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Fractionation and characterization of *Fasciola gigantica* soluble somatic antigens

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Fasciolosis caused by *Fasciola gigantica* has immense economic importance, often resulting in chronicity and sub-clinical infection in cattle. Detection of infections is usually confirmed by coprological examination and eggs can be detected at about 10th week post infection (Fagbemi *et al.* 1997) whereas, immunologically diagnosis is possible in the prepatent period (Leclipteux *et al.* 1998, Cornelissen *et al.* 1999). Several sero-diagnostic techniques using different antigenic preparations from *Fasciola* spp. have been reported for the immunodiagnosis of fasciolosis (Mousa 1994, Goubadia and Fagbemi 1995). Immuno-diagnostic assays for helminth infections are hampered by lack of specificity that is attributable to the possession of common antigens by different helminths (Fagbemi and Obarissiagbon 1991). To enhance the specificity and sensitivity, various attempts have been made to identify and isolate *Fasciola* specific antigen (Fagbemi and Guobadia 1995, Dixit *et al.* 2002). In the present study the fractionation of *Fasciola gigantica* soluble somatic protein was undertaken so as to isolate a pure antigen for sero-diagnosis of fasciolosis.

Mature *Fasciola gigantica* collected from the livers and bile ducts of buffaloes were repeatedly washed in chilled phosphate buffer saline (PBS) with 7.2 pH. They were then soaked on sterile filter paper and the flukes were triturated in PBS (1 : 1, w/v). The triturated material was centrifuged at 10.000 rpm for 10 min. and the supernatant was kept at –20°C. The protein concentration of the antigen was estimated by the method described by Lowry *et al.* (1951).

Fifteen milligram of crude somatic antigen was layered on a sephacryl S–200 column (48 cm × 1 cm) and fractionated in tris-HC1 (pH 7.2) buffer containing 0.15 M sodium chloride, 0.03 mM PMSF (phenyl methyl sulphonyl fluoride) and 0.04% sodium azide at a flow rate of 18 ml/hr. Dextran blue was used to determine the void volume (15 ml) and fractions (3 ml/tube) were collected. The absorbance of each fraction was measured in UV-spectrophotometer at 280 nm.

Six milligram of first gel-permeated fraction (GP1 and GP2) were layered on a DEAE-sepharose column (12 cm × 1 cm) separately and fractionated in 25 mM tris HC1 (pH 8.0) buffer containing 0.1 mM EDTA, 6M urea at a flow rate 15 ml/hr. At first, protein was eluted in the tris HC1 buffer having 0.15M NaC1 and subsequently fractionation was done by tris HC1 buffer containing 0.3 M NaCl. The absorption of each fraction was measured in UV spectrophotometer at 280 nM.

Five male cattle calves (3–4 month-old) were coprologically examined at regular intervals and found free of *F. gigantica* infection were maintained in stall fed conditions and used for the study. Three of 5 calves were infected orally with 600 metacercariae of *F. gigantica* per calf (infected group). The remaining 2 calves were considered as control. Sera were collected from those animals at regular intervals from 0 day through 7 weeks to assess kinetics of sero-reactivity of the antigens.

Two male adult New Zealand White rabbits (1200g) were inoculated (deep I/M) with *F. gigantica* crude somatic antigens at 7 days interval. The first injection (500 µg protein) was made with Freund's complete adjuvant (1 : 1, v/v), followed by increasing dose of proteins (up to 1200 µg) along with Freund's incomplete adjuvant (1 : 1, v/v). After 5 days of last (sixth) injection blood was collected, sera separated and stored at –20°C until further use.

To determine the immuno-reactivity of crude somatic and fractionated antigen, indirect plate ELISA was performed as per Sarkar *et al.* (2003). Crude and fractionated antigens were coated @ 2 µg per well. Sera of experimental animals at different post inoculation intervals were used as primary antibody.

It was performed as per Laemmli (1970) on vertical slab gel electrophoresis apparatus using 12.5% polyacrylamide gel. 50mA current was used for stacking gel and 100 mA current for resolving gel until the tracking dye reached the bottom. Fifty microgram proteins (crude somatic and fractionated) were loaded per lane in each case. The gel was stained by Coomassie brilliant blue R–250 staining solution

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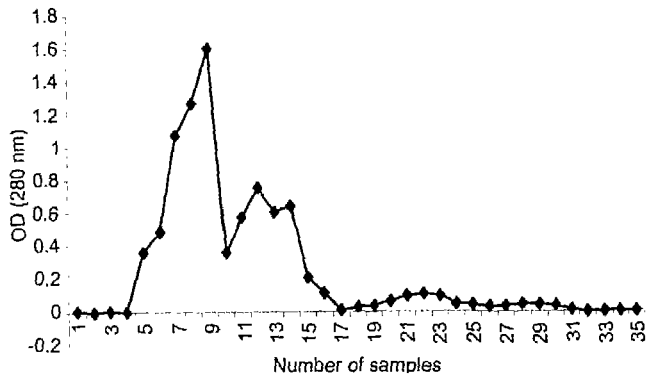

Fig. 1. Elution profile of gel filtration chromatography of *Fasciola* crude somatic antigen.

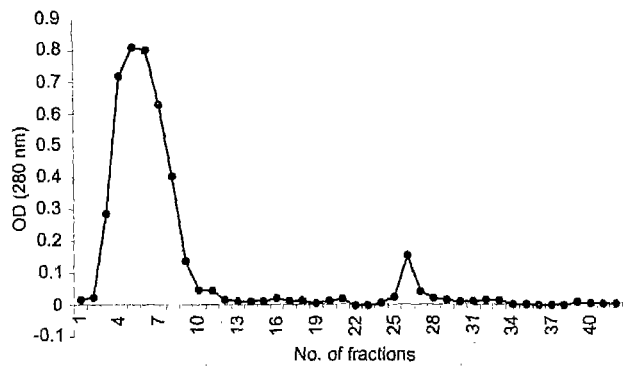

Fig. 3. Elution profile of anion exchange chromatography of gel permeated fraction (GP2) of *F. gigantica* somatic antigen by DEAE sepharose.

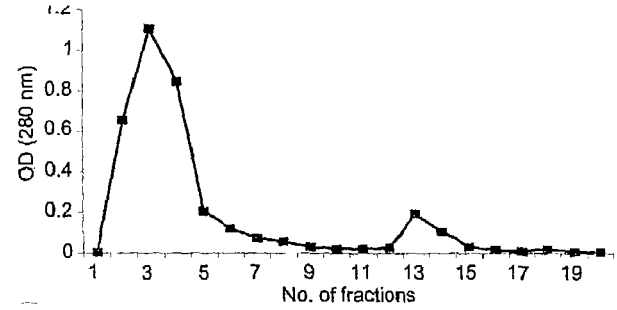

Fig. 2. Elution profile of anion exchange chromatography of gel permeated fraction (GP1) of *F. gigantica* somatic antigens.

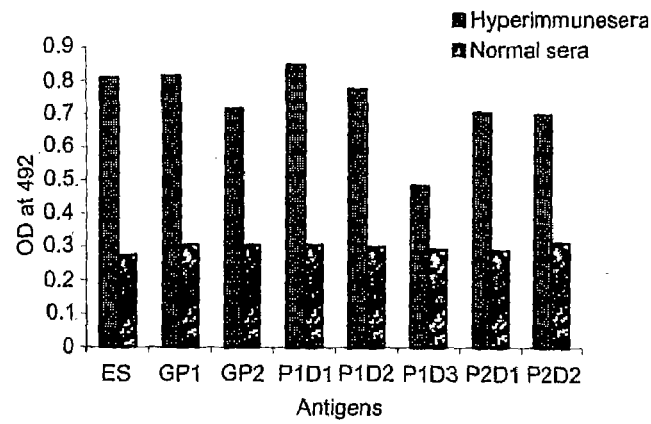

Fig. 4. Seroreactivity of crude and fractionated somatic antigens against hyperimmune and normal rabbit serum as assessed by ELISA.

and was destained subsequently by destaining solution.

Two prominent peaks were obtained in gel filtration chromatography with O D (280nm) values (Fig.1). The fractions under each peak were pooled and dialyzed against distilled water of 4°C. Pooled proteins (GP1 and GP2) were concentrated using sucrose and sterilized through membrane filter (0.22 μ), aliquoted and stored at –20°C until further use.

In anion exchange chromatography 2 prominent peaks were observed with O.D (280nm) values of GP_1 fraction (Fig. 2). Proteins of the ascending limb and descending limb of the first peak were pooled separately and were considered as P_1D_1 P_2D_2 respectively. Protein (s) of second peak was also pooled and considered as P_1D_2 and P_1D_2 respectively. Protein (s) of second peak was also pooled and considered as P_1D_3. Pooled proteins P_1D_1, P_1D_2, and P_1D_3, were concentrated using sucrose and sterilized through membrane filter, aliquoted and stored at –20°C until further use. Similarly, upon anion exchange chromatography 2 peaks were obtained from GP2 (Fig. 3). Proteins of the ascending and descending limb of the first peak were pooled and considered as P_2D_1, P_2D_2. The second peak was named as P_2D_3.

Indirect ELISA was performed using chromatographed fractions (GP1, GP2, P_1D_1, P_1D_2, P_1D_3, P_2D_1, P_2D_2, and P_2D_3) of somatic antigen with hyper immune sera (1: 200) raised in rabbits against crude somatic antigen and also with experimentally *Fasciola* infected calf sera. Sero-reactivity of P_1D_1 was slightly higher than all other fractionated antigens when hyper immune serum was used (Fig. 4). Kinetics of the seroreactivity of all the anion exchange chromatographed fractions was assessed using experimental calf sera of different post inoculation (PI) periods (0 day through 7 weeks). Anti-*Fasciola* antibody was detected from third week PI onward (Fig. 5). Amongst the fractionated antigens P_1D_1 showed higher seroreactivity in earlier PI stages.

SDS-PAGE was performed to assess the polypeptide profiles of somatic of *F. gigantica*. Seventeen major and 9 minor bands were detected after Coomassie blue staining. The molecular weight of the polypeptides calculated was 12, 14, 15, 23, 26, 28, 30, 32, 35, 36, 38, 43, 45, 47, 58, 67 and 68 kDa for major and 17, 18, 20, 40, 61, 63, 65, 66, and 97 kDa for minor bands. The highest sero-reactive fraction (P_1D_1) was observed to possess 4 polypeptides of molecular weight 26, 28, 38 and 68 kDa (Fig. 6). Earlier, several workers reported various sero-reactive antigens of *Fasciola gigantica* (Fagbemi and Hillyer 1991, Fagbemi and Guobadia 1995, Yadav and Gupta 1995 and Yadav *et al.* 2001). The kinetics

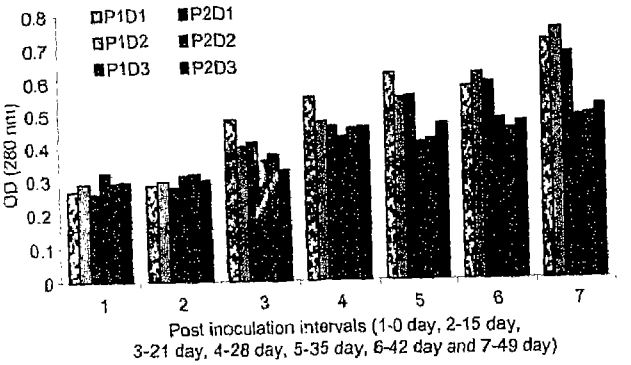

Fig. 5. seroreactivity of fractionated somatic antigens with experimentally inoculated calf sera by ELISA.

of sero-reactivity was assessed of the crude and fractionated antigens by ELISA in different post inoculation stages, it was observed that P_1D_1 could detect anti-*Fasciola* antibodies at 2–3 weeks post inoculation. Similarly, 28 kDa cysteine protease antigen of *Fasciola gigantica* recognized antibodies by Western blot in majority of infected sheep at second week post inoculation onward (Dixit *et al.* 2002). *Fasciola hepatica* antigen preparation could detect serum antibodies at fourth week post inoculation in experimentally infected sheep using dot-ELISA. Cathepsine-L-like protease was reported to recognize specific antibodies against *Fasciola gigantica* in infected sheep at fifth week post inoculation (Cornelissen *et al.* 2001). In the present study, P_1D_1 was observed to be diagnostically important antigen as it could detect anti-*Fasciola* antibodies at second week post inoculation by dip-stick ELISA (unpublished observation). The species specificity among the closely related trematodes is to be evaluated before use as a diagnostic kit for ruminant fasciolosis.

SUMMARY

The immunodominant and species-specific antigen (s) of *Fasciola gigantica* of diagnosis were prepared by column chromatographic techniques. On gel filtration chromatography, 2 prominent peaks (GP1 and GP2) were obtained. By anion exchange chromatography these fractions were further resolved into P_1D_1, P_1D_2, and P_1D_3 and P_2D_1, P_2D_2, and P_2D_3 respectively. More seroreactivity was observed in P_1D_1 than the other fractions as assessed by ELISA. P_1D_1 was observed to contain 4 polypeptides of molecular weight 26, 28, 38 and 68 kDa as assessed by SDS-PAGE. Anti-*Fasciola* antibodies in experimentally infected calves were detected by this fractionated antigen (P_1D_1) as early as second to third week post inoculation and can be used for serdiagnosis of fasciolosis.

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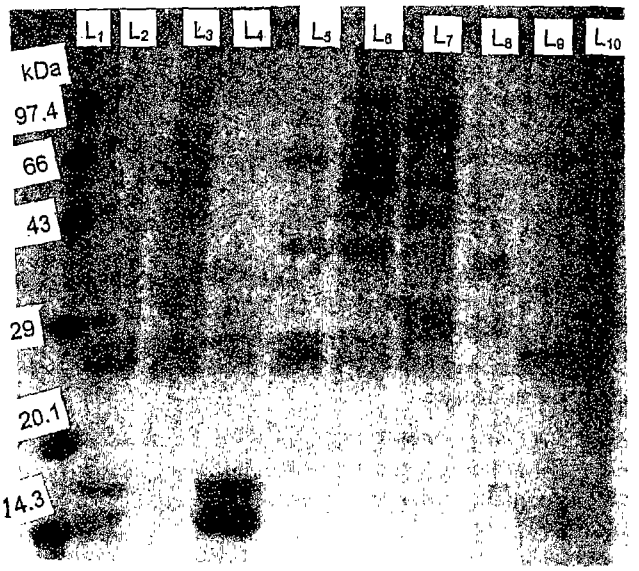

Fig. 6. SDS-PAGE profile of crude and fractionated antigens of *F. gigantica* somatic antigens—Lane–1: Standard molecular weight marker; Lane–2: Crude somatic antigen of *F. gigantica*; Lane–3: GP_1; Lane–4: GP_2; Lane–5: P_1D_1; Lane–6: P_1D_2; Lane–7: P_1D_3; Lane–8: P_2D_1; Lane–9: P_2D_2; Lane–10: P_2D_3.

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