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PURIFICATION AND CHARACTERIZATION OF A SPECIFIC FASCIOLA ANTIGEN

By

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ABSTRACT

Specific *Fasciola* antigen was prepared from homogenates of *Fasciola hepatica* adult worms. The homogenate was ultracentrifuged and the supernatant containing crude *Fasciola* antigen was then passed over a cyanogen bromide activated sepharose 4B column coupled with antiserum against *Schistosoma mansoni* adult worm surface antigen. The specific, *Schistosoma*-free *Fasciola* antigen was tested for its specificity by immunodiffusion. Characterization of the specific *Fasciola* antigen was done by gradient poly-acrylamide gel electrophoresis and immunoblotting technique. The electrophoresis migration pattern of specific *Fasciola* antigen, stained with Coomassie blue, showed 7 bands in the 12-54 kDa regions. Using the immunoblotting technique, a batch of positive fascioliasis sera recognized two specific bands at the 33 and 54 kDa regions.

INTRODUCTION

Human fascioliasis is a world-wide problem caused mainly by *Fasciola hepatica* (*F. hepatica*) and to a lesser extent *Fasciola gigantica* (Hardman et al., 1970). In Egypt, it is one of the causes of hepatobiliary disorders (Makled et al.,

1988). Numerous studies have evaluated different antigenic preparations and diverse serological tests for immunodiagnosis of *fascioliasis* (Espino et al., 1987; *Tinell et al., 1987 and Khalil* et al., 1989 & 1990). However, extensive cross reactivity has been reported between *F. hepatica* crude extracts and sera of humans and animals infected with other parasites, particularly *Schistosoma* which is prevalent in Egypt (Hillyer and Capron 1976 and Khalil et al., 1990).

This study was undertaken in a trial to characterize a specific *Fasciola* adult worm antigen that could be employed in improving the immunodiagnosis of fascioliasis.

MATERIALS AND METHODS

Preparation of crude *Fasciola antigen according* to Hillyer et al. (1979): *Adult worms of F. hepatica* were obtained from bovine livers at Cairo slaughter house. The worms were washed repeatedly in cold 0.85% saline followed by distilled water. The worms were then homogenized in a tissue grinder in 0.01 M phosphate buffered saline (PBS), pH 7.0 and centrifuged at 500 g for 10 minutes. The supernatant *was ultracentrifuged at 100,000 g for one hour* at 4°C. Protein content of supernatant was estimated by modified Lowery method (Daughaday et al., 1962) and crude antigen extract was stored at -70°C till use.

Preparation of schistosomal adult worm surface antigen (AWSA) according to McLaren et al. *(1978): Frozen S. mansoni worms were rapidly thawed in a water bath* at 37°C. After centrifugation at 800 g for 20 minutes at 4°C, the supernatant was saved and an equal volume of physiological saline was added to the precipitate. The cycle of freezing-thawing followed by low speed centrifugation was repeated. The pooled supernatant was then subjected to ultracentrifugation at *100,000 g for one hour at* 4°C. The protein content was estimated by modified Lowery method. The antigen was stored at -70°C till use.

Preparation of rabbit anti-*Fasciola* and anti-*Schistosoma mansoni* AWSA antisera according to Hillyer (1980): Newzeland rabbits (3 kg) were *hyperimmunized with either F. hepatica crude worm extract or S. mansoni* AWSA. The priming dose (1mg protein in complete Freund's adjuvant) was followed 2 weeks later by 4 booster doses, each of 0.5 mg protein in incomplete Freund's adjuvant, at weekly intervals. Antibodies' titer was tested for by

immuno-diffusion, and the rabbit was bled for collection of serum one week after reaching a good titer.

Purification of rabbit anti-*Schistosoma mansoni* AWSA antisera by ammonium sulfate precipitation of gamma-globulins according to Nowotny (1979): Ammonium sulfate was added dropwise to rabbit antisera with continuous stirring to reach a final concentration of 33.3%. The precipitate formed was collected by centrifugation for 30 minutes at 1000 g and was dissolved in physiological saline. The ammonium sulfate was removed by overnight dialysis against 0.02 M PBS, pH 7.2.

Purification of crude *Fasciola* antigen by affinity chromatography according to Axen et al. (1967): Gamma-globulins of rabbit anti - *S. mansoni* AWSA antisera were coupled to cyanogen bromide (CNBr) activated sepharose 4B (Pharmacia) according to Gottstein et al. (1983). The crude *Fasciola* antigen at 8 mg protein/g gel was added to the sepharose column. The mixture was gently rotated at 4°C for 16 hours. The unbound purified *Fasciola* antigen was eluted using 2 bed volumes of 0.07 M PBS, pH 7.2 with 0.5 M NaCl. The eluate was collected, its protein content was estimated by modified Lowery method and kept frozen at -70°C till use.

Methods of characterization of the purified *Fasciola* antigen immunodiffusion according to Ouchterlony and Nilsson (1978): Crude and purified *Fasciola* antigen fractions were added into central wells, while both anti-*Fasciola* and anti-*Schistosoma* rabbit antisera were added into the peripheral wells.

Gradient SDS- polyacrylamide gel electrophoresis according to Tsang et al. (1983): Electrophoretic migration of crude and purified *Fasciola* antigen, schistosomal AWSA, high (Sigma) and low (Bio- Rad) molecular weight protein standards was done in gel gradient 4-22.5%, using electrophoretic cell (Bio-Rad Model 595) at constant current of 30 mA for about 5 hours. The gel was stained by 0.1% Coomassie blue in methanol: acetic acid (W/V) for two hours.

Enzyme linked immunoelectrotransfer blot technique (EITB) according to Tsang et al. (1983): Purified *Fasciola* antigen resolved on SDS-PAGE was

transferred onto 0.45 µm nitrocellulose membrane by using Bio-Rad transblot cell and constant 50 V (Bio-Rad power supply model 200/20). The resolved *Fasciola* antigen was then probed with a batch of positive fascioliasis patients' sera as follows: Sera were diluted 1: 100 in working buffer (10% fat-free milk in 0.15 M PBS, pH 7.2). The nitrocellulose strips were exposed to the diluted serum overnight. The strips were washed 3 times (10 minutes each) with 0.15 M PBS, pH 7.2 / 1% Tween-20. The nitrocellulose strips were exposed for 1 hour to horseradish peroxidase-labelled anti-human IgG conjugate (1: 2000 in working buffer). The nitro-cellulose strips were again washed as before. The processed nitrocellulose strips were then exposed for 5-10 minutes to the substrate solution (diaminobenzidine/Sigma). Positive reaction showed up as grey to blue bands within 10 minutes. The strips were then thoroughly rinsed with H_2O.

RESULTS

Immunoaffinity purification of crude *Fasciola* antigen resulted into 2 peaks. The first peak represented the non-adsorbed fraction of purified antigen and the second peak represented the adsorbed cross-reacting fraction (Fig. 1). The protein concentration of purified, *Schistosoma-free Fasciola* antigen was 0.6 mg/ml corresponding to 75% of protein content of starting material.

The purified *Fasciola* antigen was evaluated for its specificity by immunodiffusion. It showed strong reaction with anti-*Fasciola* antibody. In contrast to crude *Fasciola* antigen, the purified antigen did not show cross reacting precipitation lines with anti-*Schistosoma mansoni* AWSA antisera (Fig. 2).

The electrophoretic migration pattern of crude *Fasciola* antigen was compared with that of purified antigen after resolution by SDS-PAGE using Coomassie blue stain. The crude *Fasciola* antigen showed 14 bands in the molecular weight regions of 12-130 kDa, while the purified fraction showed 7 bands in the molecular weight regions of 12-54 kDa. The crude *Schistosoma* antigen showed 14 bands in the 10-49.5 kDa regions (Fig. 3).

By using the immunoblotting technique, a batch of positive fascioliasis sera recognized two specific bands at the 33 and 54 kDa regions (Fig. 4).

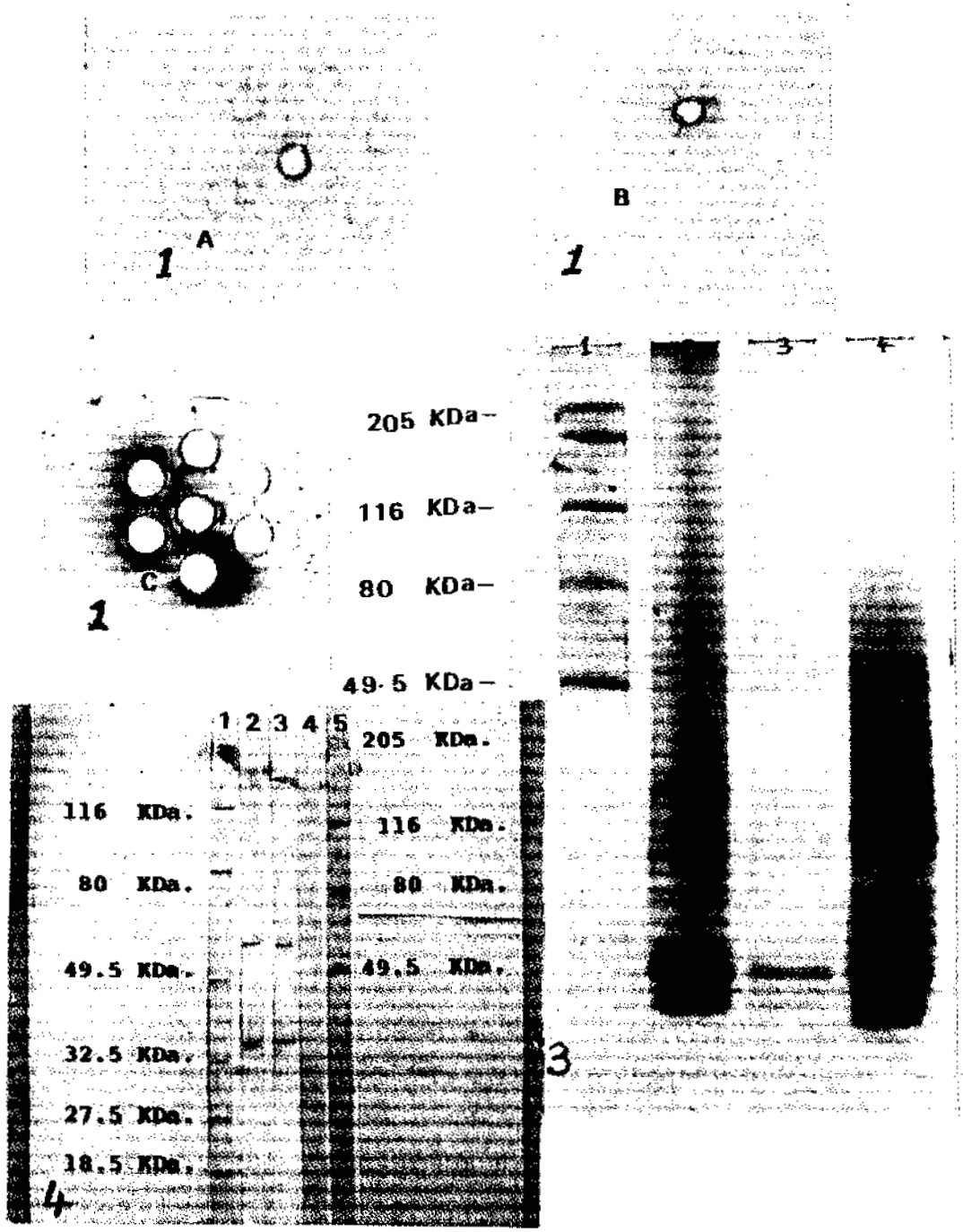

Fig. 1: Immunodiffusion reaction (a) between crude *Fasciola* antigen in central well and anti- *S. mansoni* AWSA antisera in peripheral wells (b) between purified *Fasciola* antigen in central well and anti- *S. mansoni* AWSA antisera in peripheral wells (c) between the purified Fasciola antigen in central well and anti-*Fasciola* antisera in peripheral wells.

Fig. 3: SDS-polyacrylamide gel electrophoretic migration pattern of high molecular weight standard (1), crude *Fasciola* antigen (2), purified *Fasciola* antigen (3) and crude schistosomal antigen (4), stained with Coomassie blue.

Fig. 4: Enzyme linked immunoelectrotransfer blot (EITB) of affinity purified *Fasciola* antigen probed with known positive fascioliasis patients' sera (Track 2, 3) and negative control serum (Track 4). Track 1: Low molecular weight standard marker. Track 5: High molecular weight standard.

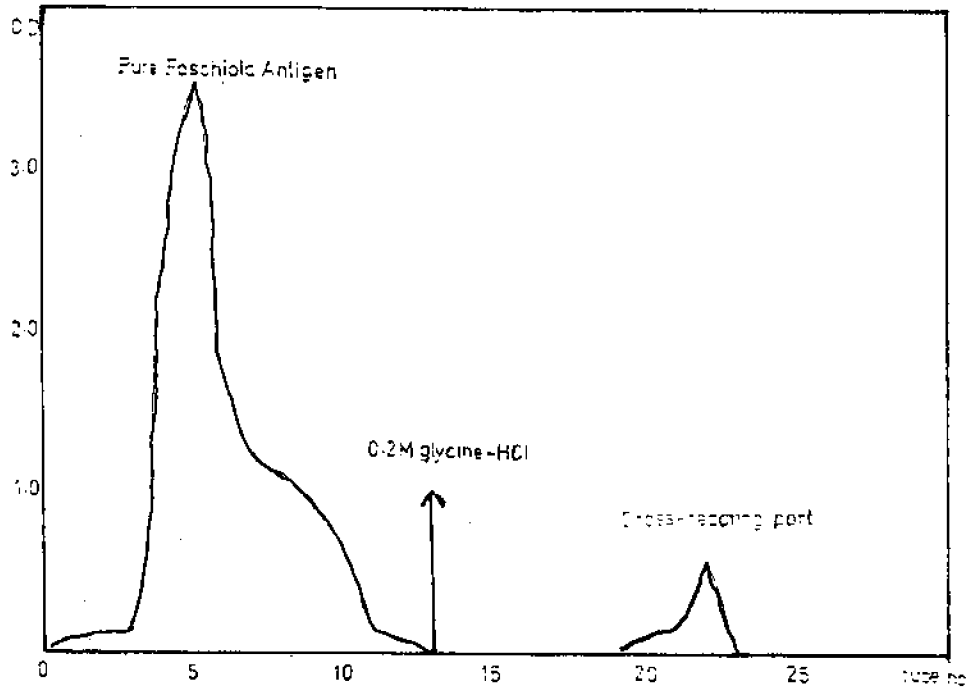

Fig.2: Elution peak of affinity chromatography column of crude Fasciola antigen.

DISCUSSION

The cross reactivity between *Fasciola* and *Schistosoma* trematode worms constitute a major problem in a country like Egypt where schistosomiasis is an endemic disease. Considering this point in this study, shared antigen fractions between *Fasciola* and *Schistosoma* worms were eliminated by affinity chromatography. Anti- *S. mansoni* gamma- globulins were coupled to CNBr-activated sepharose 4B and the crude *Fasciola* antigen was passed through the column. The unbound fraction was obtained as the purified antigen fraction and its specificity was confirmed by immunodiffusion. No precipitation band was shown between the purified antigen fraction and anti-*Schistosoma* rabbit antisera.

Characterization of the affinity purified *Fasciola* antigen was done by studying their electrophoretic migration pattern in SDS- PAGE electrophoresis. Using Coomassie blue stain; the migration pattern of the purified antigen was compared to that of the crude *Fasciola* antigen. The crude *Fasciola* antigen gave 19 bands ranging from 12-130 kDa; while the purified antigen gave 7 bands only, ranging from 12-54 kDa. Strong reaction with the 33 and 54 kDa

antigenic fractions was consistently observed when the purified *Fasciola* antigen was probed with positive sera for fascioliasis in EITB technique. The results confirm those of Santiago et al. (1986) who purified antigen by sephadex G-200 fractionation and found that the antigen fraction of 31-33 kDa was the most prominently recognized by all acute fascioliasis sera tested. Also, the 33 kDa specific fraction is close to a 30 kDa fraction recognized by Marrero et al. (1988), who fractionated *F. hepatica* excretory-secretory antigen against sera of infected rabbits, cows and sheep. The 54 kDa specific fraction is approaching a 57 kDa fraction recognized by an anti- *F. hepatica* monoclonal antibody of Hillyer (1989).

In conclusion, the employment of this specific affinity purified *Fasciola* antigen fraction is proposed for the immuno-diagnosis of fascioliasis to reach an accurate diagnosis and consequently proper management of fascioliasis.

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REFERENCES

Axen, R.; Porath, J. and Ernbach, S. (1967): Chemical coupling of peptides and protiens to polysaccharides by means of cyanogen halides. *Nature*, 214: 1302-1304.

Daughaday, W.H.; Lowery, O.H.; Rosbrough, N.H. and Fields, W.S. (1962): Determination of cerebrospinal fluid protien with the folin phenol reagent. *J. Lab. Clin. Med.*, 39: 663-665.

Espino, A.M.; Dumenigo, B.E.; Frenandez, R. and Finlay, C.M. (1987): Immunodiagnosis of human fascioliasis by enzyme-linked immunosorbent assay using excretory-secretory products. Am. *J. Trop. Med. Hyg.*, 37: 605-608.

Gottstein, B. (1983): Isolation of an antigen fraction from *Echinococcus multilocularis* with high species specificity and its identification with the western blotting technique. In: Immunoenzymatic Techniques. S. Avrameas; P. Druet; R. Masseyeff and G. Feldman, eds.. Elsevier, Amsterdam.

Hardman, E.W., Jones, R.L.H. and Davies, A.H. (1970): Fascioliasis; a large outbreak. *Br. Med.* J., 3: 502-505.

Hillyer, G.V. (1980): Isolation of *Fasciola hepatica* tegument antigens. *J. Clin. Microbiol.*, 12: 695-699.

Hillyer, G.V. (1989): *Fasciola hepatica*: Sp2/0 (helminth myeloma) hybridoma expressing parasite antigen. Am. *J. Trop. Med. Hyg.*, 41: 674-679.

Hillyer, G.V. and Capron, A. (1976): Immunodiagnosis of human fascioliasis by counterelectrophoresis. *J. Parasitol.*, 62: 1011-1013.

Hillyer, G.V., Pelly, R.P. and Del-Liano De Diaz, A. (1979): A solubilization of antigens of *Fasciola hepatica* which react with antibodies to *Schistosoma mansoni*. *J. Parasitl.*, 65: 55-60.

Khalil, H.M., Abdel Aal, T.M., Makled, M.Kh., Abdalla, H.M., Fahmy, I.M. and El-Zayyat, E.A. (1989): Sensitivity of crude and purified *Fasciola* antigens in immunological diagnosis of human fascioliasis. *J. Egypt. Soc. Parasitol.*, 19: 305-402.

Khalil, H.M., Abdel Aal, T.M., Makled M.Kh. , Abdalla, H.M., Fahmy, I.A. and El-Zayyat, E.A. (1990): Specificity of crude and purified *Fasciola* antigens in immunodiagnosis of human fascioliasis. *J. Egypt. Soc. Parasitol.*, 20(1): 87-94.

Makled, M.Kh., Khalil, H.M., Abdalla, H.M. and El-Zayyat, E.A. (1988): Fascioliasis and hepatic affection. *J. Egypt. Soc. Parasitol.*, 18(1): 1-9.

Marrero, R., Carlos, A., Santiago, N. and Hillyer, G.V. (1988): Evaluation of immunodiagnostic antigens in the execretory-secretory products of *Fasciola hepatica*. *J. Parasitol.*, 74: 646-652.

McLaren, M., Draper, C.C., Roberts, J.M., Minter-Goedloed, E., Lightart, G.S., Tessdale, C.H., Amin, M.A., Omer, A.H.S., Bartlett, A. and Voller, A. (1978): Studies on the enzyme linked immunosorbent assay (ELISA) test for *Schistosoma mansoni* infections. Ann. Trop. Med. Parasitol., 72: 243-253.

Nowotny, A. (1979): Basic Exercises in Immunochemistry. Springer Verlag Berlin Heidlberg. New York.

Ouchterlony, O. and Nilsson, L.A. (1978): Immunodiffusion and immunoelectrophoresis. In: Handbook of Experimental

Immunology. Weir, D.M. ed.. Blackwell Scientific Publications, Oxford.

Santiago, N., Hillyer, G.V., Garcia-Rosa, M. and Morales, M.H. (1986): Identification of functional *Fasciola hepatica* antigens in experimental infections in rabbits. Am. *J. Trop. Med. Hyg.*, 35: 135-140.

Tinell, M., Falagiani, P., Riva, G. and Genchi, C. (1987): RAST and ELISA in three autochonus parasitic infections in Italy: Toxocariasis, hydatidosis and fascioliasis Boll. 1st. *Sieroter Milan*, 66: 38-45.

Tsang, V.C.W., Peralta, J.M. and Simons, A.R. (1983): Enzyme linked immunoelectrotransfer blot techniques (EITS) for studying the specificities of antigens and antibodies separated by gel electrophoresis. In: Methods in Enzymology, Immunochemical Techniques. Langone J. J. and Vunakie H. V., eds. New York, Academic press.