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Proteolytic Enzymes: Evaluation as Purified Antigens in Diagnosis of Fascioliasis in Perú

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3) EXECUTIVE SUMMARY

Control of cattle and human fascioliasis is desirable, to avoid the serious economic losses and damage to human health which it causes in many rural areas of Perú and the World. The worms mature and begin to produce eggs only 50-90 days after infection; meanwhile, diagnosis depends on immunologic means. The antigens studied through this project would allow effective, early, sensitive diagnosis and treatment of affected cattle and humans, and could serve to keep this parasitosis under control. The project chose to purify proteolytic enzymes, needed by the worms for invasion and nutrition, thus important from the beginning and for the duration of the disease, as probably good marker antigens, able to indicate and accurately reflect the course of infection.

High proteolytic activity is found in the secreted/excreted products of the adult worms, and in extracts of adults and of the invasive larval forms, the metacercariæ. The activity is due to a battery of cysteine proteinases, located in the gut wall and lumen of the cæcæ of the adult parasite. Since these proteinases are involved in the nutrition of the adult parasite, they provide constant antigenic stimuli to the parasitized host. Three of these enzymes: Fas1, Fas2 and a 14.5 kDa cysteine proteinase were purified from adult worms and from their secretion/excretion products, and characterized biochemically. Fas 1 and Fas 2 are 25 kDa cysteine proteinases with different isoelectric points; both lyse hemoglobin, collagen, casein, and albumin, and Fas 2 also lysed elastin. Fas 1 and Fas 2 were precipitated by the sera of infected patients. This work showed identity of the Arc 2 precipitate band of the traditional, highly specific immunoelectrophoresis test with the Fas 1- and Fas 2-Antibody precipitates, confirmed using the radioiodinated cysteine proteinase-specific affinity inhibitor Z-(^{125}I)Tyr-Ala-CHN_2. Fas1 and Fas2 used in the Dot-ELISA format provided a sensitive assay for infected humans and experimentally infected rabbits. The Fas 1 Dot-ELISA test is a good candidate for diagnosis of chronic human fascioliasis, where as a rule there are low levels of specific circulating antibodies, usually failing to detect crude antigens in the immunoelectrophoresis tests. The purified antigens, specially Fas2, in a simple and inexpensive Dot-ELISA format may be best for reliable immunodiagnosis of human and cattle fascioliasis.

Scientific papers and 3 Theses were produced. Research in the subject began as a collaboration between two Tropical Medicine Institutes in Perú and a Biochemistry Laboratory in the U.S.A.. Dr. Dresden, leader of the U.S.A. lab, died in 1990, but this research has continued to be pursued by the Peruvian groups after his death, and the technology developed is being actively used. Four former student-researchers are now teaching staff members of the Peruvian Universities; three staff researchers (2 former student-researchers) pursue Doctorates, two in Canada, one in England.

4) RESEARCH OBJECTIVES

This research project aimed specifically at providing a means for early diagnosis of *Fasciola hepatica* infection in humans and economically valuable domestic animals, through the application of modern molecular biology. It was designed to provide important hands-on experience to Peruvian investigators in the use of biochemical technology, valuable in the study and control not only of fascioliasis, but many other parasitic diseases endemic in Perú, more specifically those caused by helminths (worms). The emphasis is in diagnosis and in the approach of using purified antigens in serodiagnosis of helminthic (worm) diseases.

Diagnosis of early infection by *Fasciola hepatica* is particularly important because of the damage which may be sustained before diagnosis is possible. The encysted larvae ingested with food (watercress and other similar plants growing in flooded areas) hatch in the intestine freeing the active juvenile worms, called metacercaria attach themselves to the intestinal wall, penetrate it and migrate to the liver, where they develop into the adult form. Tissue damage can obviously be very great, as it is proportional to the parasite load. In the case of small animals, even sometimes sheep and cattle, and more exceptionally humans, it may cause severe disease and even death. Diagnosis based on the traditional means, search for parasite eggs in feces, is impossible in the acute phase, because the worms produce no eggs until they mature, in from 50 to 90 days. Meanwhile, the developing worms continue to grow and produce more extensive tissue damage. The liver ducts, where most of the worms establish themselves, may become obstructed and produce additional complications, such as acute abdominal pain, often needing surgery in the case of humans.

We chose to concentrate in obtaining pure proteolytic enzymes, convinced that their role in the penetration of tissue and in nutrition of the worms is central to the whole survival of the parasite. This is in contrast to the work performed in other laboratories, where parasite extracts have been used to establish empiric serological tools. The procedure for the extraction of the two main 25 kDa cisteinyl proteinases, Fas1 and Fas2, has been improved and streamlined; their characterization was performed using diverse substrates and inhibitors, and their active sites determined.

The most specific of the empiric serological means, the determination of Arc 2 through immunoelectrophoresis, was shown to depend precisely on the proteolytic enzymes we studied, Fas1 and Fas2. One such cisteinyl proteinase from *Fasciola hepatica* has been cloned and sequenced already, in at least one laboratory (Yamasaki *et al.*, 1993). This opens the way to the production of artificial antigens, which would have all the advantages of the natural ones, but which would not depend on the obtention of the adult worms from infected livers.

The Fas1 and Fas2 cisteinyl proteinases offer great promise, since they are excellent in both sensitivity and specificity in the inexpensive Dot-ELISA format. Other antigens proposed for early diagnosis are crude and undefined, such as excreted/secreted products or somatic extracts (Cornelissen *et al.*, 1992), difficult to produce, such as the f2 specific antigen, which is recovered from electrophoresis runs (Levieux *et al.*, 1992) or require complex, sophisticated and expensive immunologic formats such as Western Blot (el Bahi *et al.*, 1992).

There is a 17 kDa antigen (Hillyer, 1990) which has been used in several formats, and apparently is similar in how early and specifically it could provide diagnosis of fascioliasis. He has also been working on variations of the ELISA for early diagnosis (Hillyer, 1991). In 1993, during a Congress of the Latin American Federation of Parasitologists, Dr. Hillyer visited us, and there is some possibility of future collaboration.

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5) METHODS AND RESULTS

I. PURIFICATION OF 14.5 kDa CYSTEINE PROTEINASE FROM *Fasciola Hepatica* ADULT WORMS.

A. Enzyme Extraction : Adult *F. hepatica* worms were isolated from naturally infected bovine livers ol from abattoirs in either Lima, Perú, or Houston, Texas. For enzyme extraction lyophiliz orms were resuspended in sodium citrate 0.2 M buffer containing 1 mM $HgCl_2$ at pH 5, and homogenized in a glass-Teflon homogenizer. The homogenate was centrifuged at 48,000 x g for 20 min at 4°C. After centrifugation, the supernatant and pellet fractions were separated and assayed for enzyme activity.

The activity was assayed using CBZ-Phe-Arg-AFC as substrate in 0.1 M sodium citrate, pH 5.5. After incubation at 37°C the release of the fluorescent group (AFC) was measured in a fluorometer. Measurement of general proteolytic activity was performed using Azocoll as substrate.

B.- Purification of 14.5 kDa cysteine proteinase from *F. hepatica*.

The supernatant fraction was subjected to HPLC at room temperature. The first step in purification was cation exchange chromatography using a Mono S column equilibrated with 10 mM Na-citrate, pH 5.0, containing 0.1 mM $HgCl_2$ and 1 M urea. Bound proteins were eluted with a linear gradient of 0-1.0 M NaCl dissolved in the equilibration buffer. Active fractions (1 mL) were pooled and subjected to molecular sieve HPLC.

For molecular sieve HPLC, a serial combination of two Protein Pak-124 and one Protein Pak-60 was used to produce maximal resolution of proteins over a range of 80 to 1 kDa. Samples were eluted with 10 mM Na-citrate, 0.1 mM $HgCl_2$ and 1 M urea. The native molecular weight of the proteinase was determined after calibration of the columns with proteins of known molecular weight.

Purification of *F. hepatica* proteinase using Mono S cation exchange HPLC yielded a single peak that eluted with approximately 0.5 M NaCl. Approximately 65-70 % of the activity and 19 % of the protein applied to the column was recovered in the purified fraction, resulting in a 3-fold increase in specific activity (Table 1). Active fractions from cation exchange chromatography were combined and subjected to molecular sieve HPLC. The major cysteine proteinase activity eluted as a single peak at approximately 14,500 kDa resulting in a 200-fold increase in specific activity.

SDS-PAGE according to Laemmli (1) modified by Studier (2) was employed to assess the purity of the samples at each step (Fig. 1). The purity of the protein obtained by molecular sieve HPLC was further confirmed by two-dimensional gel electrophoresis (Fig. 2). The enzyme migrated as a single band with molecular weight at 14.5 kDa.

C.- Biochemical Characterization:

Kinetic parameters of the purified enzyme were studied using CBZ-Phe-Arg-AFC as substrate. The proteinase appeared to follow typical Michaelis-Menten kinetics, giving an apparent Km of 15.54 μM, Kcat of 6.93×10^{-3} $mols^{-1}$, Kcat/Km of 0.45 $mM^{-1}s^{-1}$ under the conditions used.

Table 2 shows a comparison of specific activities using different synthetic peptide substrates. Of the substrates tested, CBZ-Phe-Arg-AFC was the most suitable. The use of specific proteinase inhibitors allowed assignment of the acidic proteinase to the class of cysteine proteinases. The activity was not inhibited by specific proteinase inhibitors as seen in Table 3.

Total inhibition of activity against CBZ-Phe-Arg-AFC was seen with the specific proteinase inhibitors Ep459, leupeptin and iodoacetic acid.

II. PURIFICATION OF 26 kDa CYSTEINE PROTEINASES: FAS1 AND FAS2.

A. Enzyme purification.

Adult *Fasciola hepatica* worms were isolated from naturally infected bovine livers obtained from abattoirs in Lima, Perú. Once in the laboratory, worms were transferred to cold distilled water in order to obtain the excretion/secretion (E/S) products .

Lyophilized E/S products were resuspended in 0.2 M sodium citrate buffer pH 5 and then cold ethanol (98 %) was added to a final concentration of 60 % and incubated 15 min at 4°C, and then centrifuged a 12,100 x g for 10 min at 4°C.

The post-dialysis supernatant was fractionated in a CM-Sephadex C-50 and bound proteins were eluted with a linear gradient 0 - 1M NaCl in 10mM sodium citrate buffer containing 1mM $HgCl_2$ pH 5.

Results of the protein purification are shown in Table 4. The chromatography step yielded two peaks of activity, one on the non-bound proteins region (Peak I), and the other eluted only when the gradient was applied to the column (Peak II). (Fig. 3).

SDS-PAGE was employed to assess the purity of the samples at each step. Peak I migrated as a single protein band corresponding approximately to 26 kDa, whereas Peak II presented an additional band of lower molecular weight (Fig. 4).

B. Kinetic Studies

For the kinetic studies Peak I was used since it migrated as a single protein in SDS-PAGE. The molar concentration of the enzyme was obtained by titration with Leupeptin and E-64.

The enzyme showed a Michaelis-Menten kinetics with the substrates of Table 5. The enzyme showed activity with substrates of the form Ac-X-Arg-pNa, when in the P_2 position were bulky residues such Phe, Leu and little activity with a charged residue such as Asp (less than 5 % from above), and it is inactive with residues such as Ala, Asn, Gln, Glu or Gly in the P_2 position (Table 6).

The enzyme is inactivated by peptidyl diazomethylketones following a pseudo-first order kinetics of inactivation as is seen in Figure 4. The inhibitors Z-Phe-Ala-CHN_2, Z-Phe-Phe-CHN_2 and Z-Phe-Tyr-CHN_2 inactivate the enzyme with kapp of 1,252, 1,696 and 1,428 $M^{-1}s^{-1}$ respectively, while Bz-Phe-Val-CHN_2 does so with 516 $M^{-1}s^{-1}$.

The pure enzyme digested extensively casein, native bovine serum albumin, and hemoglobin at pH 7 into small fragments not visualized in a 15 % SDS-Polyacrylamide gels. This activity was completely inhibited by 1 μM E-64. The enzyme detached fibroblasts in vitro.

III. EFFECT OF SDS AND BILE SALTS ON PROTEOLYTIC ACTIVITY IN *Fasciola hepatica* ADULT WORMS

A. Preparation of *Fasciola hepatica* cysteine proteinases

Adult worms collected from abattoirs in Lima, Perú were lyophilized and homogenized in 0.2 M sodium citrate, pH 4.9, containing 0.1 mM $HgCl_2$ and 1 M urea. The homogenate was centrifuged at 48,000 x g for 20 minutes at 4°C. The supernatant fraction was used to purify the major cysteine proteinase activity.

This fraction was subjected to cation exchange chromatography using CM-Sephadex. Bound proteins were eluted with a linear gradient of 0-1 M NaCl in 10 mM sodium citrate, pH 4.9, containing 0.1 mM $HgCl_2$ and 1 M urea (equilibration buffer). Absorbance at 280 nm and CBZ-Phe-Arg-AFC activity were measured in each 1 mL fraction (Fig. 5). Active fractions were pooled, dialyzed, lyophilized and stored at -20°C until used.

B. Enzyme Assays

Cysteine proteinase activity was measured at pH 7.0 by two assays. The first assay use the synthetic peptide CBZ-Phe-Arg-AFC and the second assay used Azocoll. Protein concentrations were measured according to the method of Bradford (3).

For activation assays, the proteinase samples were incubated at 37°C in 50 mM Tris-HCl, pH 7, containing 5 mM DTT and various concentrations of SDS and bile salts and 5 µL aliquots were taken to measure proteolytic activity.

C. Gel electrophoresis and autoradiography

Activation assays were followed using the radiolabelled peptide protocol of Mason *et al* (4). Samples of the semipurified enzyme were activated using various concentrations of SDS, and then incubated for 30 min at 37°C with the specific radiolabelled inhibitor, CBZ-(^{125}I)Tyr-Ala-CHN_2. Controls were incubated with 1 µM E-64 and then with the iodinated inhibitor.

After the incubation, a SDS-12.5 % Polyacrylamide gel electrophoresis was performed as described by Studier (2). Autoradiography of iodinated proteins was done with an X-ray film exposed at -70°C with an intensifying screen.

Preparations of partially purified cysteine proteinases of *F. hepatica* were activated by sodium dodecyl sulfate since a significant increase in proteolytic activity was seen using either Azocoll or CBZ-Phe-Arg-AFC as a substrate (Fig.6).

Activity increased as a function of the SDS concentration, with the highest activation at 0.05 % SDS; however, the enzyme was still active at concentrations as high as 0.2-5 % SDS. Activation is dependent on the presence of a reducing agent such as DTT, since preincubation of proteinases with SDS alone does not produce the increase in activity. Furthermore, activity in nonactivated, as well as SDS activated samples, belonged to the cysteine proteinase class, since both were completely inhibited by E-64, while PMSF, pepstatin and EDTA had no inhibitory effect (Table 7).

Activation was time dependent (Fig.7). Maximum activation ocurred in one hour of preincubation at 37oC, with only 8 % of maximum activation obtained within 30 minutes. The SDS-stimulated proteinase activity was stable for at least 3 hours.

Figure 8 shows that the enzymes were active in a broad pH range from 4.5 to 8, with maximum activity at pH 5.0 and 7.0. Furthermore, the increase in activity seen with addition of SDS was not due to a change in pH optimum.

Cysteine proteinase activity is also increased by bile salts conjugates (Fig. 9a and Fig. 9b). We used conjugated bile salts, since the non conjugated molecules are at low concentration in the biliary contents. We found that for glycine and taurine conjugates of chenodeoxycholic and deoxycholic acids the highest increase of activity (150 %) was obtained with a concentration of 0.25 %. Using conjugates of cholic acid, the highest activation was obtained with a concentration of 0.5 %, resulting in an increase of 150 % and 95% for glycine and taurine conjugates, respectively.

Figure 10 shows the identification of cysteine proteinases using an active site-specific radiolabelled inhibitor, CBZ-(^{125}I)Tyr-Ala-CHN$_2$. The nonactivated sample (lane a) showed 4 major bands with approximate molecular weights of 36, 34, 32 and 28 kDa.

Labelling of SDS-activated samples is shown in lanes "b" to "h". This was especially true with the 28 kDa band, which consistently showed maximum incorporation between 0.05 and 0.1 % SDS.

IV. *Fasciola hepatica* CYSTEINE PROTEINASES AS MARKERS OF HUMAN AND EXPERIMENTAL RABBIT FASCIOLIASIS.

A. Immunoprecipitation of CBZ-(^{125}I)Tyr-Ala-CHN$_2$ active site affinity labelled cysteine proteinases.

The peptide CBZ-(^{125}I)Tyr-Ala-CHN$_2$, a specific inhibitor for cysteine proteinases was radiolabelled according to the methodology described by Mason *et al.* (4). Crude antigen prepared according to Capron (5) containing high cysteine proteinase activity was incubated with the radiolabelled peptide. Aliquots of 10 µL of the radiolabelled sample was drawn and poured into the well of the agarose gel for immunoelectrophoresis. Controls were incubated with 1 µM Ep-459 prior to the addition of radiolabelled peptide.

Autoradiography was performed with X-ray film exposed with an intensifying screen.

Sera from positive patients (2) with the diagnosis of chronic fasciolasis by fecal parasitologic analyses and immunoelectrophoresis, and two serum samples from healthy volunteers, and pre-immune rabbitt sera and sera from experimentally infected rabbits at the 24th week of infection were used in this experiment.

Results show that human positive sera and experimental infected rabbit sera (Fig. 11), showed characteristic bands of immunoprecipitation. Inspection of the plates under ultraviolet light showed the immuno precipitation of radiolabelled inhibitor-protein complexes indicating cysteine proteinase(s). No radiolabelled protein was precipitated by the negative human and rabbit sera.

The incorporation of inhibitor is specific because the antigen preparation previously incubated with Ep-459 showed no trace of incorporation (Fig. 12). The configuration of the immunoprecipitated labelled band forms the Arc 2 band, which is a characteristic immunoelectrophoretic pattern for the diagnosis of human fascioliasis as described by Capron (5). An Ouchterlony doble diffusion showed identity of the Arc 2 antigens with the cysteine proteinase.

B. Human Fascioliasis

This work was presented at the 1st Iberoamerican Congress on Biotechnology held in April 1989 in La Habana, Cuba. We have evaluated human sera from other diseases to asses the sensitivity and specificity of the Dot-ELISA test.

The results showed that the Dot-ELISA test using a partially purified preparation of cysteine proteinases from the *F. hepatica* excretion/secretion products has the sensitivity needed for field studies of fascioliasis. It is of particular interest that the partially purified antigen (PPAg) may be sufficiently specific for a clear cut diagnosis as it is shown in Tables 8-11 and Figs. 13-14.

C. Western Blots with sera from different sources.

These experiments were carried out in order to evaluate the antigenicity of the partially purified protease with sera from infected and uninfected animals and humans.

The antigen was prepared using homogenized adult worms fractionated on a Mono S Cation Exchange column as previously described. Bovine, sheep and human sera were used in this experiment.

Gels stained with Coomassie blue showed that the partially purified pool consisted of 3 groups of bands corresponding to 14, 31 and 40 kDa.

The antigen-antibody reaction is present around 28 kDa in all the infected animal and human sera tested. In some infected animal sera there is an antigen-antibody reaction around 42 kDa, which is strong in rabbit and human sera and weak in bovine sera. There is no antigen-antibody reaction around 14 kDa. These experiments were performed with stored lyophilized sera.

D. Field Studies

The main purpose of these studies is to confirm, in the field, the advantages of the Dot-ELISA test in the immunodiagnosis of fascioliasis, using as the antigen a partially purified proteinase preparation.

The chosen place was the village of Haparquilla, 30 km from Cuzco city. Haparquilla has approximately 280 houses with 1,400 inhabitants, more than 50 % between 5 and 15 years old. The houses are built with "adobes" (blocks of mud and straw), and have earthen floors and straw and wooden roofs. Usually 4 to 5 people sleep in one or 2 bedrooms. Domestic animals, mainly dogs, are always present in each house. Tap water is supplied to half of the population. No sewage system or latrines are present in the village. Electricity is available to the population. More than 300 students are enrolled in a primary school. The main activity of the population is cattle-raising and agriculture. The village lies on a flat area with some swamps, and little vegetation. Water-cress and snails of the genus *Lymnea* are present.

It is known in the village that the cattle are highly infected by *Fasciola hepatica*, as was confirmed in the local abattoir, where almost 95% of cattle livers where infected with adult worms.

Two methods were used to determine the rates of human infection:

1. The Rapid Sedimentation Test (a method described by Lumbreras, ref. 6) of the 173 stool samples, looking for *Fasciola hepatica* eggs.

2. Serological tests: Immunoelectrophoresis of 99 samples of blood, for the "arc 2 positive" reaction. Dot-ELISA from sera of 99 samples and blood collected in filter papers.

The results of stool examination of 173 persons detected 19 cases with *Fasciola hepatica*. The rates of infection by intestinal helminths are high: *Ascaris lumbricoides* (81), *Trichuris trichiura* (35), *Hymenolepis nana* (17), *Taenia* sp. (3),. Hookworm (1), *Trychostrongylus* sp. (1). The main pathogenic protozoon detected was *Giardia lamblia* (24). Only 45 people were found free of intestinal parasites. The Dot-ELISA test showed three people with a positive reaction for fascioliasis. The low reaction rate may be due to low worm loads in chronically infected patients or to presence of ingested eggs in the persons studied.

E. Experimental Rabbit Fascioliasis

i - Collection of Metacercaria:

Naturally infected *Lymnea viatrix* were collected (about 2,000) from springs in the Mantaro Valley (a heavily endemic area) in the Peruvian Andes. Snails were carried to Lima and maintained in the animal facility at the Instituto de Medicina Tropical Daniel A. Carrión. Snails, selected according to their size, were placed in glass aquaria, where they were kept for one month with daily changes of deionized water. At that time metacercaria began to appear in the aquarium where the largest snails were kept. Some metacercaria were selected for experimental infection of rabbits.

ii. Experimental rabbit infection:

This was undertaken in order to study the normal immunological response to fascioliasis in an animal model. It is important to assess the different antigenic preparations and the diagnostic procedures.

1. Antigens: In this experiment we used crude antigen and partially purified antigen (PPAg).

2. Hyperimmune serum - Four rabbits were inoculated with crude antigen, and two with partially purified antigen according to an immunization scheme.

All rabbits were bled from the ear vein (approx. 5 mL) before the immunization and sera was collected. Samples were kept at -20°C as negative controls.

3. Animals: Ten 3 month old male rabbits (1.5-2.0 kg body weight) were selected for the experiment. A parasitologic examination and blood samples were taken before the experimental infection. Defined numbers of metacercariae were placed in gelatin capsules; for delivery, the capsule was flushed with saline through a plastic tube placed in the rabbit's esophagus. The rabbits were properly marked and kept in individual cages at the animal house at the Instituto de Medicina Tropical Alexander von Humboldt. The animals were distributed in 4 groups of 3 rabbits; each group received 10, 25 and 50 metacercariae and the control group received none.

Blood samples were obtained from the ear and used for blood smears, which were stained with Wright's stain to test for eosinophilia. Sera were obtained to perform ELISA tests, immunoelectrophoresis, and Western blot analyses. Antigens used were crude E/S products, and partially purified antigen preparations as described above. Stool examinations by the Rapid Sedimentation Test, red cell counts, eosinophilia, and body weight were checked routinely during the course of inefection.

4. Stool Examination. All infected rabbits presented *Fasciola* eggs in their faeces, starting at around 8 weeks (56 days) after inoculation, indicating the success of the infection and the establishment of the chronic phase of the disease.

5. Immunoelectroforesis. It began to show a positive immune reaction at the third week of infection onwards. Up to the 28th week there was no significant decrease in the intensity of the

reaction.

6. Dot-ELISA. It began to show positive reactions from the third week of infection onwards. On the seventh month it started to show a slight decrease in reactivity. Titers with crude antigen preparations were usually higher than with purified antigen.

7. Western Blot. Positive reactions began to appear from the third week of infection onwards; there were bands at 68 kD, 29 kD and 14.3 kD. Up to the 28th week there was no significant decrease in the reaction.

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Purification of cysteine proteinase from *Fasciola hepatica* adult worms

Step	Total Activ*	Recovery %	Total prot	Recovery %	S.A **	Purifict fold
Homog	624		26.0		24.0	
Super	562	90	6.2	24.0	90.6	3.8
MonoS	337	54	1.2	4.6	280.8	11.7
M.Sieve	686	110	0.6	2.3	1143.0	47.6

* nmol AFC/h ** nmol AFC/h x mg protein

TABLE 1

Specific activity of purified proteinase on various peptide substrates

Substrate	Specific activity +/- SEM (nmol AFC /hr x mg protein)
CBZ-Phe-Arg-AFC	396.5 +/- 19.1
CBZ-Arg-Arg-AFC	6.9 +/- 0.5
CBZ-Ala-Arg-Arg-AFC	6.2 +/- 0.4
CBZ-Gly-Arg-Arg-AFC	0
CBZ-Arg-AFC	6.9 +/- 0.5
D-Val-Leu-Arg-AFC	0
L-Leu-AFC	0

Specific activities determined (n=3) with purified enzyme 2 μg) and each substrate at 10 μg/assay. Incubation was for 4 h at 37 C.

TABLE 2

Effect of inhibitors on *F. hepatica* proteinase

Inhibitor	% control activit
None	100
Phenylmethanesulfonylflouride (1 mM)	110 +/- 4
Ethylene-diamine tetracetate (5 mM)	120 +/- 5
Pepstatin (100 µg/ml)	86 +/- 5
$CaCl_2$ (5 mM)	119 +/- 6.5
Leupeptin (2 µM)	5 +/- 1.5
Iodoacetate (1 mM)	0 +/- 0
Ep459 (1 µM)	0 +/- 0

* Purified sample was preincubated with inhibitors 37C for 15 min, and then assayed in 0.1M Na cit pH 5.5, containing 5 mM DTT using CBZ-Phe-Arg as substrate. (n=3)

TABLE 3

Purification of cysteine proteinase from Excretion/Secretion Products of *Fasciola hepatica*

Step	Total Activ*	Recovery %	Total prot	Recovery %	S.A **	Purifict fold
E/S	374.8	100	39.55	100	9.5	1
Super	100.1	26.7	4.2	10.6	23.9	2.5
Peak I	26.7	7.1	0.21	0.53	126.0	13.3

* Abs 520/h ** Abs 520/hr x mg protein

TABLE 4

TABLE 5. KINETIC CONSTANTS OF A 26 kDa CYSTEINE PROTEINASE FROM *Fasciola hepatica*

Substrate P_3-P_2-P_1	Km μM	Kcat s^{-1}	Kcat/Km ($M^{-1}s^{-1}$)
Ac-Phe-Arg-pNa	140	3.97	28,357
Sar-Phe-Arg-pNa	247	1.17	4,736
ϵNH2capPheArgpNa	276	2.69	9,747
Peg-Phe-Arg-pNa	234	2.19	9,358
Bz-Phe-Arg-pNa	23	4.48	195,782
DPhe-Val-Arg-pNa	372	1.05	2,822
DVal-Leu-Lys-pNa	129	2.37	18,372
Bz-Pro-Arg-pNA	763	2.4	3,145

TABLE 6. HYDROLYSIS OF P-NITROANILIDE SUBSTRATES BY CYSTEINE PROTEINASE FROM *Fasciola hepatica*

Substrate	Concentration mM	Enzyme activity µmol/min mg prot
Bz-Arg-pNa	1	0
Ac-Leu-Arg-pNa	1	14.8
Ac-Ala-Arg-pNa	10	0
Ac-Asp-Arg-pNa	1	0.81
Ac-Asn-Arg-pNa	1	0
Ac-Gln-Arg-pNa	1	0
Ac-Glu-Arg-pNa	1	0
Bz-Gly-Arg-pNa	1	0
Bz-Phe-Arg-pNa	1	20
Z-Gly-Pro-pNa	4	0
Suc-Gly-Pro-pNa	2	0
Suc-Gly-Pro-Phe-pNa	2	0
Suc-Leu-Leu-Val-pNa	2	0
NH_3-CapPhe(SB1)Cys-pNa	2	0
H-Phe(SBz1)Cys-pNa	2	0
Ac-Gln-Pro-Arg-pNa	2	3.3
H-(D)Phe-Val-Arg-pNa	2	3.16
Bz-Phe-Val-Arg-pNa	2	0
H(D)Val-Leu-Lys-pNa	2	7.5
Ac-Phe-Phe-Arg-pNa	1	0.46
Ac-(D)Phe-Phe-Arg-pNA	1	4
Ac-Pro-Phe-Arg-pNa	1	3.5
Ac-Gln-Phe-Arg-pNa	2	1.28
Ac-Asn-Phe-Arg-pNa	2	0.68
Ac-Gly-Phe-Arg-pNa	4	2.8

TABLE 7. INACTIVATION RATE CONSTANTS OF *Fasciola hepatica* 26 kDa CYSTEINE PROTEINASE BY DIAZOMETHYLKETONE

Inhibitor P_3-P_2-P_1	Concentration μM	$t_{1/2}$ min	kapp/I mol^{-1} min^{-1}
Z-Phe-Ala-CHN_2	0.5 1.0 1.5	17 9.5 6.5	75184 ± 553
Z-Phe-Phe-CHN_2	0.25 0.5 0.75	23 15 10	101773 ± 1635
Z-Phe-Tyr-CHN_2	0.5 0.75 2.0	16 11 4	85750 ± 1515
Z-Tyr-Phe-CHN_2	0.5	15.7	88280
Bz-Phe-Val-CHN_2	0.5 1.0 2.0	37 25 12.5	30966 ± 5622

Effect of inhibitors on activated and non activated proteinase samples

Inhibitor	% of control activity	
	non-activated	activated
None	100 +/- 4.5	100 +/- 7.90
PMSF (1 mM)	97 +/- 4.6	106 +/- 2.84
Pepstatin (100 µg/ml)	115 +/-10.7	112 +/- 0.00
EDTA (5 mM)	173 +/- 4.0	140 +/- 2.48
E-64 (1 µM)	0	0

TABLE 8

Frequence and Sensitivity of Dot Elisa with Sera of human patients at different Reciprocal Titers

Reciprocal Titer	Frequency	Relative Frequency %	Accumulative	Sensitivity
100	0	0	22	100.00
200	0	0	22	100.00
400	0	0	22	100.00
800	0	0	22	100.00
1600	7	31.81	22	100.00
3200	3	13.64	15	68.19
6400	3	13.64	12	54.55
12800	2	9.09	9	40.91
25600	4	18.18	7	31.82
51200	3	13.64	3	13.64
102400	0	0	0	0
T O T A L	22	100		

TABLE 9

Frequence and Specificity of Dot Elisa Test with healthy controls at different reciprocal titers

Reciprocal Titer	Frequency	Relative Frequency	Accumulative	Specificity
100	23	57.5	23	57.7
200	9	22.5	32	80.0
400	3	7.5	35	87.5
800	4	10.0	39	97.5
1600	1	2.5	40	100.0
3200	0	0	40	100.0
6400	0	0	40	100.0
12800	0	0	40	100.0
25600	0	0	40	100.0
51200	0	0	40	100.0
102400	0	0	40	100.0
T O T A L	40	100.0		

TABLE 10

Frequence and Specificity of Dot Elisa Test with Sera of Patients with other Diseases

Reciprocal Titer	Frequency	Relative Frequency	Accumulative	Specificity
<400	59	86.76	59	86.76
400	2	2.94	61	89.70
800	2	2.94	63	92.64
1600	2	2.94	65	95.59
3200	3	4.41	68	100.00
6400	0	0	68	100.00
12800	0	0	68	100.00
25600	0	0	68	100.00
51200	0	0	68	100.00
102400	0	0	68	100.00
T O T A L	68	100.0		

TABLE 11

Cross Reactivity of Dot-Elisa using sera from Patients with other Diseases

Disease	<400	400	800	1600	3200	>3200	n
Brucellosis	5/5	0	0	0	0	0	5
Typhoid fever	5/5	0	0	0	0	0	5
Tuberculosis	5/5	0	0	0	0	0	5
Hansen	5/5	0	0	0	0	0	5
Hystoplasmosis	2/2	0	0	0	0	0	2
Paracoccidiom.	2/3	1/3	0	0	0	0	3
Toxoplasmosis	2/2	0	0	0	0	0	2
Malaria	5/5	0	0	0	0	0	5
Chagas	2/2	0	0	0	0	0	2
Bartonellosis	4/4	0	0	0	0	0	4
Hydatidosis	6/6	0	0	0	0	0	6
Hymenolepiasis	4/4	0	0	0	0	0	4
Cysticercosis	6/6	0	0	0	0	0	6
Leishmaniasis	6/7	0	0	0	0	0	6
Paragonimiasis	0/7	1/7	1/7	2/7	3/7	0	7
T O T A L	59	2	2	2	3	0	68

TABLE 12

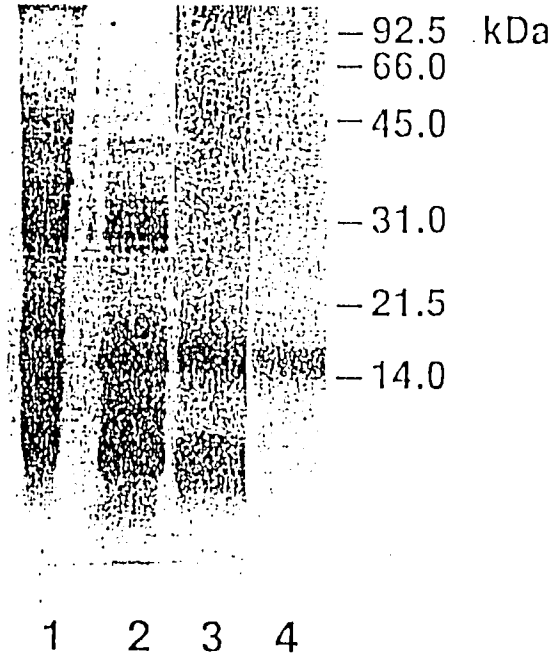

FIG. 1. SDS-PAGE of purified cysteine fractions from *F. hepatica*. Aliquots from each step in purification were boiled in SDS sample buffer containing ß-mercaptoethanol, and electrophoresed in 15% SDS-polyacrylamide gels. Gels were stained with silver nitrate. Molecular weigth standards are indicated; lane 1, homogenate (10 μg); lane 2, supernatant fraction (10 μg); lane 3, Mono S purified peak (10 μg); lane 4, molecular sieve chromatography peak (5 μg).

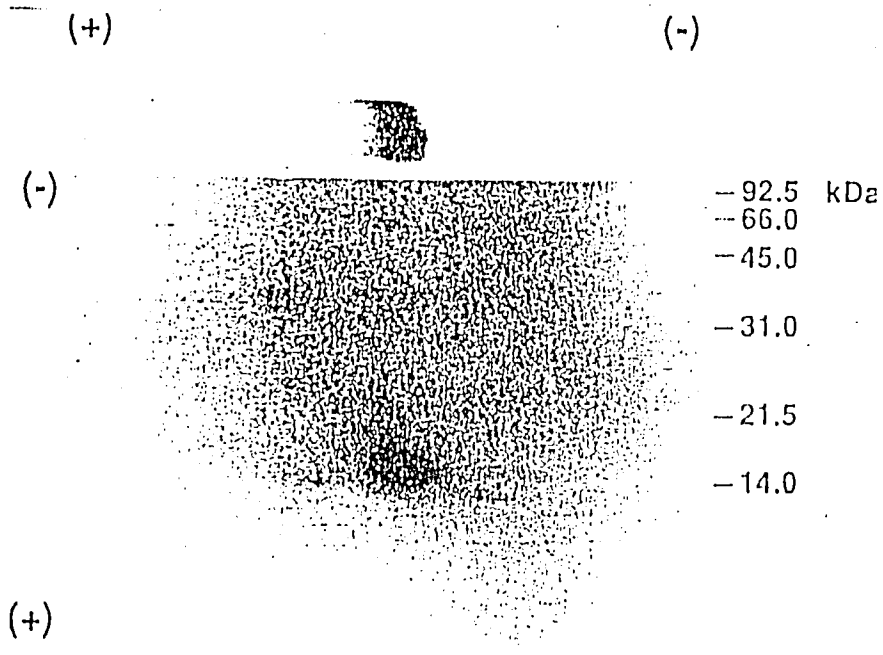

FIG. 2. Two-dimensional PAGE of purified cysteine proteinase activity from *F. hepatica*. Upper panel shows migration of the enzyme (20 μg) in a 15% acid-urea acrylamyde gel staines with Coomassie Blue R-250. Lower panel shows migration of the enzyme into the second dimension, a 15% SDS-PAGE. stained with silver nitrate.

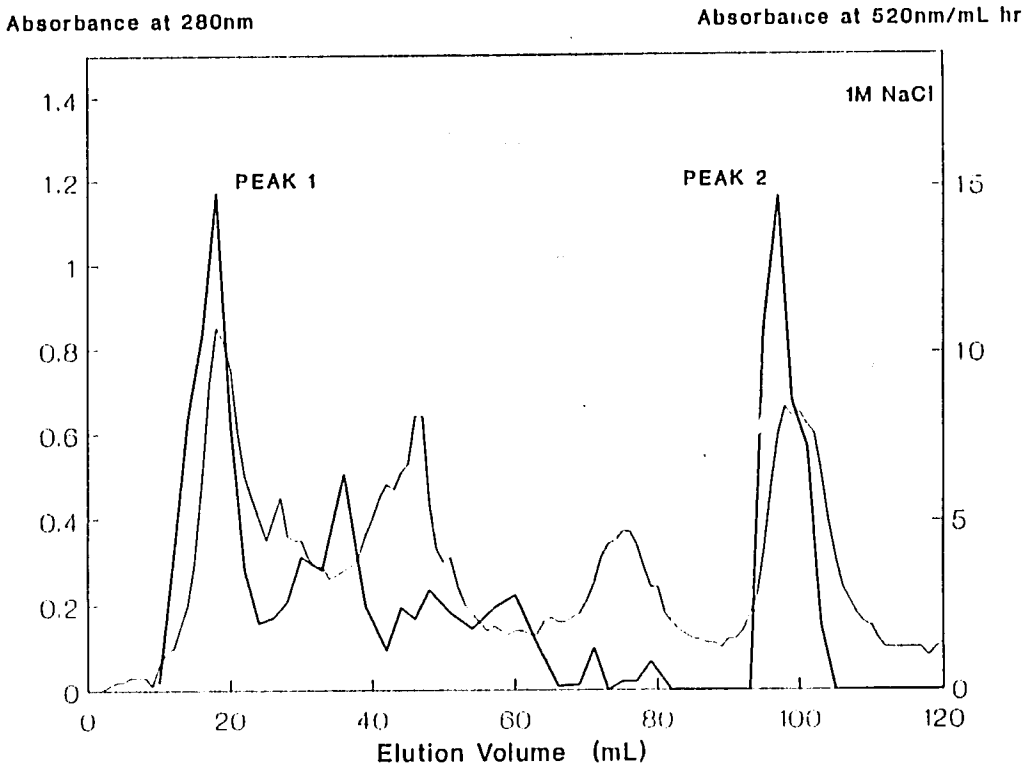

FIG. 3. Cation Exchange Chromatography of cysteine proteinase from *Fasciola hepatica*. The supernatant from ethanol precipitation was dialyzed against 10 mM sodium citrate, pH 4.9, containing 0.1 mM $HgCl_2$ and applied to a CM-Sephadex column equilibrated with the same buffer. Bound proteins were eluted with a lineal gradient of 0 - 1 M NaCl. Fractions were assayed for proteinase activity (▬) and monitored for protein content (A_{280}, ——)

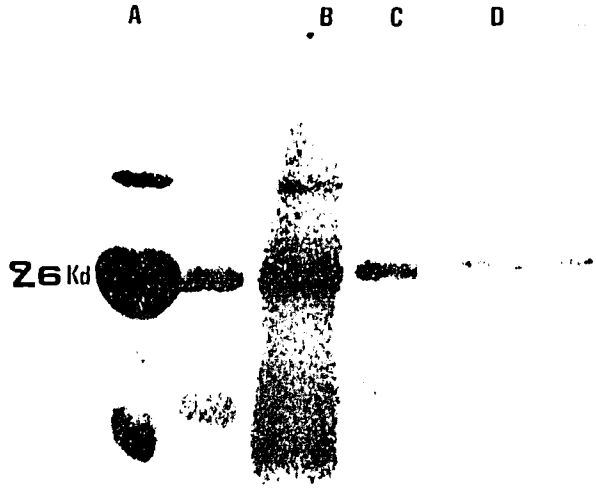

FIG. 4. SDS-PAGE of purified cysteine proteinase fractions from *Fasciola hepatica*. Aliquots from each step in purification were boiled in SDS sample buffer containing ß-mercaptoethanol, and electrophoresed in 15 % SDS-polyacrylamide gels. Gels were stained with silver nitrate. Molecular weight standards are indicated in lane A, excretion/secretion products (10 μg) in lane B, supernatant fraction of ethanol precipitation (10 μg) in lane C, cation exchange chromatography peak 1 (5 μg) in lane D.

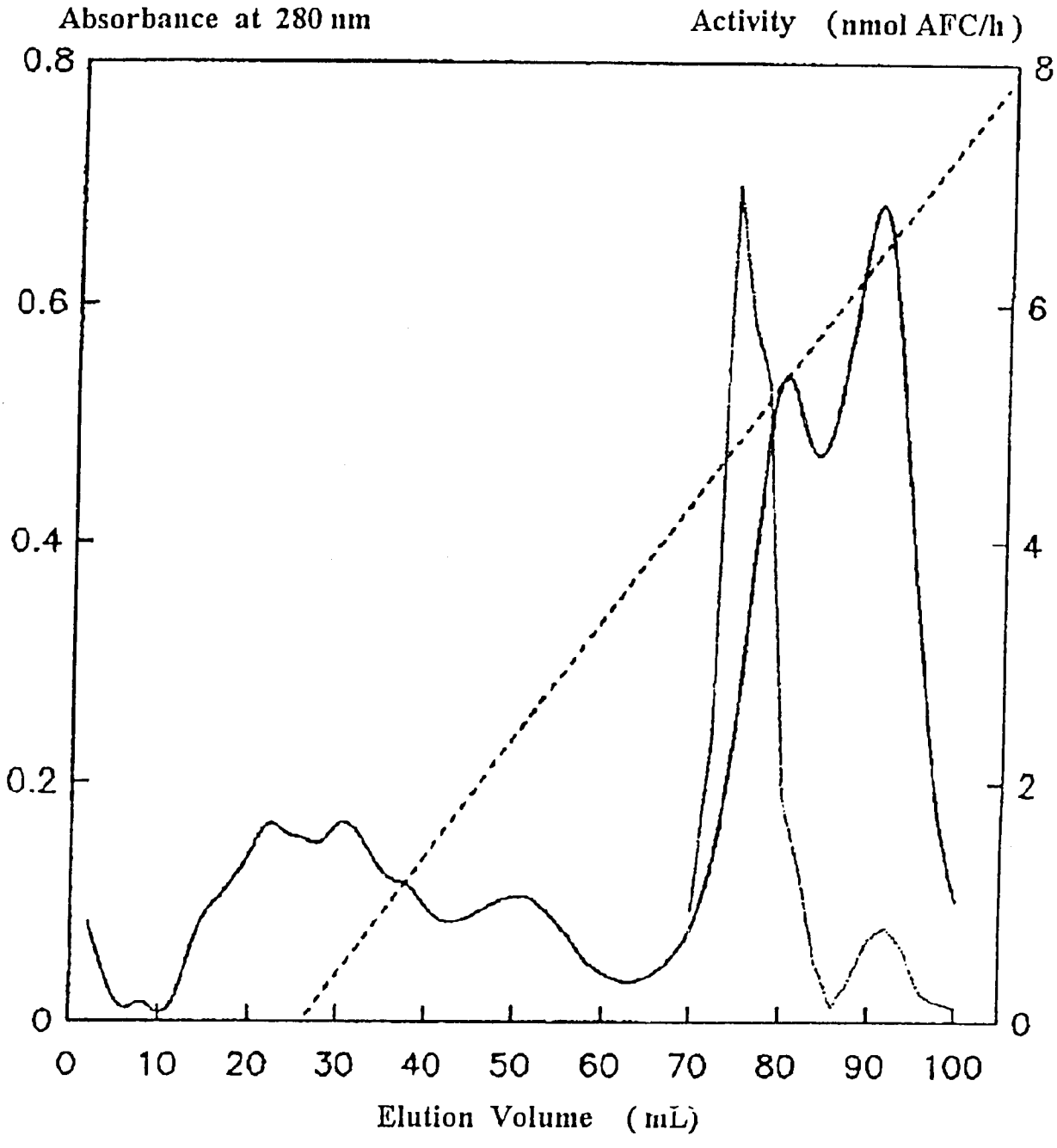

FIG. 5. Cation Exchange Chromatography of cysteine proteinase from *Fasciola hepatica*. Absorbance at 280 nm (.....), Activity (nmol AFC/hr) (▬▬), lineal gradient 1 M NaCl (——).

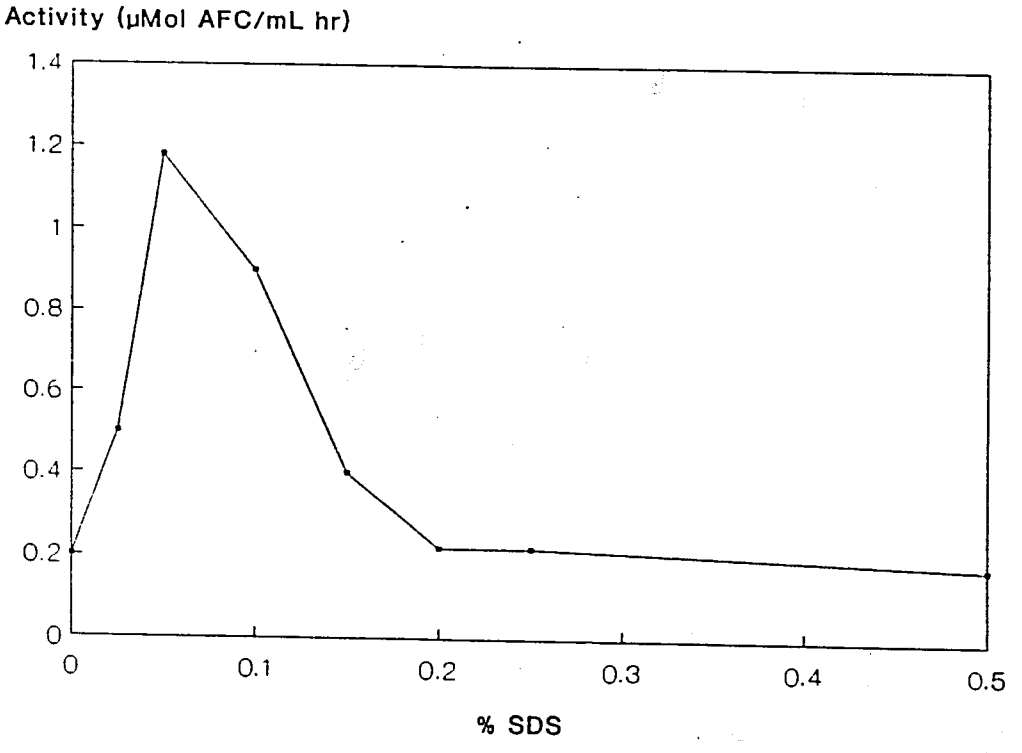

FIG. 6. Effect of SDS concentration on *F. hepatica* cysteine proteinase activity. The enzyme preparation was incubated for 2 hours at 37° C, in 50 mM Tris, pH 7 containing 5 mM DTT and different concentrations of SDS. The activity was measured using CBZ-Phe-Arg-AFC as substrate.

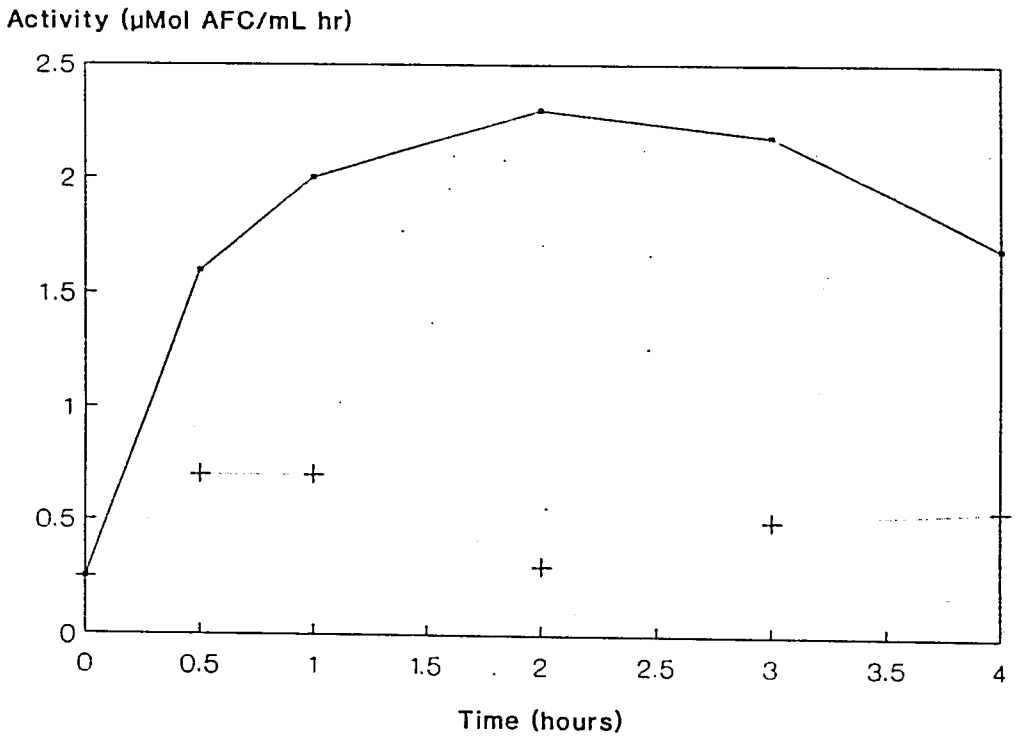

FIG. 7. Time course of activation. *F. hepatica* cysteine proteinase preparation was incubated in 50mM Tris, pH 7 containing 5mM DTT and 0.1% SDS for different periods of time at 37 ° C. The controls were incubated in the same condition but without SDS. Proteolytic activity of SDS treated (—), Control samples (·····).

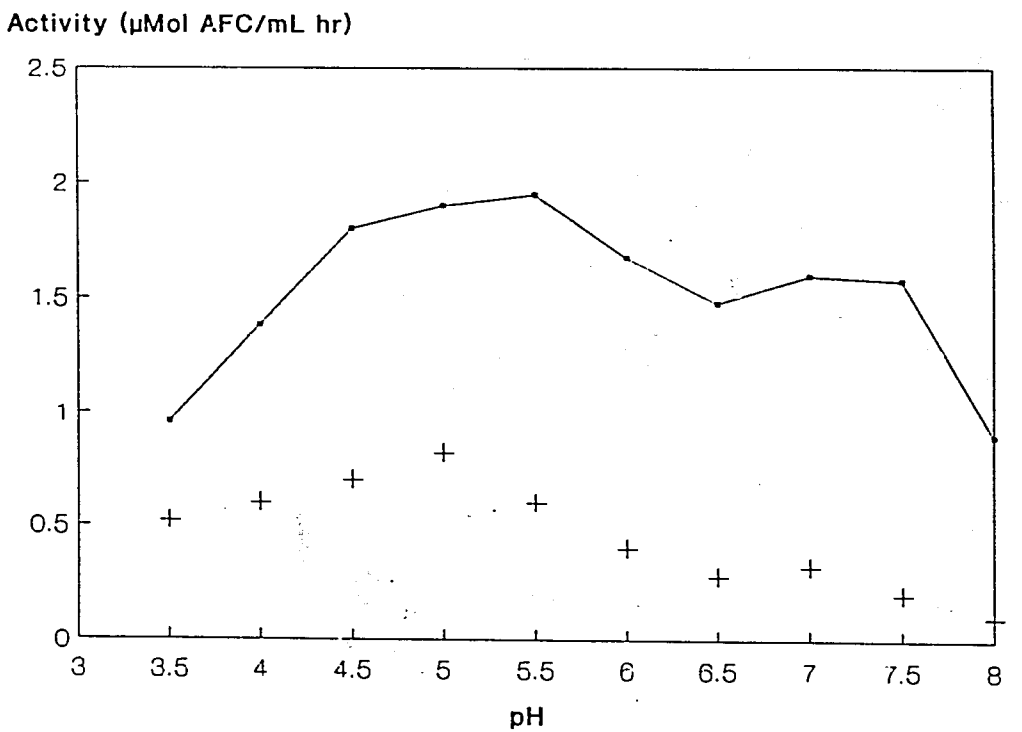

FIG. 8. SDS effect and pH dependence of *F. hepatica* cysteine proteinase activity. For activation, the semipurified preparation was incubated for 2 hours, in 0.1 M sodium citrate (pH 3.5 - 5) and 0.1 M sodium phosphate (pH 6-8) containing 5 mM DTT and 0.1% SDS, controls were incubated in the same conditions but in absence of SDS. Proteolytic activity of SDS treated (——), control samples (····).

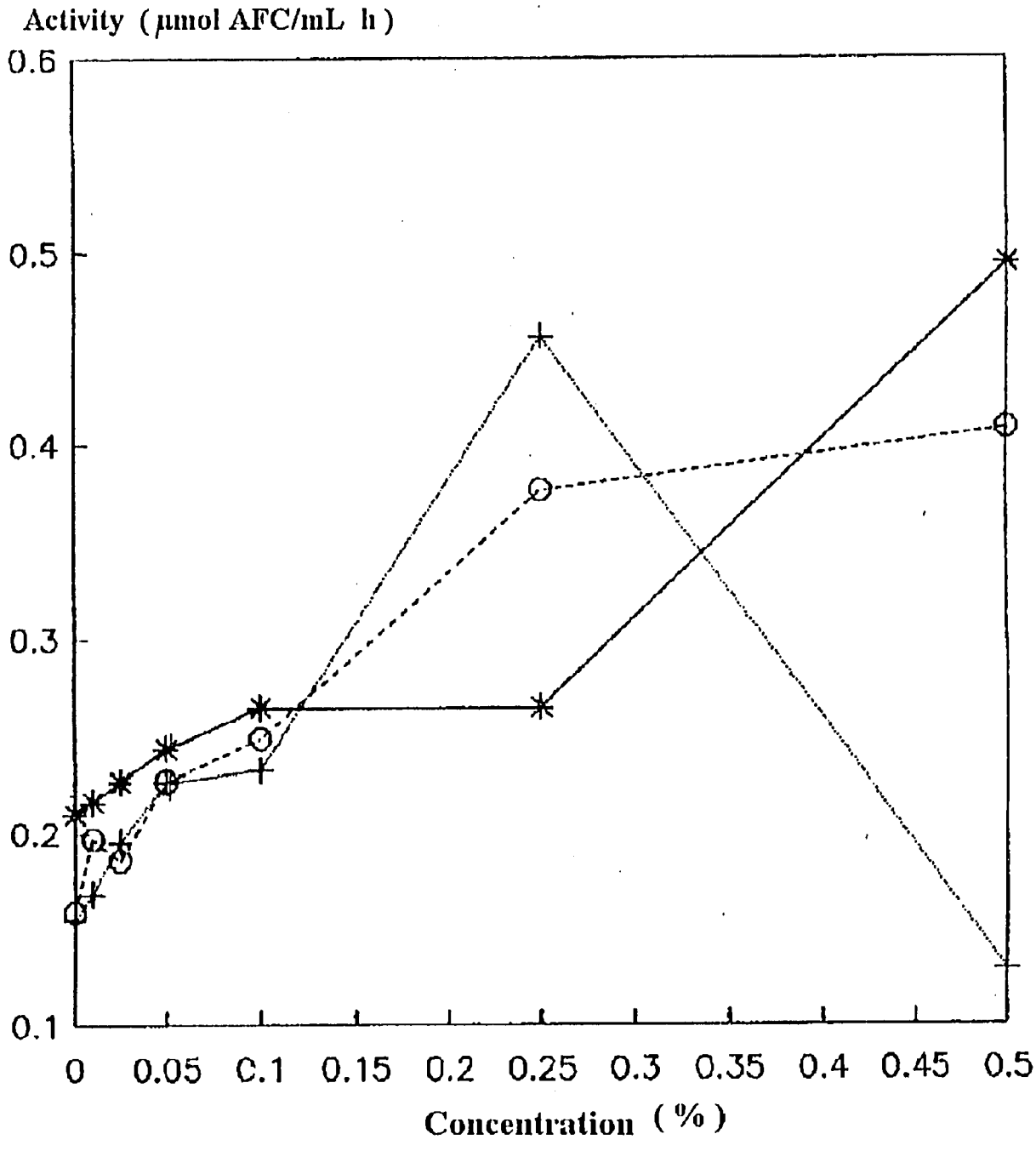

FIG. 9a. Effect of Glycine conjugates of bile salts on cysteine proteinase activity. The enzyme preparation was incubated for 2 hours at 37 ^{o} C, in 50mM Tris, pH 7 containing 5mM DTT and different concentrations of bile salt conjugates. The effects of conjugates of cholate (—), deoxycholate (----) and chenodeoxycholate (····) are shown.

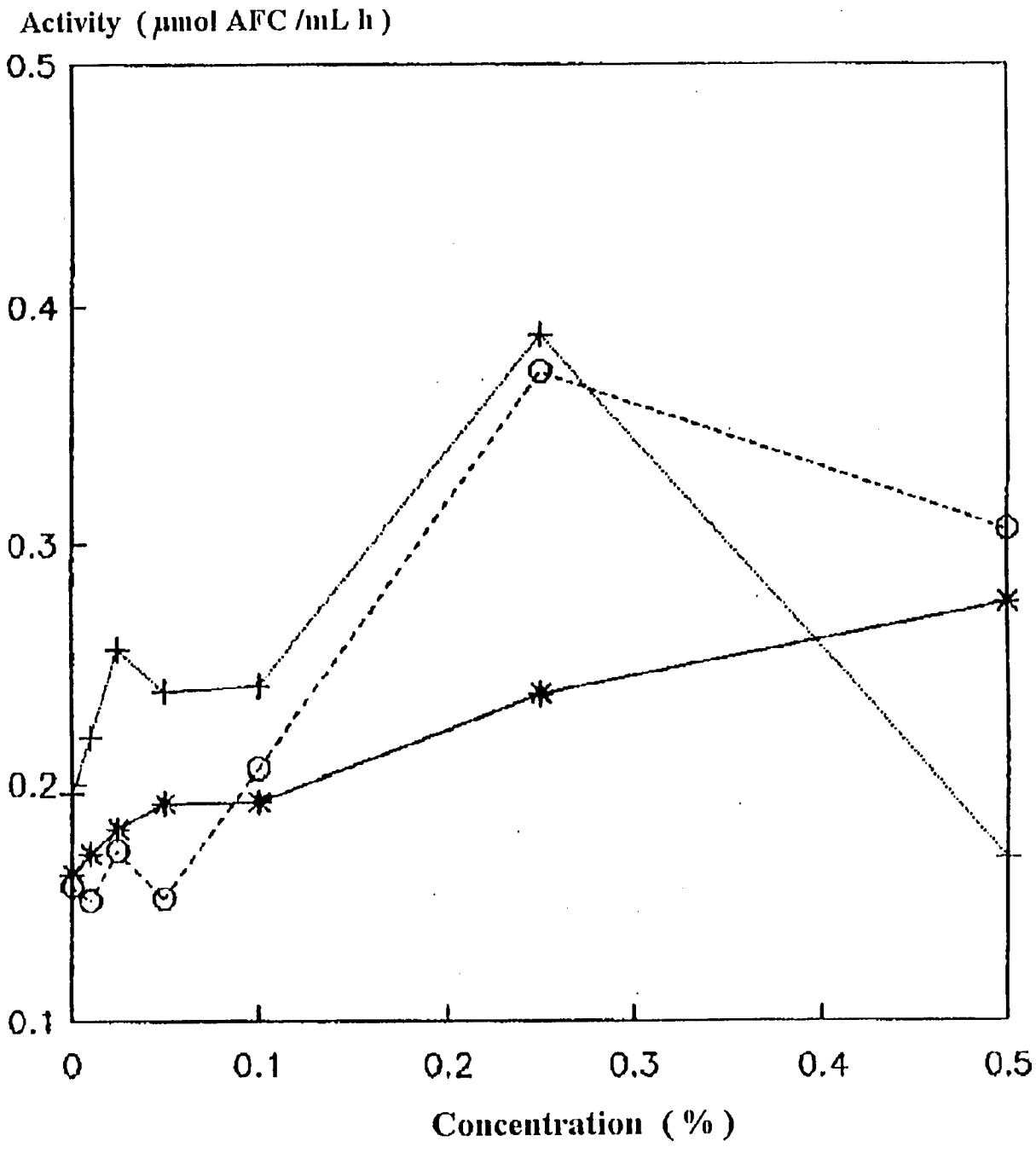

FIG. 9b. Effect of Taurine Conjugates of bile salts on cysteine proteinase activity. The enzyme preparation was incubated for2 hours at 37 ^{o} C, in 50mM Tris, pH 7 containing 5mM DTT and different concentrations of bile salt conjugates. The effects of conjugates of cholate (▬) deoxycholate (-----) and chenodeoxycholate (···).

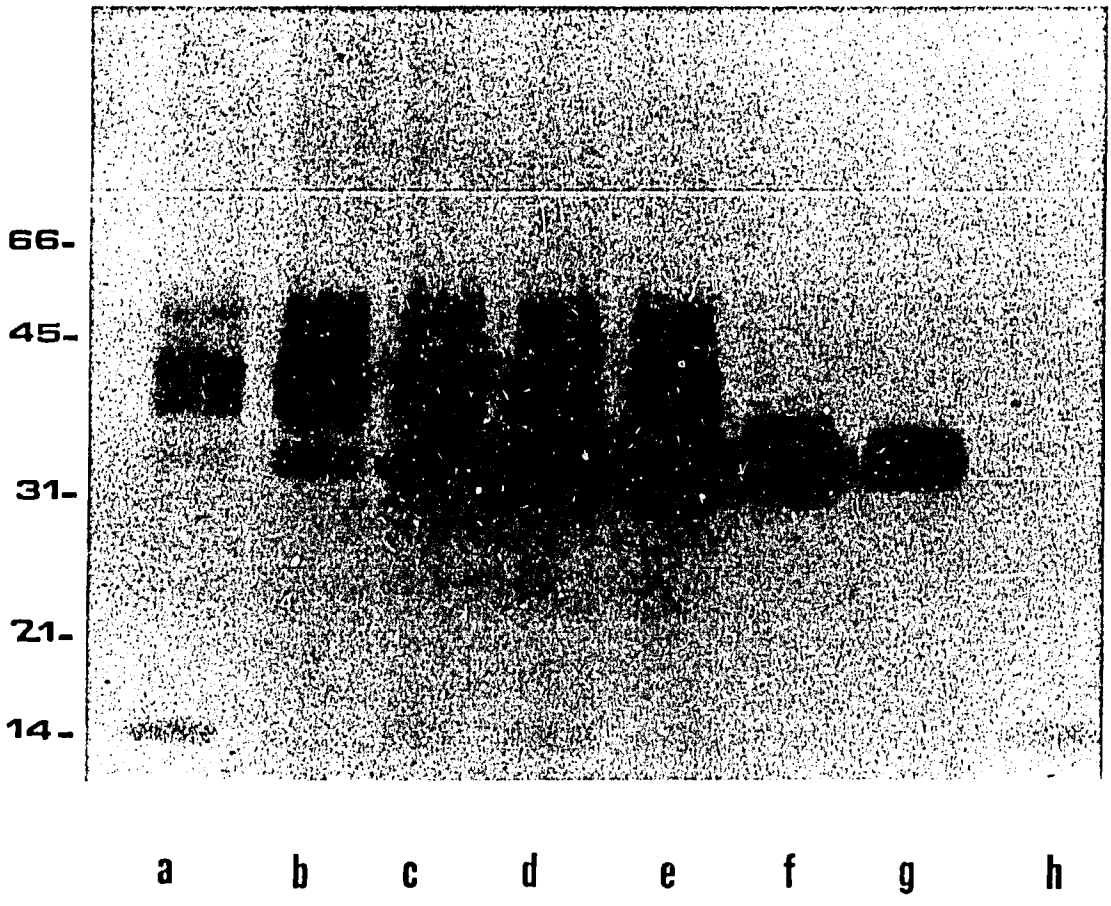

FIG. 10. Incorporation of CBZ-(^{125} I)Tyr-Ala-CHN $_2$ as a function of the SDS concentration. Line a). no SDS; b). 0.01% SDS; c). 0.025% SDS; d). 0.05% SDS; e). 0.1% SDS; f). 0.15% SDS; g). 0.2% SDS and h). 1μM E-64, 0.05% SDS.

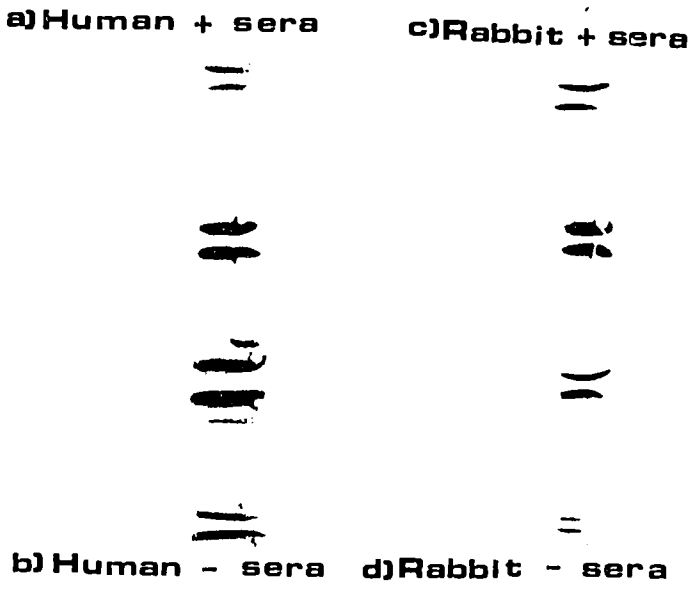

FIG. 11. Immunoprecipitation of CBZ-(^{125} I)Tyr-Ala-CHN_2 active site affinity labelled cysteine proteinases.

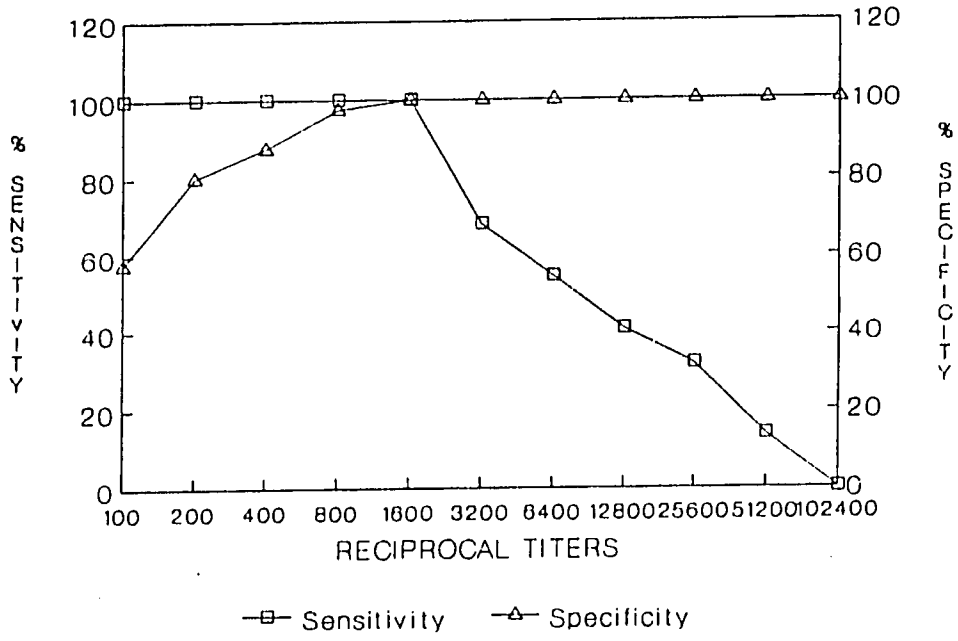

FIG. 12. Sensitivity and Specificity of dot-ELISA Test with sera of Fasciolasis patients and Controls.

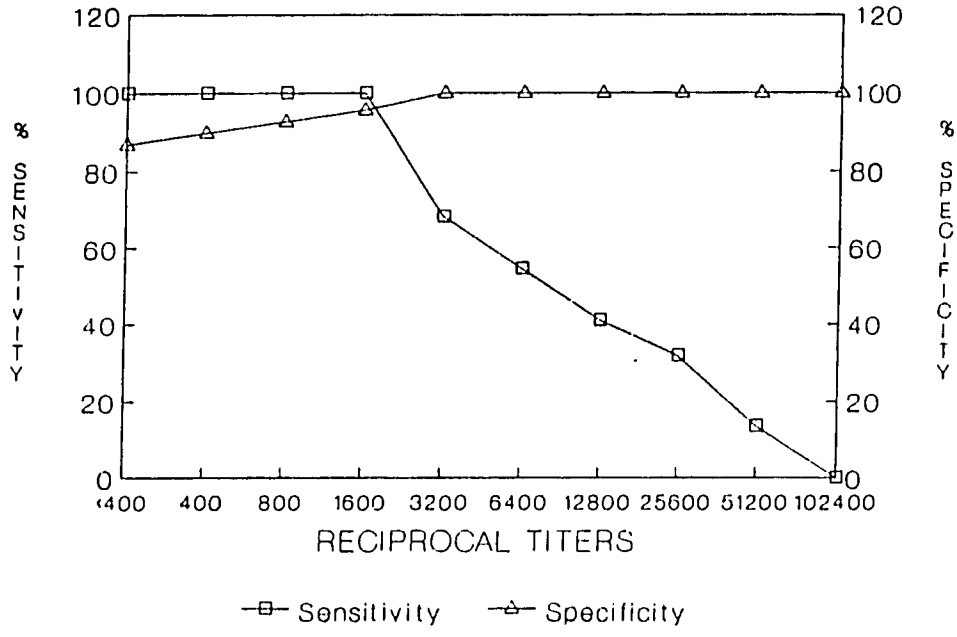

FIG. 13. Sensitivity and Specificity of dot-ELISA Test with sera of patients with Fasciolasis and other Diseases.

6) IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

The project has resulted in a number of techniques being established, improved and taught to many members of the laboratories in Perú, including trainees and studentsin general. They have been applied to other research as well, and in general have resulted in greater activity than otherwise would have been possible, in both the Peruvian Institutes and their respective Universities involved in the project.

The results of the work performed so far will make it possible to attempt to prepare a diagnostic kit for use by the cattle raisers. This kit would allow rapid diagnosis shortly after infection, and ferestall the considerable initial damage Faciola hepatica produces in the animals. The present practice calls for empiric treatment one or more times a year, when the animals could have been sustaining infection for variable periods. Moreover, the cattle themselves, when not kept free of infection, perpetuate the cycle by maintaining the local watercress and other such plants infectious to all animals. A project has been prepared to prepare a diagnostic kit, and funds are expected, to be applied in the new Laboratories for Science and Technology in the Faculty of Sciences and Philosophy of the Universidad Peruana Cayetano Heredia.

7) PROJECT ACTIVITIES/OUTPUTS

The activities were planned to be performed fundamentally by the Peruvian researchers, both in Perú and in the U.S.A. laboratory. Two Peruvian researchers spent over 6 months each training in Dr. Marc Dresden's laboratory at Baylor College of Medicine: M.Sc. José Espinoza from UPCH and Silvia Vega from UNMSM. Additionally, M.Sc. Jaime Bellatin visited for 2 weeks the laboratory at the beginning of the project.

The most unfortunate circumstance was that Dr. Dresden became ill during the second year of the project and rapidly lost strength, finally dying in February, 1990. Dr. César Náquira was able to visit Baylor College of Medicine after that, and discuss matters with Drs. Ajay Rege and Cynthia Chappel, but there was no possibility to continue the cooperation at that stage, when the funds were already gone.

In spite of all the problems, there has been an appreciable scientific production, which was possibly improved by the fact there had been a prior project also with Dr. Dresden, on the same general subject. This made it possible to build on, and improve, what had already been accomplished. The lists of publications follow, and, as well, the list of other projects which were prepared by members of the Peruvian groups and funded by other agencies, over the next few years. This succession of much smaller projects allowed completion of unfinished work and the training of more personnel. CONCYTEC, the National Council for Science and Technology, has a very limited budget, and therefore it provides funds solely for materials, reagents, consumables, and the like, not paying salaries except small sums for technical help. This, plus the very difficult economic and financial situation of Perú since 1988 have meant that the development of the laboratory has been less than planned.

Starting 1990, the main group of researchers at UPCH, Assistant Professor M.Sc. Jaime Bellatin, and former student-researchers M.Sc. Patricia Herrera and M.Sc. José Espinoza obtained training fellowships directed towards the Ph.D. degree, and left the country. This is very good for the development of the laboratory, but it has severely curtailed the available manpower for this particular project.

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Ñopo L., Herrera P., Espinoza J. 1992. Electroelución de proteasas de *Fasciola hepatica*: VII Jornadas Científicas, Universidad Peruana Cayetano Heredia, 1992. Revista Médica Herediana. 3 (supp.1): 55.
and also presented at the X Congreso Nacional de Biología, Lima, Perú.

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Cornejo W., Náquira C., López M. 1992. Properties of proteinase from *Fasciola hepatica*. VII PAABS Congress; XIX Congress of the Mexican Biochemical Society; Sociedad Española de Bioquímica, Ixtapa, México, 1992. Summary N° 103-R.

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Additional funded Research Projects, obtained to sustain the research line:

1992 - Córdova M., Arévalo J. Cistenil proteinasas de *Fasciola hepatica*: Caracterización cinética. Funded by CONCYTEC, the National Council for Science and Technology.

1992 - Ñopo L., Arévalo J. Purificación de proteasas en el estadío adulto del parásito *Fasciola hepatica* por el método de electroelución. Funded by CONCYTEC, the National Council for Science and Technology.

1993 - Herrera P., Espinoza J., Arévalo J. Proteinasas de la metacercaria de *Fasciola hepatica* : Caracterización bioquímica. Funded by the Fundación Hipólito Unanue, which depends on the national association of pharmaceutical enterprises, ALAFARPE, 1993.

1993 - Espinoza J., Córdova M. Desarrollo de un Kit de serodiagnóstico para fasciolasis humana. Funded by CONCYTEC, the National Council for Science and Technology. Selected through the annual PAHO-CONCYTEC contest.

1994 - Córdova M., Jara J. Estudio cinético del principal antígeno de 26 kDa de *Fasciola hepatica*. Funded by CONCYTEC, the National Council for Science and Technology.

1994 - Córdova M., Pacheco R. Estudio de la prevalencia de fasciolasis humana en la Comunidad de Yanama, Cusco. Funded by CONCYTEC, the National Council for Science and Technology. Selected through the annual PAHO-CONCYTEC contest.

1994 - Proteinases from *Fasciola hepatica* adult worms as markers of fasciolasis in sheep. Funded by the Third World Academy of Sciences, TWAS.

8) PROJECT PRODUCTIVITY

The original goals of the project were:

- to explore proteinase enzymes of *Fasciola hepatica* as a likely source of specific antigens.
- to find a specific *Fasciola* proteinase antigen which would be useful for a diagnostic procedure for humans and cattle.
- to provide a simpler means for targeted treatment of cattle, aiding the development of more comprehensive and cost-effective efforts toward prevention of the disease in cattle and humans.
- to contribute to the training of Peruvian graduate students in biochemistry of helminth parasites.
- to help introduce the technology of enzyme purification and characterization, and specific immunological procedures, in Peruvian laboratories belonging to two different Universities.

In addition, there were other goals which required a strong molecular biology effort, such as the preparation of monoclonal antibodies, construct a cDNA library from *Fasciola hepatica* adult worms, develop relevant cDNA clones into expression vectors and test their products as antigens for diagnosis. The inclusion of these aspects in the initial project was ambitious, and reflected the future orientation of Dr. Dresden's own work. Unfortunately, it was not possible to begin with molecular biology activities during the first year of the project at Dr. Dresden's laboratory. Later, when Dr. Dresden became ill, this component had to be dropped, and only purification, characterization, and preparation of antigens for diagnostic purposes was possible. The attempt to again produce monoclonal antibodies was fraught with difficulties, and never progressed in the group's hands in Lima beyond the initial tissue culture work. The clones which were obtained in the U.S.A. were contaminated with mycoplasmas and it was not possible to recover them.

The antigens which have been studied, characterized and purified are very likely to succeed as the basis of a practical, inexpensive diagnostic kit, of the Dot-ELISA format.

We believe that from the sections above the productivity, considering all the difficulties sustained, has been acceptable in the areas in which it was possible to work. Recapitulating, there have been 21 presentations in different scientific meetings, seven of them in countries different from Perú: the USA, Cuba, México, Brasil and India; two formal papers, one of them still to be accepted for publication, and three Master of Sciences Theses. In addition, several projects were presented to different funding agencies, and seven were successful, five with the National Council for Science and Technology, CONCYTEC (of which two were selected through a national contest, the annual PAHO-CONCYTEC contest), and one each with the local Fundación Hipólito Unanue and with the Third World Academy of Sciences, Trieste. The drive to continue to work with significant parasite problems in Perú persists.

Perhaps the most significant and permanent achievement has been the establishment of a solid cooperation between the Peruvian institutions, which will mean a great deal in the future when the general conditions in the country itself improve. It is important to note that cooperation with the Universidad de San Antonio Abad in Cusco is ongoing in the subject of the project, through the training of M.Sc. Rosa Pacheco, a Professor of Biology and Parasitology in that University.

9) FUTURE PLANS

As has been shown from the sections above, work has continued with considerable success, but at a slower pace because of the unfavorable funding situation prevalent in our country. Now, and specially starting 1996, when several of the trainees return, much of the initial project can be pursued and finalized. More research personnel within the Universities is now trained in molecular biology, a guarantee of probable success in the goals which were originally planned.

The goal of producing a kit which will allow timely diagnosis of *Fasciola hepatica* infection in cattle and in humans can be achieved, and can be reached by stages. The first stage, a commercially sustainable effort, may be very near since the Fas2 antigen specially seems well suited toward this end. A variety of immunological formats could be developed and introduced later, as well as new production procedures, including those based on molecular biology techniques. The new Laboratories for Science and Technology at the University are a most appropriate site for the pursuit of this effort.