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## Isolation and partial characterization of species-specific and cross-reactive antigens of *Echinococcus granulosus* cyst fluid

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Two parasite antigens have been isolated from *Echinococcus granulosus* hydatid cyst fluid using hydrophobic interaction chromatography, anion exchange chromatography and gel filtration chromatography. Initial characterization of the antigens indicates that both are glycoproteins, of approximately 20 and 48 kDa (Eg20 and Eg48). When the two antigens were tested with a battery of antisera from patients with heterologous parasitic infections, only Eg20 was found to be specific for *E. granulosus*. The Eg48 antigen cross-reacted with the sera of 33% of *E. multilocularis* patients. In both antigens, some of the epitopes recognized by antibodies in the sera of hydatid patients were periodate-sensitive. This suggests the involvement of carbohydrates in at least some of the antigenic determinants. Due to the abundance of the Eg48 antigen in the hydatid cyst fluid, it would be the more practically useful antigen for disease diagnosis, especially in countries where only *E. granulosus* is endemic.

**Key words:** *Echinococcus granulosus*; Hydatid cyst fluid; Purification; Specificity; Cross-reactivity; Immunodiagnosis

### Introduction

Unilocular hydatidosis is a disease caused by infection with the metacestode stage of the dog tapeworm *Echinococcus granulosus*. Although the disease is known to be widespread, exact data concerning its prevalence in man are lacking in many countries in which the disease is known to be endemic. Such information is usually obtained from hospital records, and disease endemicity is

inferred from animal studies.

In the last decade numerous techniques have been employed in the serological diagnosis of hydatid disease. Some of these techniques have also been utilized in seroepidemiological surveys [1–3]. Traditionally, the hydatid cyst fluid has been used as a source of antigen for serodiagnosis [4,5]. Analysis of the cyst fluid has revealed the presence of parasite antigens shared with other helminths, as well as host serum components [6,7]. The presence of such contaminants in the cyst fluid has limited its suitability for specific diagnosis of *E. granulosus*.

Work on the purification of the hydatid fluid led to the isolation of two major parasite antigens; a thermolabile lipoprotein, antigen 5, and a thermostable lipoprotein, antigen B [8–10]. Of these two, antigen 5 has been shown by many workers to be of diagnostic significance for *E. granulosus*. Although initial studies showed that antigen 5 was specific for *E. granulosus*, subsequent work revealed cross-reactivity with sera of patients infected with other parasites, particularly *Echinococcus multilocularis* and *Cysticercus cellulosae* [11,12].

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**Abbreviations:** AEC, anion exchange chromatography; Eg20, *Echinococcus granulosus* 20; Eg48, *Echinococcus granulosus* 48; ELISA, enzyme-linked immunosorbent assay; GFC, gel filtration chromatography; HIC, hydrophobic interaction chromatography; HRP, horseradish peroxidase; IHA, indirect hemagglutination; LPS, lipopolysaccharide; ME, mercaptoethanol; PBS, phosphate-buffered saline; OPD, *o*-phenylenediamine; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

This has stimulated the search for the identification and isolation of a specific parasite antigen. Shepherd and McManus [13], using immunoprecipitation and immunoblotting techniques, demonstrated that the subunits of antigen B are specific for *E. granulosus*. However, 23% of hydatid patients failed to react with the antigen, thus limiting its suitability for serodiagnosis.

In the present study we have isolated from the hydatid cyst fluid, and partially characterized an antigen which is specific for *E. granulosus* and another which is cross-reactive only with sera of some patients with *E. multilocularis*.

## Materials and Methods

### Crude antigen

Sheep hydatid fluid was collected from fertile lung and liver cysts obtained from infected sheep at slaughter (Irbid, Jordan). The fluid from each organ was pooled separately, centrifuged at  $2000 \times g$  for 30 min, lyophilized and stored dessicated at 4°C.

### Hydatid sera

Fifty-four serum samples from confirmed *E. granulosus* cases and 24 from patients with *E. multilocularis* were available at the Bernhard Nocht Institute. These sera had been collected between 1973 and 1980 and had been stored in small aliquots at -70°C.

### Heterologous sera

A panel of serum samples from individuals with other proven parasitic infections were also available at the Bernhard Nocht Institute, namely toxocariasis (5), fascioliasis (4), filariasis (5), paragonimiasis (2), schistosomiasis (3) and cysticercosis (15). All these sera had high antibody titers to their homologous antigens as determined by enzyme-linked immunosorbent assay (ELISA) or indirect hemagglutination (IHA). The sera were used both singly and as pools.

### Normal sera

Thirty serum samples from healthy German civil servants with no evidence of disease were used as control sera.

## ELISA

ELISA was performed according to the method of Knobloch et al. [14], with antigen in 50 mM carbonate buffer, pH 9.6, adsorbed to flat-bottomed microtiter plates (Dynatech, Denkendorf, F.R.G.). Optimal concentrations of reagents were determined by box titration. Test sera were diluted 1:100 in 0.15 M PBS, pH 7.4, containing 1% bovine serum albumin and 0.05% Tween 20. The conjugate was anti-human IgG-alkaline phosphatase (Orion, Helsinki, Finland) and the substrate was *p*-phenyl phosphate (Merck, Darmstadt, F.R.G.). The test was read at 405 nm (MR 580, Dynatech).

### Purification of sheep hydatid fluid

Lyophilized sheep hydatid fluid was reconstituted in distilled water and the protein content determined using the Bio-Rad assay (BioRad, Munich, F.R.G.). The fluid was then concentrated in an Amicon ultrafiltration cell (Amicon, Danvers, U.S.A.) using a PM 10 membrane ( $M_r$  10000 cutoff) and then reconstituted in 1.7 M ammonium sulfate solution in 50 mM PBS. The sample was then subjected to a four-step high-performance liquid chromatography procedure. It was first fractionated by hydrophobic interaction chromatography (HIC) with a linear 1.7–0 M ammonium sulfate gradient using a neopentyl column (Alkyl Superose; Pharmacia, Uppsala, Sweden). Fractions were then purified by anion exchange chromatography (AEC; Mono Q, Pharmacia) using a linear 0–1 M NaCl gradient in 20 mM Tris-HCl, pH 7.7. The third and fourth purification steps were performed by gel filtration chromatography (GFC) (TSK G4000SW, LKB, Munich, F.R.G.; and Superose 6, Pharmacia) with 50 mM PBS, pH 7.0. The peaks were detected by a computer-controlled array detector (2140 Rapid Spectral Detector, LKB). Throughout the purification procedure, the purity of the fractions was assessed with SDS-PAGE and silver staining using 8–25% and 10–15% gradient gels (Phast System, Pharmacia). The specificity of all fractions was determined by an ELISA using the sera described above.

### Antigen characterization

*Molecular weight estimation.* SDS-PAGE was

performed according to the method of Laemmli [15]. The stacking gel was 4% and the separating gel 12.5%. Before electrophoresis, the samples were diluted 1:2 with the sample buffer Tris-HCl containing 5% (v/v) 2-mercaptoethanol (2-ME) and 1% (v/v) SDS, and were boiled for 5 min. 2-ME was omitted when operating under non-reducing conditions. Electrophoresis was carried out at 125 V while in the stacking gel and at 250 V while in the separating gel. The gels were stained with Coomassie Blue. For analysis of fraction purity under reducing and non-reducing conditions, gradient gels of 10–15% and 8–25% were used (Phast System, Pharmacia). In this case, the sample buffer consisted of 10 mM Tris-HCl, 1 mM EDTA, 2.5% (v/v) SDS and 5% (v/v) 2-ME. The gels were silver-stained according to the manufacturers' instructions. The  $M_s$  of the various components in the fractions were estimated by comparison with marker proteins. Both the pre-stained high-molecular-weight markers (Sigma, Deisenhofen, F.R.G.) and the low-molecular-weight markers (Pharmacia) were used.

**Carbohydrate content.** The carbohydrate content of the samples was determined colorimetrically using periodic acid and fuchsin-sulfite (Schiff's reagent, Sigma). The assay was performed in microtiter plates as follows: 0.05 ml of a two-fold serial dilution of D-glucose (2 mg ml<sup>-1</sup>, positive control), the synthetic peptide NH<sub>2</sub>-TTQETV-COOH (2 mg ml<sup>-1</sup>, negative control), *Salmonella typhi* lipopolysaccharide (LPS; 2 mg ml<sup>-1</sup>, positive control) and the test sample, were each placed in wells of the microtiter plate. All the wells then received 0.05 ml of 0.2% periodic acid and the plates were incubated in the dark for 1 hr at RT. This was followed by the addition of 0.05 ml of Schiff's reagent to all wells and the plates were incubated in the dark for 5 h after which they were read at 570 nm.

**Pronase treatment.** The effect of pronase treatment on the epitopes recognized by the positive serum pool was carried out in microtiter plates. Plates were coated with the appropriate amounts of Eg20 and Eg48 as determined by box titration (5 µg well<sup>-1</sup>). Human IgG (5 µg well<sup>-1</sup>) and *S. typhi* LPS (0.25 µg well<sup>-1</sup>) were used as positive

and negative controls, respectively. The plates were incubated for 18 h at RT, washed in water-Tween 20 and exposed to different concentrations of pronase (500–8 µg well<sup>-1</sup> in 0.15 M PBS). They were then incubated at 37°C for 4 h. Rabbit anti-human IgG, *S. typhi* positive and *E. granulosus* positive human sera were diluted 1:100 in PBS and added to their respective wells and the plates were incubated for 1 h at RT. After washing, the wells were exposed to appropriate HRP-conjugated secondary antibodies (rabbit anti-human and swine anti-rabbit) for 1 h at RT. OPD was used as substrate and the reaction was read at 450 nm.

**Periodic acid treatment** The effect of periodic acid oxidation of Eg20 and Eg48 on their subsequent binding to antibodies in the hydatid positive serum pool was carried out according to the method of Woodward et al. [16]. Briefly, Eg20, Eg48, human IgG and *S. typhi* LPS were allowed to attach to wells of microtiter plates at the concentrations described above. The wells were washed with water-Tween 20 (0.05%) and rinsed with 50 mM sodium acetate buffer, pH 4.5, prior to exposure to periodic acid. Sets of wells were exposed to periodic acid (0–20 mM) in acetate buffer for 1 h at RT in the dark. The aldehyde groups were blocked by treatment with 1% glycine for 30 min at 23°C. The plates were then washed and the wells exposed to the appropriate HRP-conjugated secondary antibodies (rabbit anti-human and swine anti-rabbit) for 1 h at RT. The plates were washed and the substrate OPD added. The plates were then read at 450 nm.

## Results

**Purification of the crude hydatid fluid.** The SDS-PAGE profile of the crude sheep hydatid fluid revealed a complex pattern of bands ranging from 12–220 kDa. Of these, the bands at 12, 16, 33, 48, 67 and 180 kDa were the most prominent (Fig. 1, lanes B and C).

When the sheep hydatid fluid was subjected to HIC, the majority of its components was recovered in the non-adherent fraction (Fig. 1, lane D). Analysis of this fraction by SDS-PAGE revealed mainly low-molecular-weight bands (12–67

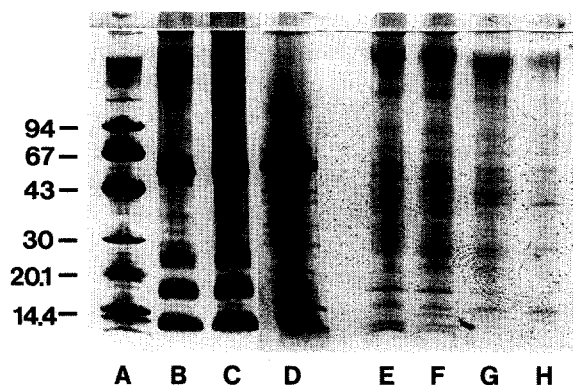


Fig. 1. SDS-PAGE of sheep hydatid fluid before (lanes B and C) and after (lanes D–H) hydrophobic interaction chromatography on 8–25% gradient gels stained with silver nitrate. Lane A, marker proteins (size in kDa is indicated); lane D, non-adherent fraction; lanes E–H, hydrophobic fractions.

kDa) which were subjected to further purification by AEC (Fig. 2). Eg48 was recovered when three pooled fractions of AEC (Fig. 2, lanes B, E and F) were subsequently purified by two steps of GFC.

The hydrophobic fractions of the cyst fluid (Fig. 1, lanes F–H) contained major bands with high molecular weight and some contaminants of the low-molecular-weight category. Each fraction was further purified by AEC and subsequent GFC without SDS-PAGE analysis. The resulting fractions were tested for sensitivity and specificity by ELISA. Only in the end, when the desired fraction was obtained (i.e., when Eg20 was recovered) was it analysed by SDS-PAGE.

A schematic diagram of the purification steps of Eg20 and Eg48 from the hydatid cyst fluid is shown in Fig. 3.

**Specificity and sensitivity studies.** Approximately 100 fractions of the hydatid cyst fluid was isolated during the various purification steps. Each fraction was tested by ELISA for its ability to discriminate between sera of patients with *E. granulosus* and those with other helminthic infections. Using antigen concentrations of 10 ng (100  $\mu$ l)<sup>-1</sup>well<sup>-1</sup>, serum dilutions of 1:100 and conjugate dilutions of 1:400, only two fractions proved to be of interest in terms of purity, sensitivity and specificity. Out of a total of 38 heterologous sera examined, the crude cyst fluid reacted with 28

(74%) while Eg48 reacted only with seven sera (18%). The latter were all *E. multilocularis* sera. Eg48 detected all confirmed *E. granulosus* cases while Eg20 detected 52 out of 54 confirmed cases (96%). In general, however, the specific serum antibody levels detected by Eg20 were lower than those obtained with the Eg48 antigen.

**Characterization of Eg20 and Eg48.** Using SDS-PAGE, the purified antigens Eg48 and Eg20 were found to be of 48 and 20 kDa, respectively (Fig. 4A,B). Both antigens were insensitive to reduction by 2-ME, and both stained positively with Schiff's reagent following oxidation with periodic acid.

The *S. typhi* LPS gave a strong reaction with Schiff's reagent following oxidation with periodic acid. The absorbance values for the different concentrations (2–0.06 mg ml<sup>-1</sup>) of *S. typhi* LPS ranged from 1.4 to 0.08. This reaction was also dose-dependent. Similarly, glucose reacted positively in this assay (*A* range 0.35–0.02). In contrast, the pure peptide gave no reaction with Schiff's reagent. Both the Eg48 and Eg20 antigens reacted positively in the assay, giving *A* values of 1.090 and 0.985, respectively. These values

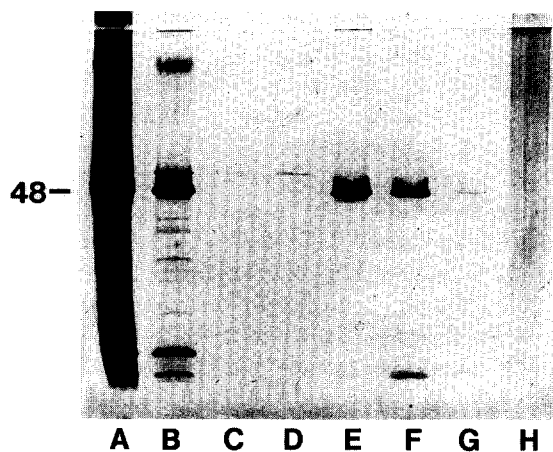


Fig. 2. SDS-PAGE of anion exchange chromatography fractions of an 8–25% gradient gel stained with silver nitrate. The fraction non-adherent to the hydrophobic interaction column (Fig. 1, lane D) was used for AEC; lane A, non-adherent fraction; lanes B–H, fractions eluted by increasing NaCl concentrations; 48 kDa is indicated; lanes B, E and F, fractions used for purification of the Eg48 antigen.

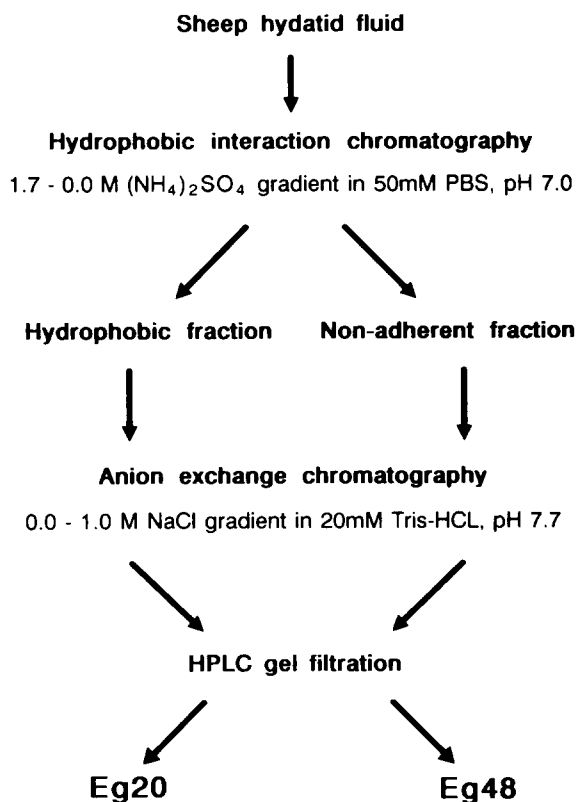


Fig. 3. A schematic diagram indicating the various steps involved in the purification of the Eg20 and the Eg48 antigens from sheep hydatid fluid.

were in the range of  $A$  given by *S. typhi* at concentrations between 0.5 and 1.0 mg ml<sup>-1</sup>.

The effect of pronase treatment on the binding of anti-*E. granulosus* antibodies to the Eg48 and Eg20 antigens was investigated. No decrease in antibody binding was obtained at any of the doses used. Similar results were obtained with *S. typhi* LPS where pronase treatment had no effect on the binding of anti-*S. typhi* LPS to its homologous antigen. In contrast, a complete loss of antibody binding to human IgG was obtained at a pronase concentration of 500 µg well<sup>-1</sup>. The binding between human IgG and rabbit anti-human IgG reached normal levels at a pronase concentration of 8 µg well<sup>-1</sup>.

Fig. 5 shows the effect of periodic acid oxidation on the binding of anti-*E. granulosus* antibodies to Eg48 and Eg20. Nearly 40% inhibition of antibody binding was obtained at a periodic acid

concentration of 20 mM. The effect of periodate on both antigens was dose dependent and inhibition of antibody binding was lost at low concentrations of periodic acid (Fig. 5, open and filled squares). The inhibition observed with periodic acid treatment of the fractions was similar to that obtained by treating *S. typhi* LPS with periodic acid (Fig. 5, filled circles). In contrast, periodic acid oxidation of human IgG had no effect on its binding to rabbit anti-human IgG at any of the doses examined (Fig. 5, open circles).

## Discussion

In the present study we were able to purify two parasite antigens from the cyst fluid. One of these antigens, designated Eg20, was shown to be a hydrophobic 20-kDa molecule with carbohydrate

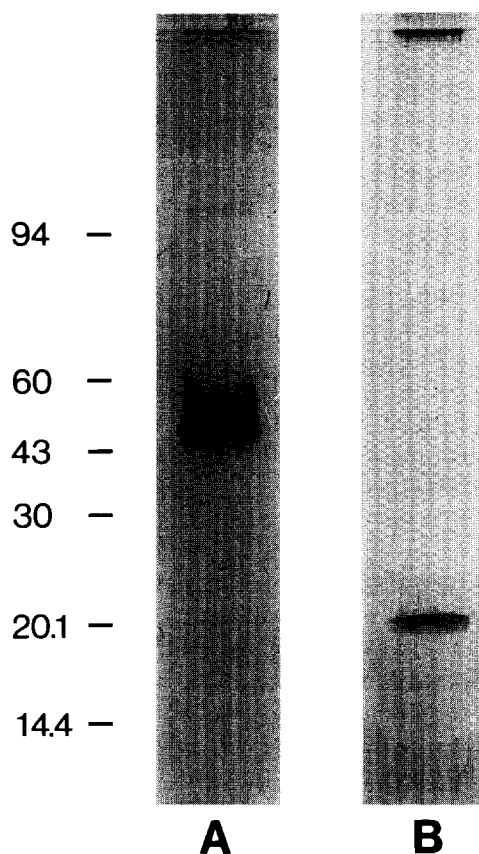


Fig. 4. SDS-PAGE showing Eg48 (A) and Eg20 (B) on 8-25% gradient gels stained with silver nitrate. Size in kDa, as obtained by marker proteins, is indicated.

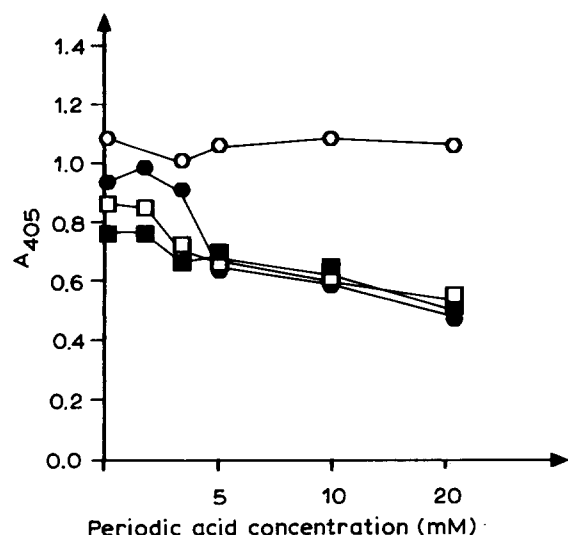


Fig. 5. Effect of periodic acid oxidation of human IgG, *S. typhi* LPS, Eg48 and Eg20 on their binding to their homologous antibodies. Open circles represent binding of rabbit anti-human IgG to human IgG; filled circles represent binding of human anti-*S. typhi* LPS to *S. typhi* LPS; open squares represent the binding of anti-*Echinococcus granulosus* antibodies to Eg48; and filled squares represent the binding of anti-*E. granulosus* antibodies to Eg20. The *A* values obtained with normal sera in the presence of periodic acid ranged from 0.20 to 0.26.

and protein constituents. It was therefore regarded as a glycoprotein. It was found to be specific for *E. granulosus*.

The properties of Eg20 were similar to those described for the subunits of antigen B. Like these subunits, Eg20 is not affected by reduction or boiling [17,18]. Moreover, it has been shown recently that antigen B is specific for *E. granulosus* [13], and we found that Eg20 did not react with any of the 38 heterologous sera examined, although all these sera had high antibody titers to their homologous antigens. Antigen B has been classified as a lipoprotein. On the other hand, it has been demonstrated that, like Eg20, it consists in part of carbohydrates [19]. These findings suggest that Eg20 is a subunit of antigen B, although we have no data to prove such a claim.

Eg48, the 48-kDa antigen purified in the present study, was also regarded as a glycoprotein, since its protein-carbohydrate composition could be demonstrated. The fact that it cross-reacts only with sera from patients with *E. multilocularis* infections makes it similar to antigen 5 [11,12]. However, it differs from antigen 5 in molecular weight. Antigen 5 has been described as being 400 kDa in size, consisting of subunits of 60–70 kDa [19,20]. EG48 is not affected by reduction, whereas antigen 5 is usually reduced to 20- and 40-kDa subunits. Finally, while the antigen described here is not affected by boiling, antigen 5 usually aggregates and precipitates out of solution once boiled [21].

Periodate oxidation and pronase digestion were used to determine the participation of carbohydrate or protein moieties in the serological reactivity of the two antigens. The results obtained suggest the involvement of periodate-sensitive carbohydrates in at least some antigenic determinants. Since the reaction was not totally inhibited by periodate oxidation, it is possible that the remaining epitopes are periodate-insensitive [16]. This is partly supported by the inability of pronase, despite its low specificity, to affect the antigenic determinants of the Eg20 and the Eg48 antigens.

The ability of Eg20 to detect 96% of sera of hydatid patients combined with its specificity makes it a suitable antigen for serodiagnosis and seroepidemiological surveys. Such surveys are important for assessment of disease prevalence in man as well as for monitoring the efficacy of control measure once implemented. Both antigens could be applied for the serodiagnosis of livestock infection, where problems of specificity are much more difficult than in man [22–24].

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