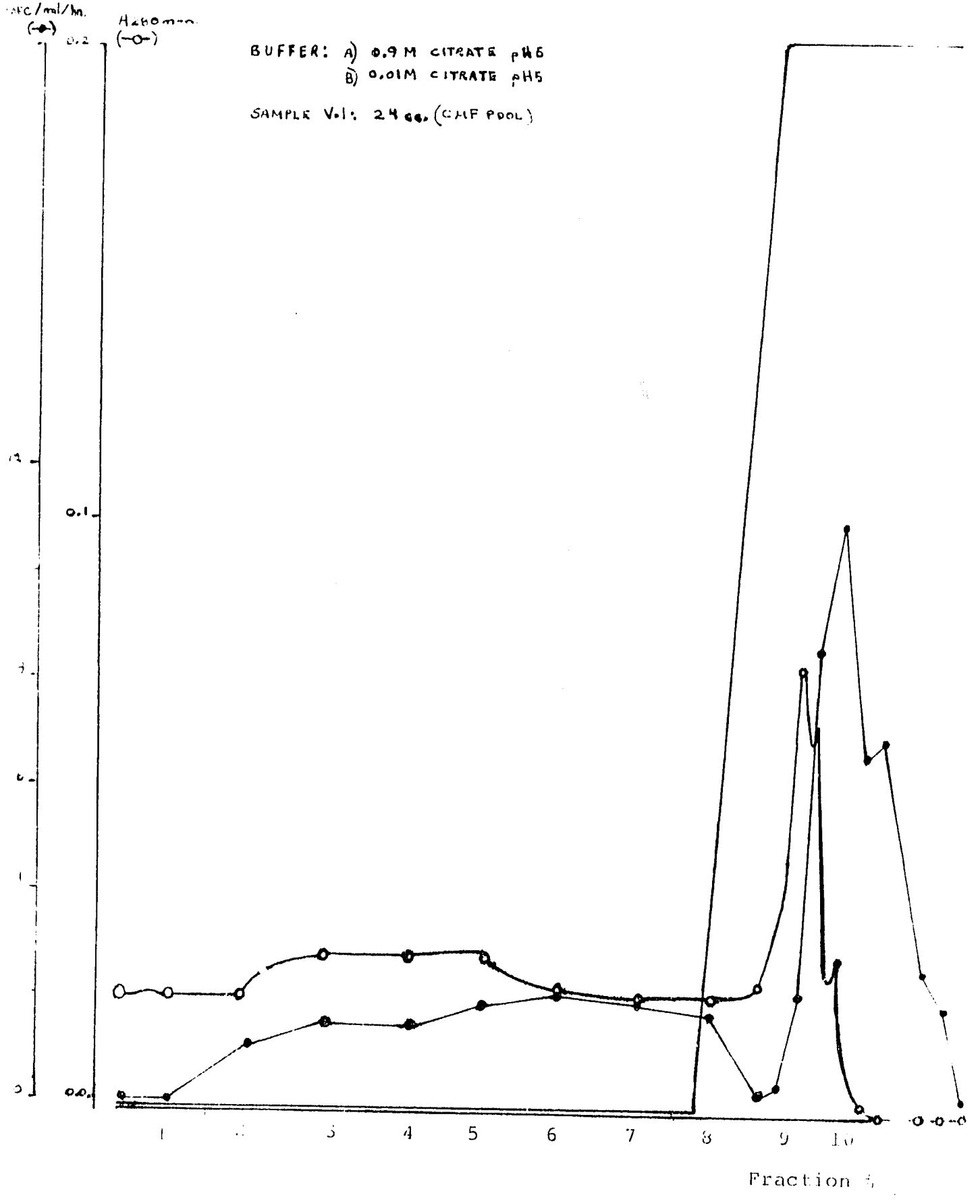


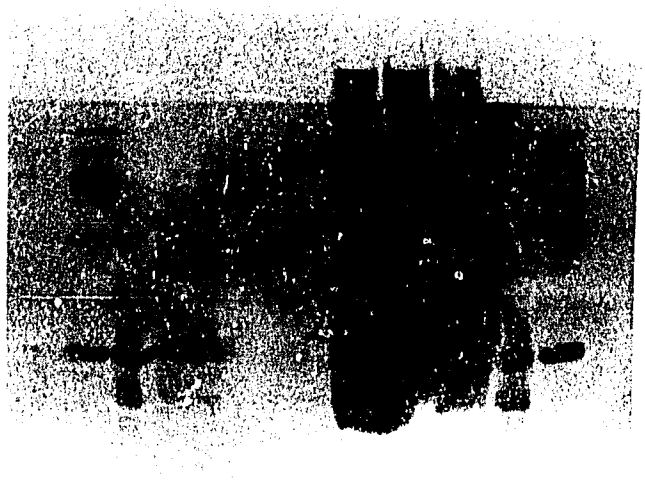
FIGURE 8

Fasciola hepatica E/S products were loaded on a Sephadex G-100 column, using 0.2 M Sodium citrate, pH 5.0 as eluting buffer. Protelytic activity was measured using Azocoll as substrate. Protein was determined as absorbance at 280 nm.

FIGURE 9

HPLC-Hydrophobic interaction chromatography (HIC): Polypropyl (C_3) column.





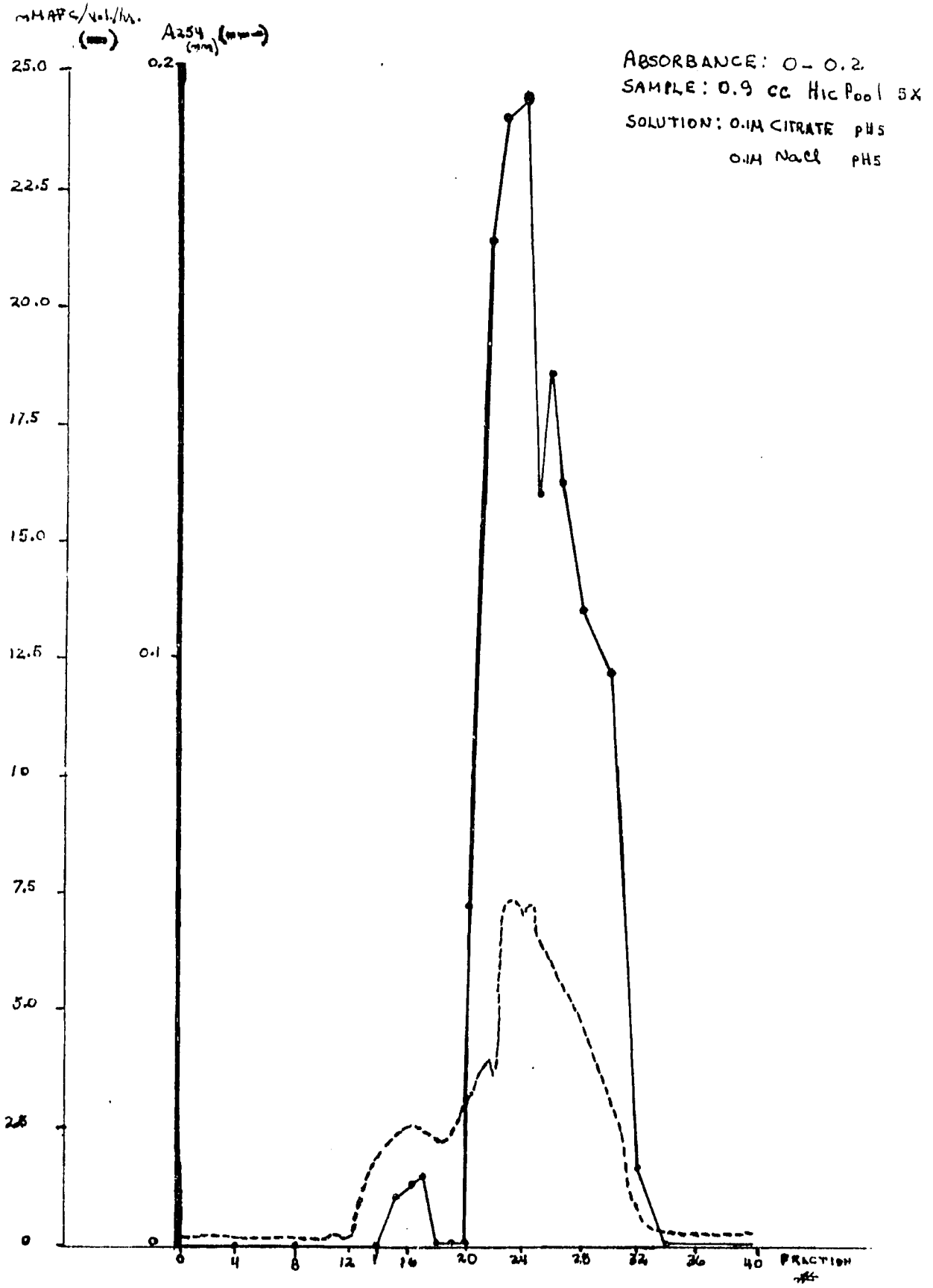
a b c d e f g h i j

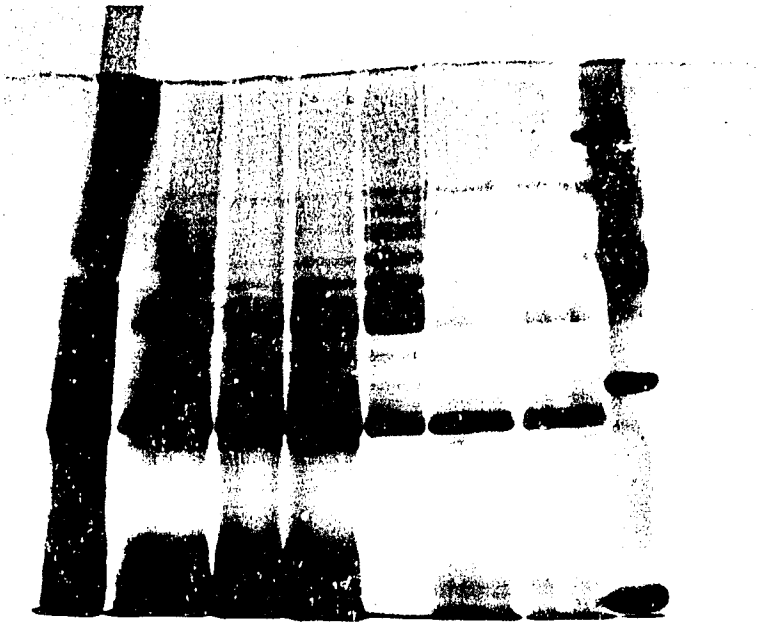
FIGURE 10

15% SDS PAGE of Fasciola hepatica fractions. A and B; molecular weight standards (MWS); C: liophilyzed HIC active peak; D: HIC tube 17; E: pre-HIC, chromatofocusing active peak (CMF); F: pre-CMF column; G: pellet; H: homogenate; I and J: MWS.

FIGURE 11

HPLC-Gel filtration: Protein pack 125





A B C D E F G H

FIGURE 12

12.5% SDS-PAGE. Fasciola hepatica S_1 fraction was loaded on HIC, and the a PPK column. A: whole homogenate; B: precolumn; C: HIC active pool; D: pre-PPK column; E: PPK-Pool 1; F: PPK-Pool 2; G: PPK-tube 24; G: molecular weight standards.

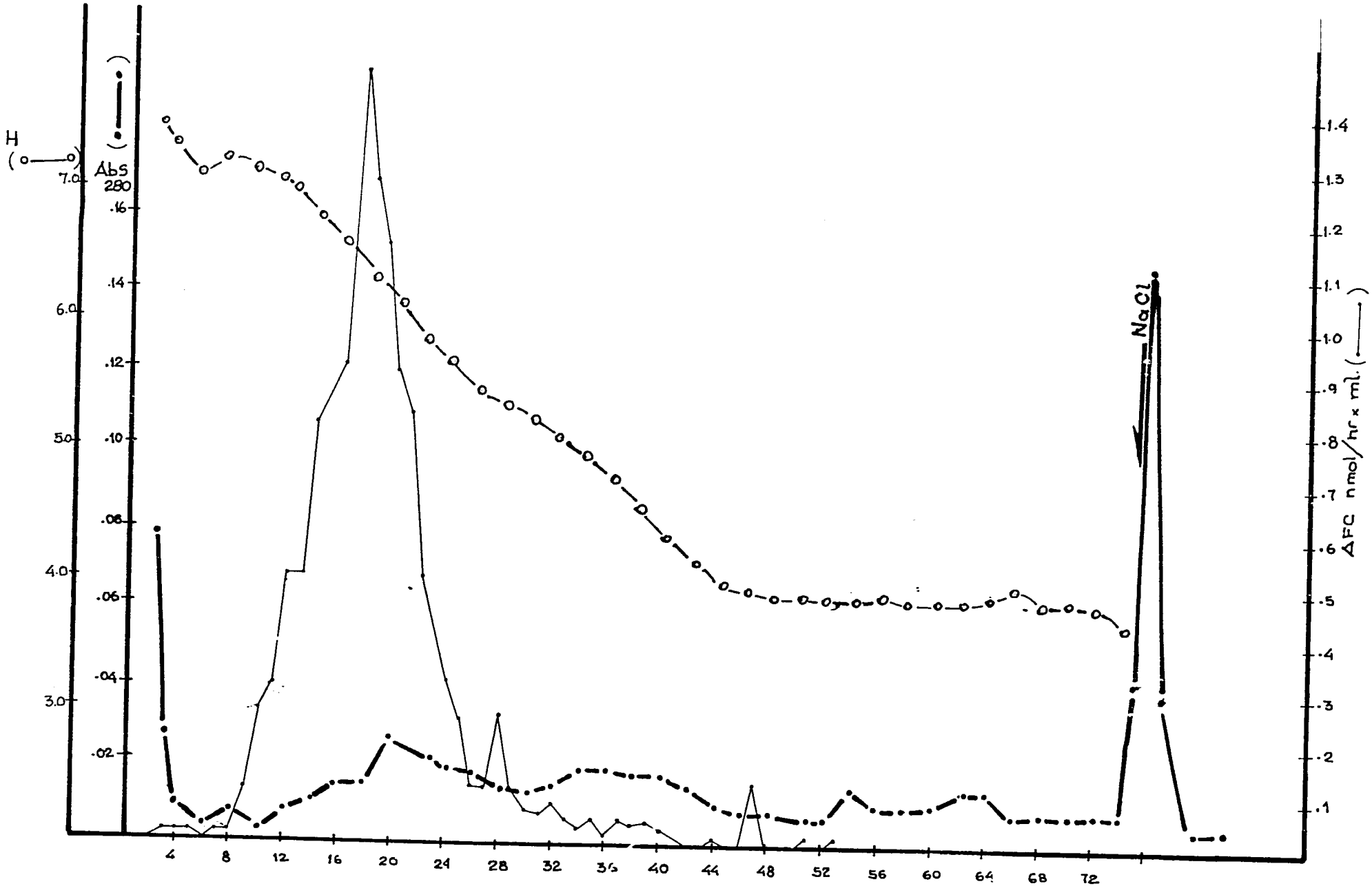


FIGURE 13

Chromatofocusing of Fasciola hepatica protease(s). Active peak eluted from an AcA 54 column was concentrated and loaded on CF column. Proteins were measured as absorbance at 280 nm; proteolytic activity was determined using CBZ-Phe-Arg-AFC as substrate; a pH gradient was formed (o-o-o)

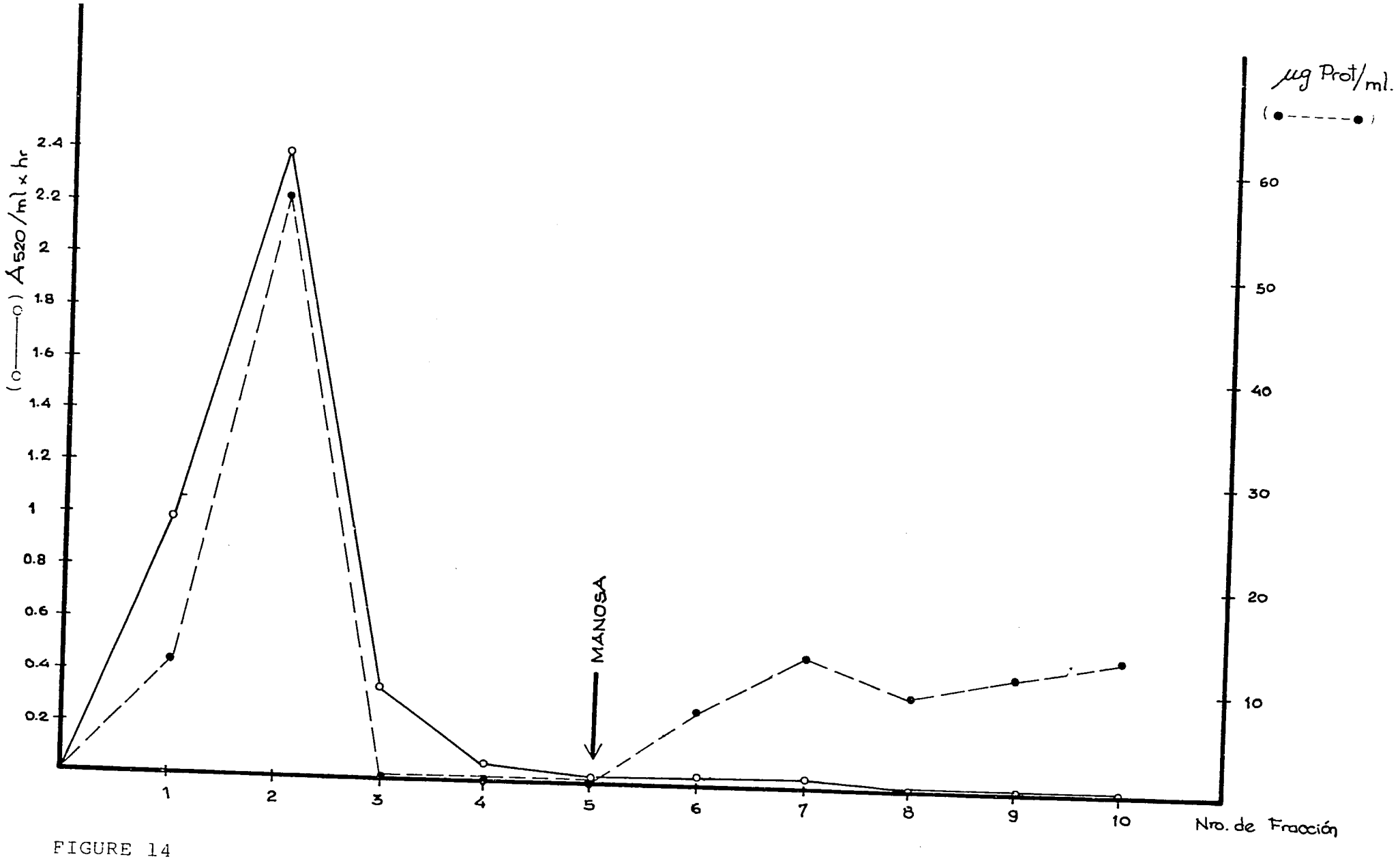


FIGURE 14

Fasciola hepatica supernatant (S_1) was loaded on an Lentil-lectin column (V_t= 0.5 ml) and eluted with appropiate buffer: fractions 1-5; then D-mannose was added. Proteins were measured using Lowry technique , and proteolytic activity was determined using Azocoll as substrate.

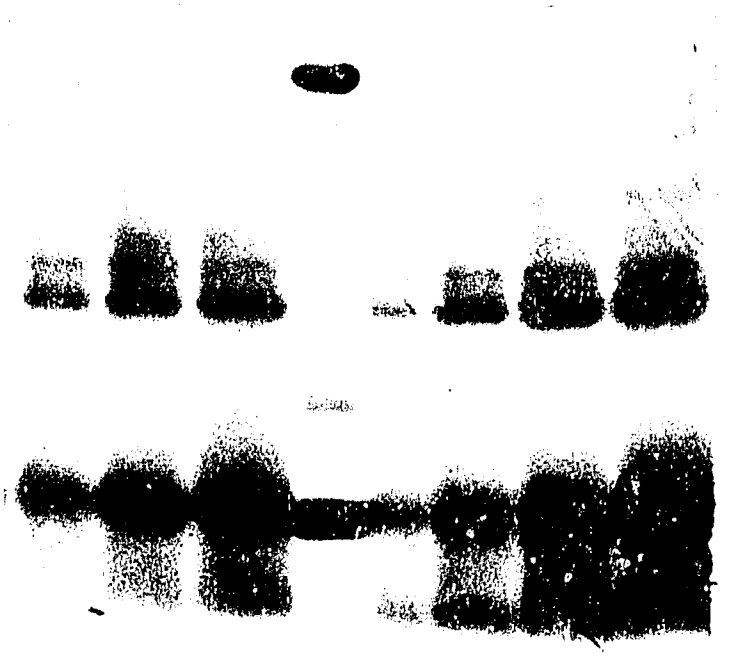


FIGURE 15

15% SDS PAGE of Fasciola hepatica homogenate and S_1 fraction.

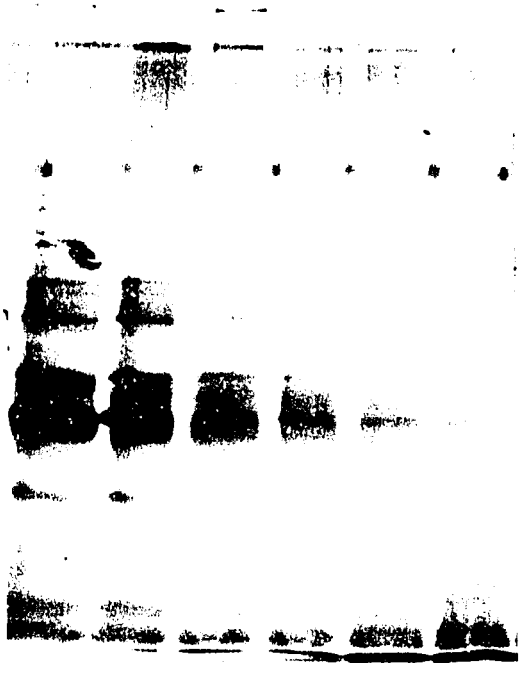


FIGURE 16

15% SDS PAGE of *Fasciola hepatica* AcA 54 active fractions

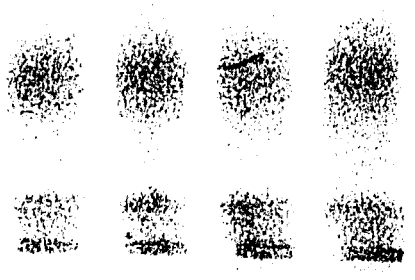


FIGURE 18

Acetate cellulose electrophoresis under non-denaturalizing conditions. Fasciola hepatica homogenate was loaded at different concentrations on an acetate cellulose sheet. See Methods for details.

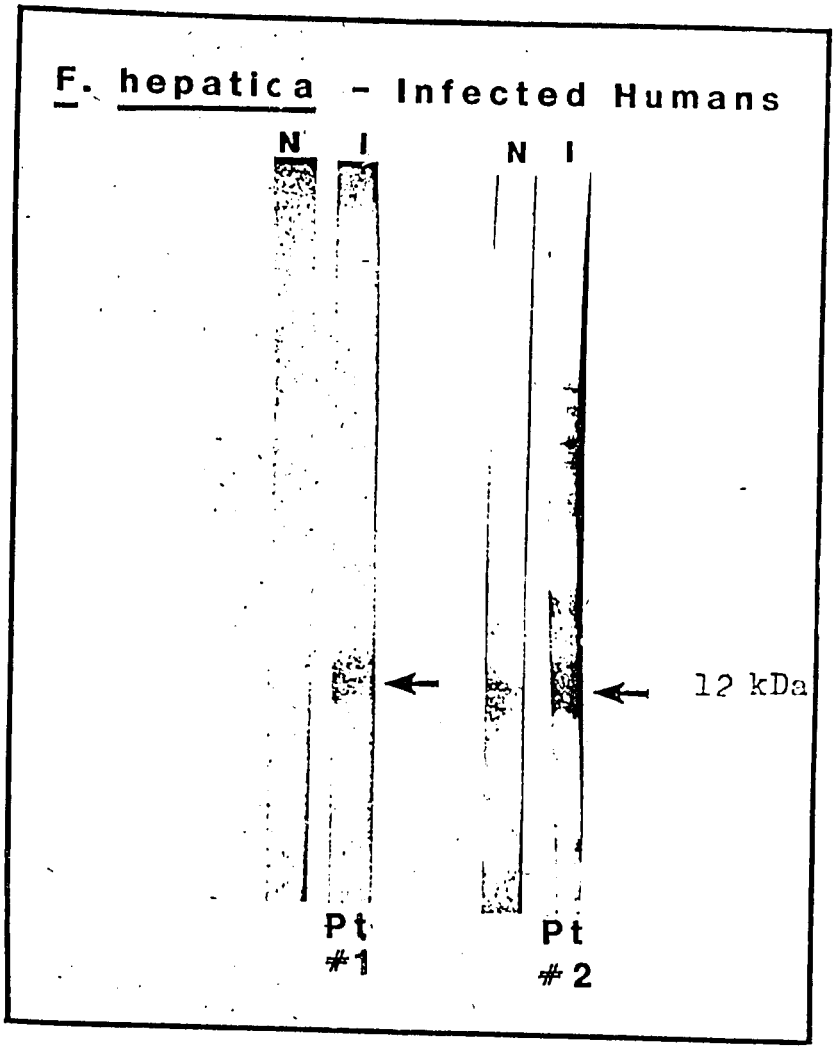


FIGURE 19

Immonogenicity of fascioliasis patients using western blot technique. Enzyme preparation on an SDS-15 % PAGE was transfered to the NC sheet and reactivity was determined using anti-human Ig-peroxidase conjugate.

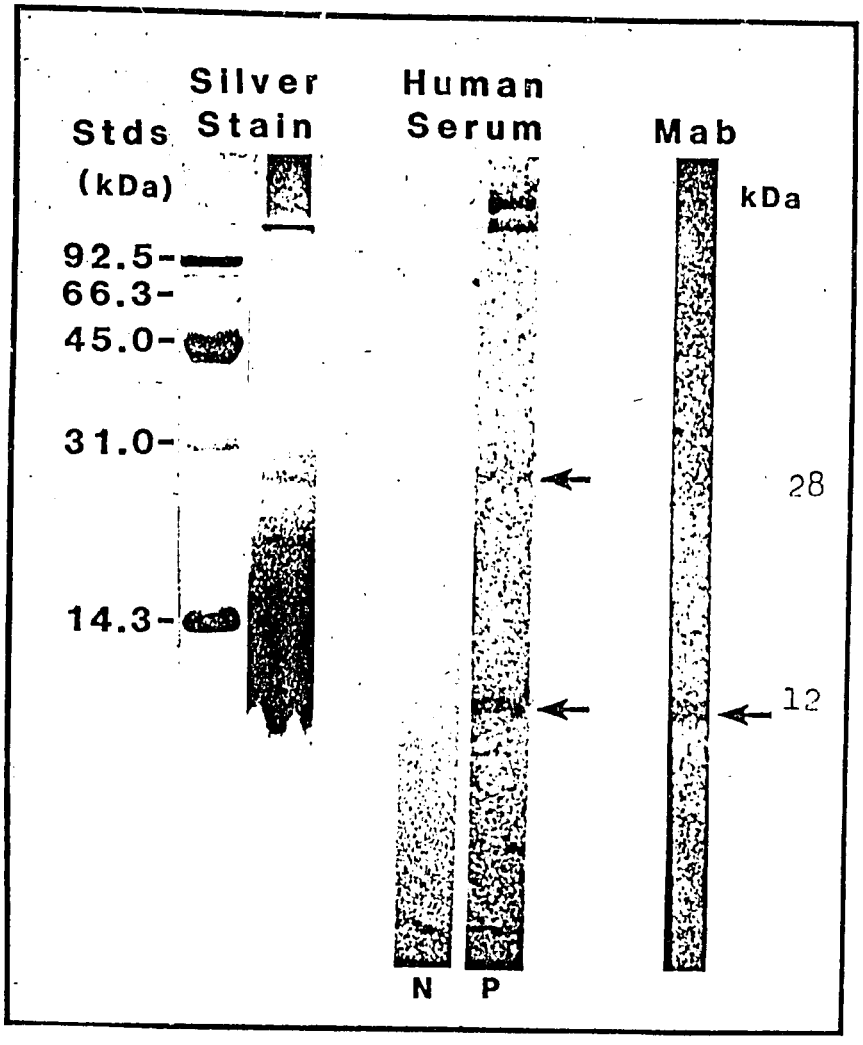
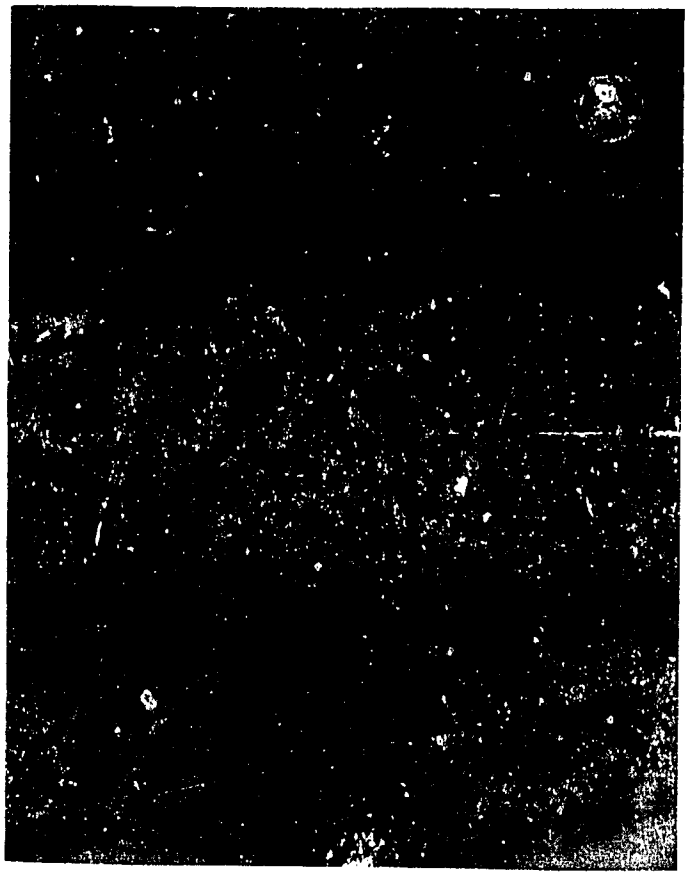
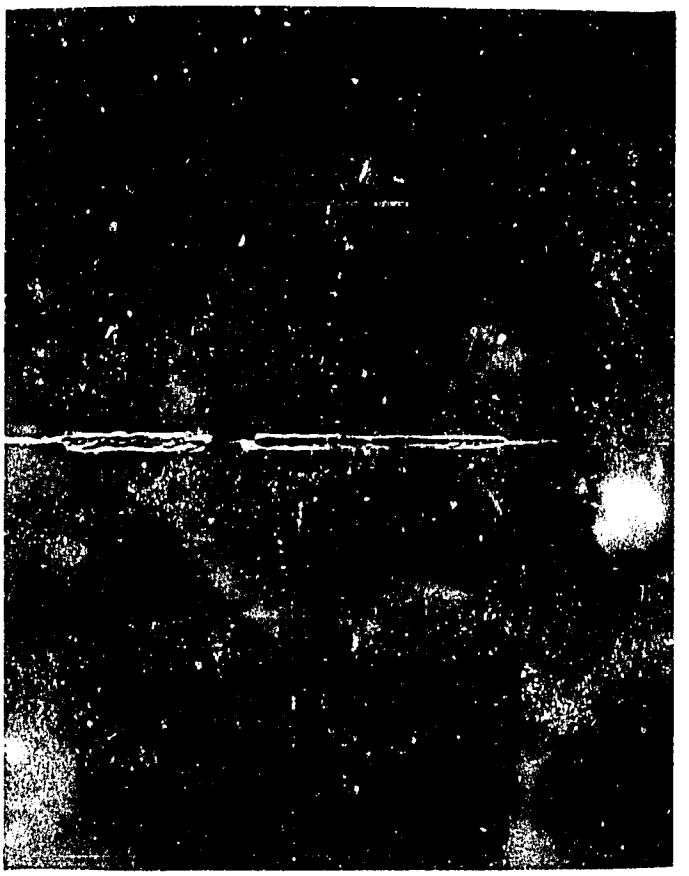


FIGURE 20

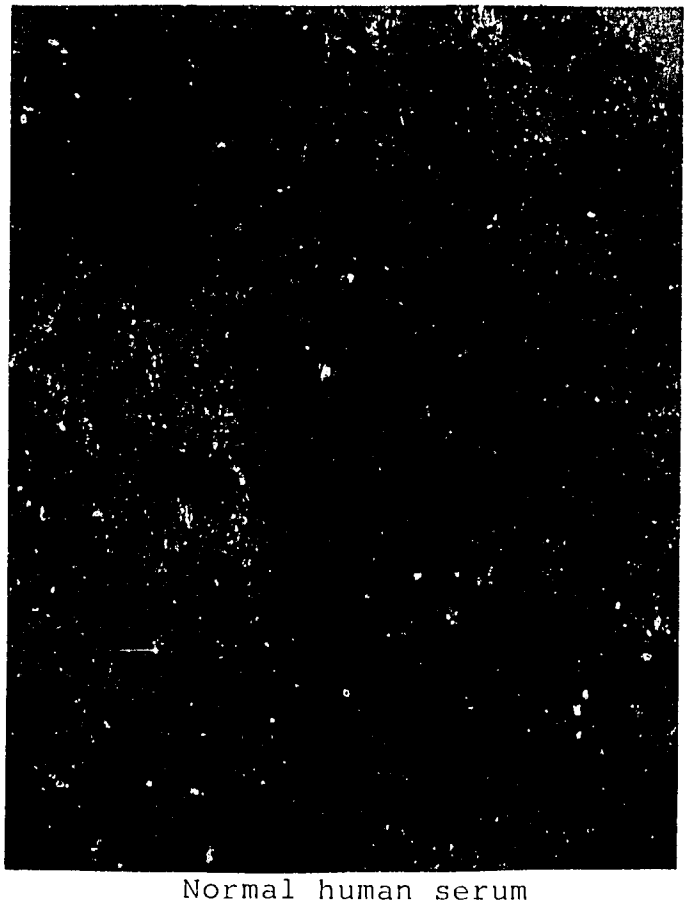
Immunogenicity of Fasciola hepatica proteinase in infected human serum and monoclonal antibody (MAb) in nitrocellulose (western blot): Enzyme preparation on a SDS-15 % PAGE was transferred to NC and reactivity was determined using the proper peroxidase conjugate.



Normal mouse serum: negative



Immunized mouse serum: gut fluorescence



Normal human serum

Infected human serum

FIGURE 21.- Indirect fluorescence: *Fasciola hepatica* sections.

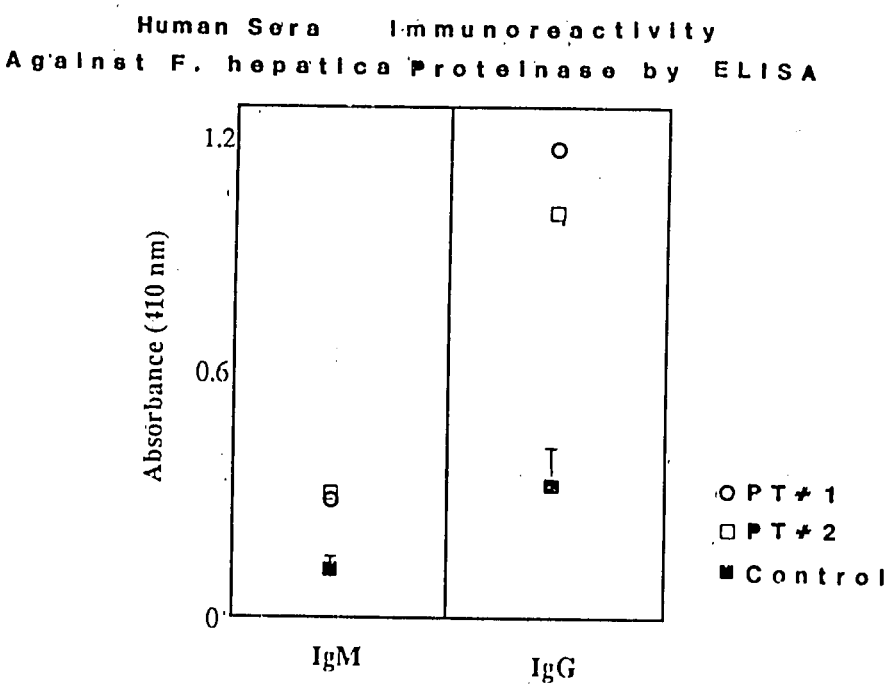


FIGURE 22

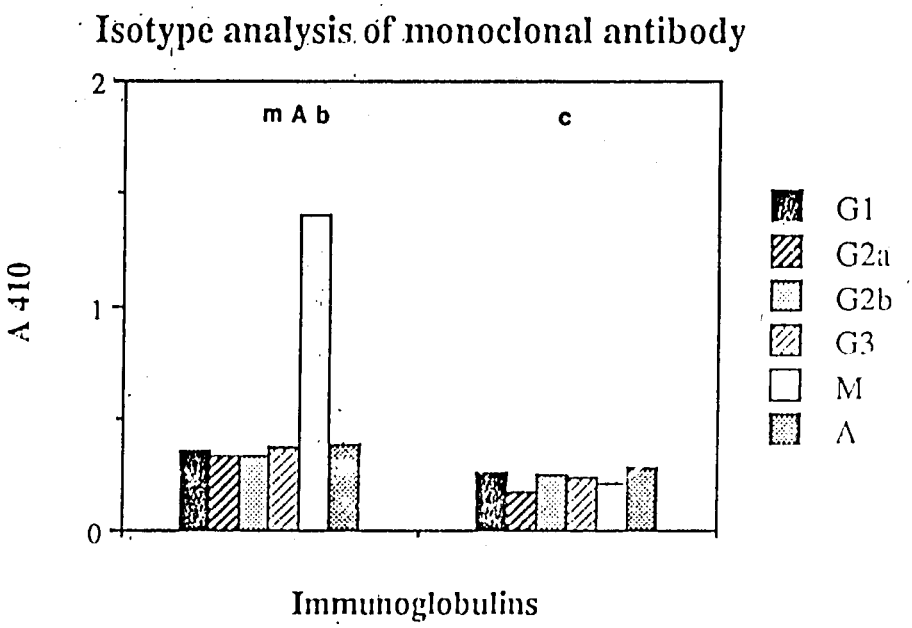


FIGURE 23

SUMMARY

Proteinase(s) from *Fasciola hepatica*

- Apparent molecular weight

  Native: 19.3 kDa, 9.8 kDa

  SDS-PAGE: 28 kDa, 12 kDa
- Immunogenic in infected animals/humans
- Developed monoclonal antibody (murine)
- Future experiments directed toward serodiagnosis