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Trichuris chilensis: physicochemical and immunological characterization of the different antigenic components

C. A. Pujol

Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellon 2, Ciudad Universitaria, 1428 Nuñez, Buenos Aires, Argentina

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Abstract. The protein pattern of *Trichuris chilensis* obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was analyzed. Complex protein band patterns covering a wide range of molecular weights were obtained. The molecular weights of the major proteins present in different tissue homogenates were estimated. Antisera raised in rabbits against homogenates of *T. chilensis* and sera from naturally infected *Ctenomys australis* were used in Western blotting, immunoelectrophoresis and passive hemagglutination to compare the antigenicity of the adult male, adult female, eggs, oocytes, stichosome and cuticle of this parasite. Specific antibodies to parasite antigens were also detected in faecal preparations and caecum mucosal extracts of *C. australis* naturally infected with *T. chilensis*.

In the course of the investigations into rodent helminthosis, a nematode belonging to the species *Trichuris chilensis* Babero, Cattan et Cabello, 1976 from Tuco-tuco, *Ctenomys australis* Rusconi, 1934 (Rodentia: Octodontidae) of Argentina was studied employing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blot (WB), immunoelectrophoresis (IE), and passive hemagglutination (PH) methodology in order to determine its protein pattern and the immunogenicity of its tissue proteins when they were injected in rabbits. In addition, specific antibodies to parasite antigens were detected in sera, faecal preparations and caecum mucosal extracts of *C. australis* naturally infected with *T. chilensis*.

MATERIALS AND METHODS

Preparation of antigenic extracts of worms

Adult males (AM) and adult females (AF) collected from the caecum of *C. australis* were washed several times and homogenized separately in 0.1 M PBS pH 7.6 in a tissue grinder held in an ice bath. For the preparation of extracts of isolated tissues, adult worms were dissected using a stereoscopic microscope into eggs (E), oocytes (O), stichosome (St) and cuticle (C) and homogenized as previously described. All preparations were passed through a fine mesh screen to remove large particulate matter and stored frozen at -20 °C. Protein concentration was estimated by the method of Lowry et al. (1951).

Sampling methods for serum, mucosal and faecal extracts

Blood for serum was collected from *C. australis* by cardiac puncture under deep anaesthesia prior to autopsy. The sera of individual animals were stored at - 20 °C before further analysis. Mucosal samples from the caecum were collected post mortem as described by Sinski and Holmes (1978). Faecal extracts were carried out according to Wedrychowicz et al. (1983).

Immunization procedures

Rabbit hyperimmune serum against whole adult *T. chilensis* (males and females) homogenate was obtained by immunizing animals intramuscularly with 10 mg of protein in complete Freund's adjuvant. Three subsequent injections of the same dose but in incomplete Freund's adjuvant were given at 15 days intervals. Immune sera against E, O, St, and C were made in a similar way. The rabbits were bled by cardiac puncture 7 days after the last injection.

Polyacrylamide gel electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the homogenates was performed according to Laemmli (1970) with slight modifications, using 13.5 % separating and 4.5 % stacking acrylamide slab gel 0.2 cm thick, 15 cm wide and 14 cm long. Briefly, the samples (50-100 μg of protein) were heated to 100 °C for two minutes in sample buffer containing in final concentrations 0.0625 M Tris-HCl (pH 6.8), 2 % SDS, 10 % glycerol, 5 % 2-mercaptoethanol and 0.001 % bromophenol blue as the dye. This procedure denatures proteins by disrupting their tertiary and quaternary structures. Under these conditions, electrophoretic mobility in acrylamide is inversely proportional to the logarithm of the molecular weight. The electrode buffer (pH 8.3) contained 0.025 M Tris, 0.192 M glycine and 0.1 % SDS. Electrophoresis was carried out with a current of 30 mA until the bromophenol blue marker reached the bottom of the gel (about 3.5 hours). Gels were either treated for staining with Coomassie blue or processed for Western blotting. Following electrophoresis, the proteins were transferred to nitrocellulose paper by pressure absorption overnight at 4 °C. The technique for immunostaining of Western blot was done following Tsang et al. (1983). Molecular weight (MW) standards included in all gels for calculation of a standard curve were: bovine albumin, MW 66.0 KDa; egg albumin, MW 45.0 KDa; glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), MW 36.0 KDa; carbonic anhydrase (bovine erythrocytes), MW 29.0 KDa; trypsinogen (bovine pancreas PMSF treated), MW 24.0 KDa; trypsin inhibitor (soybean), MW 20.1 KDa; and α - lactalbumin

(bovine milk), MW 14.2 KDa (Sigma Chemical Company). The MW of unknown proteins were estimated from the calibration curve.

Immunoelectrophoresis

Immunoelectrophoresis was performed according to Williams (1971), using 1.5 % agar in barbital buffer 0.05 M pH 8.6. A current of 5 mA per slide was mantained for 120 minutes. An aqueous solutin of 0.5 % amido Schwartz in 45 % methanol, 10 % acetic acid was used for staining. A similar mixture of alcohol and acetic acid was used to wash out excess stain before the slides were dried at room temperature.

Hemagglutination tests

Passive hemagglutination tests were carried out in U-shaped well plastic microtitre plates according to the method previously described by Pujol et al. (1988). A concentration of 1/20.000 tannic acid was used to treat sheep red blood cells (SRBC), which were then exposed to 20 µg of antigen per ml of coupling mixture in PBS pH 7.6. All sera were heat-inactivated and absorbed with SRBC. Two-fold serial dilutions of the absorbed sera were made in PBS pH 7.6 containing 0.2 % gelatin. Controls consisting of dilutions of normal serum mixed with coated cells were set up for each test. Titres were expressed as the reciprocal of the highest dilution of immune serum that resulted in complete hemagglutination.

RESULTS

Individual protein components of AM, AF, E, O, St and C were separated on a 13.5 % SDS-PAGE gel and stained with Coomassie blue (Fig. 1). Analysis of 100 µg of AM and AF antigens revealed the presence of numerous proteins over a wide range of molecular weights. A major protein band appeared at 45.0 KDa in both preparations. Analysis of 50 µg of E and O showed the presence of three bands near 42.0 KDa, three bands at 28.0, 26.0 and 24.0 KDa, a band at 22.0 KDa, a diffuse band at 15.5 KDa and a broad protein band at 14.0 KDa in the former preparation which were not present in O. Both preparations shared the bands located between 66.0 to 50.0 KDa although in O they were poorly stained. In the preparation of 100 µg of St, two closely migrating proteins were situated at 45.0 KDa. Less well stained bands at 42.0 and 29.0 KDa were also seen. When 50 µg of C was analysed, a single major protein band located at 45.0 KDa was found. Weakly stained bands at 42.0, 36.0 and 29.0 KDa were also visualized in this preparation. Peptides with MW above 66.0 KDa seem to be common to all the homogenates, except St.

Qualitative analysis by immunostaining of WB using rabbit hyperimmune serum anti whole adult *T. chilensis* (Fig. 2) revealed the presence of several antigenic peptides in the preparations corresponding to AM and AF homogenates. The polypeptides of AM that react with the immune serum were detected at 66.0, 50.0, 45.0, 36.0, 33.0, 23.0 and 16.0 KDa. In the preparation of AF, besides the polypeptides already mentioned for AM, four polypeptides located at 40.0, 27.0, 20.0 and 14.0 KDa were detected. Three bands at 29.0, 15.5 and 14.0 KDa were revealed in E. A band at 66.0 KDa was obtained with O preparation. Single bands in St and C homogenates were observed at 45.0 KDa. When sera from infected *C. australis* were used, antibodies were mainly directed towards three proteins of adult worms at 50.0, 45.0 and 33.0 KDa and the proteins present in St and C preparations at 45.0 KDa. No

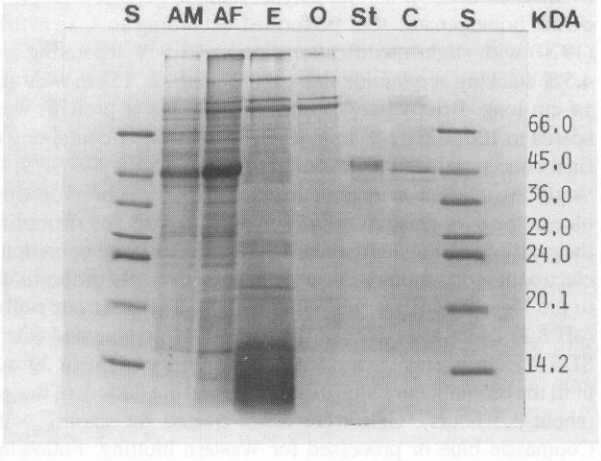

Fig. 1. Coomassie blue staining of a 13.5 % polyacrylamide gel following electrophoresis of *T. chilensis* proteins obtained from adult male parasites (AM), adult female parasites (AF), eggs (E), oocytes (O), stichosome (St) and cuticle (C). The proteins were separated with 30 mA for 3.5 hrs under reducing conditions. The position of the molecular weight standards (S) are indicated: bovine serum albumin (66.0 KDa), egg albumin (45.0 KDa), glyceraldehyde-3-phosphate dehydrogenase (36.0 KDa), carbonic anhydrase (29.0 KDa), trypsinogen (24.0 KDa), soybean trypsin inhibitor (20.1 KDa), α - lactalbumin (14.2 KDa).

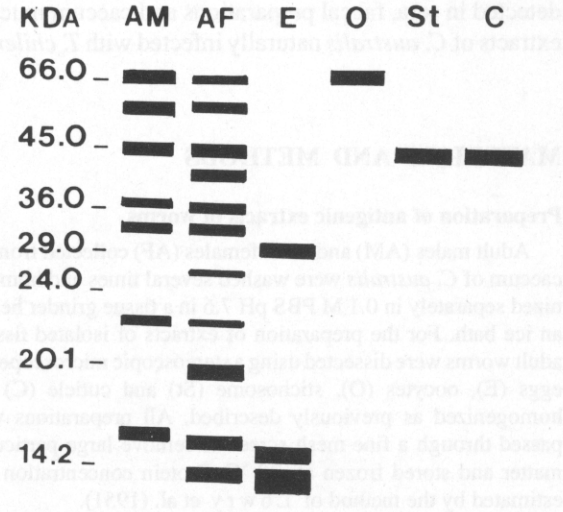

Fig. 2. Diagrammatic representation of Western blots of *T. chilensis* antigens. The proteins were separated on a 13.5 % polyacrylamide gel under reducing conditions and immunostained using rabbit antiserum against a crude homogenate from both male and female parasites. The position of molecular weight standards are indicated on the left margin (For references see legend of Fig. 1).

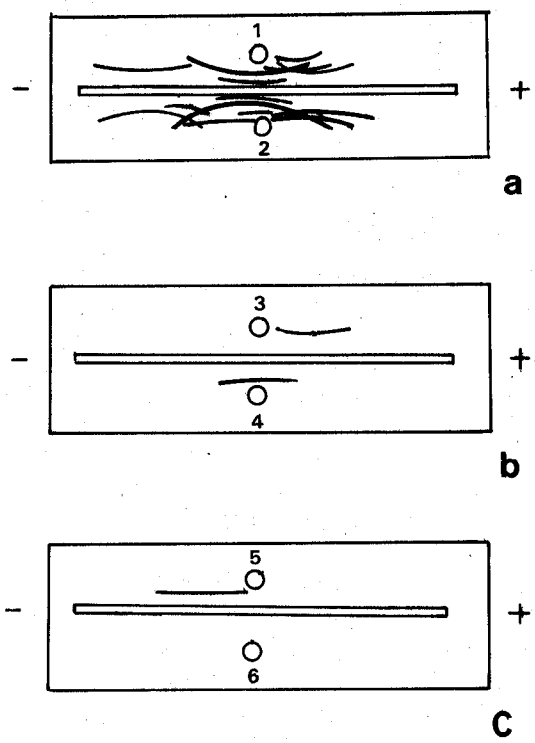

Fig. 3. Immunoelectrophoretic analysis illustrating reactions between homogenates of adult male (AM), adult female (AF), cuticle (C), stichosome (St), eggs (E), oocytes (O) and antiserum vs adult *T. chilensis*. In each case the horizontal troughs were filled with antiserum and the homogenates were separated electrophoretically as follows: a) 1-AM, 2-AF; b) 3-C, 4-St; c) 5-E, 6-O.

reaction was observed with E and O homogenates (data not shown).

Several precipitin arcs were seen by IE (Fig. 3) of the homogenates corresponding to AM and AF when they were developed with rabbit serum anti whole adult *T. chilensis*. A marked long precipitin arc was detected in both homogenates near the sample application wells. With the preparations of C, St and E a single band of precipitation was obtained in each case using the same antiserum. Nevertheless, they possessed different electropho- retic mobilities. In the case of C, it had an anodic mobility (slow α globulin region), St remained near the well demonstrating a slower electrophoretic mobility (β globulin region) and E migrated towards the cathode (γ globulin region). No demonstrable reaction was observed with rabbit serum anti whole parasite and O homogenate, although an arc with cathodic mobility was obtained when O proteins were developed with specific serum anti O (data not shown).

The results of PH using rabbit hyperimmune sera and tanned SRBC sensitized with C, St, E and O proteins are shown in Table 1. When rabbit hyperimmune serum against the whole parasite was used, titres of 25,600 and 6,400 were obtained with SRBC coupled to C and St antigens, respectively. Titres with E and O antigens were lower (3,200 and 400 respectively) than those obtained with St and C. With immune sera raised against C, St, E and O preparations higher titres were always obtained with the homologous antigen. Nevertheless, with anti C and anti St antisera an intense cross reaction was observed when SRBC were coupled either with St or C proteins. Controls of normal rabbit sera and coupled SRBC were all negative.

Table 1. Passive hemagglutination using different antisera and SRBC coupled with stichosome (St), cuticle (C), egg (E) and oocyte (O) antigens. Titres are expressed as the reciprocal of the highest dilution of serum that yields complete hemagglutination.

Rabbit antiserum	Antigen coupled to SRBC			
	St	C	E	O
anti - total	6,400	25,600	3,200	400
anti - C	6,400	12,800	400	100
anti - St	12,800	6,400	100	0
anti - E	100	100	3,200	200
anti - O	0	0	100	1,600

The hemagglutinating antibody titres in sera and faecal and mucosal extracts of eleven *C. australis* parasitized with *T. chilensis*, employing St antigens coupled to SRBC, are shown in Table 2. When cuticle proteins were coupled to sheep erythrocytes, results differed in no more than one dilution. No demostrable hemagglutinating reaction was observed when other parasite antigens were coupled to SRBC nor with the sera and faecal and mucosal extracts of five uninfected *C. australis*.

Table 2. Hemagglutinating antibody titres in serum, caecum mucosa and faeces of *C. australis* using tanned SRBC coupled with stichosome antigen. Titres are expressed as the reciprocal of the highest dilution that yields complete hemagglutination. The number of parasites found in the caecum of each rodent is also shown.

C. australis number	Number of parasites in caecum	Hemagglutinating titres		
		Serum	Caecum	Faeces
1	16	400	160	32
2	24	400	320	64
3	7	400	80	8
4	27	200	320	8
5	7	50	ND	2
6	12	400	320	ND
7	14	50	ND	4
8	2	50	20	8
9	27	400	160	ND
10	64	80	20	20
11	42	320	160	32

ND = not done

DISCUSSION

Antigenic proteins from whole worm extracts have been characterized by different biochemical and immunological methods (Jenkins and Wakelin 1983, Dorzok et al. 1989, Fujii 1989). In the present study, the proteins corresponding to the homogenates of whole *T.*

chilensis as well as those from homogenates of different anatomical structures were analyzed by SDS-PAGE, WB, IE and PH.

As it was suspected, in the case of whole parasite homogenates, complex protein band patterns covering a wide range of molecular weights were obtained when stained with Coomassie blue. Immunoperoxidase staining of WB using rabbit serum anti whole worm showed great antigenicity of the polypeptides present in these preparations, although fewer bands were seen when compared with those obtained by SDS-PAGE. Even fewer polypeptides were detected by WB using sera from naturally infected *C. australis*. Only three proteins of adult worms at 50.0, 45.0 and 33.0 KDa and the proteins present in St and C preparations at 45.0 KDa reacted with these sera. It is possible that a number of potentially antigenic proteins were simply not available for the immune response to infection (Dorzok et al. 1989). In the case of rabbits immunized with whole worm homogenates, "internal antigens" of the parasite were able to elicit an antibody response, but they were not exposed in living parasites so as to be recognized by the immune system of the host. Other explanations may be: (1) either some antigens were masked during the infectious cycle (Maizels et al. 1982) or (2) the immune response to some antigens was actively suppressed by products of the living parasites (Philipp and Rumjaneck 1984).

Besides the homogenates of whole worms, different organe and structures of the parasite obtained by dissection were homogenized separately to characterize the different antigenic components. In particular, trichuroid nematodes have a stichosome which occupies most of the anterior region of the body. Mechanical separation of the whole of this anterior stichosome region from the rest of the body is a simple initial method of fractionation and soluble antigens extracted from the stichosome of *T. muris* have been used to vaccinate mice very effectively (Wakelin 1973). When St homogenate was analyzed by SDS-PAGE, a major protein band was detected at 45.0 kDa. This was the only band revealed by WB in St homogenate using either rabbit hyperimmune serum or serum from naturally infected *C. australis*. This result could be correlated with a previous report about a 48.0 kDa beta stichocyte granule antigen present in *Trichinella spiralis* and in adult *Trichuris trichiura* homogenates that was recognized by monoclonal antibodies and serum from mice with patent *T. muris* worm burdens (Roach et al. 1988). This St homogenate gave a single precipitin arc by IE when rabbit serum anti whole parasite homogenate was used. In a similar work on *T. muris* a stichosome extract, when analyzed by immunodiffusion and immunoelectrophoresis, also gave a single precipitin line which showed identity with a single line in soluble material secreted by the worms (Jenkins and Wakelin 1977). The same antigen could be detected among several other components in whole worm extracts.

Cuticle homogenate also presented a major protein of 45.0 kDa recognized in WB by both rabbit hyperimmune serum and serum from infected host, but it differed from St antigen in its immunoelectrophoretic mobility.

Despite the fact that a complex protein pattern was seen in E homogenate when analyzed by SDS-PAGE, few bands were observed in WB using rabbit hyperimmune serum and a single precipitin line was developed by IE using the same antiserum.

Different stage-specific antigens were detected when O and E homogenates were compared, thus revealing the importance of the analysis of these antigens in different developmental stages. It was also found that E and O antigens could not elicit a serum antibody response in naturally infected hosts.

Antibodies against O antigens were readily detectable by the passive hemagglutination technique in rabbit serum using SRBC sensitized with O proteins. This antigen showed lower titres than those obtained when St, C and E antigens were coupled to SRBC. It can be concluded that O antigens are less immunogenic than the other antigens assayed. Besides, taking into account the results of the PH test we can say that the rabbit antibody response when injected with a whole worm homogenate was mainly directed to antigens present in St and C.

For the production of antisera, the rabbits were injected for a long period of time (two months) in order to obtain antibodies of high affinity that could recognize all the antigenic substances present in the homogenates. However, it is important to mention that the specificity of such antibodies decrease along the immunization period and so cross reactions between the different antigens are more probable.

On the other hand, anti worm antibodies have been demonstrated in faecal preparations, intestinal extracts and serum from infected animals (Wedrychowicz et al. 1983, Maclean et al. 1986). Using PH methodology, circulating antibodies in the sera of eleven *C. australis* parasitized with *T. chilensis*, with titres ranging from 50 to 400, were detected. Local antibody response was also detected at the caecum mucosa (titre=20 to 320) and in faecal extracts (titre=2 to 64) of infected rodents employing the same methodology. No antibody reaction was observed when other parasite's antigens different from St and C were coupled to SRBC.

Functional antigens, responsible for stimulating a protective immune response, have been detected in the stichocyte secretory cells associated with the oesophagus of trichuroid nematodes (Clegg and Smith 1978). Because of the above mentioned data, we could also postulate the cuticular proteins to be functional antigens too.

Unfortunately, it was difficult to correlate the number of parasites found in each rodent with their hemagglutinating antibody titre because we do not know if they were recently infected or chronically parasitized at the time they were captured. Besides, the capacity of mammalian hosts to respond to gastrointestinal nematodes is a function of

the age, nutritional and reproductive status and genotypic variations of the host (Else and Wakelin 1988, 1989). Nevertheless, it is surprising that those animals heavily parasitized showed similar hemagglutinating antibody titres to those with few nematodes in their caecum.

From the above mentioned data, the following conclusions may be drawn: as judged from the presence of hemagglutinating antibodies detectable in serum and faecal and mucosal extracts, the immune response in naturally infected *C. australis* is directed to antigens present in St and C. Both antigens have a molecular weight of 45.0 KDa but they are distinguishable from each other by their immunoelectrophoretic mobility. These antigens, among others, may be functional antigens of the parasite.

Much more biochemical and immunological analysis of parasite antigens, with protein and peptide purification, localization of antigenic sites and structural determination will become extremely important for understanding structure-function relationships.

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