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Purification of *Anisakis simplex* antigen by affinity chromatography

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Abstract In order to improve the specificity and sensitivity of the techniques for the diagnosis of human anisakidosis, a method of affinity chromatography for the purification of species-specific antigens from *Anisakis simplex* third-stage larvae (L3) has been developed. New Zealand rabbits were immunized with *A. simplex* or *Ascaris suum* antigens or inoculated with *Toxocara canis* embryonated eggs. The IgG-specific antibodies were isolated by means of protein A-Sepharose CL-4B bead columns. IgG anti-*Anisakis simplex*, anti-*Ascaris suum* and anti-*T. canis* were coupled to CNBr-activated Sepharose 4B. For the purification of the larval *Anisakis simplex* antigens, it was loaded into the anti-*A. simplex* column and bound antigens were eluted. For the elimination of the epitopes responsible for the cross-reactions, the *A. simplex*-specific proteins were loaded into the anti-*Ascaris suum* and anti-*T. canis* columns. To prove the specificity of the isolated proteins, immunochemical analyses by polyacrylamide gel electrophoresis and immunoblotting were carried out. Likewise, immunoaffinity columns were prepared using specific IgG from patients with *Anisakis simplex* sensitization, previously diagnosed by fluoro-enzymo-immunoassay. The protein patterns of antigen after purification by the human columns were similar to those obtained using the rabbit columns.

Introduction

Humans acquire anisakidosis by eating raw seafood dishes or undercooked fish and squid dishes (Sakanari and McKerrow 1989). Based on the location of the lesion, anisakidosis is divided into gastric, intestinal and heterologous anisakidosis (Ishikura et al. 1993). Acute symptoms of anisakidosis could be caused by a type I allergic reaction in the gastrointestinal wall (Suzuki et al. 1979) with elevated specific IgE. The earliest method for immunodiagnosis of anisakidosis was a complement fixation test (Daniels 1962). An immunofluorescence test was more sensitive than complement fixation, but cross-reacted with sera from toxocariosis patients (Ruitemberg 1970). Immunoelectrophoresis on starch demonstrated cross-reactivity to antigens from both *Toxocara* and *Ascaris* (Suzuki 1968). An indirect fluorescence-antibody test was specific but with purified haemoglobin (Suzuki et al. 1974). The radioallergosorbent test (Desowitz et al. 1985) did not cross-react with *Ascaris* or *Toxocara*. Takahashi et al. (1986) established monoclonal antibodies directed against *Anisakis* larvae which were assessed by the ELISA method as well as by immunofluorescence test on the frozen sections of larvae.

Iglesias et al. (1996) used an ELISA and immunoblotting to investigate antigenic cross-reactivity between *Anisakis simplex* and *Ascaris suum*, *Toxocara canis*, *Hysterothylacium aduncum*, *Trichinella spiralis* and *Trichuris muris*, to assess the potential diagnostic value of somatic, excretion-secretion, pseudocoelomic fluids and cuticular antigen preparations. The murine sera raised by immunization with an ascaridoid somatic substance reacted even more strongly with the *A. simplex* antigen than with the corresponding homologous one. The serum raised by immunization with the *A. simplex* somatic substance reacted strongly with the *A. simplex* excretor-secretor. The sera raised by infection with non-ascaridoids reacted only very weakly with the *A. simplex* antigen preparations. However, extensive homology between both somatic and excretor-secretor antigens of *A. simplex* and other ascaridoid nematodes has been reported (Kennedy et al. 1989) using radioimmunoprecipitation, an extremely sensitive technique. By means of immunoblotting, Iglesias et al. (1996)

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demonstrated that several somatic (11–18 kDa) and pseudocoelomic fluid (22 and 27 kDa) components appear to be specific for *A. simplex* in mice. Iglesias et al. (1997) developed five monoclonal antibodies specific for *A. simplex* which recognized antigens that are good candidates for serodiagnostic purposes.

In this work the specificity and sensitivity of *A. simplex* antigens prepared by affinity chromatography were assayed by polyacrylamide gel electrophoresis and immunoblotting using sera from immunized animals and human patients.

Materials and methods

Hyperimmune sera

New Zealand rabbits of about 3 kg body weight were immunized with *Anisakis simplex* or *Ascaris suum* crude extract (CE) prepared as previously described (Del Águila et al. 1987; Cuéllar et al. 1990; Perteguer and Cuéllar 1998) as multiple doses of 3 ml antigen in Freund's complete adjuvant(1,000 µg/ml in final volume) intramuscularly given weekly for 3 weeks. Other New Zealand rabbits were inoculated with multiple doses of 2,000 embryonated eggs of *Toxocara canis* weekly, during 4 weeks, by oral administration with gastric tubing. Animals were bled weekly postimmunization (p.i.) from the 1st inoculation (week 0). Specific antibodies were detected by ELISA against their homologous antigens (Cuéllar et al. 1990; García et al. 1996).

Human sera

Human anti-*Anisakis* sera were obtained from patients previously investigated (Montoro et al. 1997; Perteguer et al. 2000). They were residents of Madrid and had attended the Immunology and Allergy Service of the Hospital del Aire (Madrid) with recurrent acute urticaria. The concentration of circulating anti-*Anisakis*-specific IgE in human sera was measured by means of Pharmacia CAP System RAST FEIA (Pharmacia AB, Uppsala, Sweden), according to the directions for use.

Purification of *Anisakis simplex* CE products by affinity chromatography

Protein A-Sepharose CL-4B bead (Pharmacia Biotech) columns were prepared according to the manufacturer's instructions. Rabbit anti-*Anisakis simplex*, anti-*Ascaris suum* or anti-*Toxocara canis* antibodies, as well as human anti-*Anisakis* sera, in sample buffer [0.05 M Tris, 0.5 M NaCl (pH 8.0)] were loaded into the columns. Fractions of 1 ml were then collected. Unbound immunoglobulins were washed with washing buffer (0.05 M Tris, 0.5 M NaCl). Bound immunoglobulins then were eluted with glycine buffer (0.2 M glycine, 0.5 M NaCl, pH 2.8). Fractions were collected onto 100 µl of collection buffer (Tris-base 1 M, pH 8.5) and read on a spectrophotometer at A_{280} for calculation of rabbit or human IgG concentration. Protein A affinity-isolated IgG anti-*Anisakis simplex*, at a concentration of 5 mg/ml, in 0.1 M $NaHCO_3$ with NaCl 0.5 M (pH 8.5) was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions (Pharmacia Biotech). *A. simplex* CE antigen in sample buffer was loaded into the column and incubated for 3 h at room temperature. Fractions of 1 ml were then collected. Unbound antigens were washed with washing buffer and bound antigens then were eluted with glycine buffer followed by 50 mM diethylamine in saline, pH 11.5, collecting into glycine to neutralize eluted fractions. Fractions were read at A_{280} and the purity of eluted proteins was assayed by SDS-PAGE followed by silverv staining. The same procedure was carried out using columns prepared with rabbit IgG anti-*A. suum* and anti-*T. canis*, as well as antibodies from human patients.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970) and revised by Hames (1986) using a Mini Protean II cell (Bio Rad). The gels consisted of a 4% stacking gel and a 5–20% gradient separating gel. Samples were dissolved in a sample buffer (50 mM Tris-HCl buffer, pH 8.6, containing 2% SDS, 20% glycerol and 0.02% bromophenol blue) diluted 1:1 in electrode buffer (25 mM Tris, 192 mM glycine, pH 8.3), containing 1% SDS. Electrophoresis was performed for 2 h at a constant 100 V in Tris-glycine electrode buffer (see above). Broad range molecular weight markers (6,500–205,000 or 7,200–209,000 Da, Bio Rad) were incorporated into each electrophoretic run. Gels were stained with silver.

Immunoblot

Following the SDS-PAGE of the larval CE antigen, the protein bands were transferred onto a 0.22 µm pore size nitrocellulose membrane (Pharmacia) in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) with 25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3. The transblot was carried out at a constant 100 V for 1 h. The membrane was blocked for 3 h at room temperature with PBS containing 5% non-fat dry milk, prior to immunorecognition by incubation for 2 h with the rabbit or human sera diluted in PBS-Tween, containing 1% non-fat dry milk. Each paper was then washed with PBS-Tween 20 (3×5 min) and incubated for 3 h with affinity-isolated, peroxidase-conjugated, goat anti-rabbit or anti-human immunoglobulins (Caltag Laboratories, San Francisco, Calif., USA), at the appropriate dilution in PBS-Tween 20, containing 1% non-fat dry milk. To visualize bands the nitrocellulose was washed with PBS-Tween 20 (3×5 min) and reacted with the substrate (PBS, containing 0.006% H_2O_2:methanol, containing 0.03% 4-chloro-1-naphtol). The reaction was stopped by exhaustive washing with distilled water.

Results

Protein A affinity-isolated rabbit and human IgG anti-*Anisakis simplex* were coupled to CNBr-activated Sepharose 4B. *A. simplex* CE antigens were loaded into the columns, the unbound antigens were washed and bound antigens were then eluted with glycine buffer followed by diethylamine. All the eluted fractions were mixed and electrophoresed in order to study differences in the antigen composition before and after purification across the rabbit IgG anti-*A. simplex* column. Both antigen preparations were similar. The proteins of 205 kDa and 120 kDa were maintained. The doublet of 66–45 was observed in a smaller proportion while higher quantities of 40 kDa protein and doublets between 31–21 kDa and 21.5–14.4 kDa were present.

The human antigenic pattern was similar to those obtained when rabbit IgG was used. Differences were seen in the range of 45–31 kDa which appeared with a higher intensity than those observed with the rabbit IgG column (Fig. 1A).

In order to detect the proteins responsible for cross-reactions, an immunoblotting of *A. simplex* CE antigen was carried out using sera from rabbits immunized with

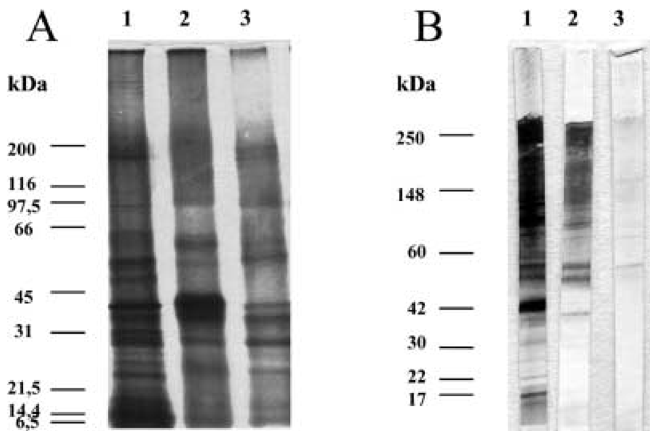

Fig. 1 **A** SDS-PAGE. *Lane 1 Anisakis simplex* CE antigen. *Lane 2 A. simplex* CE antigen eluted from a CNBr-activated Sepahrose 4B coupled to IgG from human patients sensitized to *A. simplex*. *Lane 3 A. simplex* CE antigen eluted from a CNBr-activated Sepharose 4B coupled to IgG from rabbits immunized with *A. simplex* CE antigen. **B** Immunorecognition patterns against the CE antigen of *Anisakis simplex* under non-reducing conditions. *Lane 1* Serum from rabbits immunized with larval *A. simplex* CE antigen. *Lane 2* Serum from rabbits immunized with adult *Ascaris suum* CE antigen. *Lane 3* Serum from rabbits inoculated with *Toxocara canis* embryonated eggs

A. simplex, *Ascaris suum* or *Toxocara canis*. Proteins of high molecular weight between 250 and 148 kDa were recognized by the anti-*A. suum* serum, as well as a doublet around 60 kDa also present using the anti-*A. simplex* serum and small bands situated at 120, 40 and 14 kDa. In the case of *T. canis* the number of proteins responsible for cross-reactions were few with a protein around 60 kDa also present using *Ascaris* antiserum and another of 14 kDa (Fig. 1B).

Likewise, *A. simplex* purified antigens were loaded into the column prepared with anti-*A. suum* rabbit IgG. Unbound antigens (*A. simplex* specific) were washed and bound antigens (cross-reacting) eluted. Further, immunoblotting analysis of both antigens was carried out using sera from rabbits immunized with *A. simplex* CE antigen. In the immunorecognition patterns (Fig. 2A) proteins of 205 and 120 kDa appeared, as well as two doublets of about 84–40 and 32–18.5 and two bands of 40 and 14 kDa, which were increased in intensity after passing across the anti-*Ascaris* column. The same procedure was carried out using sera from patients previously diagnosed with *Anisakis* sensitization. We observed proteins in the range of 205–25 kDa (Fig. 2B) with intense bands at 60 and 25 kDa and a doublet around 40 kDa. After purifying the antigen by anti-*A. suum* column this protein of 60 kDa was increased in intensity, while in the eluted fractions bright bands of high molecular weight of about 209 and 150 kDa appeared and there was no immunorecognition of the 60 kDa protein.

In Fig. 3A, electrophoretic patterns obtained with the above–mentioned antigens by SDS-PAGE are shown. In the antigen purified by anti-*A. simplex* column the pre-

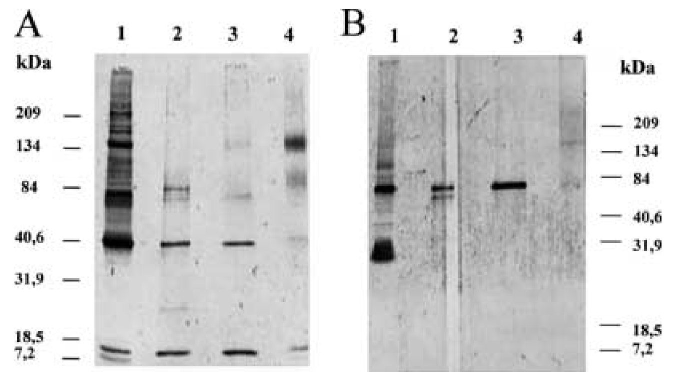

Fig. 2 **A** Immunorecognition patterns of IgG from rabbits immunized with larval *Anisakis simplex* CE antigen against: *lane 1 A. simplex* CE antigen; *lane 2 A. simplex* CE antigen eluted from a CNBr-activated Sepharose 4B coupled to IgG from rabbits immunized with *A. simplex* CE antigen; *lane 3 A. simplex* CE antigen after loading into a CNBr-activated Sepharose 4B coupled to IgG from rabbits immunized with adult *Ascaris suum* CE antigen; *lane 4 A. simplex* CE antigen eluted from the anti-*A. suum* column. **B** Immunorecognition patterns of immunoglobulins from human patients sensitized to *Anisakis simplex* against: *lane 1 A. simplex* CE antigen; *lane 2 A. simplex* CE antigen eluted from a CNBr-activated Sepharose 4B coupled to IgG from rabbits immunized with *A. simplex* CE antigen; *lane 3 A. simplex* CE antigen after loading into a CNBr-activated Sepharose 4B coupled to IgG from rabbits immunized with adult *Ascaris suum* CE antigen; l*ane 4 A. simplex* CE antigen eluted from the anti-*A. suum* column

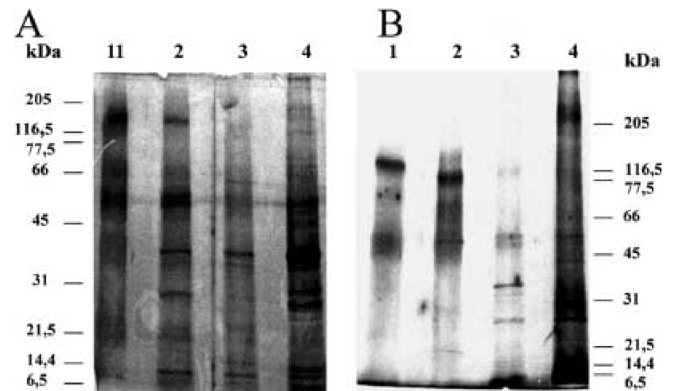

Fig. 3 **A** SDS-PAGE. *Lane 1 Anisakis simplex* CE antigen eluted from a CNBr-activated Sepharose 4B coupled to IgG from rabbits immunized with adult *Ascaris suum* CE antigen. *Lane 2 A. simplex* CE antigen after loading into the anti-*A. suum* column. *Lane 3 A. simplex* CE antigen eluted from a CNBr-activated Sepharose 4B coupled to IgG from rabbits immunized with *A. simplex* CE antigen. *Lane 4 A. simplex* CE antigen. **B** SDS-PAGE. *Lane 1 Anisakis simplex* CE antigen eluted from the anti-*Toxocara canis* column. *Lane 2 A. simplex* CE antigen after loading into a CNBr-activated Sepharose inoculated with *T. canis* embryonated eggs. *Lane 3 A. simplex* CE antigen eluted from a CNBr-activated Sepharose 4B coupled to IgG from rabbits immunized with *A. simplex* CE antigen. *Lane 4 A. simplex* CE antigen

viously mentioned doublet is seen. In the unbound fractions obtained by purification using the anti-*A. suum* column the 120 kDa protein increased in concentration, and there was a doublet of about 66–45. The 40 kDa

protein was maintained but the 25 kDa protein increased in intensity, and there was a a doublet between 21.5 and 14 kDa. In the eluted fractions debris of rabbit IgG was observed at 97–66 kDa. Likewise, very bright proteins of ≈35 kDa and 7 kDa were present. In Fig. 3B, electrophoretic patterns of fractions obtained by purification using the anti-*T. canis* column are shown. The 120 kDa protein has disappeared, a doublet of 66–45 kDa is maintained and the 40 and 25 kDa proteins are diminished in intensity.

Discussion

In this study we have used the two paths that affinity chromatography offers for the purification of antigens using antibodies to parasitic antigens bound to Sepharose 4B beads.

The first path consisted of binding the larval *Anisakis simplex* antigen to homologous antibodies from experimentally immunized rabbits and eluted from the column. This purification is necessary because the only treatment to eliminate cross-reactivity with closely related parasites cannot eliminate the possibility of cross-reactivity of the antigen with bacteria or viruses (Su and Prestwood 1990). Moreover, allergenic cross-reactivity between third-stage larvae of *Hysterothylacium aduncum* and *A. simplex* has been reported by Fernández et al. (1998) and cross-reactivity between IgE binding proteins from *Anisakis*, *Daphnia*, chironomid spp., Atlantic shrimp (*Pandalus borealis*) and German cockroach (*Blatella germanica*) (Pascual et al. 1997).

Successively, this antigen was purified across the rabbit IgG anti-*A. simplex* column and we observed that both patterns were similar, but several proteins appeared in different proportions and, in the case of the human column, the 40 kDa protein was very concentrated. The antigenic pattern of *A. simplex* antigen eluted from a column of anti-*A. simplex* human IgG was similar to those obtained when rabbit IgG was used. Because of this, we propose the use of the column of anti-*A. simplex* rabbit IgG for further purifications.

We observed cross-reaction between *Anisakis simplex* CE antigen and sera from rabbits immunized or inoculated with *Ascaris suum* or *Toxocara canis* embryonated eggs. The molecular mass of ABA-1 is controversial: it has been previously estimated at 14,000 (Christie et al. 1990), but mass spectrometry analysis indicated that there were five components of similar size, with the major species being 14,643.2 ± 1.4 Da with a high degree of similarity amongst ascaridid parasites (Christie et al. 1993). Yahiro et al. (1998) cloned the cDNA of TBA-1, the nematode polyprotein allergen of *T. canis* and found it to be most similar to ABA-1, the *Ascaris* nematode polyprotein, on the basis of the amino acid sequence. They observed a transient TBA-1 IgG antibody response during the infection that could explain the failure of Kennedy et al. (1988) to find anti-TBA-1 antibodies in animals infected with *T. canis* for prolonged periods. Also different forms of ABA-1 have been reported by Kato and Komatsu (1996), which could explain the different antibody recognition such that two different forms of TBA-1 may be expressed in a stage-specific manner. Also, Kennedy et al. (1988) observed evidence that a M_r 14,000 component of *A. simplex* has a homologue in *A. suum*, *Ascaris lumbricoides* and *T. canis*, but did not elicit an antibody response in anisakiasis.

In the case of *T. canis* antisera, the number of cross-reacting proteins was low with strong reactions at 60 kDa and 14 kDa. Zarnowska and Jastrzebska (1994), by SDS-PAGE of larval ES products from *T. canis*, showed polypeptides of molecular weights ranging from 19 to 200 kDa. However, an additional polypeptide, not observed on stained gels, resolving at 14 kDa, was detected by Western blot. Sera from patients with *A. lumbricoides* recognized polypeptides of 74, 75 and 160 kDa. According to these authors cross-reactions occur with the ES proteins of molecular weight 39–160 kDa. Iglesias et al. (1996) confirmed by immunoblotting the high degree of cross-reactivity between the somatic antigens of *A. simplex* and somatic antigens of the ascaridoids *A. suum*, *T. canis* and *H. aduncum*, although several *A. simplex* components in the 11–18 kDa range were only recognized by sera from mice infected with *A. simplex*. Tanaka et al. (1983) developed a radioimmunassay for *A. suum* protein and observed that *T. canis* had a high concentration of a substance partially cross-reactive with *A. suum* protein. Also, small amounts of substance cross-reacting with *A. suum* protein were exhibited by *Anisakis* larvae. High concentrations of *A. suum* protein were observed in sera from patients with ascariasis (64.5 ± 18.8 ng/ml), anisakiasis (75.2 ± 28.0 ng/ml) and toxocariasis (78.4 ± 31.3 ng/ml). Nunes et al. (1997) detected at least one band with molecular weight around 55–66 kDa that seemed to be responsible for the cross-reactivity between *T. canis* and *A. suum* once it disappeared when previous absorption of serum samples with *A. suum* antigens had been performed. Kennedy et al. (1989) observed, using radioimmunoprecipitation and SDS-PAGE, that there was a significant antigenic similarity between the antigens of *A. suum* and *T. canis*. Among the cross-reactive components, these authors found a 14 kDa internal protein which has a homologue in the two parasites, observing that it was the subject of an IgG antibody response in *Ascaris* infection, but there was no measurable response to it in toxocariasis. McWilliams et al. (1987) observed that *A. suum* cross-reacted allergically with *T. canis* and that the cross-reacting allergens were predominantly of high molecular weight.

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