# Partial purification and characterization of *Gigantocotyle explanatum* somatic antigens

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## Abstract

Soluble extracts of *Gigantocotyle explanatum*, isolated from the liver of buffalo *Bubalus bubalis* were fractionated on Sephadex G-200 columns. Nine major fractions referred to as F1, F2, F3, F4, F5, F6, F7, F8 and F9 were separated. Each fraction was tested by ELISA for antigenicity using sera from *G. explanatum*-infected field buffaloes. Fractions F1 and F2 were highly antigenic, F3, F4, F6 and F7 were moderately antigenic and F5, F8 and F9 were poorly antigenic. Analyses by SDS–PAGE revealed that each fraction comprised several polypeptide(s) in the molecular weight range of <29 to >205 kDa. Results of Western blotting indicated that not all polypeptides which appeared in the SDS–PAGE were antigenic. The antigenic molecules of each fraction were mostly in the low molecular weight range of <14 to >94 kDa with the polypeptides in the range of >14, 14, 18, 21–25 and 34–36 kDa.

## Introduction

*Gigantocotyle explanatum* (Fukui, 1929) is a member of the family Paramphistomidae which constitutes one of the most common and abundant groups of digenetic trematodes of domesticated livestock, especially in tropical and subtropical areas of the world. Many genera of adult paramphistomes inhabit the fore stomach of ruminants, but *G. explanatum* inhabits the bile duct. The life cycle of *G. explanatum* resembles that of *Fasciola hepatica* (Dunn *et al.*, 1985). The disease paramphistomiasis, caused by massive infections of the small intestine with immature paramphistomes, is characterized by sporadic epizootic outbreaks of acute parasitic gastroenteritis with high morbidity and mortality rates, particularly in young stock (Horak, 1971). Adult *G. explanatum* causes haemorrhaging, pronounced periductal fibrosis and other hyperplastic changes. Many outbreaks of paramphistomiasis have been reported in the past from India (Dutt, 1980).

Despite the considerable economic importance of paramphistomes, no comprehensive attempt has been made to analyse the antigens of this group of helminths although a number of studies on the taxonomy, ultrastructure and biochemical composition of paramphistomes have been published (Dunn *et al.*, 1985, 1987; Awharitoma *et al.*, 1988; Khan *et al.*, 1990; Mattison *et al.*, 1994; Abidi & Nizami, 1995). However, more recently Saifullah *et al.* (2000a) analysed excretory–secretory (ES) and somatic antigens of a rumen amphistome *Gastrothylax crumenifer* and demonstrated a number of antigens in its ES and somatic extracts. Furthermore, Saifullah *et al.* (2000b) partially purified and characterized the somatic antigens of *G. crumenifer* by gel filtration chromatography, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western immunoblotting using rabbit hyperimmune sera but no attempt has been made to characterize antigens of *Gigantocotyle explanatum*. The diagnosis of *G. explanatum* is normally carried out by routine microscopic examination of eggs in faeces, which is time consuming and only adult parasite infections can be detected by this method. The identification, isolation, purification and characterization of parasite antigens are required either for immunodiagnosis or immunoprotection of the disease. The present study was therefore

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undertaken to partially purify and characterize the *G. explanatum* somatic antigens to assist in the immuno-diagnosis of liver paramphistomiasis.

## Materials and methods

### Collection of parasites and serum

Adult worms of *Gigantocotyle explanatum*, collected from the liver of Indian water buffalo (*Bubalus bubalis*) slaughtered at the local abattoir, were quickly washed with Hanks' balanced salt saline (HBSS) without glucose, prewarmed at 37°C at a pH of 7.4. Worms were ground at 4°C in 10% phosphate buffered saline (PBS), pH 7.4 and subjected to ultrasonication (5 mm probe) for 1 min at 4°C and centrifuged at 10,000 g for 15 min. The supernatant was stored at −20°C with a 0.02% NaN$_3$ preservative. The protein concentration was determined by the method of Spector (1978).

Blood samples from normal and *G. explanatum*-infected buffaloes were collected from slaughtered animals at the local abattoir. The blood was allowed to coagulate and the serum was isolated after centrifugation.

### Column chromatography

Phosphate buffered saline (0.15 M, pH 7.4) was used for washing and equilibrating a Sephadex G-200 (Pharmacia, Sweden) gel filtration column (2 × 90 cm). Five millilitres of a concentrated crude antigen (CA) of *G. explanatum* containing 100 mg protein was applied to a Sephadex G-200 column. Each 4 ml fraction was collected and monitored at 280 nm for protein concentration. Individual peaks of eluant were collected separately, packed into cellulose tubing (Mr cut-off 3500, Sigma, USA), dialysed against distilled water and lyophilized in a freeze dryer (DC 41, Yamato, Scientific Co. Ltd. Tokyo, Japan). The freeze dried material was stored at −20°C until used.

### Enzyme-linked immunosorbent assay (ELISA)

The ELISA of partially purified antigens of *G. explanatum* was performed as previously described by Ahmad & Nizami (1998). Flat-bottomed polystyrene microtitre plates (Dynatech Immulon 1) were coated with 50 µl per well of various purified fractions in 15 mM Na$_2$CO$_3$, 35 mM NaHCO$_3$ buffer (protein concentration of 12 µg/ml$^{-1}$) and left overnight at 4°C. Wells were then washed three times with PBS containing 0.1% Tween (PBST), blocked with 150 µl of 5% skimmed milk in PBS and incubated at room temperature for 1 h. The plates were again washed three times with PBST. Serially diluted 100 µl normal or *G. explanatum*-infected buffalo sera were added to each well and incubated for 3 h at room temperature in a humid chamber. The plates were washed again for 3 × 10 min with PBST. Thereafter, 50 µl of 1:1000 diluted anti-bovine IgG conjugated with alkaline phosphatase was added to each well and again incubated for 3 h at room temperature. After incubation, the plates were washed for 3 × 10 min with PBST. The reaction was developed by adding 100 µl of substrate, p-nitrophenyl phosphate (Sigma, USA) in a concentration of 1 mg ml$^{-1}$ in 10% diethanolamine (DEA) buffer and incubated at room temperature for 20 min. Finally, the reaction was stopped by the addition of 50 µl of 3 N NaOH solution, and the optical density (OD) was recorded at 405 nm with an ELISA Reader (SLT Lab Instruments, Austria).

### SDS−PAGE and Western blot analysis

For the analysis of protein profiles, SDS−PAGE was performed following the method of Laemmli (1970) in a separating 7−15% gradient slab gel and 4% stacking gel using a vertical slab gel system (Pharmacia, LKB, Sweden). A soluble protein sample containing 70 µg protein was mixed with an equal volume of Laemmli's sample buffer (0.625 M Tris-HCl, pH 6.8), containing 20% SDS and 5% (v/v) β-mercaptoethanol, and the sample was boiled for 8 min at 100°C. Standard high molecular weight marker proteins used in the present study were purchased from Pharmacia, (LKB). The electrophoresis was carried out at 6°C using a Multitemp II, thermostatic circulator (Pharmacia, LKB, Sweden) by applying a constant current of 30 mA/slab gel until the marker tracking dye reached 1 cm above the gel before the end of electrophoresis.

After electrophoresis, one gel was Coomassie stained while another gel was transferred onto 0.45 mm pore size Immobilon p membrane (Millipore, USA) by a semi-dry technique using the Nova Blot assembly (Pharmacia, LKB) as described by Towbin *et al.* (1979). Non-specific binding sites were blocked with blotto (i.e. 5% skim milk, Anikspray, India). Blots were incubated in normal or *G. explanatum*-infected buffalo sera with blotto in a ratio of 1:100 for 3 h. Subsequently, the strips were thoroughly washed with Tris buffered saline, pH 7.4 containing 0.02% Tween 20. The strips were then incubated in anti-bovine IgG conjugated with alkaline phosphatase at a dilution of 1:2000 in blotto for 3 h. Finally, the antigenic polypeptides were visualized by bromochloro-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) chromogen. All steps were carried out at room temperature with continuous shaking.

## Results

### Partial purification of G. explanatum *somatic proteins*

A total of nine protein profiles were obtained (fig. 1) and hereafter referred to as F1–F9. Fractions F1, F2 and F9 appeared as very prominent and sharp peaks while the rest of the fractions are distinct but small peaks. The protein concentration in the individual peaks were 18.60, 7.81, 2.32, 3.41, 5.80, 1.34, 11.82, 1.21 and 24.85 mg, respectively. Thus the total protein recovery from the column was 77.16%. Each protein profile was dialysed against distilled water and lyophilized for further use.

### ELISA

A cut-off point (OD value 0.175) was calculated after reacting non-infected buffalo serum with all the antigens. The cut-off value for ELISA was the mean OD value of the control serum plus 2 SD. Fractions F1 and F2 were highly antigenic (ELISA titres 6400 and 12800, respectively with infected buffalo sera). Fractions F3, F4 and F6 were
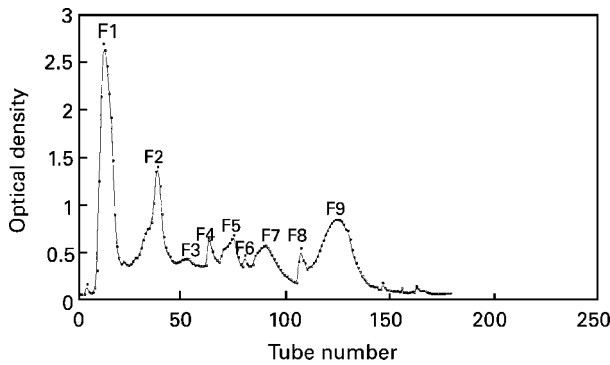
Fig. 1. Protein profiles (F1–F9) of somatic extracts of *Gigantocotyle explanatum* from a Sephadex G-200 gel filtration column.

moderately antigenic (titres 3200), F7 was less antigenic (titre 1600) and F5, F8 and F9 were poorly antigenic (titres 800) (fig. 2).

### SDS-PAGE and Western blot analysis

The results of Coomassie stained SDS–PAGE revealed that each of the nine fractions consisted of several polypeptide(s) of varying molecular weights (fig. 3). The F1 fraction comprised of 18 bands ranging from >205 to < 29 kDa, whereas the F2 and F3 contained 13 and 12 polypeptides respectively ranging from 102 to < 29 kDa. The F4, F5, F6, F7, F8 and F9 fractions comprised 3, 3, 3, 2, 1 and 2 polypeptides with molecular weights ranging from 36 to < 29 kDa.

Since all nine fractions obtained from Sephadex G-200 column reacted positively in the ELISA, these fractions were subjected to Western blotting for further analysis of their antigenicity. Western blots of the nine fractions probed with normal buffalo serum showed that the non-infected serum did not contain IgG anti-*G.explanatum* antibody. However, when these blots were reacted with *G. explanatum* infected buffalo serum, a number of SDS–PAGE separated polypeptides were observed to be antigenic and detected by the infected buffalo serum. A total of 11, 7, 6, 2, 2, 2, 4, 4, 1 and 1 antigenic bands were



Fig. 3. Polypeptide profiles of nine fractions (1–9) of somatic extracts of *Gigantocotyle explanatum* as revealed by Coomassie staining. Lane M indicates standard molecular weight markers.

observed in the Western blots of F1, F2, F3, F4, F5, F6, F7, F8 and F9 fractions respectively. Overall analysis of data revealed that the antigenic molecules of all nine fractions were mostly of low molecular weight ranging from > 94 to < 14 kDa (fig. 4). But the antigenic polypeptides of <14, 14, 18, 21–25, and 34–36 kDa appeared to be dominant.

### Discussion

An analysis of individual antigens, their abundance and immunogenicity is necessary to facilitate the preparation of specific antigens suitable for immuno-diagnosis. The present study aimed to identify and partially purify antigenic polypeptides of *Gigantocotyle explanatum* that may help in the immunodiagnosis of paramphistomiasis. There is no detailed information on
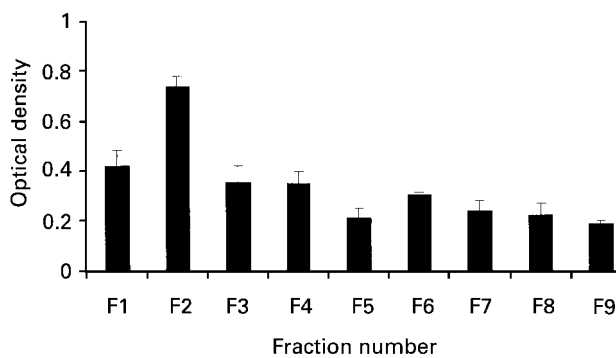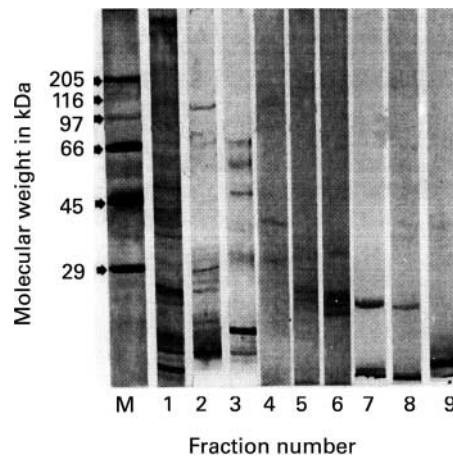


Fig. 2. Absorbance values of Sephadex G-200 purified antigens of *Gigantocotyle explanatum* detected by a 1:3200 dilution of infected buffalo sera. The optical density values for all antigens tested are a mean of five replicates.
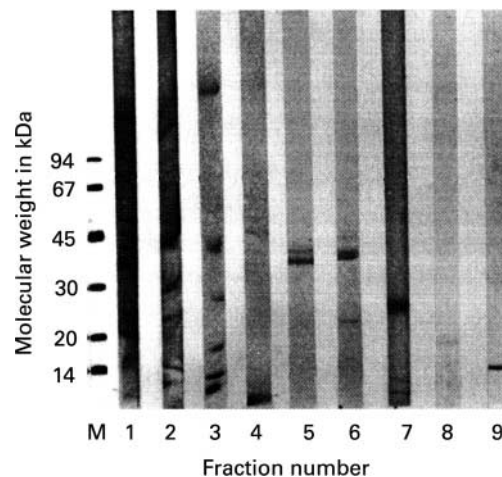


Fig. 4. Western immunoblotting of nine fractions of somatic extracts of *Gigantocotyle explanatum* obtained by Sephadex G-200 gel fractionation and reacted with naturally infected buffalo serum. (M = standard molecular weight markers.)

the antigenic polypeptides of this parasite, although Maji *et al.* (1997a) investigated antigenic cross-reactivity amongst three species of paramphistomes using the Ouchterlony double diffusion test. In another study, Maji *et al.* (1997b) analysed the polypeptides of *Paramphisto-mum epiclitum*, *Gastrothylax crumenifer* and *Gigantocotyle explanatum*. These workers identified a total of 21 polypeptides in Coomassie stained gels in the molecular weight range of 19.9 to 125.8 kDa. However, Maji *et al.* (1997a,b) did not analyse the antigenicity of individual polypeptides identified by Coomassie staining. The present study is different from the previous work because the antigenic polypeptides of *G. explanatum* were partially purified using more sensitive and specific tests (i.e. ELISA and Western blotting). In addition, this is the first time that antigenic polypeptides of this amphistome species have been investigated using sera from naturally infected field buffaloes. However, a number of reports are available on the antigens of the related bile duct trematode *Fasciola hepatica* (Spithill *et al.*, 1997).

The present results, using infected buffalo serum, identified antigenic components in all nine fractions obtained from gel filtration columns but the number of antigenic polypeptides varied from fraction to fraction. Most of the antigenic polypeptides were observed in the low molecular weight range, which further supports our previous work (Saifullah *et al.*, 2000a,b) that antigenic polypeptides of amphistome parasites are of low molecular weight in the range of <14 to 50 kDa. However, only one component in the fraction F3 was >90 kDa molecular weight.

Santiago & Hillyer (1988) identified some prominent somatic antigens of the liver trematode *Fasciola hepatica* with an apparent molecular weight range of 30 to 38 kDa, with diffuse bands of 56, 64 and 69 kDa also being observed. These workers assumed that the development of antibodies to low molecular weight antigens might depend on the genetic constitution of the host, and hence the antigens are either prominently recognized or not at all. In the present study, antigens of apparent low molecular weight in the range of <14 to 25 kDa were very prominent in all gel filtration fractions except for F5 which consisted of 34 and 36 kDa antigenic polypeptides.

Some investigators have attempted to characterize the antigenic polypeptides of another trematode *Paragonimus heterotremus*. Indrawati *et al.* (1991) studied partially purified antigenic polypeptides of *P. heterotremus* using Western blotting and identified a 35 kDa antigen which showed 100% sensitivity and specificity for the detection of paragonimiasis. Other workers (Maleewong *et al.*, 1992) have analysed the metabolic products of *P. heterotremus* by SDS–PAGE and immunoblotting. A total of 11 antigenic polypeptides of this parasite have been identified by Western blot in the molecular weight range of 12.3 to 144 kDa. Although in the present study antigenic polypeptides in the range of <14 to 25 kDa apparent molecular weight reacted strongly with *G. explanatum* infected buffalo sera, it is too early to assign any specific immunodiagnostic role for these polypeptides until their potential importance is investigated using more serum samples from naturally infected buffaloes. It was also observed that the F1 (Mr range 19–70 kDa) and F2 fraction (Mr range <14 to 85 kDa) from the Sephadex G-200 column could detect IgG antibodies even up to a dilution of 1:6400 and 1:12800 of primary sera in the ELISA. This shows that there are certain immunodominant antigens which can detect IgG molecules in the ELISA up to such a high dilution of primary sera. It is thus concluded that soluble extracts of *G. explanatum* consist of a number of polypeptides, several of which are antigenic but the polypeptides in the Mr range of <14 to 36 kDa appeared to be more prevalent.

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