Echinococcus granulosus coproantigens: chromatographic fractionation and characterization

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SUMMARY

Dogs infected with adult tapeworms of *Echinococcus granulosus* release antigens (coproantigens) in faeces which can be detected by a capture ELISA. Supernatants prepared from *E. granulosus*-infected dog faecal samples were fractionated by size-exclusion fast protein liquid chromatography (FPLC) on a Superose-6 column. Coproantigen ELISA and Western blotting were used to demonstrate the immunoreactivity of eluted fractions. Two main FPLC peaks of antigenic activity were detected and designated as fraction F1 and fraction F2 with approximate relative molecular weights >670 kDa, and in the range of 146 to 440 kDa respectively. These two antigenic fractions (F1 and F2) fractionated from infected dog faeces were heat stable and largely protease-insensitive, but were highly sensitive to sodium periodate treatment, which strongly suggested the involvement of carbohydrates. Capture IgG antibodies against *E. granulosus* proglottis somatic extracts, detected a molecule with an approximate molecular weight of 155 kDa in fraction F2 after immunoblotting. The 155 kDa antigen could be completely ablated by sodium periodate treatment, but not after protease or lipase treatment. A surface tegument preparation of adult *E. granulosus* tapeworms contained large amounts of antigen that corresponded in size range and antigenicity to that observed in the FPLC fraction F2. There was also a peak of antigenic activity at >670 kDa corresponding to fraction F1 from a culture derived excretory–secretory (E–S) adult tapeworm preparation. The involvement of carbohydrate moieties in coproantigen activity present in the FPLC fractions F1 and F2 from faecal supernatants of *E. granulosus*-infected dogs was confirmed by lectin-binding assays and exoglycosidase treatment, which showed that α-D-mannose and/or α-D-glucose, β-galactose and *N*-acetyl-β-glucosamine residues were the most important carbohydrate components in putative coproantigens present in both fractions. *N*-acetyl-β-glucosamine and sialic acid residues were also contained in coproantigen molecules present in fraction F2. These results suggested that coproantigens detected in faeces of *E. granulosus*-infected dogs are large molecular weight molecules that may be derived from the carbohydrate-rich surface glycocalyx of adult worms, and are shed, released or secreted during the life-span of the tapeworm.

Key words: *Echinococcus granulosus*, coproantigen, FPLC, periodate, lectin, exoglycosidase.

INTRODUCTION

Echinococcus granulosus is an important zoonosis which is the causative organism of cystic echinococcosis in humans and animals that occurs throughout the world (WHO/OIE, 2001). Accurate diagnosis of the infection in the common definitive host, the dog, plays a potentially central role in epidemiological studies and for surveillance of hydatid control programmes. An enzyme-linked immunosorbent assay (ELISA) has been developed and applied successfully to detect *E. granulosus* antigens (coproantigens) in stool samples from definitive hosts (Allan *et al*. 1992; Deplazes *et al*. 1992; Jenkins *et al*. 2000). Only limited information is available about the biochemical nature and the immunological identity of the coproantigens detected by the capture ELISA (Fraser & Craig, 1997; Elayoubi *et al*. 2003).

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Isolation and characterization of cestode-derived antigens of infected hosts have been advocated with regard to elimination of cross-reactivity to improve immunodiagnostic tests (Ito & Smyth, 1987). For coproantigen detection characterization of defined parasite antigens in faeces should permit potential improvement in assays at genus-specific and even to species-specific levels. Maass, Delgado & Knobloch (1992) identified a 60 kDa antigen in human stools from *Taenia solium* carriers by SDS–PAGE and Western blot using a polyclonal anti-*T. solium* IgG. Using membrane ultrafiltration techniques, antigen(s) in the faeces of *Taenia saginata*-infected humans detected by a capture ELISA were shown to have an approximate molecular weight of >100 kDa (Machnicka, Dziemian & Zwierz, 1996). Western blot analysis of *Hymenolepis diminuta*-infected rat faeces showed a number of specific antigens with molecular weights >30 kDa (Allan & Craig, 1994). Preliminary studies on *Echinococcus* coproantigens indicated that they were heat stable, resistant to degradation by faecal enzymes, they

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were protease-insensitive, but susceptible to degradation by periodate treatment, and remained active after faecal samples were stored in 10% formal-saline (Allan *et al*. 1992; Sakai *et al*. 1995; Sakashita *et al*. 1995; Fraser & Craig, 1997; Guarnera *et al*. 2000; Elayoubi *et al*. 2003). Furthermore biochemical characterization of antigens in faecal supernatants from *E. granulosus*-infected dogs indicated that they comprised carbohydrates with α-D-mannose and/or α-D-glucose, β-galactose and *N*-acetyl-β-glucosamine residues (Elayoubi *et al*. 2003).

Echinococcus granulosus coproantigens are clearly demonstrable by capture ELISA but have not yet been fully characterized. The current study was aimed at fractionation of immunodiagnostic-relevant *E. granulosus* antigens directly from faecal samples of infected dogs or from adult worm extracts using size-exclusion fast protein liquid chromatography (FPLC), and the subsequent biochemical characterization of the partially purified antigens.

MATERIALS AND METHODS

Faecal sample

Faecal samples from dogs naturally or experimentally infected with *E. granulosus* were obtained from different sources, i.e. Welsh fox hounds (UK), culled stray dogs (Kenya) or experimentally infected dogs (Australia) (Jenkins *et al*. 2000). The infection was confirmed by necropsy or arecoline purgation as well as coproantigen capture ELISA. Control negative faecal samples were from pet dogs from UK (Lancashire). The control samples were tested individually by coproantigen capture ELISA. Raw unfixed faecal samples were kept frozen at −20 °C until used.

Preparation of faecal supernatants

Raw frozen faecal samples were thawed and mixed (1:2 w/v) with 0·15 M phosphate-buffered saline (PBS), pH 7·2 containing 0·3% Tween-20 (PBST–0·3%). The mixture was shaken vigorously and then clarified by centrifugation at 2000 ***g*** for 30 min at room temperature (22–25 °C). Faecal supernatants were collected and stored at −20 °C until tested or fractionated in FPLC. On the same day prior to separation on FPLC, faecal supernatants were recentrifuged and filter sterilized using a 0·22 μm syringe filter (Gelman, USA).

Adult worms

Echinococcus granulosus adult worms were recovered at necropsy from naturally and/or experimentally infected dogs as described elsewhere (Craig, 1997; Jenkins *et al*. 2000). Worms were washed several times with 0·15 M PBS, pH 7·2 containing 100 IU/ml penicillin and 100 μg/ml streptomycin by centrifugation at 800 ***g*** to remove as much debris as possible and were stored frozen in PBS at −70 °C. Washed worms were thawed and brought into suspension in PBS containing protease inhibitor (1 mM phenylmethylsulphonyl fluoride, PMSF; Sigma) and used to prepare *E. granulosus* antigen extracts.

Preparation of adult whole worm extract antigen (EgWWE)

This was prepared according to the method described by Sakai *et al*. (1995). Briefly, adult worms in PBS containing 1 mM PMSF were frozen/thawed twice and then homogenized on ice with 20–30 strokes using a hand-held glass homogenizer (Merck, USA). Subsequently, the mixture was sonicated in an ice bath for 10 sec in 10 bursts with a 10 sec pause at medium amplitude on an ultrasonic disruptor. The suspension was left to stand on ice for 1 h with agitations and then centrifuged at 1500 ***g*** for 30 min at 4 °C. Supernatants containing the EgWWE extracts were aliquoted and stored at −70 °C until used.

Preparation of E. granulosus *surface and somatic antigens*

Echinococcus granulosus adult worms in PBS containing 1 mM PMSF protease inhibitor were frozen/thawed, vortexed and allowed to settle for 10 min. This was repeated several times until the supernatant became cloudy. The supernatant containing the surface extracts (SAg) was recovered and the remaining pellet, which represented the somatic extracts (CAg) material, was re-suspended in PBS containing 1 mM PMSF and homogenized thoroughly using a glass homogenizer immersed in an ice-bath. Both extracts were sonicated on ice using an ultrasonic disruptor in 3 bursts for SAg and 6 bursts for CAg, each of 10 sec duration with a 10 sec pause. The sonicates were centrifuged at 1500 ***g*** for 30 min at 4 °C and the supernatants containing the antigenic extracts were stored separately at −70 °C in aliquots until used.

Preparation of adult worm excretory–secretory products (E–S)

Living adult *E. granulosus* tapeworms were recovered post-mortem from the small intestines of naturally infected dogs in Turkana (Kenya) according to the method described by Craig (1997). The worms were immediately washed thoroughly with PBS containing antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). Approximately 500 *E. granulosus* worms were incubated at 25 °C for 20 h in a 75 cm^2 tissue culture flask containing 30 ml of

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Dulbecco's Modification of Eagle's Medium (DMEM), pH 7·4 mixed with 1% glucose, and penicillin (100 IU/ml) and streptomycin (100 μg/ml). Afterwards, culture fluid was removed and kept frozen at −20 °C until brought to the lab (UK) where it was centrifuged at 1500 ***g*** for 20 min at 4 °C. The supernatant from conditioned media was collected, dialysed against PBS for 24 h at 4 °C and concentrated using an Amicon ultrafiltration unit with a 10 000 Mol. wt (YM-10) membrane. The solution was stored in aliquots at −70 °C for further studies.

Fractionation of coproantigens and worm extracts by size exclusion FPLC

Chromatography was performed on a fast protein liquid chromatography (FPLC) system (Pharmacia, Sweden) using a size-exclusion gel filtration column (Superose-6 HR 10/30, Pharmacia, Sweden). The column was equilibrated overnight with vacuum-filtered PBS (0·22 μm filter) after which 100 μl of filter-sterilized *E. granulosus* positive or negative faecal supernatant or adult worm extracts (EgWWE, CAg, SAg, E/S) were applied to the column using a 1 ml syringe at 0·2 ml/min flow rate. Forty-eight fractions were collected in 0·25 ml volumes and tested for antigenic activity in capture ELISA. Fractions containing antigenic activity were pooled to comprise 2 discrete peaks of antigenicity and a third fraction that was non-antigenic, i.e. fractions F1, F2, and F3, respectively. Fractions were stored at −20 °C until further analysis.

Standard proteins of different molecular weights were used for column calibration, i.e. blue dextran (Pharmacia Fine Chemicals), 2000 kDa; thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; human IgG, 146 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; trypsin inhibitor, 20 kDa; cytidine, 0·25 kDa (all from Sigma). The value of the column void volume was based on the elution volume of blue dextran.

Coproantigen capture ELISA

The method used was essentially that of Allan *et al.* (1992). Briefly, flat-bottomed microtitre plates (Immulon 4, Dynex) were coated at 100 μl/well with an optimal dilution (1/4000) of rabbit IgG anti-*E. granulosus* adult whole worm extract (anti-EgWWE) in 0·05 M bicarbonate–carbonate buffer (BCB, pH 9·6) and left overnight at 4 °C. Plates were washed 3 times with PBST-0·1%, pH 7·2 and blocked with 100 μl/well of PBST-0·3% for 1 h. The plates were washed again and then FPLC fractions were incubated neat in duplicate for 1 h at 100 μl/well. Peroxidase conjugated rabbit IgG anti-EgWWE at optimal dilution (1/2000) in PBST-0·3% was incubated for 1 h and after washing as above substrate solution (TMB – tetramethyl benzidine, PharMingen, UK) was added to each well and incubated for 20 min in the dark. Finally, the plates were spectrophotometrically read at 630 nm on MR5000 microtitre plate reader (Dynatech, USA). Each plate contained the original faecal supernatant before centrifugation, after centrifugation, and after filtration as a positive control. A standard negative control faecal sample was also included. The optimal dilutions of the capture antibody and antibody-peroxidase conjugate were previously determined by a checkerboard titration using negative and positive control dog stool supernatants.

Lectin-binding assays

The basic sugar composition of *Echinococcus* coproantigens was evaluated by lectin-binding ELISA according to the method of Osinaga *et al.* (1994), with slight modifications. The following biotinylated lectins were used in this study: concanavalin A (Con A), *Bandeiraea simplicifolia* (BS-1), *Ulex europaeus* (UEA-1), *Triticum vulgaris* (WGA), *Lens culinaris* (LCA), *Dolichos biflorus* (DBA), or *Arachis hypogaea* (PNA) (all from Sigma). Briefly, flat-bottomed microtitre plates (Immulon 4, Dynex) were coated overnight at 4 °C with 100 μl/well of an optimal dilution (1/4000) of rabbit IgG anti-EgWWE in BCB, pH 9·6. The wells were then washed 3 times with PBST-0·1% and blocked with 100 μl/well of PBST-0·3% for 1 h at 37 °C. After 3 washes with PBST-0·1%, 100 μl of each FPLC fraction (F1 and F2) were incubated neat in duplicate for 1 h at 37 °C. The wells were then washed with PBST-0·1% and incubated (100 μl/well) for 1 h at 37 °C with either 5 μg/ml of lectins or 5 μg/ml of lectins plus 0·2 M of the corresponding inhibitory sugars (α-D-mannose or α-D-glucose for Con A and LCA, *N*-acetyl-β-glucosamine for WGA, α-L-fucose for UEA, *N*-acetyl-α-galactosamine for DBA, α-D-galactose for BS-1, and β-D-galactose for PNA), all diluted in blocking buffer. Prior to use, the lectins and the inhibition control mixture had been incubated in a water bath for 1 h at 37 °C. After washing PBST-0·1%, streptavidin-alkaline phosphatase (Sigma) diluted 1/2000 in PBST-0·3% was added and incubated for 1 h at 37 °C. After 3 washes with PBST-0·1%, the plates were developed by incubation for 30 min in the dark with substrate solution (100 μl/well), which was prepared by dissolving 1 tablet (5 mg) of *p*-nitrophenyl phosphate, pNPP (Sigma) per 5 ml of diethanolamine buffer (pH 9·8). The plates were read spectrophotometrically at 405 nm.

Heat treatment

To examine the stability of FPLC fractionated faecal supernatants, the pooled fractions that represented

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fraction F1 and F2, which contained *E. granulosus* coproantigen activity were boiled for 30 min. Antigen activity before and after boiling was tested in the coproantigen capture ELISA.

Biochemical treatment

The two FPLC fractions (F1 and F2) of dog faecal supernatants that contained *E. granulosus* antigen activity were treated with 20 mM sodium periodate, 10 U/ml lipase or 2·5 U/ml protease and the antigenic activity of fractions (before and after treatment) analysed by coproantigen capture ELISA and Western blot as previously described (Elayoubi *et al.* 2003). In addition, *E. granulosus*-positive dog faecal supernatants were also treated in the same manner prior to fractionation by FPLC. Collected fractions were tested for coproantigen activity in the coproantigen capture ELISA.

SDS–PAGE and Western blotting

Three FPLC fractions (F1, F2, F3) were separated by SDS–PAGE under reducing or non-reducing conditions according to the method described by Laemmli (1970) using discontinuous polyacrylamide vertical slab minigels (Mighty Small System, Hoefer, USA). A 4% stacking gel and 12% linear separating gels (0·75 mm thick) were used. Prestained standard molecular weight markers (range 4–250 kDa) (Novex, USA) were run on each gel. Gels were stained using a silver staining kit (Novex, USA), as described by the manufacturer, and dried onto cellulose sheets (Novex, USA) or electrotransferred to a nitrocellulose membrane. Gels were blotted onto nitrocellulose membrane (0·45 μm pore size; Sartorius, Göttingen, Germany) according to Towbin, Staehelin & Gordon (1979) using a miniblotter (Hoefer) at 35 mA overnight at RT. After the transfer, membranes were blocked with 5% (w/v) skimmed milk in PBST-0·3% (PBSTM) for 1 h on a rocking platform, rinsed 3 times for 5 min each with PBST-0·1% and incubated for 1 h in hyperimmune IgG rabbit anti-EgWWE diluted 1/2000 in PBSTM. Membranes were washed as before and then incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) at a 1/2000 dilution in PBST-0·3% for 1 h. The membranes were washed again and then equilibrated for 5 min with alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris base, pH 9·6) before development with substrate solution (0·66% bromo-chloro-indolyl phosphate (BCIP) and 0·33% nitro blue tetrazolium (NBT) per 100 ml of alkaline phosphatase buffer, pH 9·6) (Sigma). After incubation for 5–10 min in the dark, the reaction was stopped with repeated washes in distilled water.

Exoglycosidase treatment

The two major FPLC fractions (F1 and F2) of faecal supernatants were treated individually with a panel of enzymes specific for certain carbohydrates: α-glucosidase (from rice), β-glucosidase (from almonds), α-mannosidase (from jack beans), β-mannosidase (from snail acetone papowder), α-galactosidase (from green coffee beans), β-galactosidase (from bovine testes), β-*N*-acetylglucosaminidase (from bovine kidney), α-L-fucosidase (from bovine kidney), β-fructosidase (from yeast), or neuraminidase (from *Vibrio cholerae*) (all from Sigma, except β-fructosidase and neuraminidase from Roche). The sensitivity of the coproantigen to exoglycosidase digestion was determined by incubation of 1 U/ml of each enzyme with fraction F1 or fraction F2 for 24 h at 37 °C for α-glucosidase, β-glucosidase and neuraminidase and at 25 °C for the other enzymes used. Control samples were treated in the same way but instead of adding enzymes, PBS was added. Coproantigen activity of the treated and untreated fractions was analysed by the coproantigen capture ELISA.

Statistical analysis

Wilcoxon's test for matched pairs was used to determine the significant differences in *E. granulosus* coproantigen activity (ELISA OD values) between treated and untreated FPLC fractions (F1 or F2).

RESULTS

FPLC fractionation of faecal supernatants

Echinococcus granulosus coproantigen-positive and negative faecal supernatants were fractionated using size-exclusion fast protein liquid chromatography (FPLC) on a Superose-6 column that separates molecules on the basis of molecular weight. Forty-eight fractions were collected and analysed for antigenic activity by coproantigen capture ELISA. Two main peaks of antigenic activity were detected which indicated that 2 major components with different molecular weight ranges were present in *E. granulosus*-positive dog faeces but not in negative dog faeces (Fig. 1). The fractions corresponding to these 2 peaks were pooled separately and designated, fraction F1 and fraction F2 respectively. Comparison with elution profiles of proteins of known molecular weight, indicated that fraction F1 was eluted corresponding to size >670 kDa and fraction F2 within the molecular weight range of 146 to 440 kDa. In addition, fractions corresponding to molecular weight <66 kDa that had no antigenic activity in ELISA were pooled, designated as fraction F3, and used as an internal negative control. All the *E. granulosus*-positive faecal samples tested showed the same FPLC profile of the 2 peaks of coproantigen activity.

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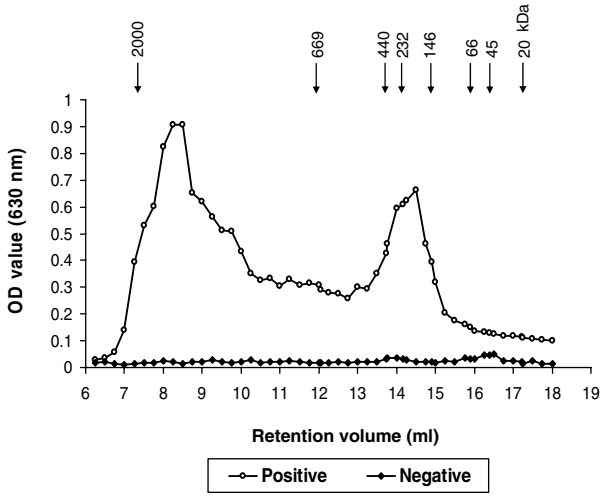

Fig. 1. Coproantigen capture ELISA analysis of *Echinococcus granulosus*-positive and negative faecal samples fractionated by size-exclusion FPLC on a Superose-6 column. Two main peaks of coproantigen activity (F1 and F2) were detected in *E. granulosus*-positive faecal sample at approximately >670 kDa and 146–440 kDa respectively but not in *E. granulosus*-negative sample. The position of the elution of the protein standards (20–2000 kDa) is indicated by the arrows.

No significant antigenic activity was demonstrated in the equivalent for 48 FPLC fractions of all negative faecal samples tested.

Heat treatment

The effect of heat treatment for 30 min at 100 °C on *E. granulosus* coproantigen activity as detected in the 2 FPLC fractions, F1 and F2, was analysed by coproantigen capture ELISA. Results showed that coproantigen activity in fraction F1 and fraction F2 reduced only marginally by 5·6% and 17·1% respectively after boiling compared with unheated fractions.

SDS–PAGE and immunoblotting

SDS–PAGE separation under reducing condition of molecules in FPLC fraction F1, fraction F2, and fraction F3 from *E. granulosus* coproantigen-positive and negative faecal samples did not show any bands (data not shown). Under non-reducing conditions, silver staining indicated a specific, but negatively stained, band at approximately 155 kDa in fraction F2 from coproantigen-positive faecal samples, but not from coproantigen-negative faecal samples (Fig. 2A). Some bands were stained in fraction F1, however, they were not specific as they were also found in fraction F3 (FPLC negative antigen control) from either positive or negative faecal samples.

Western blotting using hyperimmune anti-whole worm *E. granulosus* (anti-EgWWE) IgG indicated antigenic recognition of the 155 kDa band in fraction F2 from *E. granulosus*-positive faecal samples. No detectable bands were, however, observed in fraction F1 (Fig. 2B). These results suggest that the 155 kDa molecule associated with FPLC fraction F2, could be a large putative carbohydrate-rich antigenic

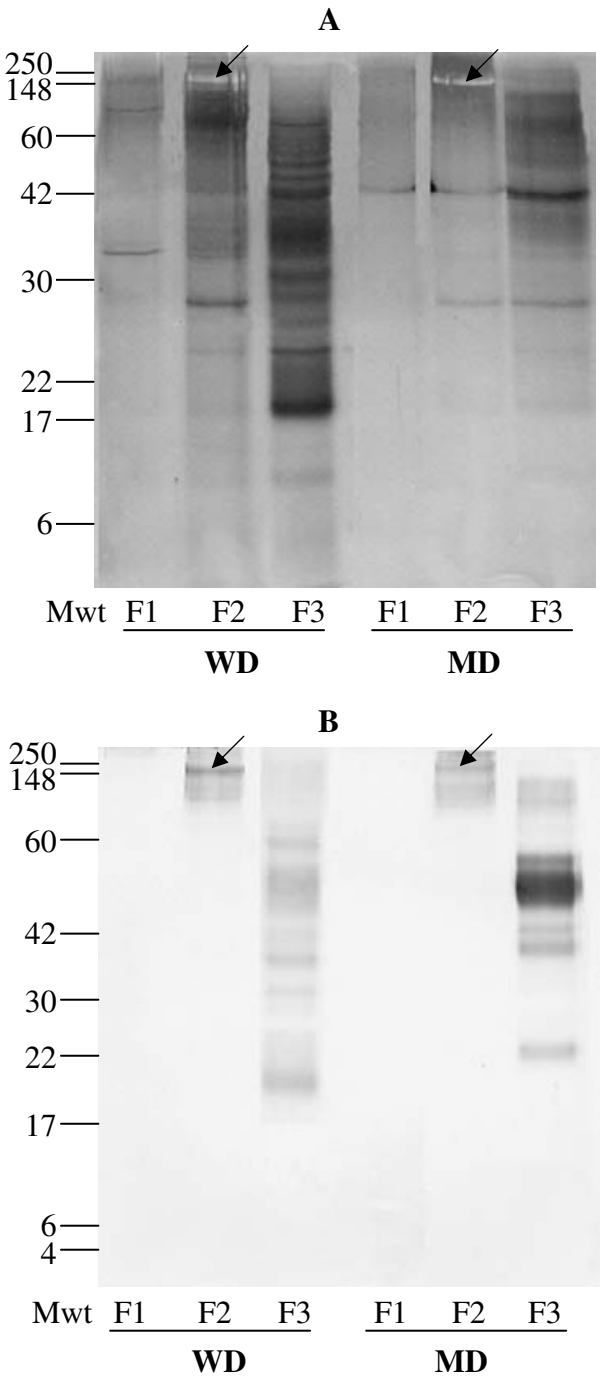

Fig. 2. FPLC fractions (F1, F2, F3) of *Echinococcus granulosus* coproantigen-positive dog faecal samples (WD, MD) separated by SDS–PAGE on a 12% minigel under non-reducing conditions and either (A) stained with silver stain or (B) transferred to nitrocellulose membrane and probed with capture IgG antibodies against *E. granulosus* whole worm extract (rabbit anti-EgWWE). A specific band at 155 kDa (pointed arrow) was detected. The molecular weight markers (kDa) are shown on the left.

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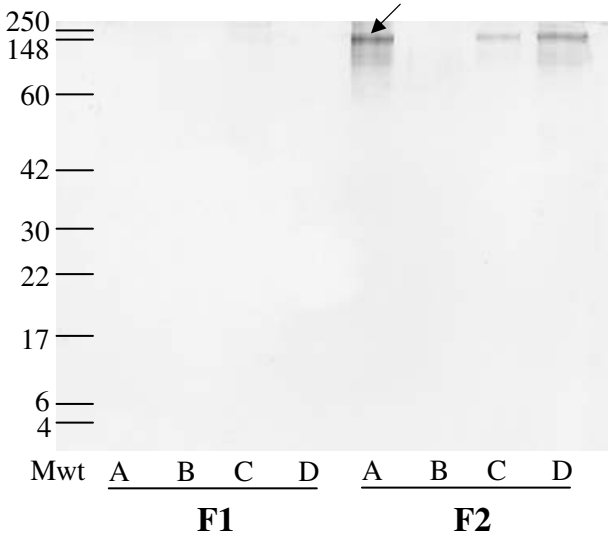

Fig. 3. The effect of periodate and enzymatic treatment on 155 kDa antigen. The FPLC fractions F1 and F2 from *Echinococcus granulosus*-positive dog faecal samples untreated (A) or treated with 20 mM sodium periodate (B), 2·5 U/ml protease (C), or 10 U/ml lipase (D) and separated by SDS–PAGE before immunoblotting and probing with anti-EgWWE antibodies. The 155 kDa band (pointed arrow) disappeared after the periodate treatment, but not after protease or lipase treatments. The molecular weight markers (kDa) are shown on the left.

molecule that contributed to *E. granulosus* coproantigen activity.

Sodium periodate and enzymatic treatment analysis

Changes in coproantigen activity of FPLC fraction F1 and fraction F2 from 8 different faecal supernatants were evaluated by coproantigen capture ELISA and Western blotting before and after sodium periodate, protease or lipase treatment. The average reduction in ELISA OD values after 20 mM sodium periodate treatment was 82·7% and 71·8% for fraction F1 and fraction F2 respectively, in comparison to untreated fractions which were statistically significant ($P<0·05$ in both cases). Treatment with protease or lipase had a less significant effect ($P<0·05$ in both cases). Protease treatment removed 12·8% and 15·3% of antigen activity in fraction F1 and fraction F2 respectively. Lipase treatment removed 15·4% and 17·3% of the antigencity of fraction F1 and fraction F2 respectively.

Western blot analysis showed that periodate treatment of fraction F2 completely ablated the binding of anti-EgWWE antibodies to the 155 kDa antigen. This strongly suggested that carbohydrate moieties significantly contributed to the antigenicity of the 155 kDa coproantigen molecule and may be crucial in immune recognition of *E. granulosus* coproantigens (Fig. 3).

In order to confirm the effect of periodate treatment on FPLC fractions F1 and F2, coproantigen-positive faecal supernatants were first treated with periodate (or enzymes) and then fractionated by FPLC. Coproantigen activity of the FPLC fractions before and after the treatments was tested by ELISA as shown in Fig. 4. Periodate treatment resulted in significant loss of the antigenic activity of fraction F2, but not of fraction F1. This finding confirmed the Western blot results that carbohydrate-rich antigenic molecules in fraction F2, probably including a molecule with an approximate molecular weight of 155 kDa, are important in immunorecognition of *E. granulosus* coproantigens. Protease or lipase treatment of faecal supernatants before FPLC separation did not result in any effect on either fraction F1 or fraction F2 after separation.

FPLC fractionation of E. granulosus *adult worm extracts*

In order to identify the source of adult tapeworm derived coproantigens, the FPLC profiles of *E. granulosus* coproantigen-positive faecal supernatants were compared with the fractionation profiles of different *E. granulosus* adult worm extracts. These were an adult whole worm extract (EgWWE), a surface antigen extract (SAg), a somatic antigen extract (CAg), and an excretory–secretory preparation (E–S) (Fig. 5). The EgWWE separated by FPLC showed an antigenic profile by ELISA with exactly the same 2 main antigenic peaks (F1 and F2) that were observed in *E. granulosus*-positive dog faecal supernatants. Somatic extracts (CAg), which were prepared in the same way as EgWWE extract but with the surface material excluded, showed stronger antigenicity in the first peak (fraction F1) than in the second peak (fraction F2). This suggests that most of the antigenic activity in fraction F2 was probably related to adult worm surface antigens. This was further confirmed by the presence of greater antigenic activity in fraction F2 than in fraction F1 for the surface antigen preparation (SAg). In addition, ELISA results showed that most of the antigenic activity of the E–S material was contained in fraction F1 rather than in fraction F2. These results suggested that the *E. granulosus* coproantigen activity within FPLC fraction F1 and fraction F2 may be related primarily to adult worm E–S material and to adult surface antigens respectively. Also that the larger carbohydrate or glycan molecules (>670 kDa) were probably released from the worms during *in vitro* maintenance, while smaller glycans (146–440 kDa) were more likely retained on the adult tapeworm surface.

Lectin-binding analysis

Biotinylated lectins were used to assess the presence of specific carbohydrate epitopes on antigens reactive to *E. granulosus* antibodies, in FPLC fractions.

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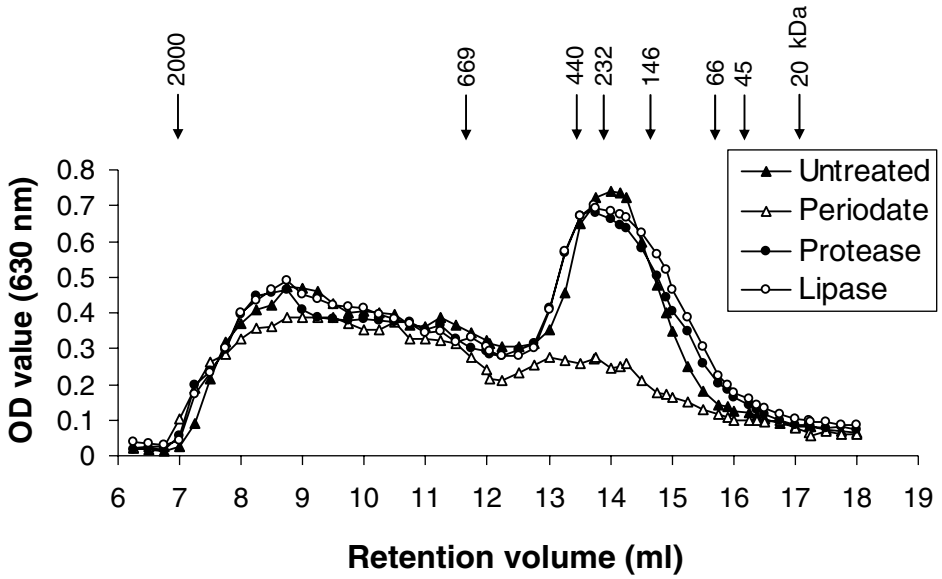

Fig. 4. Coproantigen capture ELISA results for FPLC fractions from a Superose-6 column used to separate *Echinococcus granulosus* coproantigen positive dog faecal samples previously treated with 20 mM sodium periodate, 2·5 U/ml protease or 10 U/ml lipase. The position of the elution of the protein standards (20–2000 kDa) is indicated by the arrows. The second peak (fraction F2) was significantly reduced after periodate treatment.

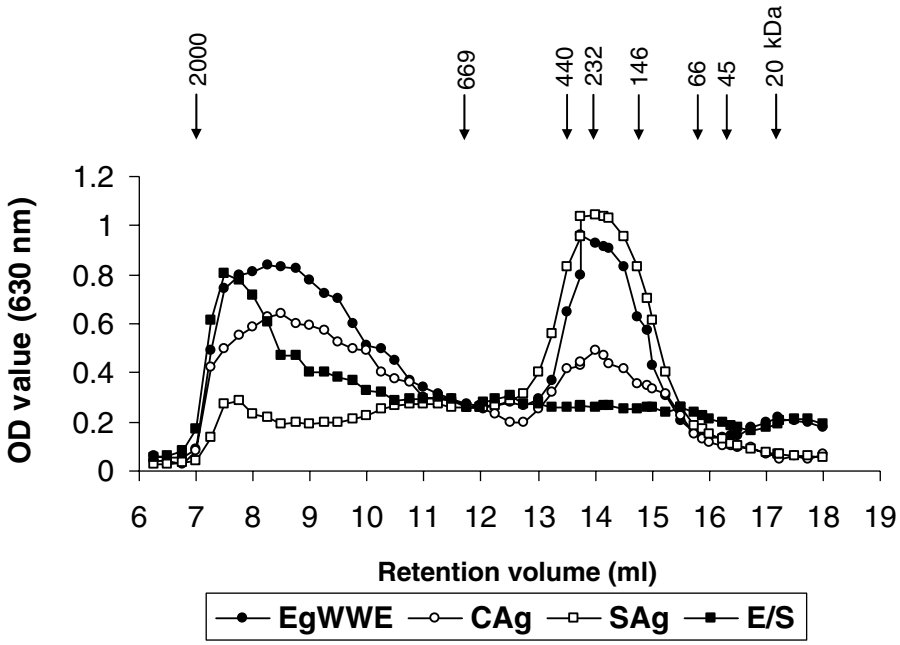

Fig. 5. FPLC separation patterns (Superose-6 column) by coproantigen capture ELISA of different *Echinococcus granulosus* adult worm extracts: whole worm extract (EgWWE), surface extract (SAg), somatic extract (CAg) and excretory/secretory (E/S) preparation. The position of the elution of the protein standards (20–2000 kDa) is indicated by the arrows.

Both fractions F1 and F2 showed strong binding in the capture ELISA to Con A and LCA lectins, and moderate binding to PNA lectins. In addition, WAG lectin bound to fraction F2, which has affinity for D-*N*-acetylglucosamine and sialic acid residues. No binding was observed with UEA-1, BS-1 and DBA lectins (Fig. 6). Binding of each lectin was specific and could be blocked by the addition of a 0·2 M solution of a suitable inhibitory sugar to the reaction mixture. These results indicated that coproantigen molecules in the 2 FPLC derived antigenic peaks (fractions F1 and F2) from positive faeces contained sugars that included α-D-mannose and/or α-D-glucose, β-D-galactose, and *N*-acetyl-β-galactosamine. The *N*-acetyl-β-glucosamine and sialic acid residues also constituted antigenic molecules in fraction F2 but not in fraction F1. The sugars, α-L-fucose, α-D-galactose and *N*-acetyl-α-galactosamine were not specifically detected in any of the fractions tested.

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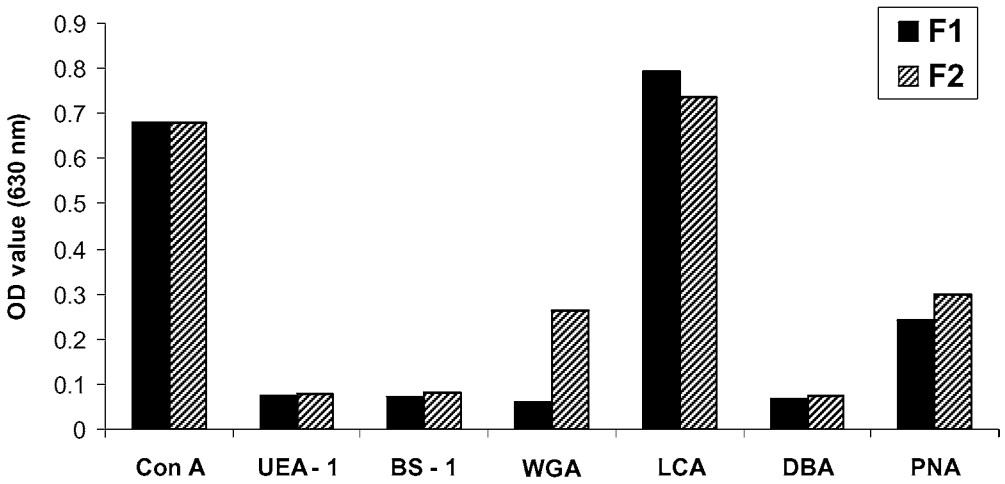

Fig. 6. Coproantigen capture ELISA results of lectin-binding analysis of FPLC fraction F1 and fraction F2 from *Echinococcus granulosus* coproantigen positive dog faecal samples. Values expressed are the mean of 8 samples tested.

Exoglycosidase treatment

Further characterization of the carbohydrate moieties associated with *E. granulosus* coproantigen activity in FPLC fraction F1 and fraction F2 from positive faeces was undertaken by enzymatic treatment using a panel of exoglycosidases. Changes in antigenicity after the treatment were assessed by coproantigen capture ELISA. The assays showed that, antigen activity in fractions F1 and F2 was retained after nearly all enzymatic treatments, except after treatment with β-*N*-acetylglucosaminidase, galactosidase or neuraminidase. Comparison with untreated fractions indicated that the coproantigen activity in ELISA was reduced by 5% and 37% when fractions F1 and F2 were incubated with β-*N*-acetylglucosamine respectively. Incubation with neuraminidase reduced the antigenic activity of fractions F1 and F2 by 4·9% and 27% respectively. In addition, terminal galactose residues on coproantigens were shown to be β-linked, as antigenicity was reduced by 11·9% and 36·9% after incubation of fractions F1 and F2 with β-galactosidase respectively but not with α-galactosidase. These data indicate that β-*N*-acetylglucosamine, β-galactose and sialic acid residues appear to contribute to the antigenicity of *E. granulosus* coproantigen molecules which were mainly present in fraction F2. Furthermore, it suggests that they are present in surface-specific glycans within a molecular weight range from 146 to 440 kDa.

DISCUSSION

Coproantigen ELISA represents a very useful, sensitive and essentially genus-specific diagnostic approach for detection of *Echinococcus* antigens in stool samples from infected dogs (Allan *et al*. 1992; Deplazes *et al*. 1992). Existing coproantigen assays for canine echinococcosis are based on the detection of a potentially broad array of largely undefined *Echinococcus* antigens, some of which might prove to cross-react with antigens of other intestinal parasites and possibly also with faecal contaminants (Allan *et al*. 1992; Deplazes *et al*. 1992; Malgor *et al*. 1997; El-Shehabi *et al*. 2000). For the purposes of standardizing such assays, determining their limits of sensitivity, and ensuring genus and species specificity for *Echinococcus*, will be important for the identification and characterization of specific antigens present in faecal samples around which such coproantigen assays were empirically developed (Craig, 1997). To date there have been few previous reports on the characterization of *Echinococcus* coproantigens, though recent studies broadly identified a role for carbohydrates in *E. granulosus* adult tapeworms (Casaravilla, Malgor & Carmona, 2003) and faecal supernatant from *E. granulosus*-infected dogs (Elayoubi *et al*. 2003). The present investigation is the first attempt to isolate and characterize parasite-specific antigenic components directly from the faeces of dogs infected with *E. granulosus*.

FPLC results indicated that at least 2 major antigenic components or fractions (F1 and F2) with different large molecular weights (>670 kDa and 146–440 kDa respectively) were specifically present in faecal supernatants from *E. granulosus*-infected dogs as demonstrated by coproantigen capture ELISA. These 2 high molecular weight fractions probably represent the effective coproantigen activity that is detectable by the *Echinococcus* coproantigen ELISA. These results, in general, support previous findings by Machincka *et al*. (1996) who reported that *T. saginata* coproantigens from infected human faeces had relative molecular weights >100 kDa, as estimated by selective ultrafiltration techniques.

Echinococcus granulosus coproantigens appear to be remarkably resistant, and can remain functionally unaltered by climatic effects, faecal enzymes, heat and formalin treatments, and even contamination by

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bacteria and fungi (Allan *et al*. 1992; Sakai *et al*. 1995; Guarnera *et al*. 2000; Jenkins *et al*. 2000). Heat-stability of *E. multilocularis* coproantigen has also been reported (Kohno *et al*. 1995; Sakashita *et al*. 1995). The present observations also support this, as it was demonstrated that *E. granulosus* coproantigen activity within the 2 major FPLC fractions (F1 and F2) remained stable after boiling for 30 min. Heat stability of *Echinococcus* coproantigens may be due to their highly glycosylated nature, which may also protect any protein core. Such stability is of practical importance in epidemiological studies of *E. granulosus* in domestic and wild canids, as faecal scats can be collected from the environment and successfully tested for coproantigens (Jenkins *et al*. 2000). In addition, faeces could be heated before testing to render them bio-safe for handling and preventing accidental infection as has been considered for *E. multilocularis* in foxes (Sakai *et al*. 1995; Sakashita *et al*. 1995).

Analysis of FPLC fractions from positive faecal supernatant by both SDS–PAGE and Western blot revealed a specific molecule with approximate molecular weight of 155 kDa in fraction F2, but not from coproantigen negative faecal samples. In addition, the negative silver stain appearance of the 155 kDa molecule may indicate its non-proteinaeous content. The effect of periodate treatment as observed by ELISA on the coproantigen activity of fractions F1 and F2 strongly suggested the involvement of carbohydrates in the antigenic determinants of *E. granulosus* coproantigens. This was also supported by immunoblotting analysis that showed the binding of polyclonal anti-EgWWE antibodies (IgG) to a 155 kDa antigen was completely ablated by periodate treatment.

It is of interest to note that in the current study, both *E. granulosus* coproantigen-positive dog faecal samples and adult whole worm extracts (EgWWE) demonstrated the same antigenic profile after FPLC fractionation as determined by coproantigen capture ELISA. This is possibly explained by the high degree of sensitivity and specificity using antibodies against somatic antigens (anti-EgWWE) in coproantigen ELISA for detection patent and pre-patent *E. granulosus* infections in dogs as reported by Allan *et al*. (1992) and Jenkins *et al*. (2000). There is, however, some evidence to suggest that improved test specificity of *Echinococcus* coproantigen ELISA may occur with the use of capture antibody directed against adult taeniid excretory–secretory (E–S) antigens, or parasite surface antigens rather than whole worm extracts (Deplazes *et al*. 1992, 1999).

It has been known for some time that the adult cestode tegument plasmalemma, i.e. the glycocalyx, is made up of a coat rich in carbohydrates (Lumsden, Oaks & Alworth, 1970; Lumsden, 1975). In addition, the release of tegumentary material in large amounts from adult cestodes has been observed (Whitfield, 1979). The present study showed that FPLC separation of a surface antigen preparation from *E. granulosus* adult worms contained large amounts of antigen, which corresponded in size (146–440 kDa) and antigenicity to putative coproantigen detected in the fraction F2. There was also a peak of antigenic activity (fraction F1) at >670 kDa from an *E. granulosus* adult E–S preparation. This suggested that coproantigens may be derived from the carbohydrate-rich surface glycocalyx of adult worms, and are shed or secreted during the life-span of the worm. These observations are consistent with previous suggestions by Allan *et al*. (1992) and Nonaka *et al*. (1996) who considered that taeniid coproantigens present in host faeces would be associated with parasite surface turnover and/or its excretory–secretory (E–S) products. Surface antigen study is also supported by other reports, which showed that rabbit antibodies to *T. saginata* surface antigen had the greatest ability to detect *T. saginata* antigens in human faeces in comparison with antibodies raised against somatic or metabolic antigens (Machnicka *et al*. 1996). Furthermore, increased specificity for *E. multilocularis* coproantigen detection was obtained when adult surface antibodies were incorporated into a sandwich ELISA (Deplazes *et al*. 1999).

A panel of lectins was used in the current study to assess the presence of specific carbohydrate epitopes in *E. granulosus* coproantigen enriched fractions (F1 and F2) which revealed that the dominant carbohydrate moieties were α-D-mannose and/or α-D-glucose, β-D-galactose and *N*-acetyl-β-D-galactosamine. *N*-acetyl-β-D-glucosamine and sialic acid residues were also present in putative coproantigen molecules of FPLC fraction F2 as binding to WAG lectin was observed with this fraction but not with fraction F1. In addition, exoglycosidase treatment confirmed the presence of β-galactose, *N*-acetyl-β-glucosamine and sialic acid in fraction F2, but not in fraction F1. This result may suggest that the coproantigen molecule(s) eluted from positive faeces in FPLC fraction F1 are different from those present in fraction F2 in respect to their carbohydrate composition. It is also possible that due to the high molecular weight (>670 kDa) and the complexity of the carbohydrate moieties of coproantigen molecule(s) found in fraction F1 have prevented the enzymes from reaching their cleavage sites and similarly the lectins from reaching their specific surface sugar residues. Recently, Casaravilla *et al*. (2003) reported that mannose is a highly expressed component of the *E. granulosus* adult glycans as shown by widespread reactivity of ConA in tegument and parenchyma components, including the reproductive system. In addition, they showed that reproductive structures are also rich in *N*-acetyl-D-glucosamine (GlcNAc)-*N*-acetyl neuraminic acid (NeuAc) and galactose as demonstrated by their strong reactivity with WGA and PNA, respectively.

The lack of binding of UEA-1, BS-1 and DBA lectins as detected by ELISA, suggested that terminal α-L-fucose, terminal α-D-galactose and α-*N*-acetylgalactosamine residues respectively, are not common in carbohydrates present in *E. granulosus* coproantigens. These residues may be positioned in the oligosaccharide chain at sites unavailable for lectin binding or alternatively may not be present in glycoproteins of *E. granulosus* coproantigens. Casaravilla *et al*. (2003) reported that UEA-1 failed to bind to any *E. granulosus* adult worm tissues as determined by lectin fluorescence. This is generally in agreement with information available on the carbohydrate composition of other types of cestodes (Friedman *et al*. 1982; Sandeman & Williams, 1984; Schmidt & Peters, 1987; Schmidt, 1988), but was at variance with the studies of Berrada-Rkhami *et al*. (1990), who showed that the tegumental surface of the adult pseudophyllidean tapeworm *Bothriocephalus gregarious* had contained α-*N*-acetylgalactosamine, α-D-galactose and fucose residues.

In summary, the FPLC study indicates that *E. granulosus* coproantigens comprised at least 2 major antigenic components (designated fraction F1 and F2) with approximate molecular weights >670 kDa and in the range of 146–440 kDa respectively. Heat stability and sodium periodate sensitivity of these 2 faecal antigenic components strongly suggested the involvement of carbohydrates in *E. granulosus* coproantigens. A carbohydrate-rich 155 kDa antigenic molecule was identified in FPLC fraction F2 that may be derived from the carbohydrate-rich surface glycocalyx of adult worms. However, the antigenic molecules present in fraction F1 were likely to be derived from adult worm E–S products. Carbohydrate moieties associated with coproantigen molecules present in fractions F1 and F2 were found to be composed of α-D-mannose and/or α-D-glucose, β-galactose and *N*-acetyl-β-galactosamine residues. *N*-acetyl-β-glucosamine and sialic acid residues were also contained in coproantigen molecules present in fraction F2. This initial study on the involvement of carbohydrate moieties in coproantigen antigenicity will now serve as a basis for a further characterization of the corresponding glycoproteins.

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