Accepted manuscript. González-Fernández J, Rivas L, Luque-Ortega JR, Núñez-Ramírez R, Campioli P, Gárate T, Perteguer MJ, Daschner A, Cuéllar C. Recombinant vs native Anisakis haemoglobin (Ani s 13): Its appraisal as a new gold standard for the diagnosis of allergy. Exp Parasitol. 2017 Oct;181:119-129. doi: 10.1016/j.exppara.2017.08.010.
Full text available at: https://www.sciencedirect.com/science/article/pii/S0014489417304654

Recombinant *vs* native *Anisakis* haemoglobin (Ani s 13): its appraisal as a new gold standard for the diagnosis of allergy

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Note: Supplementary data associated with this article

Accepted manuscript. González-Fernández J, Rivas L, Luque-Ortega JR, Núñez-Ramírez R, Campioli P, Gárate T, Perteguer MJ, Daschner A, Cuéllar C. Recombinant vs native Anisakis haemoglobin (Ani s 13): Its appraisal as a new gold standard for the diagnosis of allergy. Exp Parasitol. 2017 Oct;181:119-129. doi: 10.1016/j.exppara.2017.08.010.
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Abstract

Recombinant allergens are currently the best option for serodiagnosis of human anisakiasis in terms of sensitivity and specificity. However, previous reports showed high rates of anisakiasis patients who were negative to Ani s 7 and especially to Ani s 1. Recently, *Anisakis* haemoglobin was described as a major allergen (Ani s 13). Although Ani s 13 belongs to a conserved protein family, it seems not to be a cross-reacting antigen because of the absence of IgE recognition against *Ascaris* haemoglobin in *Anisakis* patients. The aim of this study is to develop a more sensitive and specific diagnosis tool for *Anisakis* based on the recently discovered allergen Ani s 13. We obtained and purified recombinant *Anisakis* haemoglobin (rAni s 13) and the native form (nAni s 13). The recognition of both recombinant and native haemoglobins by anti-haemoglobin IgE from patients' sera was assessed by indirect ELISA and immunoblotting using 43 *Anisakis* sensitised patients and 44 non-*Anisakis* sensitised patients. Native Ani s 13 was also treated with periodate to study if oxidation of glycans destroys antibody binding. Furthermore, it was structurally characterised by negative staining electron microscopy and analytical ultracentrifugation. Recombinant Ani s 13 was only recognised by four patients with gastro-allergic anisakiasis (GAA) and immunoblotting analyses showed no bands. However, nAni s 13 was detected by 72.1% of *Anisakis* sensitised patients measured by indirect ELISA. Particularly, 18 (90%) out of 20 GAA patients were positive. Tetramers and octamers were the most abundant homomers of nAni s 13 but octamers had higher content of bound heme. None of the non-*Anisakis* sensitised patients were positive. Combined use of purified native form of Ani s 13 with current gold standards would improve the sensitivity and specificity for diagnosing anisakiasis.

Keywords: Allergy, Ani s 13, *Anisakis*, Fish parasites, Haemoglobin, IgE, Purification, Recombinant.

1. Introduction

Anisakis simplex is a nematode that infects humans in its third larval stage (L3) (Nieuwenhuizen and Lopata, 2013). Humans are incidental hosts that after infection may show different symptomatology accounting for the different phenotypes of anisakiasis, disease first described in the 60's (Van Thiel, 1962). Gastric anisakiasis, the most frequent, is characterised by an acute reaction caused by the invasion of the gut by the nematode, characterised by epigastric pain, nausea and vomiting within 12 hours after eating fish with live larvae (Daschner et al., 2000; Sugimachi et al., 1985). Gastro-allergic anisakiasis (GAA) includes also acute allergic symptoms ranging from urticaria or angioedema to anaphylaxis (Daschner et al., 2000) surmised to be involved in a defence mechanism aimed to expel the larvae in the first hours post-infection (Daschner and Cuéllar, 2010). Chronic urticaria (CU) has been associated to *Anisakis* sensitisation and constitutes a different clinical and immunological phenotype (CU+) (Daschner et al., 2013, 2005).

Gastro-allergic anisakiasis (GAA), which is always considered a secondary infection (as witnessed by the presence of specific IgE antibodies), is diagnosed after a detailed clinical history concerning raw or undercooked fish intake up to 48 hours prior to the start of the symptoms plus a positive Skin Prick Test (SPT) as well as specific IgE against *Anisakis* (Daschner et al., 2000). But in spite of the acute character of an IgE-mediated hypersensitivity reaction, in this entity the onset of symptoms is often not immediate and it makes the diagnosis difficult when the attending physician does not ask explicitly for raw fish intake (Daschner et al., 2000). In addition, false negatives may exist even though GAA patients generally have positive SPT and specific IgE anti-*Anisakis*. In the above mentioned study, two (5%) out of 40 GAA patients showed negative SPT against *Anisakis* crude extract and only one (2.5%) lacked specific anti-*Anisakis* IgE (Daschner et al., 2000). On the contrary, SPT and specific IgE to *A. simplex* crude extract to a certain extent are falsely positive in healthy population. In the previous study, four (19%) out of 21 subjects from the control group had positive SPT to *A. simplex*, even without a history of hypersensitivity reaction (Daschner et al., 2000). There are some studies involving larger number of patients in our region that support this statement. In a Spanish multicentric study, 56 (13.1%) out of 427 of subjects without any episode of angioedema or urticaria were sensitised to *Anisakis* (Fernández de Corres et al., 2001). The high rate of subclinical sensitisation has thus been interpreted as previous undiagnosed parasitic episodes (Daschner et al., 2012). Moreover a recent report showed positivity to *Anisakis* by SPT in 25 (3.2%) out of 789 mite allergic patients, four (25%) out of 16 shellfish allergic patients and three (8.3%) out of 36 mite and shellfish allergic patients, being excluded patients with anisakiasis (López-Matas et al., 2016).

In addition to the reported clinical history, SPT, and specific IgE by ImmunoCAP®; recombinant allergens such as Ani s 1 and Ani s 7 are currently the best option for serodiagnosis of human anisakiasis in terms of sensitivity and specificity (Anadón et al., 2010). A recombinant fragment of Ani s 7 allergen (t-Ani s 7) was considered as a specific indicator of contact with *Anisakis* live larvae (Anadón et al., 2009). Nonetheless, 17.9% of GAA patients and 57.5% of CU+ patients were negative to Ani s 1; and 7.1% of GAA patients and 7.5% of CU+ patients were negative to Ani s 7 (Cuéllar et al., 2012). Hence, development of new and more specific diagnosis tools for *Anisakis* is needed.

Recently, *Anisakis* haemoglobin was described as a major allergen (Ani s 13) with absence of IgE cross-reaction to *Ascaris* haemoglobin in *Anisakis* patients. In addition, Ani s 13 presented high rates of recognition (80.9% of the GAA patients) by a specific antigen-capture ELISA (González-Fernández et al., 2015). This previous demonstration of recognition justifies the production of the recombinant protein and the purification of the native form to measure anti-Ani s 13 IgE levels in *Anisakis* sensitised patients' sera. In addition, it will contribute to clarify the controversial issue on the use of recombinant *vs* native allergens for allergy diagnosis.

In this study, we expressed and purified recombinant *Anisakis* haemoglobin (rAni s 13) and purified *Anisakis* L3 in its native form (nAni s 13). The recognition of both recombinant and native

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haemoglobins by anti-haemoglobin IgE from patients' sera was assessed by indirect ELISA and immunoblotting.

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2. Material and methods

2.1. Patients and serum samples

Two main groups of patients were studied: 43 *Anisakis* sensitised patients and 44 non-*Anisakis* sensitised patients. The first group encompasses two clinical subgroups: 20 patients with gastro-allergic anisakiasis (GAA) and 23 patients with *Anisakis* sensitisation associated chronic urticaria (CU+). All patients were recruited prospectively within the same time frame were the same as we used except one patient #80 from the GAA group, all of them were included in our previous study (González-Fernández et al., 2015). GAA was diagnosed when a typical history (acute urticaria/angioedema or anaphylaxis of less than 48 h duration within 48 h of raw or undercooked-fish intake) was accompanied by further positive Skin Prick Test (SPT) and specific IgE against *Anisakis*. SPT was performed with *Anisakis simplex* extract (Lab. ALK-Abelló, Madrid, Spain) following standard protocols and was considered positive with a mean wheal diameter ≥ 3 mm. Histamine (1%) and saline solution 0.9% NaCl were considered as positive and negative controls, respectively. CU+ patients were included if recurrent wheals were present at least twice in a week for at least six weeks and they displayed positive SPT as well as specific IgE against *Anisakis* >0.35 kU/L. Patients were excluded from this study if the urticarial reaction was elicited mainly by physical stimuli. The second group of non-*Anisakis* sensitised patients is clinically subdivided in 22 chronic urticaria patients without SPT and specific IgE against *Anisakis* (CU-) and 22 healthy subjects not referring urticaria or currently *Anisakis* associated symptoms (HS). Written consent was obtained from all studied subjects. The project was approved by the Ethics Committee of the University Hospital La Princesa, Madrid.

2.2. Recombinant Anisakis haemoglobin (rAni s 13)

2.2.1. cDNA synthesis and cloning of A. simplex haemoglobin gene

An *A. simplex* cDNA collection was prepared with the Marathon cDNA amplification kit (Clontech, Mountain View, CA, USA) using mRNA obtained from the L3 with Fast Track mRNA isolation kit (Invitrogen, San Diego, CA, USA). The *A. simplex* haemoglobin gene was obtained by PCR using the following forward 5′- ATGCACTCGTCACTAGTAGCTTTGGCC-3’ and reverse 5’-TCAGTGGTGTTCCTCCTTGTGCTCT-3’ primers. The thermocycler conditions were 94ºC for 1 min; 30 cycles of 94ºC - 5 s, 50ºC - 30 s, 72ºC - 2 min; and a final extension at 72ºC for 7 min. The PCR product was gel-purified, cloned into pGEM®-T Easy cloning vector (Promega, Madison, WI, USA) and sequenced by Sanger method using ABI PRISM® 377 (Applied Biosystems, Foster City, CA, USA; Unidad de Genómica, CNM-ISCIII, Madrid Spain).

2.2.2. *Subcloning of* A. simplex *haemoglobin gene*

A. simplex haemoglobin lacking the signal sequence was then amplified with primers 5´-GAGAGAGACCCGGGTCAAAAACGCGA-3´ (forward) and 5´-TCTCTCTCCTGCAGTCAGTGGTGTTCCTCC-3´ (reverse), introducing 5´ *SmaI* and 3´ *PstI* restriction sites. The PCR reaction (50µl) contains 3 ng of template DNA, 10 µM of each primer, 1.5 µl of 10 mM dNTPs mix, and 1 µl of Taq DNA polymerase (1 U/µl) (Biotools, Madrid, Spain) in reaction buffer. PCR conditions were: 94 °C, min; five cycles, 94 °C, 30 s; 72°C, 4 min; five cycles of 94 ºC, 30 s; 70°C, 4 min; 25 cycles, 94 °C, 20 s; 68°C, 4 min; and a final extension at 72 °C, 10 min. The pQE32 vector was digested with *SmaI* and *PstI* and afterwards the amplified DNA haemoglobin was cloned into this vector. The digestion products were incubated for 30 min with T4 ligase at room temperature and transformed into XL1-Blue MRF´ *E. coli* (Agilent Technologies, Santa Clara, CA, USA) (Sambrook and Russell, 2001). All recombinant plasmids were verified by DNA sequencing as described above.

2.2.3. *Protein expression and purification of the* A. simplex *recombinant haemoglobin*

M15 (pREP4) *E. coli* were transformed with the recombinant haemoglobin plasmids. A single positive colony was inoculated into 100 mL of Luria–Bertani medium, containing ampicillin (100 µg/ml) and kanamycin (25 µg/mL) and further incubated at 37°C and 200 rpm. The recombinant protein was induced with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), at 37°C for 4 h. After protein expression, cells were harvested by centrifugation and resuspended in 20 ml B-PER© Bacterial Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA), incubated for 20 min, and then centrifuged for 20 min at 38280 *g*. The supernatant was discarded and the resulting pellet obtained was purified using a Ni-NTA affinity column Ni Sepharose™ 6 Fast Flow (GE Healthcare, Amersham, UK) under denaturing conditions. The polypeptides were eluted with 250 mM imidazole in 0.15 M Tris, pH 10.5. Recombinant *A. simplex* haemoglobin was analysed by SDS-PAGE in 12.5% polyacrylamide gels. The protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA), with BSA as a standard (Smith et al., 1985). The recombinant protein was stored at -80 °C until use.

2.3. Immunisation of female BALB/c mice

Female BALB/c mice were purchased from Charles River Laboratories (Barcelona, Spain). All animals were handled and housed in strict accordance with the Spanish law 53/2013 in which Directive 2010/63/EU was transposed. Three female BALB/c mice were immunised i.p. with purified *Anisakis* recombinant haemoglobin (rAni s 13). Briefly, mice were immunised with 200 µl of recombinant protein (30 µg) or PBS (negative control) mixed with complete Freund's adjuvant (Sigma, St. Louis, MO, USA) on day 0 and 21. Sera from mice were obtained weekly after the 21st day within two months after immunisation.

Similarly, as per a previous study (Perteguer et al., 2003), *Anisakis* crude extract immunised mouse serum (AniM) was obtained from BALB/c mice which were single inoculated i.m. with 1 mg of *A. simplex* crude extract mixed with complete Freund's Adjuvant.

2.4. Native Anisakis haemoglobin (nAni s 13)

Separation of *Anisakis* haemoglobin was carried out as per the methodology followed to purify *Ascaris suum* haemoglobin (Darawshe et al., 1987) with modifications. Approximately 200 *Anisakis* L3 were harvested manually from naturally parasitised blue whiting (*Micromesistius poutassou*). After washing the larvae thoroughly using double distilled water, they were drained and carefully homogenised in a ceramic mortar using PBS. Then, the extract was centrifuged at 6700 *g* for 15 min. The supernatant was collected and filtered (0.22μm). This sterile solution (*Anisakis* extract) was centrifuged at 175000 *g* for 6 h and the supernatant was discarded (S1). The tube containing the pellet was stored 12 h at 4ºC in contact with cold pyrogen-free distilled water. The contents of the tube were centrifuged at 63000 *g* for 30 min to separate the red solution of haemoglobin from the white insoluble material. The haemoglobin solution was centrifuged again at 175000 *g* for 6 h discarding the supernatant (S2) and the red pellet was dissolved in 50 mM NaCl/25 mM Tris-HCl buffer, pH 7.5 (loading buffer). This solution was loaded into Poly-Prep® columns (Bio-Rad, Hercules, CA, USA) with DEAE-Sephacel® (GE Healthcare) equilibrated with loading buffer. Elution was carried out step-wise in 2 ml fractions with 50, 100, 150, 200, 250, 500 mM and 2 M NaCl/25 mM Tris-HCl buffer, pH 7.5. The eluted fractions containing haemoglobin, according to heme absorbance, measured at 415 nm were centrifuged at 175000 *g* for 6 h to be concentrated. The protein content was quantified by the Bradford protein assay (Bio-Rad). Native *Anisakis* haemoglobin was stored at -20ºC until use.

2.5. Immunoblotting

SDS-PAGE was carried out as was previously described (Hames, 1986; Laemmli, 1970) using a Mini Protean® III cell (Bio-Rad). Proteins and extracts 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). They were loaded (400 μg for single-well gels or 25 µg per well for multi-lane gels) onto 12% gels with 5% stacking gels and electrophoresis was performed for 2 h at a constant 100 V in Tris-glycine electrode buffer. Broad range molecular weight markers (10-250 kDa Kaleidoscope®, Bio-Rad) were incorporated into each electrophoresis run. Following the SDS-PAGE of antigens, the protein bands were transferred onto a 0.22 µm pore size nitrocellulose membrane (Hybond® ECL, GE Healthcare) 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 membranes were blocked over night at 4ºC with PBS containing 5% skimmed milk. Nitrocellulose membranes were cut into strips. Each membrane strip was then washed with PBS-Tween 20 (PBS plus 0.05% Tween 20) (3 x 5 min) and incubated for 3 h with the mAb 4E8g anti-*A. pegreffii* haemoglobin (2 µg/ml) diluted in PBS-Tween containing 1% skimmed milk (henceforth used as diluent for all antibody and sera dilutions). Each strip was then washed with PBS-Tween 20 (3 x 5 min) and incubated for 2 h with goat anti-mouse IgG1 (γ1), horseradish peroxidase conjugate (HRP) (Molecular Probes Inc., Eugene, OR) at 1/1000. To visualize bands the nitrocellulose was washed with PBS-Tween 20 (3 x 5 min) and the strips were treated with a solution of 0.5 mg/ml of 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) with 0.05% hydrogen peroxide in PBS.

For mouse IgG1 determination, after blocking step, the mice sera were diluted at 1/25 and incubated for 3 h. Each strip was then washed with PBS-Tween 20 (3 x 5 min) and incubated for 2 h with goat anti-mouse IgG1 HRP conjugate (see above) at 1/500.

For human IgE determination, after blocking step, strips were incubated for 3 h with sera diluted at 1/10. After that, nitrocellulose strips were washed with PBS-Tween 20 (3 x 5 min) and incubated for 2 h with a murine mAb against human IgE (IgG1k, E21A11; INGENASA, Madrid, Spain) at 1/500 followed by the goat anti-mouse IgG1 HRP conjugate (see above) at 1/500. Finally, all the strips were equally treated with DAB solution (see above).

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2.6. IgG1 inhibition experiment

A pool of the three rAni s 13 immunised mice sera was diluted at 1/5 and preabsorbed with 10 µg of recombinant *Anisakis* haemoglobin per 1 ml of serum dilution or, for control purposes, with 40 µg/ml BSA at 4ºC overnight . The amount of recombinant allergen was selected to be in excess of specific IgG1 antibodies. After centrifuging, immunocomplexes were discarded and supernatants were incubated with strips containing blotted *Anisakis* crude extract following the above mentioned mouse IgG1 determination by immunoblotting.

2.7. Periodate treatment

Nitrocellulose-blotted stripes were treated with periodate before sera incubation as described (Woodward et al., 1985). Briefly, nitrocellulose strips were incubated with 50 mM sodium acetate buffer, pH 4.5, with or without 20 mM sodium periodate ($NaIO_4$) at room temperature for 1 h. Strips were then washed three times with PBS-Tween 20. Immunoblotting was carried out as described above.

2.8. Structural characterisation of nAni s 13

2.8.1. Analytical ultracentrifugation analysis

Samples at concentrations ranging from 0.25 to 1.0 mg/mL, in 25 mM Tris and 150 mM NaCl, were loaded (400 µL) into analytical ultracentrifugation cells. The experiments were carried out at 20ºC and 48000 rpm in a XL-A analytical ultracentrifuge (Beckman-Coulter, Brea, CA, USA) equipped with an UV-VIS absorbance detection system, using an An-50Ti rotor, and 12 mm Epon-charcoal standard double-sector centrepieces. Sedimentation profiles were recorded both at 280 nm and 415 nm. Differential sedimentation coefficient distributions were calculated by least-squares boundary modelling of sedimentation velocity data using the continuous distribution *c*(s) Lamm equation model as implemented by SEDFIT (Schuck, 2000). These *s* values were corrected to standard conditions

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(water, 20 °C, and infinite dilution) (Van Holde, 1985) using the program SEDNTERP (Laue et al., 1992) to get the corresponding standard *s* values ($s_{20,w}$).

2.8.2. *Electron Microscopy and image processing*

Native protein was adsorbed on 400 mesh carbon-coated grids at different concentrations, stained using 2% uranyl acetate and observed using a JEOL-1230 operated at 100 kV. Images of single molecules were obtained automatically using a TVIPS F416 CMOS at a nominal magnification of x40000. A total of 18,531 images at 5.68 Å per pixel were automatically selected and extracted from the micrographs, and then classified and averaged using methods implemented in EMAN2 (Tang et al., 2007) and XMIPP (De la Rosa-Trevín et al., 2013). Image classification was used to remove those images that did not correspond to molecule images but were incorrectly selected by the unsupervised automatic particle picking. Reference-free averages were calculates using CL2D algorithm (Sorzano et al., 2010).

2.9. *ELISA and statistics*

2.9.1. *Anti-Anisakis haemoglobin IgE and mice IgG1 determinations by indirect ELISA*

Each well of a 96-well microtitre plates (Costar, Corning, NY, USA) was coated with 100 µl of 5 µg/ml rAni s 13 or nAni s 13. Duplicate dilutions of sera at 1/100 in PBS-Tween 20 containing 0.1% BSA (this diluent was used for all next dilutions) were added and incubated, and IgG1 quantified using the goat anti-mouse IgG1 HRP conjugate (Molecular Probes Inc.) at 1/500. For the IgE determination, test sera at a 1/2 dilution were added in duplicate. Samples were incubated with a murine mAb against an epsilon human IgE chain (IgG1κ, E21A11, INGENASA, Madrid, Spain) at 1/500, followed by the goat anti-mouse IgG1 HRP conjugate (Molecular Probes Inc.) at 1/500 (see above).

After incubation at 37 ºC and subsequent washing steps, bound Ig-HRP was detected by incubation with o-phenylenediamine (OPD; Sigma) in phosphate-citrate buffer (pH 5.0) with 0.04% hydrogen peroxide. The reaction was stopped with 3 N sulphuric acid. The O.D. at 490 nm was calculated by subtracting the O.D. values of the same serum in the absence of antigen. Experiments were done in duplicate. For data analyses, sera were considered positive if the O.D. was more than X + 3SD, where X and SD are the corresponding mean O.D. and the S.D. obtained with 14 *Anisakis* negative control sera.

2.9.2. Statistical analysis

Median values and interquartile ranges (IQR) were calculated for specific antibody levels and compared using a Mann–Whitney U test. The Spearman correlation coefficient was used for correlation studies. All statistics were calculated using IBM SPSS Statistics Version 20. Significance was established at $P < 0.01$, 2-tailed.

3. Results

3.1. Sequence of *Anisakis simplex* haemoglobin

The open reading frame of the cDNA identified for *Anisakis simplex* haemoglobin (GenBank accession no. **KX457669.1**) was 999 bp long. The translated sequence of 332 amino acids showed 10 different amino acids with respect to *Anisakis pegreffii* haemoglobin sequence (GenBank accession no. **AFY98826.1**) (Fig. 1). Three of them (positions 93, 94, 98) were similar amino acids. The percentages of similarity and identity between haemoglobins from both *Anisakis* species were 97.7% and 96.7%, respectively. Other haemoglobin sequences from nematodes as *Pseudoterranova decipiens* (GenBank accession no. **CAA77743.1**) and *Ascaris suum* (GenBank accession no. **AAA29374.1**) were aligned together with the *Anisakis* ones (Fig. 1). *A. simplex* haemoglobin sequence showed a N-glycosylation site Asn-Xaa-Ser/Thr (where Xaa is not Pro) in the position 217 (Fig. 1) with a high probability score 0.7756 (http://www.cbs.dtu.dk/services/NetNGlyc/). None of O-GalNAc (mucin type) glycosylation sites were positively predicted (Steentoft et al., 2013). Thr 70 showed a low probability of O-β-GlcNAc glycosylation (score 0.4472 with a threshold of positivity of 0.4098) (Gupta and Brunak, 2002).

The partial recombinant *Anisakis simplex* haemoglobin (rAni s 13) presented three mutations after subcloning: C175Y, F253L and I265V (Fig.1).

3.2. Specific IgE against recombinant Ani s 13

Specific IgE was measured against rAni s 13 by indirect ELISA. The positivity cutoff was an O.D. of 0.022. It is noteworthy that we run out of serum from patient 319 (GAA group) and from patient 10 (CU- group) only for carrying out the indirect ELISA against rAni s 13. The sera of the groups CU+ (*n*=23), CU- (*n*=21) and HS (*n*=22) were negative except four (21%) out of 19 GAA patients tested (Supplementary Fig. S1). These positive four sera presented low O.D.'s (0.033, 0.035, 0.054, 0.078). Immunoblotting analyses with rAni s 13 showed no bands in any of 20 GAA and 23 CU+

Anisakis sensitised patients (data not shown). However, anti-*Anisakis* haemoglobin mAb 4E8g (IgG1), IgG1 from immunised mice using rAni s 13, and IgG1 from immunised mice using *Anisakis* crude extract, recognised rAni s 13 (Fig. 2).

3.3. Immunisation of mice using recombinant Ani s 13

Recombinant Ani s 13 is immunogenic. The specific IgG1 antibodies induced by rAni s 13 in mice recognised rAni s 13 and native haemoglobin present in the *Anisakis* crude extract by indirect ELISA. The ratio of Ani s 13 in the crude extract measured are lower and consequently the levels of recognition are decreased (Fig. 3). Inhibition immunoblotting results confirmed that the immunised mice recognised both native and recombinant Ani s 13 (Supplementary Fig. S2).

3.4. Purification of nAni s 13

The anionic exchange chromatography of the red pellet obtained from the *Anisakis* L3 by ultracentrifugation allowed separation of the haemoglobin fractions after step-wise elution from DEAE-Sephacel. Haemoglobin eluted at 150 mM NaCl/25 mM Tris-HCl buffer (peak 3). All peaks obtained (1 to 6) were again analysed by SDS-PAGE together with the initial *Anisakis* extract and the two supernatants (S1 and S2) after the 6 h ultracentrifugation steps (Fig. 4). Peaks 1 and 3 with a 37 kDa band (suspected to be haemoglobin) were transferred for immunoblotting to be checked with anti-haemoglobin 4E8g mAb and with a positive human serum to Ani s 13. Native haemoglobin (nAni s 13) mostly appeared at the fractions from peak 3 while absent in those pertaining to peak 1. A small amount of nAni s 13 was lost in the first supernatant (S1) (Supplementary Fig. S3).

3.5. Analytical ultracentrifugation and electron microscopy

Under the conditions assayed, sedimentation velocity assays at 280 nm revealed that nAni s 13 comprised four main oligomeric species with experimental sedimentation coefficients of 5.1 S ($s_{20,w}$ = 5.3 S), 7.6 S ($s_{20,w}$ = 8.0 S), 9.5 S ($s_{20,w}$ = 10.0 S), and 11.5 S ($s_{20,w}$ = 12.1 S), compatible with the globular dimer, tetramer, hexamer and octamer of the protein, respectively (Fig. 5). These peaks were accompanied by a small fraction (< 5% of total protein) of unspecific aggregates at higher S. The readouts at 415 nm, corresponding to the heme group absorbance, gave a sedimentation profile with only three main peaks at 7.6 S ($s_{20,w}$ = 8.0 S), 9.5 S ($s_{20,w}$ = 10.0 S), and 11.5 S ($s_{20,w}$ = 12.1 S), compatible with the globular tetramer, hexamer and octamer of the protein, respectively (Figure 1) as well as a small fraction (2.5% of total protein) of unspecific aggregates.

In summary, the peak ascribed to the Ani s 13 dimer lack absorbance at 415 nm, supported an absence of heme group for this protein. Besides, the tetrameric form of Ani s 13, accounting for a 43.8% of the total protein content, only accounted for a 14.4% of the total heme absorbance within the sample. On the contrary, the peak corresponding to the octamer of Ani s 13, which represents a 35.7% of the total protein content of the sample, accounted for the 64.1% of the total heme absorbance of the sample.

Negative staining of the fractions eluted from peak 3, showed in electron microscopy particles of different shapes and sizes, consistent with data obtained by analytical ultracentrifugation (Fig. 6).

3.6. Periodate treatment and cross-reactivity with Ascaris

Anti-haemoglobin 4E8g mAb (Supplementary Fig. S4, lanes 1) recognised a common glycosylated epitope of *Anisakis* and *Ascaris* haemoglobins because periodate treatment severely affected their recognition. However, periodate treatment did not affect epitopes recognised by rAni s 13 immunised mice IgG1 (lanes 3). The binding of patient’s IgE and *Anisakis* crude extract immunised mice IgG1 (Lanes 2 and 4) have not been as affected as mAb 4E8g by periodate.

IgE from *Anisakis* haemoglobin-positive human serum and IgG1 from rAni s 13 immunised mice did not recognised *Ascaris* haemoglobin (Supplementary Fig. S4, panel C, lanes 2 and 3).

3.7. Specific IgE against native Ani s 13

Specific IgE antibodies were measured against purified native Ani s 13 using indirect ELISA. Levels of anti-Ani s 13 IgE were higher in the GAA group compared with the CU+, CU- and HS groups ($P = 0.001$, $P < 0.001$, and $P < 0.001$, respectively). Similarly, CU+ group presented higher levels of anti-Ani s 13 IgE than CU- and HS groups ($P < 0.001$). CU- and HS groups did not show differences in their Ani s 13 IgE recognition (Fig. 7). Anti-Ani s 13 IgE values obtained by indirect ELISA correlated significantly ($P < 0.001$) with anti-Ani s 13 IgE values obtained previously (González-Fernández et al., 2015) by antigen-capture ELISA (Rho 0.835, $n= 87$).

The positivity cutoff was an O.D. of 0.037. IgE against nAni s 13 was detected in 31 (72.1%) out of 43 *Anisakis* sensitised patients. When analysed separately, 18 (90%) out of 20 GAA patients and 13 (56.5%) out of 23 CU+ patients were positive. None of the individuals from CU- and HS groups (non-*Anisakis* sensitised) were positive.

Immunoblotting analyses of IgE binding to nAni s13 were less sensitive than ELISA as 16 (80%) out of 20 GAA patients and eight (34.8%) out of 23 CU+ patients were positive. Immunoblotting showed as patient 36 (CU+ group) recognised other different bands (Fig. 8).

4. Discussion

In this work, native form of *Anisakis* haemoglobin (nAni s 13) has shown higher sensitivity in its recognition by IgE from patients using indirect ELISA than using previously developed antigen-capture ELISA, not being recognised by non-*Anisakis* sensitised patients.

Firstly, we studied Ani s 13 sequence *in silico* for possible glycosylation sites because previous studies reported a different recognition between native Ani s 7 [captured by the mAb UA3] and the recombinant t-Ani s 7 by IgE from rats immunised with dead *Anisakis* L3 (Anadón et al., 2009). The authors discussed that native captured Ani s 7 could be cross-reacting by means of common epitopes like O-glycans, present in protease-resistant *Anisakis* antigens from dead L3. These O-glycans have been reported as a source of cross-reactivity in human serological determinations of *Anisakis* (Lorenzo et al., 2000). There are three main types of glycosylation in eukaryotic organisms, N-linked, O-linked GalNAc (mucin type) and O-β-linked GlcNAc (intracellular/nuclear). In addition, as it was mentioned in section 3.1., different prediction methods based on neural networks have been designed for each glycosylation type. Our results gave no virtual probability of O-linked GalNAc, one O-β-linked GlcNAc glycosylation site on the first haemoglobin domain (Thr 70) with low probability and one N-glycosylation (Asn 217) shared by other nematode haemoglobins such as *Ascaris suum* (De Baere et al., 1992). In contrast, the substitution of N-terminal asparagine in *Ascaris* haemoglobin by serine in Ani s 13 precludes the same type of glycosylation described for the former. Concerning the mutation C175Y present in the recombinant protein (rAni s 13), inter- and intramolecular disulfide bonds have been reported in other haemoglobins (Bykova et al., 2006). Although *E. coli* expression system has been successful to obtain haemoglobins (Kabbua et al., 2014), this mutation may affect the correct folding of the molecule modifying conformational IgE epitopes.

For the sake of comparison, it is noteworthy that we tested rAni s 13 with the same patients sera as in the previous study (González-Fernández et al., 2015) except for patient number 319 (GAA group) and 10 (CU- group) as we ran out of serum only for carrying out the indirect ELISA against rAni s 13. We observed that rAni s 13 was recognised by four GAA patients with high IgE and IgG4 levels against captured Ani s 13 in the above referred study. Although O.D's obtained from these four patients were considered positive, the recognition was more limited against rAni s 13. In a recent study of allergenicity of native *vs* recombinant major allergens of *Dermatophagoides* mites using 30 patients with mite allergy (Sookrung et al., 2016), recombinant allergens presented lower percentages of IgE binding than native allergens. These authors claimed that lower capacity to be recognised by IgE of tested recombinants rDer p 1 and rDer f 1 was related to the fact these allergens had strictly conformational IgE-binding epitopes. In our case, *Anisakis* haemoglobin is a globular protein in which conformational IgE-binding epitopes have been previously predicted (González-Fernández et al., 2015). This could explain why rAni s 13 is limitedly recognised by patients' IgE. On the other hand, IgG1 from immunised mice with rAni s 13 or with *Anisakis* crude extract as same as anti-*Anisakis* haemoglobin mAb 4E8g, showed important reactivity against rAni s 13 by immunoblotting and ELISA, which suggest that IgG of patients should be tested in future works to confirm the existence of different IgG/IgE binding epitopes. In accordance with this statement, we confirmed that native and recombinant Ani s 13 had similar IgG binding epitopes by inhibition immunoblotting.

In this point, we had obtained a recombinant protein that was purified under denaturing conditions and was not recognised by patients' IgE. Even using eukaryotic biofactories to express recombinant allergens, sometimes it is necessary to purify the native allergens (Van Oort et al., 2004). Therefore, with the aim to elucidate whether natural glycosylation and folding were essential to the IgE binding; we purified the native forms of Ani s 13.

Several protocols have been developed to purify different extracellular haemoglobins of invertebrates. The first methods developed to purify *Anisakis* haemoglobin were based on precipitation with ammonium sulphate (Suzuki and Ishida, 1979). For haemoglobin from the crustacean *Artemia salina* combined ultracentrifugation with precipitation (Moens and Kondo, 1976) was used while others such as *Ascaris suum* haemoglobin was obtained just using ultracentrifugation (Darawshe et al., 1987). The last methodology was applied to *Anisakis* in order to preserve the quaternary structure of Ani s 13. Purified fractions obtained from peak 3 contained all the different Ani s 3 homomers, however the purity of the haemoglobin was demonstrated by the exclusive band of the monomer at 37 kDa after SDS-PAGE. The presence of higher molecular weight homomers was also described for *Ascaris* haemoglobin (Darawshe et al., 1987; González-Fernández et al., 2015). The elution peaks and the absorbance ratios at 280 nm and 415 nm reminded those obtained with the purification of *Artemia* haemoglobin (Moens and Kondo, 1978).

After a confirmation of Ani s 13 by mAb 4E8g and a positive human serum, we characterise the fractions from peak 3 by analytical ultracentrifugation and electron microscopy.

Analytical ultracentrifugation confirmed that 300 kDa native Ani s 13 octamer had an sedimentation coefficient $s_{20,w}$ = 12.1 S. This value was calculated previously as 12.4 S for *A. simplex* larvae and 12.0 S for for *A. physeteris* (Suzuki and Ishida, 1979). Although the calculated sedimentation coefficient is closer to *A. physeteris*, the values are rather similar. Even though both species can be distinguished morphologically (*A. simplex* larvae have a ventriculus longer than broad and often sigmoid in shape) and have enough genetic differentiation (Mattiucci and Nascetti, 2008), haemoglobins are highly conserved proteins that show similar sedimentation coefficients even between the genus *Anisakis* and *Ascaris*. The variable heme/protein absorbance ratio (A_{415}/A_{280}) was also present in the purification of *Ascaris* haemoglobin (Darawshe et al., 1987). These authors confirmed that purified fraction did not contain protein contaminants, thus this variable absorbance ratio obeyed to heme content. Haemoglobin from *Pseudorerranova*, which is an anisakid nematode, also showed different heme:protein ratio accounting for a variable number of empty heme binding pockets that give rise to different colour of the nematodes, which varied from reddish brown to white larvae (Dixon et al., 1993). Ani s 13 was not only present as octamers, as in the case of *Ascaris* and *Pseudoterranova* haemoglobins (Minning and Goldberg, 1998), but also hexamers, tetramers and

Accepted manuscript. González-Fernández J, Rivas L, Luque-Ortega JR, Núñez-Ramírez R, Campioli P, Gárate T, Perteguer MJ, Daschner A, Cuéllar C. Recombinant vs native Anisakis haemoglobin (Ani s 13): Its appraisal as a new gold standard for the diagnosis of allergy. Exp Parasitol. 2017 Oct;181:119-129. doi: 10.1016/j.exppara.2017.08.010.
Full text available at: https://www.sciencedirect.com/science/article/pii/S0014489417304654

dimers with lower content of bound heme. This fact could be due to the lack of a charged C-terminal tail in the Ani s 13 sequence, present in the above mentioned nematode haemoglobins. This tail seems to serve as intramolecular chaperone facilitating multimeric assembly because if deleted, haemoglobin aggregates and monomers became the major forms, being the octamer expressed in a small amount (Goldberg, 1999).

Native Ani s 13 negative staining electron microscopy pictures showed similar structures as those described for native *Ascaris* haemoglobin (Darawshe and Daniel, 1991; De Baere et al., 1992). Since most homomers are tetramers and octamers, the 300 kDa particle (octameric haemoglobin) should have a size at least around 10 nm (Erickson, 2009), as for the Dam1 200 kDa complex (Wang et al., 2007); the smallest and most abundant particles observed by electron microscopy are surmised to be tetramers. The larger particles are compatible with projections of hexamers, octamers or other aggregates. Improvement in the homogeneity of the different complexes in the sample will allow us to assess a better structural description by negative stain electron microscopy or cryo-electron microscopy.

Periodate treatment conditions (pH 4.5, 1 h) were carefully chosen to prevent additional damage to the polypeptide aside from removal of glycosylations (Barletta et al., 1998). Some authors reported ranges from 12.5 mM to 100 mM of periodate and times ranging from 1h to overnight for nitrocellulose strips (Bugajska-Schretter et al., 1998; Ghosh et al., 2015; Moneo et al., 1997; Nayak et al., 2013; Su et al., 2003). Periodate oxidation treatment did seriously affect the binding of mAb 4E8g, strongly supporting the recognition of a O-glycosylated epitope as N-glycosylations are impervious to periodate oxidation (Gong et al., 2015). In contrast, periodate did not affect binding of rAni s 13 immunised mice IgG1, raised against non-glycosylated rAni s 13; neither were IgE or IgG1 antibodies from a polyclonal stimulation in human and mouse.

Generally speaking, *Anisakis* sensitised patients have cross-reactive immunoglobulins against *Ascaris* (González-Fernández et al., 2015). However, it is not the case for *Ascaris* and *Anisakis* haemoglobins when IgE from *Anisakis* sensitised patients is determined (González-Fernández et al., 2015). These previous results are consistent with our immunoblotting. Although the different sequences of haemoglobin have billions of years of evolution, the functional acquisition of a similar tertiary and quaternary structures determined by x-ray crystallography were significantly similar adopting a common classical globin fold (Hardison, 1996). However, surface of IgE binding epitopes seems to be modified in *Ascaris* haemoglobin (González-Fernández et al., 2015), maybe to evade the host immune system, avoiding IgE binding.

Therefore, although Ani s 13 belongs to a conserved protein family, it seems not to be a panallergen. In addition, mice immunised with rAni s 13 did not generated IgG1 antibodies against epitopes from *Ascaris* haemoglobin, which confirms that immunogenic epitopes from Ani s 13 specific. Furthermore, mice immunised with the whole *Anisakis* crude extract recognised *Ascaris* cross-reactive proteins with a different pattern of molecular weights from the one observed against the *Anisakis* crude extract.

Ani s 13, as it was mentioned in the introduction, showed high rates of positivity in *Anisakis* sensitised patients by antigen-capture ELISA (González-Fernández et al., 2015). However the improved indirect ELISA using directly the purified native protein showed better results in terms of sensitivity. The new technique showed a high correlation with values obtained with the antigen-capture ELISA. Recognition of nAni s 13 was also higher in GAA and allowed differencing between GAA and CU+ groups of patients as well as it happens with other allergens like Ani s 1 (Cuéllar et al., 2012). Advantages of this method are: time/costs saving because of the lack of monoclonal capturing antibody, total absence of basal noise (the cutoff of captured-ELISA=0.293 *vs* indirect ELISA=0.037) and the fact that no patient from the HS (n=22) or CU- (n=22) groups were positive. These advantages further accompanied by an increased sensitivity of 9.1% in the GAA group and 8.7% in the group of CU+ (combined increase 7.8% when analysing *Anisakis* associated allergic disease).

Immunoblotting showed as two GAA patients (3 and 319) could not be detected as positive because of the lack of sensitivity of this technique. Patient 36 showed an intense and different profile of bands. This patient had O.D. of 0.109 by ELISA and was negative to Ani s 13 by the previous capture ELISA (González-Fernández et al., 2015). The reason for its false positivity might be the existence of trace quantities of other *Anisakis* protein due to the purification process or traces of Ani s 13 fragments with highly reactive exposed epitopes for this patient.

To conclude, combined use of nAni s 13 with current gold standards would improve the sensitivity and specificity for diagnosing anisakiasis.

Acknowledgements

We thank Natalie Nieuwenhuizen and Andreas Lopata for generously donating the 4E8g antibody. This work was supported by ISCIII: RETICS FEDER RICET [RD12/0018/0007 to LR] and [RD12/0018/0011 and PI14CIII/00076 to TG and MJP]. PC is supported by a PhD fellowship from ISCIII (RETICS-RICET RD12/0018/011). JGF is supported by a PhD fellowship from Universidad Complutense de Madrid. These founding sources had no involvement in the research and preparation of the article.

References

Anadón, A.M., Rodríguez, E., Gárate, M.T., Cuéllar, C., Romarís, F., Chivato, T., Rodero, M., González-Díaz, H., Ubeira, F.M., 2010. Diagnosing human anisakiasis: recombinant Ani s 1 and Ani s 7 allergens versus the UniCAP 100 fluorescence enzyme immunoassay. Clin. Vaccine Immunol. 17, 496–502.

Anadón, A.M., Romarís, F., Escalante, M., Rodríguez, E., Gárate, T., Cuéllar, C., Ubeira, F.M., 2009. The Anisakis simplex Ani s 7 major allergen as an indicator of true Anisakis infections. Clin. Exp. Immunol. 156, 471–478.

Barletta, B., Tinghino, R., Corinti, S., Afferni, C., Iacovacci, P., Mari, A., Pini, C., Di Felice, G., 1998. Arizona cypress (Cupressus arizonica) pollen allergens. Identification of cross-reactive periodate-resistant and -sensitive epitopes with monoclonal antibodies. Allergy 53, 586–593.

Bugajska-Schretter, A., Elfman, L., Fuchs, T., Kaplotis, S., Rumpold, H., Valenta, R., Spitzauer, S., 1998. Parvalbumin, a cross-reactive fish allergen, contains IgE-binding epitopes sensitive to periodate treatment and Ca2+ depletion. J. Allergy Clin. Immunol. 101, 67–74.

Bykova, N. V., Igamberdiev, A.U., Ens, W., Hill, R.D., 2006. Identification of an intermolecular disulfide bond in barley hemoglobin. Biochem. Biophys. Res. Commun. 347, 301–309.

Cuéllar, C., Daschner, A., Valls, A., De Frutos, C., Fernández-Fígares, V., Anadón, A.M., Rodríguez, E., Gárate, T., Rodero, M., Ubeira, F.M., 2012. Ani s 1 and Ani s 7 recombinant allergens are able to differentiate distinct Anisakis simplex-associated allergic clinical disorders. Arch. Dermatol. Res. 304, 283–288.

Darawshe, S., Daniel, E., 1991. Molecular symmetry and arrangement of subunits in extracellular hemoglobin from the nematode Ascaris suum. Eur. J. Biochem. 201, 169–173.

Darawshe, S., Tsafadyah, Y., Daniel, E., 1987. Quaternary structure of erythrocruorin from the nematode Ascaris suum. Biochem. J. 242, 689–694.

Daschner, A., Alonso-gómez, A., Cabañas, R., Suarez-de-Parga, M., López-Serrano, M.C., 2000. Gastroallergic anisakiasis : Borderline between food allergy and parasitic disease — Clinical and allergologic evaluation of 20 patients with confirmed acute parasitism by Anisakis simplex. J. Allergy Clin. Immunol. 105, 176–181.

Daschner, A., Cuéllar, C., 2010. The hidden sense of symptoms: urticaria can be beneficial. Med. Hypotheses 75, 623–6.

Daschner, A., Cuéllar, C., Rodero, M., 2012. The Anisakis allergy debate: does an evolutionary approach help? Trends Parasitol. 28, 9–15.

Daschner, A., Fernández-Fígares, V., Valls, A., Frutos, C. De, Rodero, M., Ubeira, F.M., Cuéllar, C., 2013. Different fish-eating habits and cytokine production in chronic urticaria with and without sensitization against the fish-parasite Anisakis simplex. Allergol. Int. 62, 191–201.

Daschner, A., Vega de la Osada, F., Pascual, C.Y., 2005. Allergy and parasites reevaluated: wide-scale induction of chronic urticaria by the ubiquitous fish-nematode Anisakis simplex in an endemic region. Allergol. Immunopathol. (Madr). 33, 31–37.

De Baere, I., Liu, L., Moens, L., Van Beeumen, J., Gielens, C., Richelle, J., Trotman, C., Finch, J., Gerstein, M., Perutz, M., 1992. Polar zipper sequence in the high-affinity hemoglobin of Ascaris suum: amino acid sequence and structural interpretation. Proc. Natl. Acad. Sci. U. S. A. 89, 4638–4642.

De la Rosa-Trevín, J.M., Otón, J., Marabini, R., Zaldívar, A., Vargas, J., Carazo, J.M., Sorzano, C.O.S., 2013. Xmipp 3.0: An improved software suite for image processing in electron microscopy. J. Struct. Biol. 184, 321–328.

Dixon, B., Kimmins, W., Pohajdak, B., 1993. Variation in colour of Pseudoterranova decipiens (Nematoda; Anisakidae) larvae correlates with haemoglobin concentration in the pseudocoelomic fluid. Can. J. Fish. Aquat. Sci. 50, 767–771.

Erickson, H.P., 2009. Size and shape of protein molecules at the nanometer level determined by sedimentation, gel filtration, and electron microscopy. Biol. Proced. Online 11, 32–51.

Fernández de Corres, L., Del Pozo, M., Aizpuru, F., 2001. Prevalencia de la sensibilización a Anisakis simplex en tres áreas españolas, en relación a las diferentes tasas de consumo de pescado. Relevancia de la alergia a Anisakis simplex. Alergol. e Inmunol. Clínica 16, 337–346.

Ghosh, N., Sircar, G., Saha, B., Pandey, N., Bhattacharya, S.G., 2015. Search for allergens from the pollen proteome of sunflower (Helianthus annuus L): A major sensitizer for respiratory allergy patients. PLoS One 10, e0138992.

Goldberg, D.E., 1999. Oxygen-Avid Hemoglobin of Ascaris. Chem. Rev. 99, 3371–3378.

Gong, W., Huang, F., Ma, Y., Bai, H., Yin, L., Li, J., Chen, C., Xu, X., Chen, X.-P., 2015. Protective immunity against Schistosoma japonicum infection can be provided by IgG antibodies towards periodate-sensitive or periodate-resistant glycans. Parasit. Vectors 8, 234.

González-Fernández, J., Daschner, A., Nieuwenhuizen, N.E., Lopata, A.L., Frutos, C. De, Valls, A., Cuéllar, C., 2015. Haemoglobin, a new major allergen of Anisakis simplex. Int. J. Parasitol. 45, 399–407.

Gupta, R., Brunak, S., 2002. Prediction of glycosylation across the human proteome and the correlation to protein function. Pacific Symp. Biocomput. 7, 310–22.

Hames, B.D., 1986. An introduction to polyacrilamide gel electrphoresis, in: Gel Electrophoresis in Proteins. IRL Press, Oxford.

Hardison, R.C., 1996. A brief history of hemoglobins: Plant, animal, protist, and bacteria. Proc. Natl. Acad. Sci. U. S. A. 93, 5675–5679.

Kabbua, T., Anwised, P., Boonmee, A., Subedi, B.P., Pierce, B.S., Thammasirirak, S., 2014. Autoinduction, purification, and characterization of soluble alpha-globin chains of crocodile (Crocodylus siamensis) hemoglobin in Escherichia coli. Protein Expr. Purif. 103, 56–63.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Laue, T.M., Shah, B.D., Ridgeway, T.M., Pelletier, S.L., 1992. Computer-aided interpretation of analytical sedimentation data for proteins, in: Harding, S.E., Rowe, A.J., Horton, J.C. (Eds.), Analytical Ultracentrifugation in Biochemistry and Polymer Science. Royal Society of Chemistry, Cambridge, UK, pp. 90–125.

López-Matas, M.A., de Larramendi, C.H., Moya, R., Sánchez-Guerrero, I., Ferrer, A., Huertas, A.J., Flores, I., Navarro, L.A., García-Abujeta, J.L., Vicario, S., Andreu, C., Peña, M., Carnés, J., 2016. In vivo diagnosis with purified tropomyosin in mite and shellfish allergic patients. Ann. Allergy. Asthma Immunol. 116, 538–543.

Lorenzo, S., Romarís, F., Iglesias, R., Audícana, M.T., Alonso, J.M., Leiro, J., Ubeira, F.M., 2000. O-glycans as a source of cross-reactivity in determinations of human serum antibodies to Anisakis simplex antigens. Clin. Exp. Allergy 30, 551–559.

Mattiucci, S., Nascetti, G., 2008. Advances and trends in the molecular systematics of anisakid nematodes, with implications for their evolutionary ecology and host-parasite co-evolutionary processes. Adv. Parasitol. 66, 47–148.

Minning, D.M., Goldberg, D.E., 1998. Determinants of Ascaris hemoglobin octamer formation. J. Biol. Chem. 273, 32644–32649.

Moens, L., Kondo, M., 1978. Evidence for a dimeric form of Artemia salina extracellular hemoglobins with high-molecular-weight subunits. Eur. J. Biochem. 82, 65–72.

Moens, L., Kondo, M., 1976. The structure of Artemia salina haemoglobins. A comparative characterisation of four naupliar and adult haemoglobins. Eur. J. Biochem. 67, 397–402.

Moneo, I., Audicana, M., Alday, E., Curiel, G., del Pozo, M., García, M., 1997. Periodate treatment of Anisakis simplex allergens. Allergy 52, 565–569.

Nayak, A.P., Green, B.J., Sussman, G., Berlin, N., Lata, H., Chandra, S., Elsohly, M.A., Hettick, J.M., Beezhold, D.H., 2013. Characterization of Cannabis sativa allergens. Ann. Allergy, Asthma Immunol. 111, 32–37.

Nieuwenhuizen, N.E., Lopata, A.L., 2013. Anisakis-a food-borne parasite that triggers allergic host defences. Int. J. Parasitol. 43, 1047–1057.

Perteguer, M.J., Águila, C., Fenoy, S., Guillén, J.L., Cuéllar, C., 2003. Cross-reactivity induced by Anisakis simplex and Toxocara canis in mice. J. Helminthol. 77, 331–334.

Sambrook, J., Russell, D.W., 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, N.Y.

Schuck, P., 2000. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. Biophys. J. 78, 1606–1619.

Smith, P., Krohn, R., Hermanson, G., Mallia, A., Gartner, F., Provenzano, M., Fujimoto, E., Goeke, N., Olson, B., Klenk, D., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

Sookrung, N., Choopong, J., Seesuay, W., Indrawattana, N., Chaicumpa, W., 2016. Allergenicity of native and recombinant major allergen groups 1 and 2 of Dermatophagoides mites in mite sensitive Thai patients. Asian Pacific J. Allergy Immunol. 34, 51–58.

Sorzano, C.O.S., Bilbao-Castro, J.R., Shkolnisky, Y., Alcorlo, M., Melero, R., Caffarena-Fernández, G., Li, M., Xu, G., Marabini, R., Carazo, J.M., 2010. A clustering approach to multireference alignment of single-particle projections in electron microscopy. J. Struct. Biol. 171, 197–206.

Steentoft, C., Vakhrushev, S.Y., Joshi, H.J., Kong, Y., Vester-Christensen, M.B., Schjoldager, K.T.-B.G., Lavrsen, K., Dabelsteen, S., Pedersen, N.B., Marcos-Silva, L., Gupta, R., Bennett, E.P., Mandel, U., Brunak, S., Wandall, H.H., Levery, S.B., Clausen, H., 2013. Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. EMBO J. 32, 1478–88.

Su, S.N., Peng, H.J., Yang, S.Y., Tsai, L.C., Chow, L.P., Huang, S.W., 2003. Purification and characterization of a novel isoallergen of a major Bermuda grass pollen allergen, Cyn d 1. J. Biomed. Sci. 10, 111–119.

Sugimachi, K., Inokuchi, K., Ooiwa, T., Fujino, T., Ishii, Y., 1985. Acute Gastric Anisakiasis. Analysis of 178 cases. J. Am. Med. Assoc. 253, 1012–13.

Suzuki, T., Ishida, K., 1979. Anisakis simplex and Anisakis physeteris: physicochemical properties of larval and adult hemoglobins. Exp. Parasitol. 48, 225–234.

Tang, G., Peng, L., Baldwin, P.R., Mann, D.S., Jiang, W., Rees, I., Ludtke, S.J., 2007. EMAN2: An extensible image processing suite for electron microscopy. J. Struct. Biol. 157, 38–46.

Van Holde, K.E., 1985. Physical biochemistry, 2nd ed. Prentice-Hall, Englewood Cliffs, New Jersey.

Van Oort, E., De Heer, P.G., Dieker, M., Van Leeuwen, A.W., Aalberse, R.C., Van Ree, R., 2004. Characterization of natural Dac g 1 variants: An alternative to recombinant group 1 allergens. J. Allergy Clin. Immunol. 114, 1124–1130.

Van Thiel, P., 1962. Anisakiasis (Abstract). Parasitology 52, 16P–17P.

Wang, H.-W., Ramey, V.H., Westermann, S., Leschziner, A.E., Welburn, J.P.I., Nakajima, Y., Drubin, D.G., Barnes, G., Nogales, E., 2007. Architecture of the Dam1 kinetochore ring complex and implications for microtubule-driven assembly and force-coupling mechanisms. Nat. Struct.& Mol. Biol. 14, 721–726.

Woodward, M.P., Young, W.W., Bloodgood, R.A., 1985. Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. J. Immunol. Methods 78, 143–153.

Figures

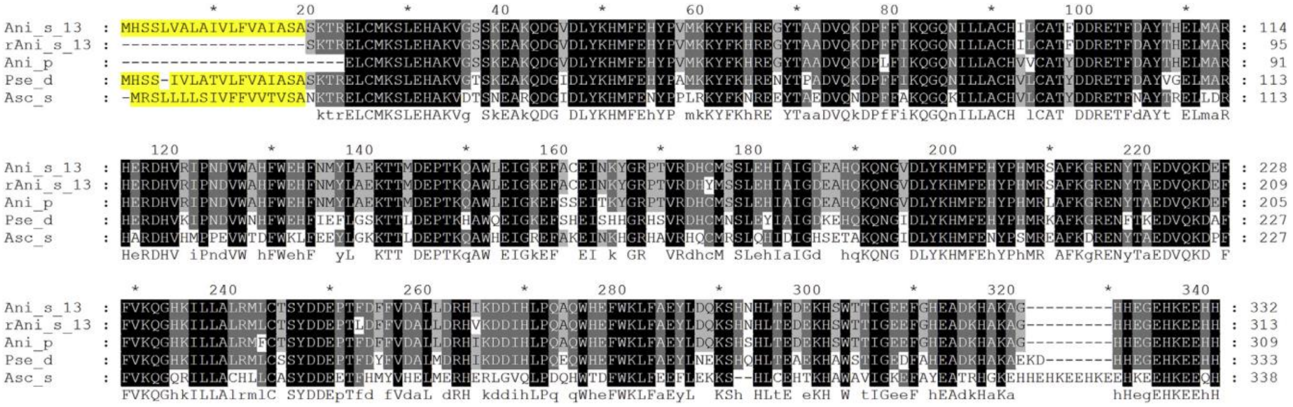

Fig. 1. Alignment of complete (Ani_s_13) and partial recombinant (rAni s 13) *Anisakis simplex* haemoglobins with haemoglobins from *Anisakis pegreffi* (Ani_p), *Pseudoterranova decipiens* (Pse_d), and *Ascaris suum* (Asc_s). Signal peptides were highlighted in yellow (alignment positions 1-19). Alignment edited with GeneDoc v2.7, Nicholas KB and Nicholas HB (1997) https://www.psc.edu/index.php/user-resources/software/genedoc.

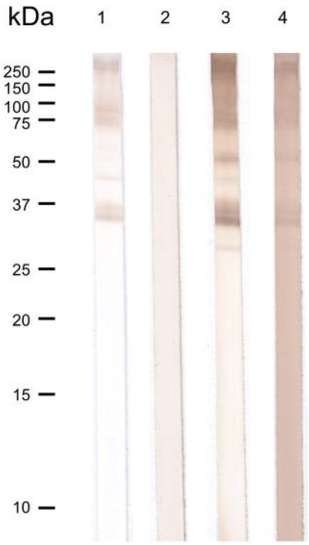

Fig. 2. Immunoblotting analysis of rAni s 13. Lanes: 1: monoclonal antibody 4E8g against *Anisakis* haemoglobin (IgG1), 2: *Anisakis* haemoglobin positive human serum IgE, 3: rAni s 13 immunised mice serum IgG1, 4: *Anisakis* crude extract immunised mice serum IgG1.

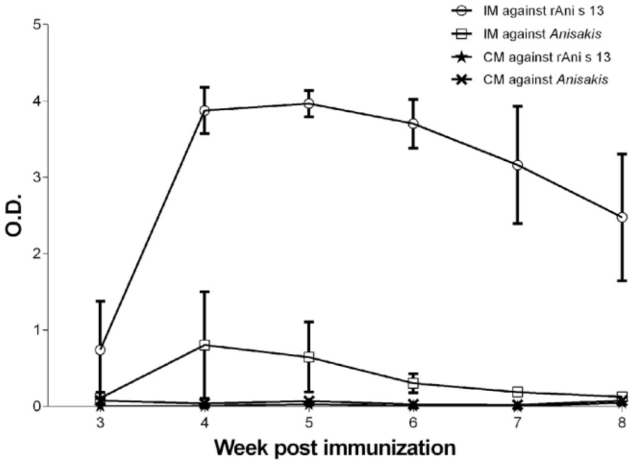

Fig. 3. IgG1 antibody levels induced by rAni s 13. BALB/c mice (*n*=3) were inoculated intraperitoneally with purified recombinant *Anisakis* haemoglobin (rAni s 13). Serum samples were obtained weekly and ELISA were carried out against rAni s 13 or *Anisakis* crude extract. The values are mean O.D. at 490 nm ± S.D. IM: Immunised mice. CM: Control mouse.

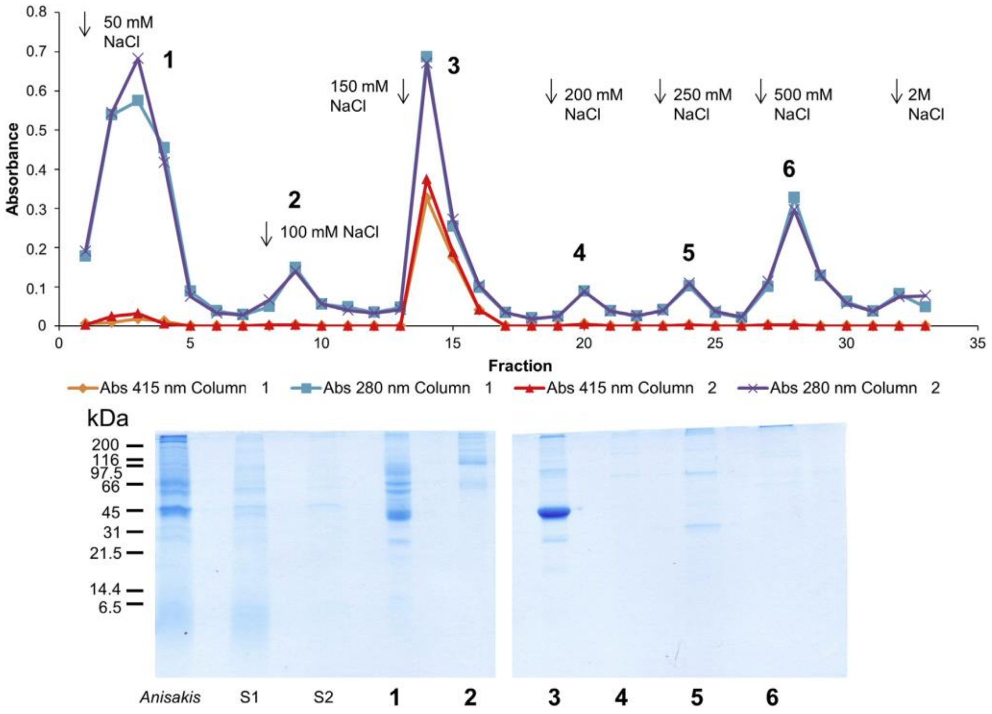

Fig. 4. Anionic exchange chromatography of the red pellet obtained from ultracentrifugation steps of *Anisakis* extract and SDS-PAGE. Absorbance of the different fractions was measured at 280 nm (protein) and at 415 nm (heme). Fractions eluted with 150 mM NaCl contained all the materials absorbing at 415 nm and showed a clear 37 kDa band matching with monomeric Ani s 13.

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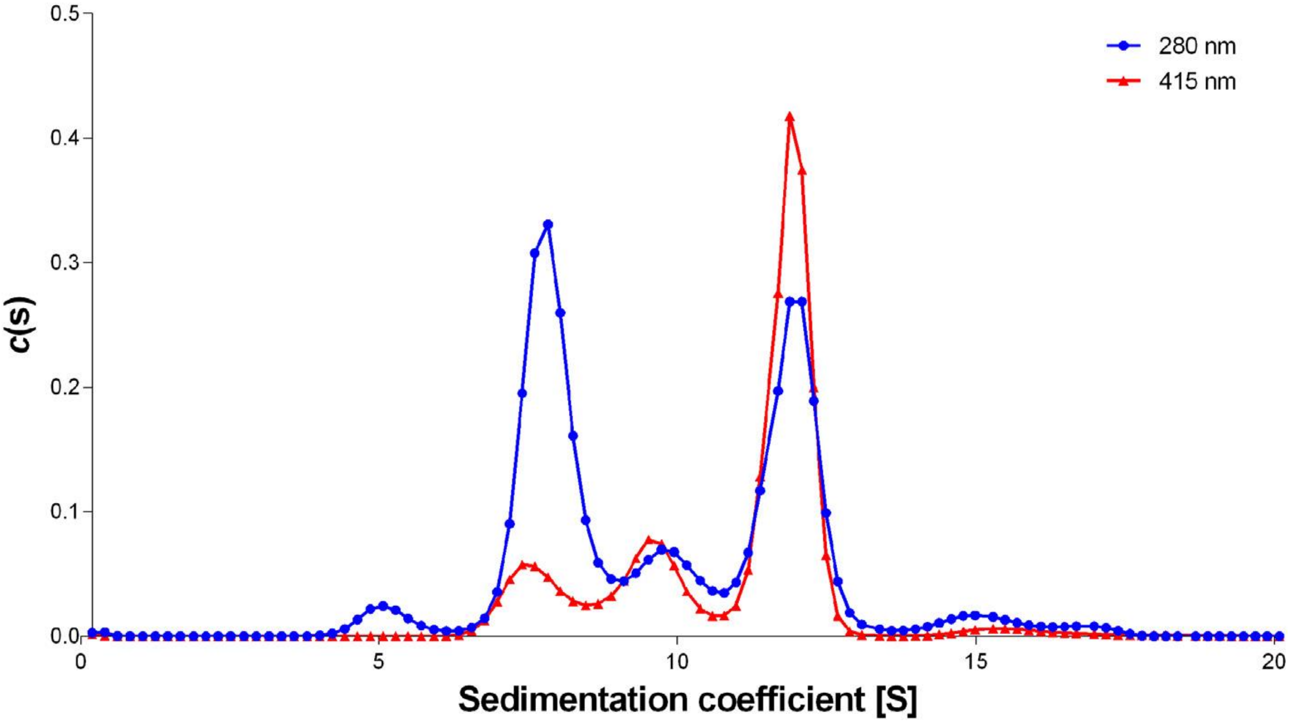

Fig. 5. Analytical ultracentrifugation analysis of *Anisakis simplex* haemoglobin (Ani s 13). Sedimentation coefficient distributions *c*(s) profiles of 0.5 mg/ml Ani s 13 obtained by sedimentation velocity at 170000 *g*. Absorbance was measured at 280 and 415 nm.

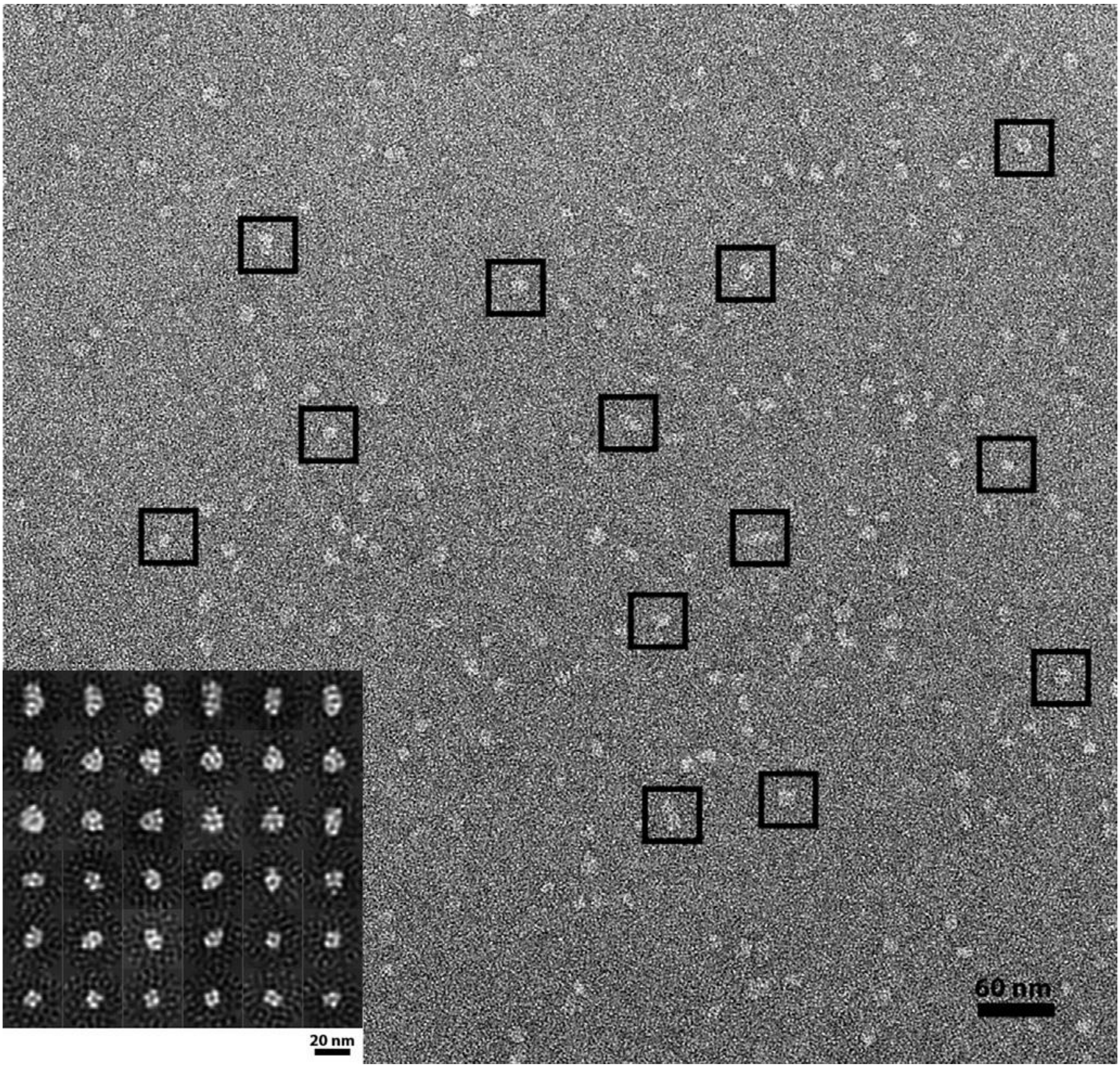

Fig. 6. Negative-stain electron microscopy of Ani s 13 homomers. Well-dispersed particles of Ani s 13 observed by negative staining. Pictures left bottom corner are class averages after classification and alignment in two dimensions.

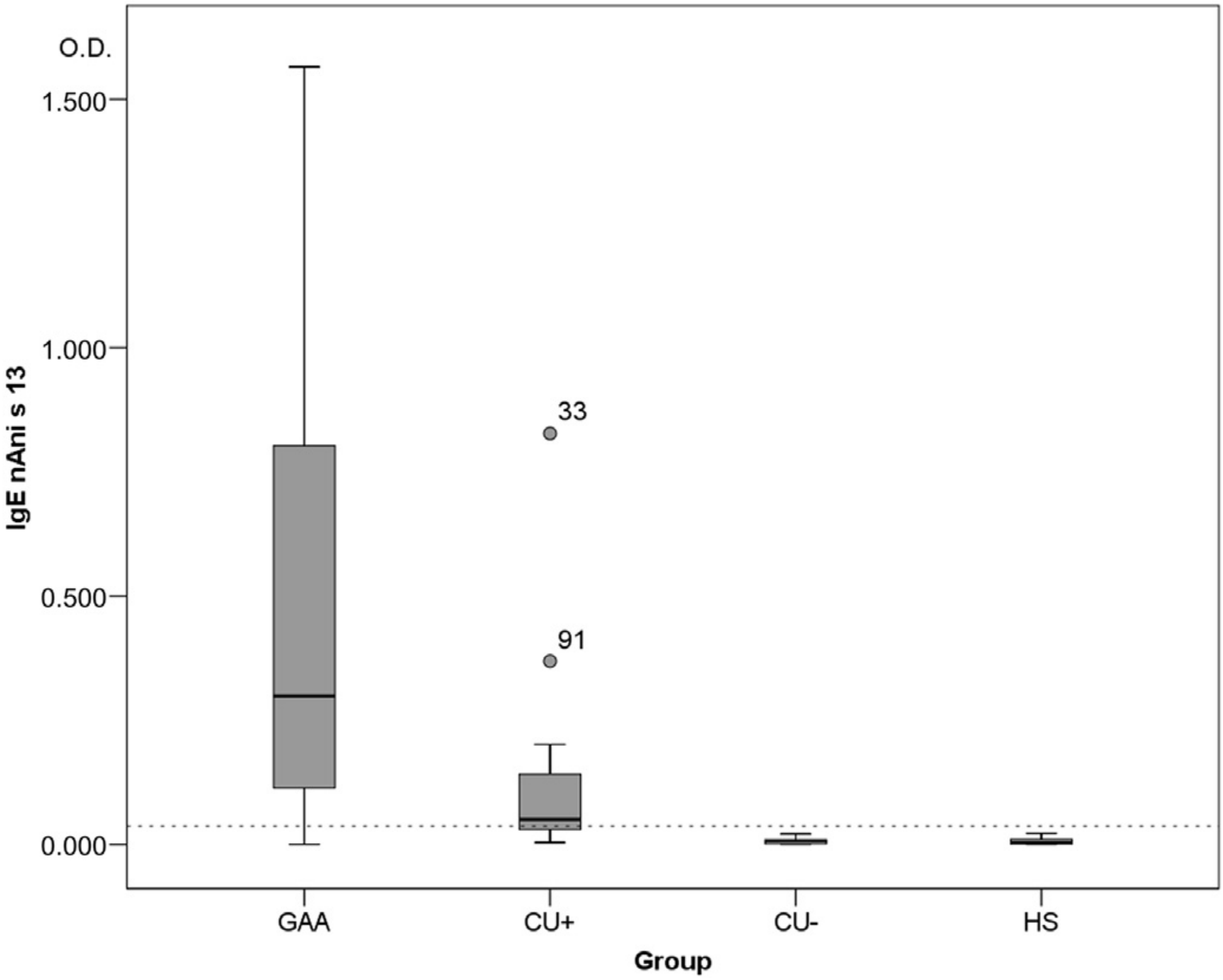

Fig. 7. Median and interquartile ranges of anti-*Anisakis* native haemoglobin (nAni s 13) IgE in patients with gastro-allergic anisakiasis (GAA) (*n*=20), *Anisakis* sensitisation associated chronic urticaria (CU+) (*n*=23), chronic urticaria without *Anisakis* sensitization (CU-) (*n*=22) and in healthy subjects (HS) (*n*=22). The positivity cutoff was indicated with a dashed line.

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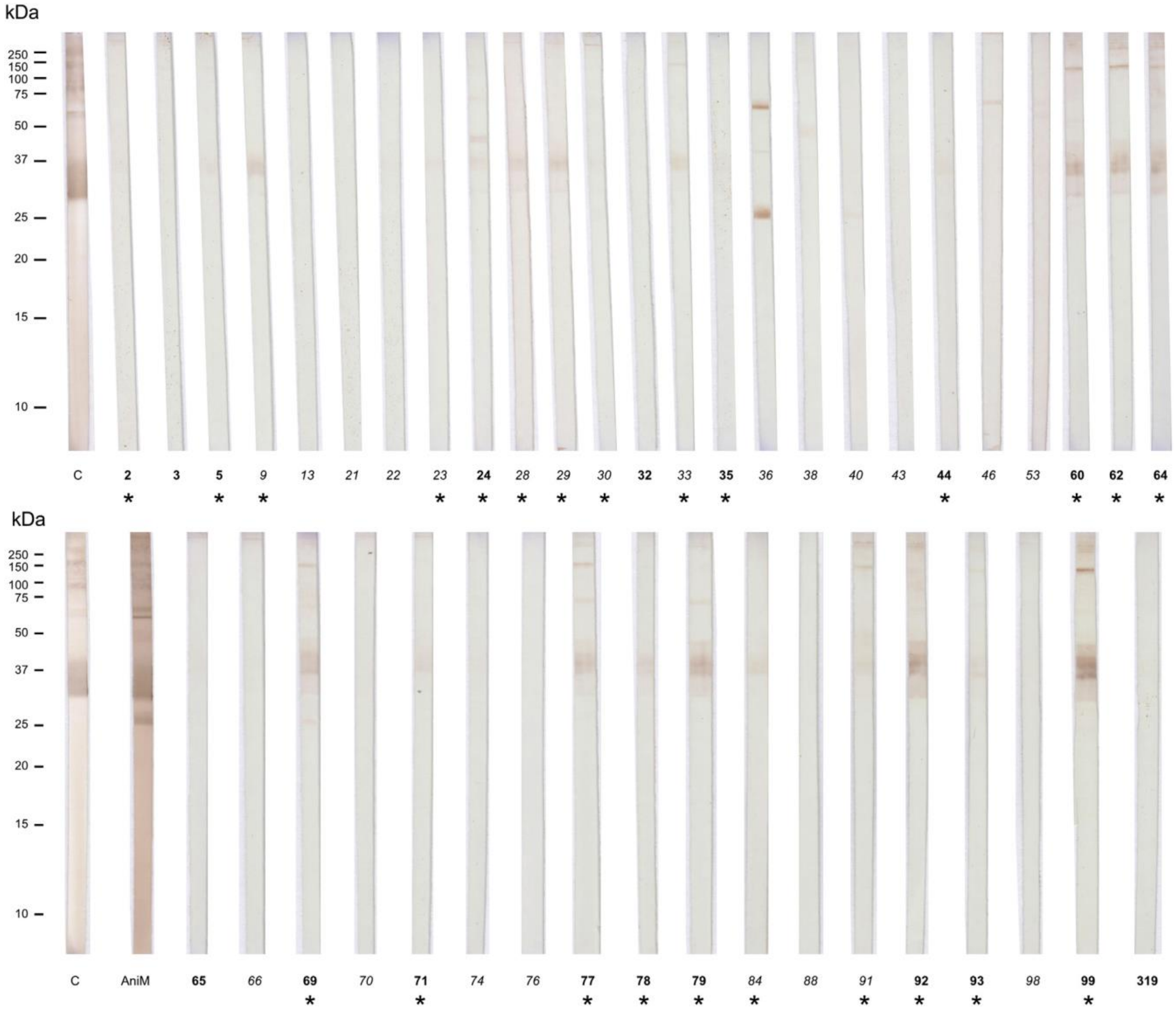

Fig. 8. Immunoblotting analysis of IgE binding to native *Anisakis* haemoglobin (nAni s 13) with sera from *Anisakis* sensitised individuals. C: rAni s 13 immunised mice serum IgG1. AniM: *Anisakis* crude extract immunised mice serum IgG1. *Denotes positive sera. Gastro-allergic anisakiasis patients are marked in bold type. Chronic urticaria patients are marked in italic type.

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Supplementary figure legends

Supplementary data related to this chapter can be found at http://dx.doi.org/10.1016/j.exppara.2017.08.010

Supplementary Fig. S1. Median and interquartile ranges of anti-*Anisakis* recombinant haemoglobin (rAni s 13) IgE in patients with gastro-allergic anisakiasis (GAA), *Anisakis* sensitisation associated chronic urticaria (CU+) (*n*=23), chronic urticaria without *Anisakis* sensitization (CU-) (*n*=21) and in healthy subjects (HS) (*n*=22). The positivity cutoff was indicated with a dashed line.

Supplementary Fig. S2. IgG1 inhibition immunoblotting. Serum from immunised mice with rAni s 13 was inhibited with rAni s 13 and supernatant was incubated with *Anisakis* crude extract blotted strip. I: Inhibited mice serum. N.I.: Non-inhibited mice serum.

Supplementary Fig. S3. Presence of Ani s 13 in different steps of the purification process. A: *Anisakis* extract before centrifuging, B: first supernatant (S1), C: chromatography peak 1, D: chromatography peak 3.

Supplementary Fig. S4. Periodate sensitivity of antibody-binding to purified native Ani s 13 (A), *Anisakis simplex* (B) and *Ascaris suum* (C) crude extracts. Lanes: 1: monoclonal antibody 4E8g against *Anisakis* haemoglobin (IgG1), 2: *Anisakis* haemoglobin positive human serum IgE, 3: rAni s 13 immunised mice serum IgG1, 4: *Anisakis* crude extract immunised mice serum IgG1. N: Non-oxidised. Ox: oxidised.