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UNIVERSIDAD PERUANA CAYETANO HEREDIA
INSTITUTO DE MEDICINA TROPICAL
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Charles Mantione
Health and Nutrition
Agency for International Develpment
Pte.

Dear Mr. Mantione:

Please find enclosed the First Scientific Report corresponding to 1989 for the project "Proteolytic Enzymes: Evaluation as purified antigens in diagnosis of Fasciolasis in Perú". Grant No. 936-5542-G-00-7296-00. Project 936-5542. Obligation No. 7361249.

Sincerely yours,

Dr. Humberto Guerra
Director

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PROTEOLYTIC ENZYMES: EVALUATION AS PURIFIED ANTIGENS IN DIAGNOSIS OF FASCIOLASIS IN PERU.

GRANT NO. 936-5542-G-00-7296-00.

PROJECT 9365542.

OBLIGATION No. 7361249.

FIRST REPORT 1,989

2.

I. Experimental fascioliasis

In human infections by Fasciola hepatica, the specific antibody titer decreases in the chronic stage of the disease. The timing and degree of this process has implications for the usefulness of immune diagnosis. It is probable that the failure of detect circulating antibodies against Fasciola antigens is due to the low sensitivity of the techniques or antigens used. In our experience with the Dot-ELISA method using the partially purified antigen preparations, we obtained positive reactions with sera of all the 40 patients parasitologically positive, that is in the chronic stage of the disease where the adult parasites were producing eggs (see Figure 1). We have used rabbits as experimental animals for the disease in order to study the serological evolution using a sensitive technique such as as ELISA, as well as with conventional methods of immunoelectrophoresis. The information obtained will allow us to plan field trials with populations from endemic areas, both human and cattle.

II. Collection of Metacercaria:

Naturally infected Lymnea viatrix were collected from springs in the Mantaro Valley (heavily endemic area) in the Peruvian Andes. Snails were carried to Lima and maintained in the animal facility at Instituto de Medicina Tropical D. A. Carrión. Fifty to sixty days later metacercaria were collected as described in previous reports. Some metacer-

caria were selected for experimental infestation of rabbits, and the rest were frozen for later immunological studies.

III. Experimental Fascioliasis in Rabbits

The study was undertaken in order to study the normal immunological response to fascioliasis in an animal model. It will be important to assess the different antigenic preparations and the diagnostic procedures. Some of these have been tested as described below, and a collection of sera has been frozen to test future modifications of the procedures.

Ten 3 month old male rabbits were selected for the experiment. Defined numbers of metacercaria were placed in gelatin capsules. A plastic tube was introduced in the rabbit esophagus and the capsule was flushed with saline. The rabbits were marked properly and kept in individual cages at the animal house at Instituto de Medicina Tropical Alexander von Humboldt. The animals were distributed in 4 groups:

Group 1 (3 rabbits): infested with 10 metacercaria

Group 2 (3 rabbits): infested with 25 metacercaria

Group 3 (3 rabbits): infested with 50 metacercaria

Group 4 (1 rabbit) : control only saline was given

Blood samples were taken previous to infestation. The following procedures were performed weekly for 28 weeks.

a. Collection of blood samples: Samples were obtained from the ear and used for blood smears, stained with Wright to

test for eosinophilia. Sera were colleted to perform ELISA tests, immunoelectrophoresis, and Western blots. Antigens used were crude E/S products,and partially purified antigen preparations as described in our last report (February 1989).

1. Western blot: It began to show positive reactions from the third week of infection onwards. Up to the 28th week there was no significant decrease in the reaction.

2. ELISA: Goat Anti-rabbit IgG peroxidase conjugate was used. 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. Further bleedings are being performed in order to have information about the serological status of the chronic disease.

3. Immunoelectrophoresis: It began to show positive reaction (arc 2) from the third week of infection onwards. Up to the 28th week there was no significant decrease in the reaction.

4. Parasitological examination: Faeces of rabbits were collected, previous to infestation, and periodically after infestation. They were analyzed by a modification of the rapid sedimentation method of Lumbreras. All infested rabbits presented Fasciola eggs in their faeces, indicating the success of infestation and the establishment of the chronic phase of the disease.

5. Other examinations such as weight and hematocrit were performed to assess the general health of the rabbits.

A complete analysis of all the data obtained from this experimental disease is underway and will be presented in the next report.

IV. Dot ELISA Test:

The Dot ELISA test performed with sera from human patients has been described in detail in the previous report. This work was presented at the 1st Iberoamerican Congress on Biotechnology held in April 1989. We are enclosing a copy of the abstract. We have continued evaluating human sera from other diseases to confirm the great potential of this simple procedure as an specific test for fascioliasis. The current status of this research is presented in Tables 1-4 and Figures 1 and 2. At present we are following for bovine fascioliasis the same steps as for the human disease.

V. Western Blots with Sera from Different Sources

These experiments were carried out at Baylor 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. The active fractions were pooled and concentrated before a 15% PAGE analysis. Proteins from the gel were electrotransferred to nitrocellulose membrane.

Bovine, sheep and human sera were used. The nitrocelullose strips were incubated with different sera for 1 hour at 37 °C, before incubating with the corresponding antiserum peroxidase conjugate and color development.

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

The antigen antibody reaction is present around 28 kD in all the infected animal and human sera tested. In some infected animal sera there is an antigen-antibody reaction around 42 kD, which is strong in rabbit and human sera and weak in bovine sera. There is no antigen-antibody reaction around 14 KD.

These experiments were performed with stored lyophilized sera so new tests are being performed with fresh samples in order to confirm and extend these findings.

VI. Enzyme Characterization

A. Ammonium sulfate precipitation: Lyophilized adult worms (20 mg dry weight/ml) were homogenized in 0.2 M citrate buffer, pH 4.9 and centrifuged at 12000 g x 30 minutes. Proteins of the supernatant were precipitated with 20% ammonium sulfate. After centrifugation the pellet was dialyzed against 0.01 M citrate buffer, pH 4.9 (See Figure 3). Proteolytic activity was tested in the different fractions obtained (Table 5). A 15% SDS PAGE was performed using 25 ul of samples from different fractions containing

0.4 mg protein/ ml and silver staining was performed according to Wray et al.

Results show that S_1 is rich in a 14 kD protein; S_2 has a band at 28 kD and S_3 has bands at 28, 14 and 6 kD.

S was filtered through Sephadex G-100 and Sulfopropyl Sephadex (cationic exchanger) but most of the activity was lost. Material passed through Sephadex G-100 was used to immunize animals in order to use antibodies for future use of Affinity chromatography for further purification of the enzyme.

B. Enzyme Stability

E/S material maintained for 3-4 hours at 4 C and 37 C was loaded on a SDS 15% PAGE and stained with Coomassie blue showing:

- At 4 C 38-40 kD and 28-14 kD bands are present
- At 37 C only 28 and 14 kD bands are present

C. Enzyme Activation . Sodium dodecyl sulfate and bile salt conjugates increase the cysteine proteinase activity in partially purified preparations from homogenates of *Fasciola hepatica* adult worms. Sodium dodecyl sulfate increases the proteolytic activity 5-7.5 times at concentrations near its critical micellar concentration, while the glycine and taurine conjugates of bile salts are less effective. The proteinase activity is dependent on reducing agents and the activation is completely inhibited by E-64, suggesting that cysteine proteinases are exclusively involved in this

process. Detergent activated enzymes are active over a broad range of pH (4.5-8).

1. Preparation of cysteine proteinases

Adult worms collected from abattoirs in Lima, Peru were rinsed with PBS and lyophilized. Twenty five mg of this material were suspended in 1 ml of 0.2 M sodium citrate, pH 4.9, containing 0.1 mM $HgCl_2$ and 1 M urea, and homogenized in a glass-teflon homogenizer. The homogenate was centrifuged at 48,000 x g for 20 minutes at 4 C. The supernatant was dialyzed overnight at 4 C against 100 volumes of 10 mM sodium citrate, pH 4.9, containing 0.1 mM $HgCl_2$ and again centrifuged as above. After centrifugation the supernatant was used to purify the major cysteine proteinase activity.

The supernatant fraction was assayed for enzyme activity and then subjected to cation exchange chromatography using CM-Sephadex. The column (1 cm x 30 cm) was previously equilibrated with 10 mM sodium citrate, pH 4.9, containing 0.1 mM $HgCl_2$ and 1 M Urea. The bound proteins were eluted with a linear gradient of 0-1 M NaCl in equilibration buffer. Absorbance at 280 nm and CBZ-Phe-Arg-AFC activity were measured in each 1 ml fraction. Active fractions were pooled, dialyzed as before, and concentrated by ultrafiltration using a stirred cell concentrator and YM10 filters (molecular weight cut-off 10,000). The partially

purified, concentrated enzyme pool was lyophilized and stored at -20 C until use.

2. Enzyme Assays.

Activity was measured at pH 7.0 by two different assays. The first utilized CBZ-Phe-Arg-AFC and the second used Azocoll suspended in 50 mM Tris-HCl, pH 7, containing 5mM DTT, and incubated at 37 C. Incubation was terminated by centrifugation and dye release was monitored by measuring absorbance at 520 nm.

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

Preparations of partially purified proteinases were incubated with various concentrations of sodium dodecyl sulfate (Fig. 4). A significant increase in proteolytic activity was seen using either Azocoll or CBZ-Phe-Arg-AFC as substrate. Activity increased as a function of the concentration of SDS, with the highest activation at 0.05 % SDS; however the enzyme was still active at concentrations as high as 0.2 % 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 non-activated 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 6).

Activation was time dependent (Figure 5). Maximum activation ocurred in one hour of preincubation at 37 C, however 80% of maximum activation was within 30 minutes. The SDS stimulated proteinase activity was stable for at least 3 hours.

Figure 6 shows that the enzymes were active in a broad range of pH from 4.5 to 8, with maximum activity at pH 5.0 and 7.0. Furthermore, the increase in activity by SDS was not due to a change in pH optimum.

Cysteine proteinase activity of F. hepatica is also increased by conjugates of bile salts (Fig 7a and b). We used conjugated bile salts, since the non conjugated molecules are 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.

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
TOTAL	22	100		

TABLE 1

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
TOTAL	40	100.0		

TABLE 2

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
TOTAL	40	100.0		

TABLE 3

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
Hymenoleplasis	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
TOTAL	59	2	2	2	3	0	68

TABLE 4

Proteolytic Activity of Different Fractions after Ammonium Sulphate Precipitation

	So	S1	S2	S3
Total Volume	10.0	2.0	19.0	3.7
Prot Mg/ml	1.475	0.274	0.133	0.415
Total Protein	14.75	0.548	2.527	1.535
Total Activity	0.009	0.121	0.0009	0.028
Spec.Activity	0.616	4.425	0.676	1.283
Yield	100	718	109	320
Fold Purific.	1	7	-	2

TABLE 5

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 ug/ml)	115 +/-10.7	112 +/- 0.00
EDTA (5 mM)	173 +/- 4.0	140 +/- 2.48
E-64 (1 uM)	0	0

TABLE 6

SENSIBILITY AND SPECIFICITY OF DOT ELISA TEST WITH SERA OF FASCIOLIASIS PATIENTS AND CONTROLS

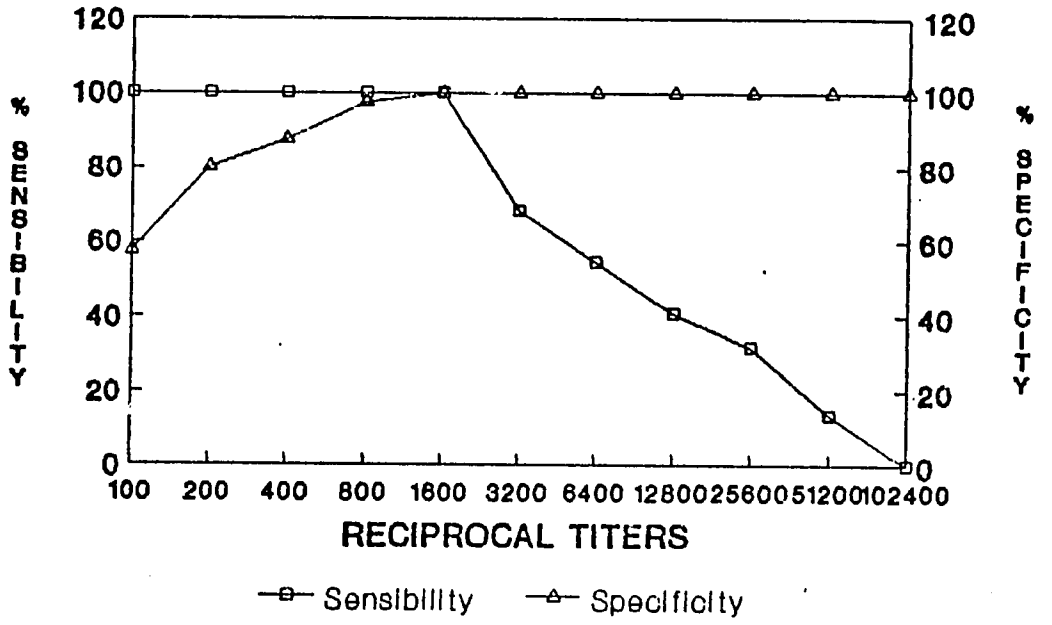


FIGURE 1

SENSIBILITY AND SPECIFICITY OF DOT ELISA WITH SERA OF FASCIOLIASIS AND OTHER DISEASES

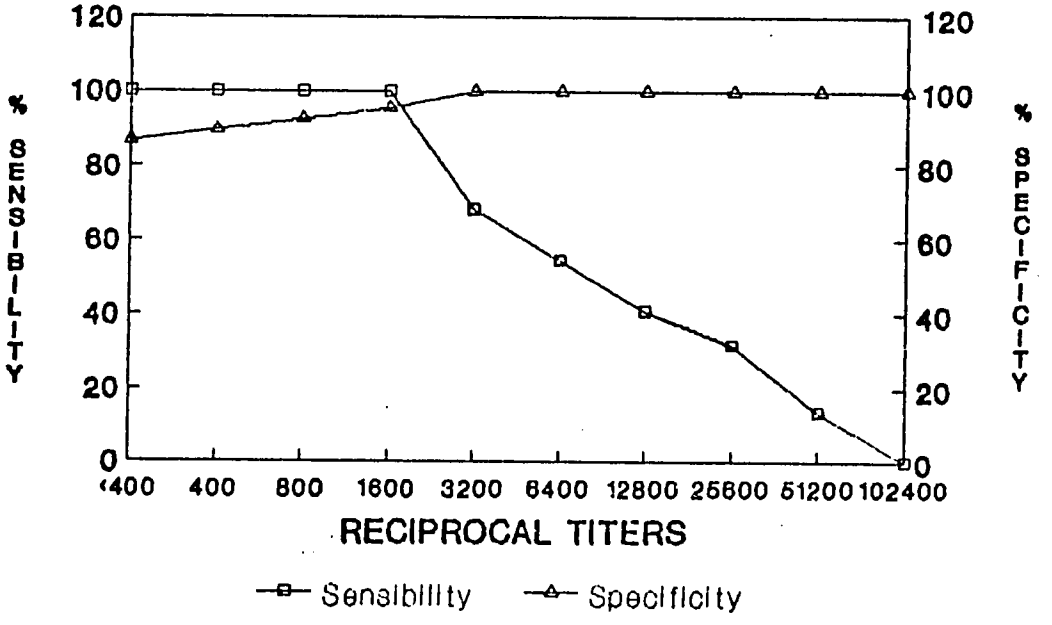


FIGURE 2

Fractionation Scheme of adult worms using Ammonium sulphate

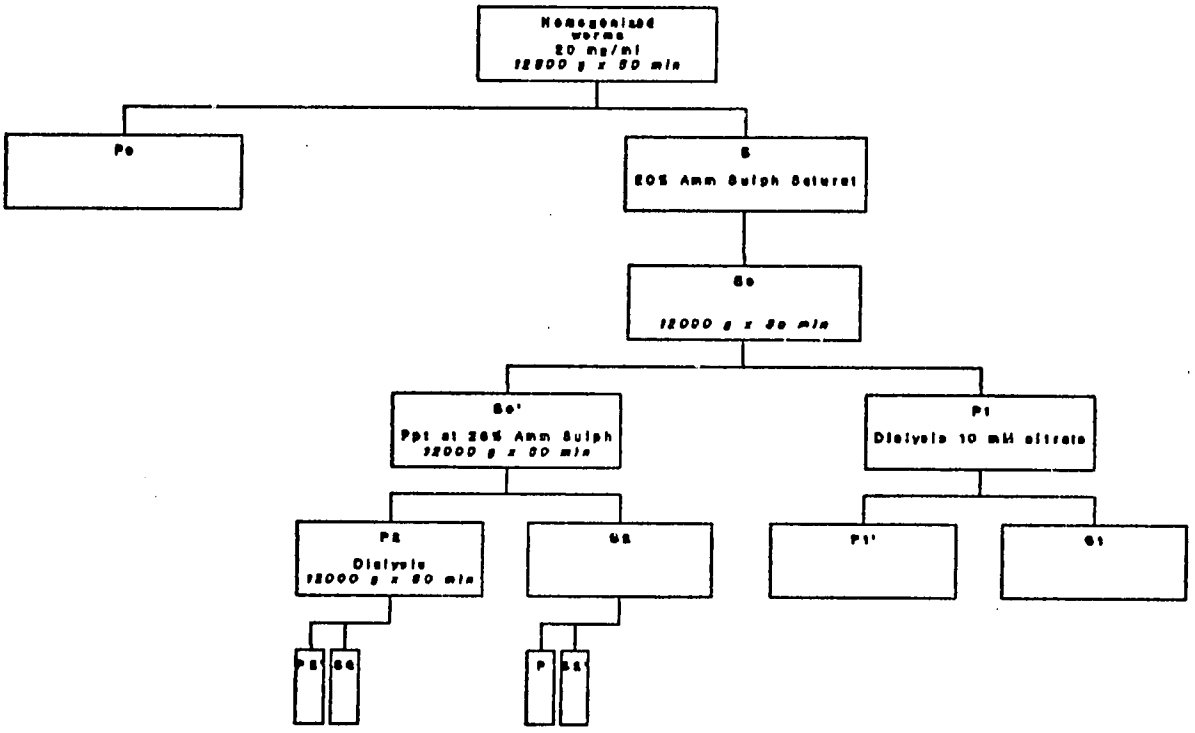
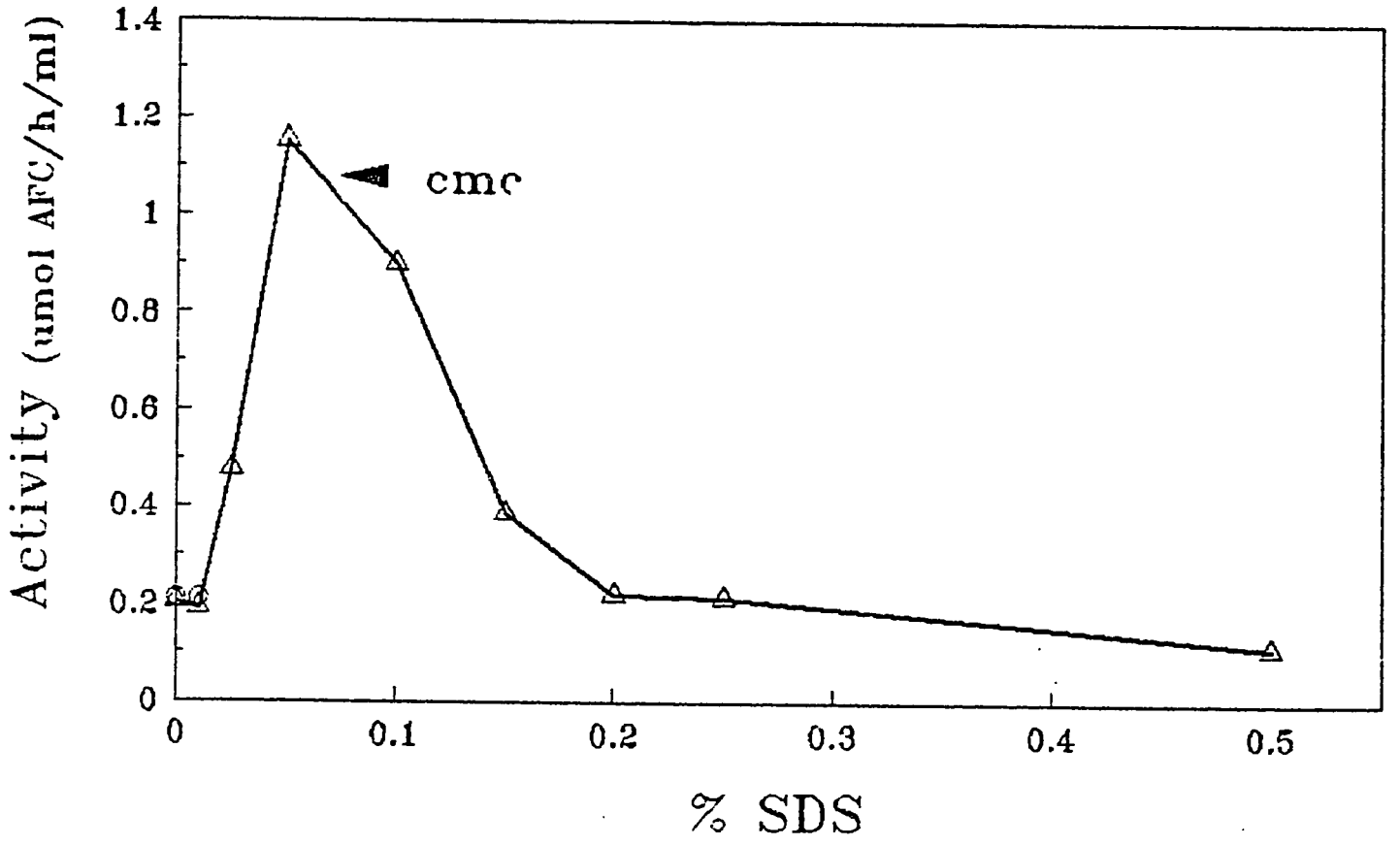


Figure 3



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 5mM DTT and different concentrations of SDS. The activity was then measured using CBZ-Phe-AFC as substrate as described in Materials and Methods.

FIGURE 4

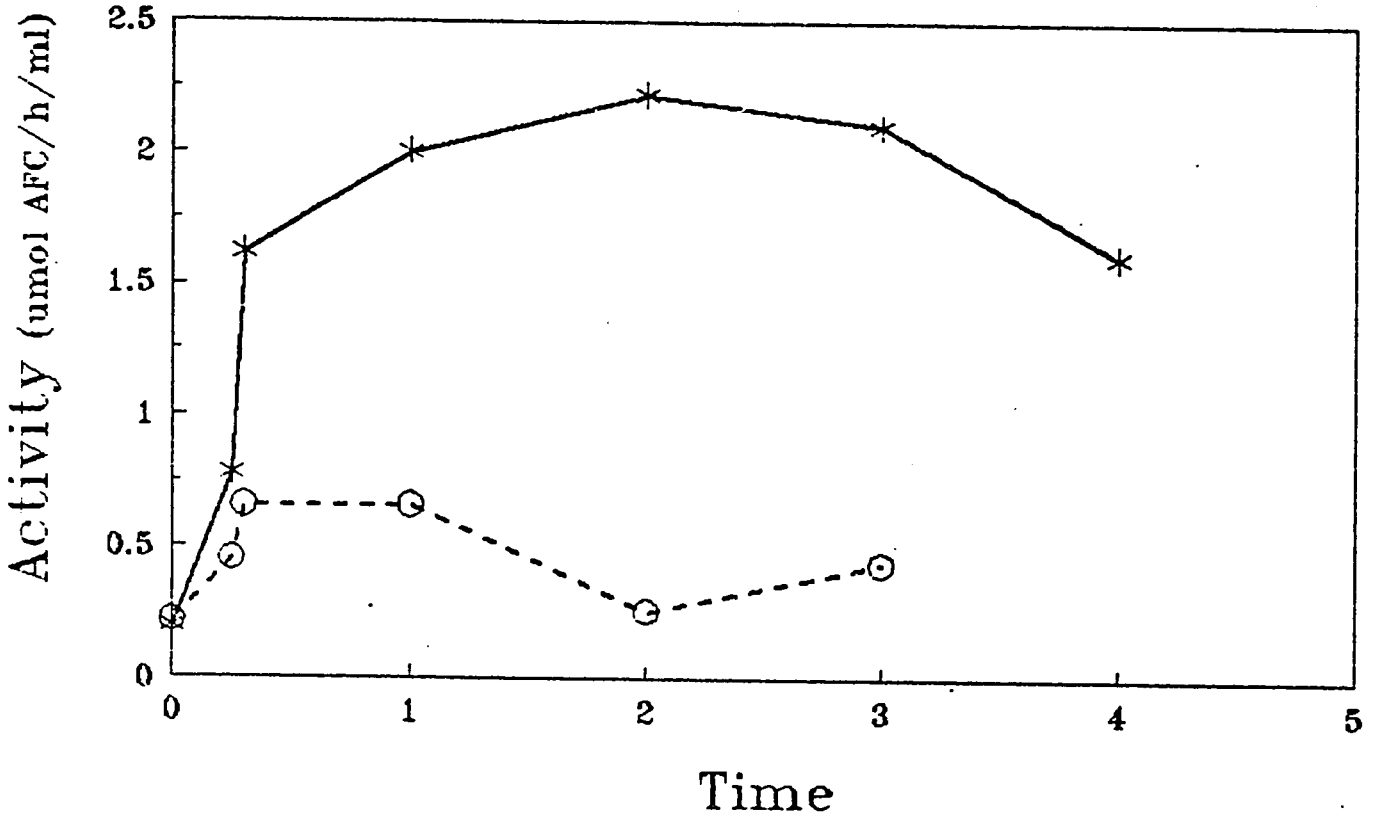
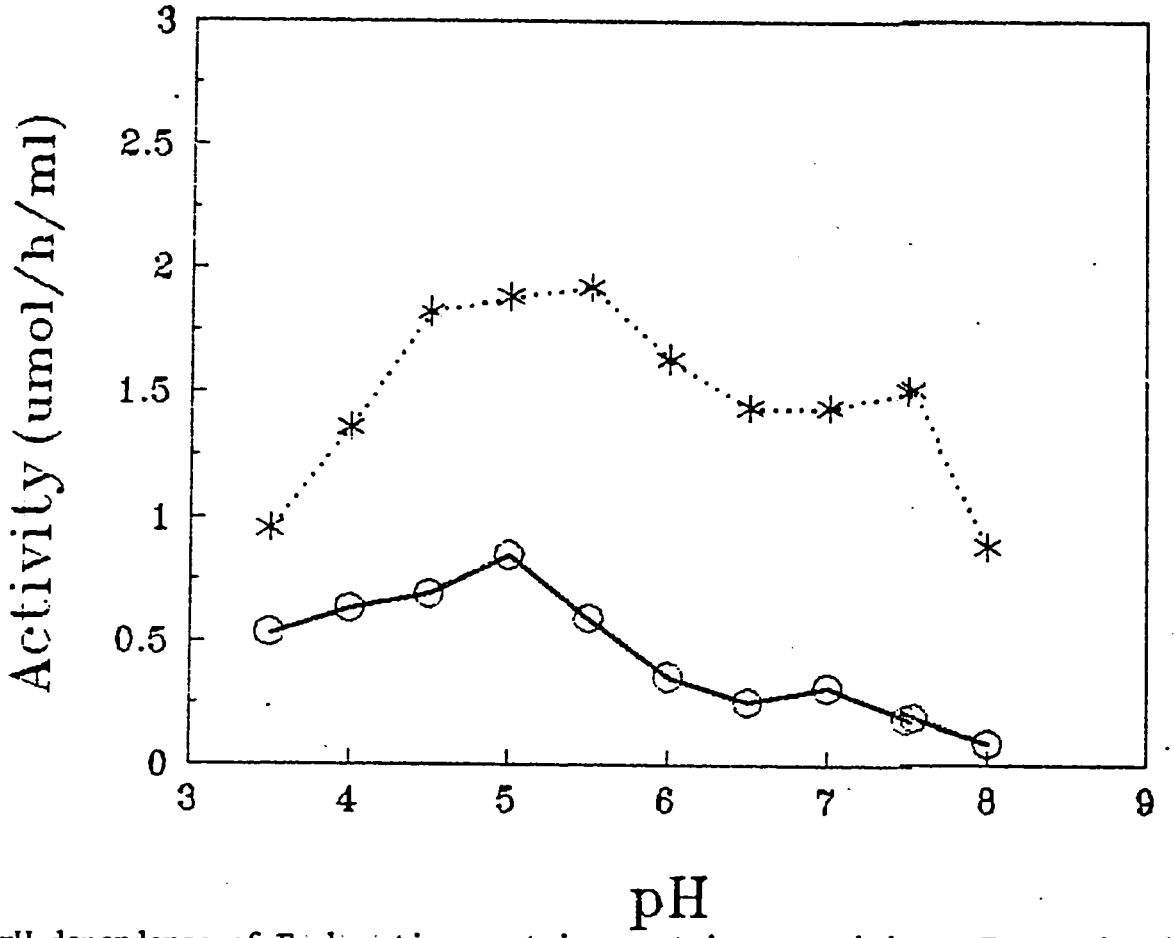
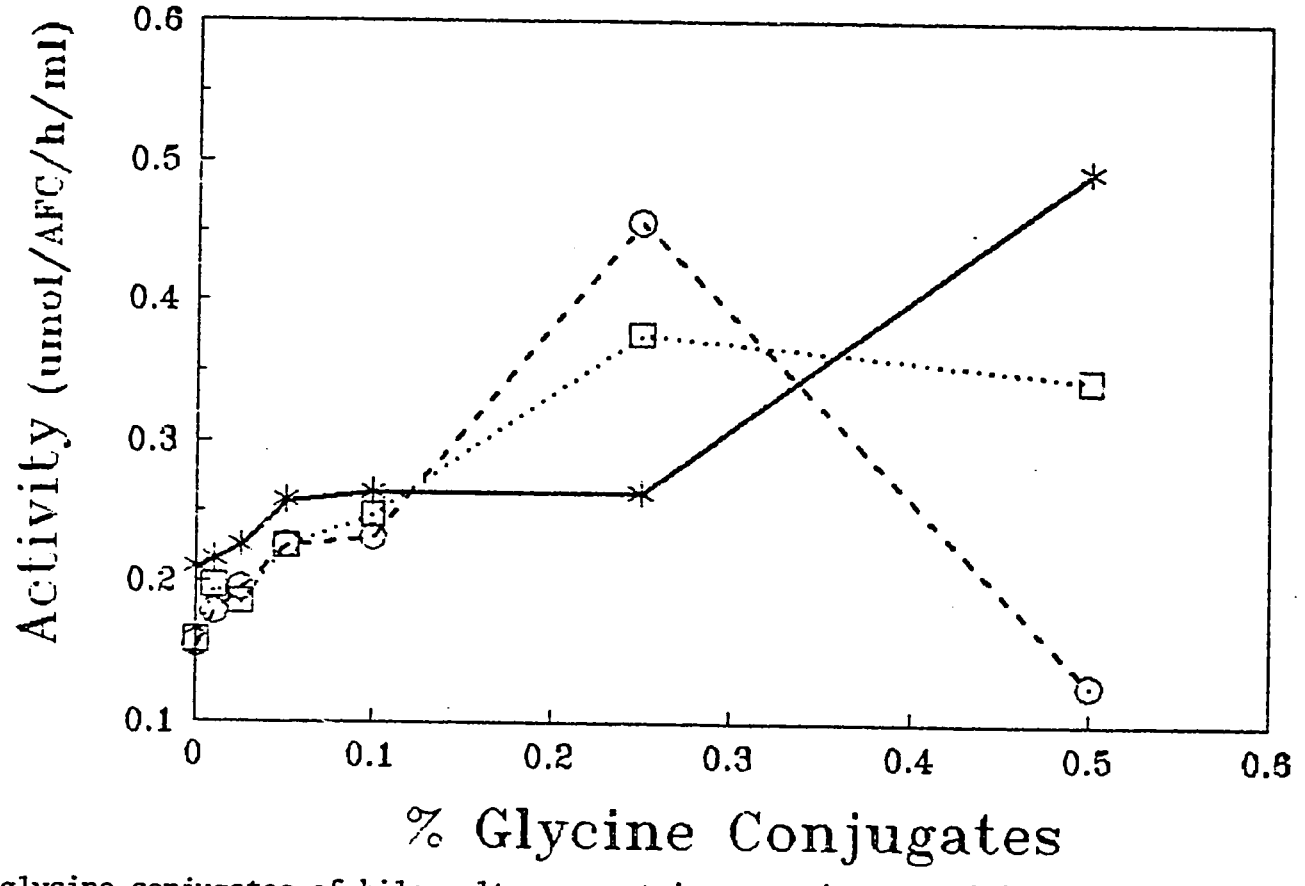


FIGURE 5 Time course of activation. F. hepatica cysteine proteinase preparation was incubated in 50 mM Tris. pH 7, containing 5mM DTT and 0.1% SDS for different periods of time at 37 C. The controls were incubated in the same condition but without SDS. The cysteine proteinase activity of of the SDS treated (*) and control samples (o) was then measured as described



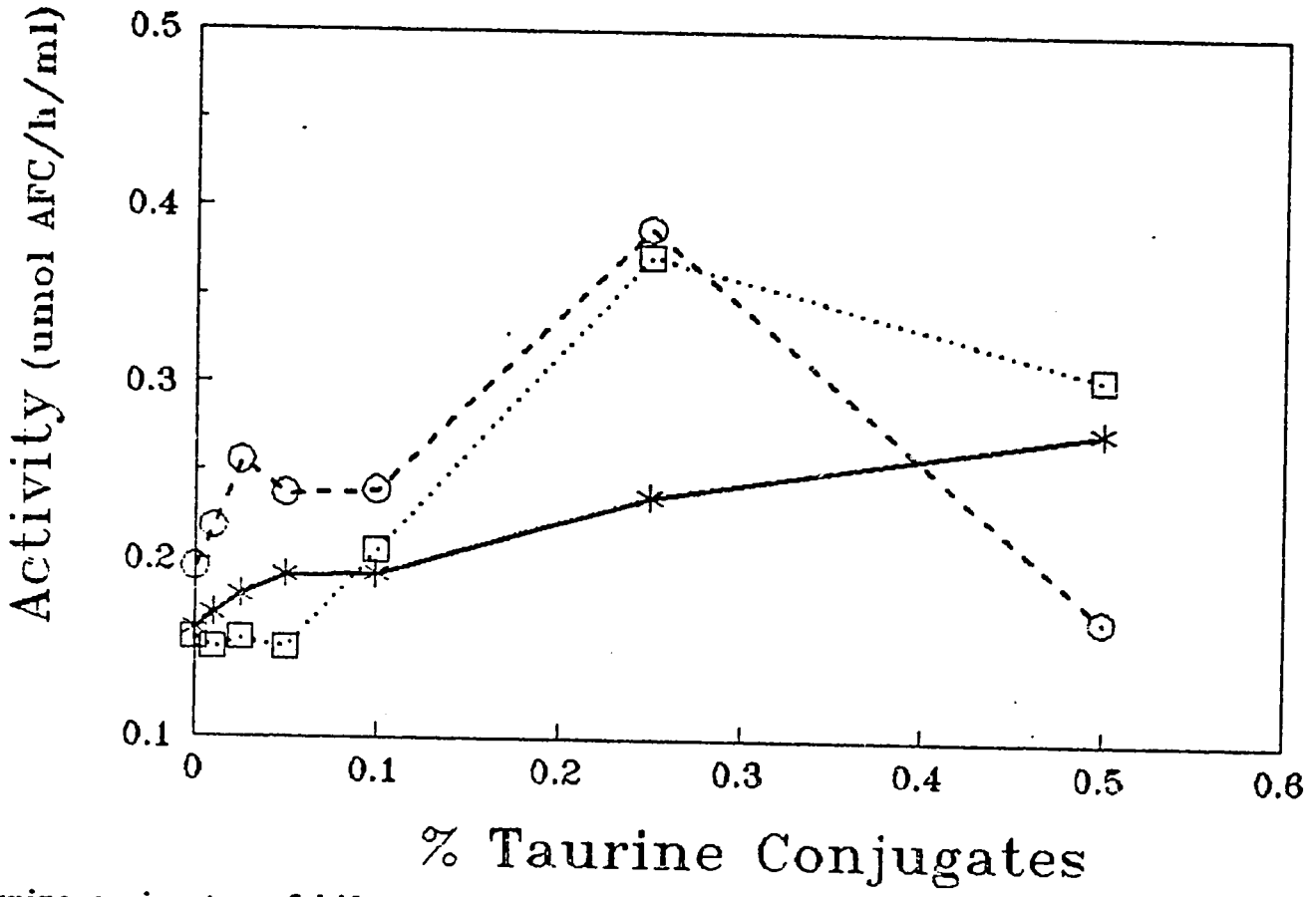
SDS effect and pH dependence of F. hepatica cysteine proteinase activity. For activation, the semipurified preparation was incubated for 2 hours at 37C, in 0.1 M sodium citrate (pH 3.5-5) and 0.1 M sodium phosphate (pH 6-8) containing 5 mM DTT and o.1% SDS, controls were incubated in the same conditions buy in absence of SDS. Proteolytic activity of SDS treated (*) and control samples (o) was determined as described before.

FIGURE 6



Effect of glycine conjugates of bile salts on cysteine proteinase activity. The enzyme preparation was incubated for 2 hours at 37C, in 50 mM Tris, pH 7, containing 5 mM DTT and different concentrations of bile salt conjugates. The enzyme activity was measured as previously described. The effects of conjugates of cholate (*), deoxycholate (o) and chenodeoxycholate ( ) are shown

FIGURE 7a



Effect of taurine conjugates of bile salts on cysteine proteinase activity. The enzyme preparation was incubatd for 2 hours at 37 C, in 50 mM Tris, pH 7, containing 5 mM DTT and different concentrations of bile salt conjugates. The enzyme activity was measured as previously described. The effects of conjugates of cholate (*) (*) deoxycholate (o) and chenodeoxycholate () are shown.

FIGURE 8