

Molecular Cloning of Paramyosin, a New Allergen of *Anisakis simplex*

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Key Words

Anisakis simplex · Paramyosin · Nematodes ·
Recombinant allergens · Anisakiasis

Abstract

Background: *Anisakis simplex* is a fish parasite that, when accidentally ingested by humans, may cause allergic reactions in sensitized individuals. The main objectives of our study were to: (1) construct a cDNA expression library of *A. simplex*; (2) identify clones producing specific IgE binding protein antigens, and (3) produce and purify the protein/s codified by the isolated clones produced in *Escherichia coli*. **Methods:** An expression cDNA library from the third stage larvae (L3) of *A. simplex* was constructed. This library was first screened with a rabbit anti *A. simplex* hyperimmune serum. The positive clones, identified using the rabbit serum, were re-screened with a pool of human sera containing high titers of IgE antibodies against *A. simplex*. **Results:** Two positive clones were isolated carrying the genes which codify for paramyosin. The paramyosin protein was produced in *E. coli* and purified. The partial sequence of a second paramyosin gene was also identified. The frequency of specific IgE binding to the recombinant and native forms of paramyosin using the sera of 26 *A. sim-*

plex-sensitive individuals was 23 and 88%, respectively. Both paramyosins were able to inhibit 11% of the specific IgE binding to a total extract. **Conclusions:** We describe the primary structure of a paramyosin of *A. simplex*. It can be considered as an allergen based on its IgE binding capacity. We suggest that the recombinant protein does not maintain the complete allergenic properties of the native paramyosin, considering its lower IgE binding capacity of the recombinant protein. However, both proteins have the same specific IgE inhibition capacity. The recombinant protein can be produced in large quantities in *E. coli*. We propose the term Ani s 2 for this allergen.

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Introduction

A great number of parasites can be present in fish, but only a few species are capable of infecting humans. The most important helminths acquired by humans when eating fish are the anisakid nematodes, particularly *Anisakis simplex* and *Pseudoterranova decipiens*, and some cestodes and digenetic trematodes [1]. The third larval stage (L3) of *A. simplex*, which occurs in fish and cephalopods, is the cause of the human-related illnesses. When in-

gested, *A. simplex* can cause several pathologies in humans, namely a parasitic disease (acute or chronic) named anisakiasis, and hypersensitivity reactions which take place after ingestion of parasited fish. In most cases, these pathologies occur when the fish is ingested raw, marinated or undercooked [2–5].

A. simplex has a worldwide distribution and has been isolated from many fish species [2]. It has been reported that patients who develop allergic symptoms (urticaria/angioedema and anaphylaxis) after the ingestion of fish are frequently sensitized to allergens of this nematode [2, 6–8]. Occupational conjunctivitis [9] and asthma [10], supposedly induced by *A. simplex*, have also been reported.

There is increasing evidence that only the ingestion of live larvae is responsible for symptoms associated with sensitivity to *A. simplex* [11–13]. The antigens, released by *A. simplex* during tissue penetration, may cause the allergic symptoms observed in gastroallergic anisakiasis, since *A. simplex*-sensitive patients tolerate the ingestion of deep-frozen seafood.

Several authors have investigated the allergenic composition of *A. simplex*. Del Pozo et al. [14] identified several IgE binding protein/peptides from total extracts of this fish parasite. Most sera recognized a group of 4 bands with molecular weights between 30 and 40 kD. Several bands of 14–30 kD and >45 kD were also recognized. Akao et al. [15] demonstrated that several antigens ranging from 50 to 120 kD are recognized by the sera of patients with gastric anisakiasis. Similar results have been obtained by Moneo et al. [16], who identified IgE binding bands in the molecular weight range from <17 kD to >65 kD. Several monoclonal antibodies have been produced against *A. simplex* proteins. These monoclonal antibodies recognize proteins of 48, 67, 139 and 154 kD [17]. A recent study by del Pozo et al. [18] suggested that a Th2 mechanism plays an important role in the inflammatory infiltrate produced by the anchorage of *A. simplex* to the gastrointestinal wall of patients suffering from a gastrointestinal infection. The allergens accountable for these Th2 responses have not been identified.

Cross-reactivity between allergens of *A. simplex* and of another fish parasite has been documented [19]. It has also been shown that the sera of *A. simplex*-sensitized individuals recognize allergenic proteins from *Ascaris lumbricoides*, *Ascaris suum*, *Toxocara canis*, *Blatella germanica* and *Chironomus* spp. [20, 21].

As far as we know, the sequence of two antigenic proteins of *A. simplex* has been published [22, 23]. One of these molecules, Ani s 1, has homology with troponin of

nematodes and was recognized by the IgE of 20% of *A. simplex*-sensitized individuals.

Methods

Collection of Larvae

Live *A. simplex* larvae were recovered with forceps from viscera of Couch's whittings (*Micromesistius poutassou*) as previously described [19]. The parasites were washed 4 times with 0.85% NaCl, frozen in liquid N₂ and stored at –80 °C until use.

Subjects and Serum Samples

The patient population consisted of 26 individuals who had experienced several allergic reactions (urticaria/angioedema, abdominal pain or anaphylaxis) after eating marinated fish. Patients had positive skin tests to an *A. simplex* extract (CBF LETI, SA, Madrid, Spain) and a positive specific IgE determination by CAP (Pharmacia & Upjohn, Sweden). The sera from 5 atopic individuals with high specific IgE levels to common aeroallergens and negative to *A. simplex* were used as controls.

Construction of the Anisakis simplex cDNA λ Zap Library

Total RNA and poly A⁺ RNA were isolated following the instructions of total and poly (A)⁺ RNA Quick mRNA isolation kit from Stratagene (Stratagene Cloning Systems, La Jolla, Calif., USA). The cDNA λ Zap library was made with the Zap-cDNA synthesis kit from Stratagene. The packaged library was plated on XL1 Blue *Escherichia coli* cells to produce a library containing 10⁵ recombinant clones.

Isolation of A. simplex cDNA Clones Coding for Allergens from a cDNA λ Zap Library, DNA Sequencing and Analysis

A preliminary screening directed to identify clones coding for immunogenic proteins was done using the serum of a rabbit immunized against a whole *A. simplex* extract. Immunoscreening was done following standard methods [24]. Once positive phages were isolated, the *E. coli* containing the λ zap-positive phages were coinfecting with a helper phage following the manufacturer's protocol. The coinfection resulted in the production of a plasmid containing the cDNA insert. The *E. coli* cultures bearing each plasmid were induced to overproduce proteins. Cellular lysates from these cultures were electrophoresed, transferred to nitrocellulose membranes and tested for IgE binding using a pool of strongly reacting human sera. Two clones were selected. Both strands of the DNA insert from the positive clones were sequenced by cycle sequencing and read in an ABI Prism DNA sequencer (PE Applied Biosystems, Calif., USA). The sequences obtained were compared with the sequences present in the GenBank using the BLAST program [25].

Subcloning and Purification of Recombinant Paramyosin

The DNA insert of one of the plasmids (clone 1) was 3,200 bp in length. The coding region was subcloned into the pMalc2 vector (New England Biolabs (NEB) Inc., Beverly, Mass., USA) to produce a genic fusion between maltose binding protein and paramyosin (MBP-paramyosin). This region was amplified by PCR [26] using Pfu polymerase (Stratagene) and the following oligonucleotides:

5' CATCAGGAATTCATGTCTGATACTCTCTACAGA 3' (sense)
5' TCGATCGAATTCGGCGTGTTATTCAAC 3' (antisense)

These oligonucleotides include EcoRI cutting sites (underlined) to allow the cloning of the amplified fragment in the EcoRI cut site of pMalc2. The cloning was done following standard procedures [22]. The resultant plasmid was called pMparal.

The MBP-paramyosin fusion was expressed in *E. coli* XL1 Blue cells bearing the pMparal clone. Bacteria were grown at 37°C in Luria broth (LB) to 0.6 OD₅₉₅ units. Isopropyl-1-thio-β-galactopyranoside (IPTG) was added to the culture (1 mM) to induce the expression of the fusion protein. After 2.5 h of induction, the culture was harvested. The cellular pellet was resuspended in 20 mM Tris HCl 200 mM NaCl 1 mM EDTA, pH 7.4. After three cycles of freezing and thawing, the cells were sonicated under mild conditions. The MBP-paramyosin protein was purified using amylose resin following NEB recommendations. The fusion protein was cut with the Xa factor to produce a protein with 4 extra amino acids at the NH₂ extreme (Ile-Ser-Glu-Ser). An equal volume of 1 M NaH₂PO₄ was added to the mix containing both molecules to isolate the paramyosin from MBP. After 1 h of gentle agitation, the mix was centrifuged for 15 min at 12,000 g. The supernatant containing the MBP portion was discarded. The pellet was resuspended in 10 mM phosphate buffer, pH 8. After 1 h of incubation the sample was centrifuged for 15 min at 12,000 g. This protocol was performed twice to obtain the soluble r-paramyosin.

Purification of Native Paramyosin

The procedure followed for the purification of the protein was a modification of the method described by Laclette et al. [27]. Soluble extracts of *A. simplex* L3 were obtained by homogenization in a solution containing 0.05 M NaCl and 10 mM phosphate, pH 7.2. The homogenate was centrifuged at 12,000 g for 15 min and the pellet was extracted overnight in the same buffer containing 0.9 M NaCl. The extract was clarified by centrifugation, brought to 0.4 M NaCl and loaded onto a DEAE-Sepharose column. To lower the pH of the unbound fraction, which contained paramyosin, 0.15 vol of 1 M NaH₂PO₄ was added. After 30 min of incubation the mix was centrifuged at 12,000 g for 15 min. The supernatant was discarded and the pellet was resuspended in 10 mM phosphate, pH 7.2. The sample was loaded again on a DEAE-Sepharose column equilibrated with 10 mM NaH₂PO₄/Na₂HPO₄, 0.05 M NaCl, pH 7.2. The column was washed with 3 vol of 0.16 M NaCl, pH 7.2. The native paramyosin (n-paramyosin) was eluted with 0.4 M NaCl.

Specific IgE Binding to Both Paramyosins

The prevalence of specific IgE antibodies against n- and r-paramyosin was determined by ELISA following standard procedures [19]. Microtiter plates (Immulon, Chantilly, France) were coated with 500 ng of either one of the paramyosins using carbonate/bicarbonate buffer at pH 9.2. The protein concentration was estimated by densitometry [28]. The sera were used at a 1:5 dilution. The assays were done in duplicate; 1 µg/ml of mouse monoclonal anti-human IgE was added to each well (Ingenasa, Madrid, Spain). A result was considered positive when a serum bound more than the mean plus 5 standard deviations of the negative controls.

Inhibition of Specific IgE Binding to a Total *A. simplex* Extract

Variable quantities of both paramyosins and of *A. simplex* extract were used to inhibit specific IgE binding to a total extract of *A. simplex* [19].

SDS-PAGE and Immunoblotting

SDS-PAGE was performed as described by Laemmli [29]. The separated proteins were electrotransferred onto a nitrocellulose membrane (Millipore Corp., Bedford, Mass., USA) and visualized by chemiluminescence (ECL, Amersham International, UK) after incubation with the appropriate antibody.

Purification of Rabbit Monospecific Antibodies

Rabbit monospecific antibodies against MBP-paramyosin were purified following the method of Weinberger et al. [30]. The fusion protein was fixed to a nitrocellulose filter and incubated overnight with the anti-*A. simplex* rabbit serum diluted 1:100 in PBS, 5% non-fatty milk 0.1% Tween-20. After washing the filter in PBS 0.1% Tween-20, the antibodies were eluted with 5 mM glycine-HCl (pH 2.3). Then, the pH was adjusted to 7.5 with 1 M Tris pH 7.

Results

Sequence and Homologies of *A. simplex* Paramyosin

Figure 1 shows the 3,200-bp-long nucleotide sequence of the complete paramyosin gene (clone 1, GenBank accession No. AF173004). This fragment includes a 5' non-coding region of 251 bp, a 3' untranslated region of 342 bp and an open reading frame (ORF) of 2,607 bp. The translation of this ORF originates from an 869-amino-acid-long protein (fig. 1) with a predicted molecular weight of 100 kD and a pI of 5.21. The sequence has a potential Asn glycosylation site (NLTA) at position 649 and several sites of potential phosphorylation. The analysis of the sequence by the nearest-neighbor method [31] indicates that, with the exception of the amino- and carboxyl-terminals, the rest of the molecule adopts a hypothetical α helical coil similar to that postulated for the paramyosin of the nematode *Caenorhabditis elegans* [32]. Amino acid sequence similarity searches revealed that the complete clone of *A. simplex* paramyosin has an 89% identity with the paramyosin of the filaria *Onchocerca volvulus* [33, 34] (Swiss-Prot Q02171) and 87% with the paramyosin of the free-living nematode *C. elegans* (Swiss-Prot P10567) [32]. The identity with other paramyosins decreases outside of the phylum Nematoda. In an arthropod, such as *Drosophila melanogaster*, the identity is 48% (Swiss-Prot P35415) [35]. In a trematode, such as *Schistosoma japonicum* (Swiss-Prot Q05870), the identity is 33% [36, 37]. The multiple alignment of the above-mentioned proteins is shown in figure 2. It may be observed that the amino- and carboxy-terminal ends of the molecule are the less conserved regions. It is interesting to notice that all of these nematode paramyosins maintain a potential Asn glycosylation site in the same region.

CGGGCCCGCCCGGATCGTGTGTGCTGTTGTTGGCCGCTAGATTTCGTTAGCAGTAATCGTGTGTT 60
ATTATTAAAGAGCCAGCAGTAGCTGCTGCAAGATAAACATTGCGCTTGCAGACTTATCCGCGCAC 120
TAGTGTTCAGTCAACCACTATTATTCGCGAATACTAATCTTCTTCTTCTTCTTCTTCTTCTTCTT 180
TCATGCCAGACGAGCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTT 240
ATTCAATCGAATAAATAATGTTCTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCT 300
T S D L S M I
AGCACTGCTGATATGGGCGCTTTAACGTCAATGAGTGTAGCCGATTGGGGAAGTTTAAACA 360
S T A D M O A L T S M L T S V A D L S V A D L S V A D L S V A D L S V A D L S V 420
CGATTGGAGGATAAGATTGCGCTGCTGCAAGATGACTTCGAATCAGAACCGCAACTTCGA 480
R L E D K I R L L Q D D F E L R L L Q D D F E L R L L Q D D F E L R L L Q D D F 540
AATCGTATTGAACGTGAACGTGCTGATTGAGCGTTGAGTGTGATCGCTCTCACTGATCGT 600
H R I E S E V L A D L S V L Q D D F E L R L L Q D D F E L R L L Q D D F E L R L L 660
TTACAGGATGCTGAATGCTGCGAATGCTGAATGCTGAATGCTGAATGCTGAATGCTGAATGCTGA 720
L Q D A E C A A T D S Q I E S R R K R E V L Q D A E C A A T D S Q I E S R R K R 780
GAGCTGTCAAACTACGTAAACTGCTGGAAGAAATCACAGTTGGAAAATGAAGATGCCATG 840
E L S K L R K L L E S S Q L E N G S D A M M 900
AATGTGTGTCGTAAGAAAATCAAGATGTTTGTCTTGACTACACCGCAAAATTTGAACAA 960
N V L R K K K H Q D V C L D Y T E Q I E S R R K R E V L Q D A E C A A T D S Q I 1020
CTCCAGAAAGAACTCGAAGATCGATCGTGAACGACAACTGCTTCAAGATGAAGTGAAT 1080
L Q R K M S K I D R L S V L Q D D F E L R L L Q D D F E L R L L Q D D F E L R L L 1140
GAACATAACGCGCCACATTTGATCAATCTCAAAAGGACAACTGCTGAGAAAATGCGC 1200
E L T A T I D Q L Q K D H V A D K M A 1260
CAAAAAATTCGAACCAAAACCATTGAGTTGTCTGAATAAAGTGGAAAGACTTGAACAAACAT 1320
Q K F E Q Q T I E L S D N K V E D L S H H 1380
GTGAACGATTGCGCAACCAACGCAACCGTCTCCAAAGCCGAGAACGATATTGCTGGCT 1440
V N D L A Q Q R Q L L Q D A E C A A T D S Q I E S R R K R E V L Q D A E C A A T 1500
GAAATTCACGATCAAAAGGTACAACTCGAAGATGCTGCAACATGTCAAAATATCAGTTGGCA 1560
H I Q D Q V Q L E S V A L H E E V E D L 1620
CAACAAATGGAAAGATCTCGTCTGTTAGAAAGATGCTGAACGTTGAAGATGCTGCAAAATG 1680
Q Q L E S R R R L L E D A E R R S Q Q M 1740
CAAGCAAACTTCATCAGGTACAACTTGAGCTTGATTGAGTTCGTTGCTGCTAGATGAA 1800
Q A Q L H Q V Q L L D S V R V A L D E 1860
GAAAGTGCAGCGTGTGTAAGCCGAAACACAAAGTTATCGTTGGCAACCGGAAATCACA 1920
E S A A R V E H K L L S L A M T E I T 1980
CAATGGAGAGTAAATTCATGCTGAAGTGGCTTTACATCAGGAAGATTTGAAGTGAATTTA 2040
Q M K F D A E V A L E S V A L H E E V E D L 2100
CGCAAGAGATGATGTCAGAAACAACTGAAATATGAAGAACAACTGAAATATGCTCCAA 2160
R K K M M O K Q A E Y L E I M L L Q 2220
AAGGTATCACAGCTCGAAAAGCCAAAGCTCGTCTACAAAGTGAAGTTGAAGTTCTCTATC 2280
K V S Q L E K A K A R L L Q D D F E L R L L Q D D F E L R L L Q D D F E L R L L 2340
GTTGATTGTAAGAAAGCTCAGAAATCAATCGCAATTTTGGAAACGTTGCTTGAAGAACAACT 2400
V D L K E T I A E I L E S Q L E S V A L H E E V E D L 2460
GAGAACCAAGTGCCTGAGATGAGTCTGAAGTGGCTTTACATCAGGAAGATTTGAAGTGAAT 2520
H K Q V L E M K R I D E L V L E S V A L H E E V E D L 2580
GCACAACTGTAAGCCGCGCAGCACTTCCGGAACCTCAGAAAGTGAAGATGCTGCAAAATG 2640
A Q R E A R A L A E L Q K M K Q L Y E 2700
AAAGCCGTGTAACAAAAGAAAGCTTGCGACGTGAGAACAAAGAACTTCAAGATGACCTTA 2760
K A V E Q K E A L A R E N K K L Q D D F E L R L L Q D D F E L R L L Q D D F E L 2820
CATGAAGCTAACGAGCTCTTGCAAGATGCTAACCGCAAAATGTCACGAACTGACCTTGAG 2880
H E N E T I A E I L E S Q L E S V A L H E E V E D L 2940
AATGCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 3000
N A R L A E I R D L Q V S H A 3060
GCAAGACGTGATGCCGAAGCTCGAGCTCAACGCGCTCTCTGCTGAGCTACAAACAACTCCGT 3120
A R R D A E A R A Q R A L A E L Q K M K Q L Y E 3180
ATTGAGATGGAACGCTGTTTGCAGAGAAAGAAAGAAATGGAAGCCCTGCGTAAATCA 3240
I E M E R R L Q E K E L E S V A L H E E V E D L 3300
ATGCAATTCGAAATGATCGTCTTACCGCAGCACTTGTCTGATGCTGAAGCAAGTGAAG 3360
M Q I D R L S V L Q D D F E L R L L Q D D F E L R L L Q D D F E L R L L Q D D F E 3420
GCCGAAATTCGACGCTCTTGCAGAAAGATATCAACCGGAGATGCTGCAAAATGGAAGTGA 3480
A E I A R L R K K Y Q A M I A E L E M T 3540
GTTGATAACTGAATCGTGCAAACTTGAAGCCGAGAAAGCAATCAAGAAACAGAGCGAA 3600
V D N L N R A N L E A Q K T I K K Q S E 3660
CAAATCATTCAATGCAAGCCAACTCTCGAAGATACGCAACGCCAATTTGAGCAGACTCTC 3720
Q I I Q L Q A M L E D T Q R Q L Q Q T L 3780
GATCAGTACGCACTGCGCAACCGTAAGATTTCGGCGTTGAGCGCTGAAATTTGAGGAAATGC 3840
D Q Y A L A Q R R I S A L S A E L E C 3900
AAGACCCGATTGGACAACCGGATCGTGCACGCAAGCAAGCCGAAGCTGATCTTGAAGAG 3960
K T A L D N A I R A R K D Q A L L Q D D F E L R L L Q D D F E L R L L Q D D F E 4020
GCACATGTACGCATCTCGGACTTGCATCGATCAACAGCAACTTGACTTCAATCAAGAAT 4080
A H V R I S D L T S I N S N L T A I K N 4140
AACTCGAAACCGAGCTATCGACGGCAACAGCAGATTGTTGACGAGGTCACAAAGAGTTG 4200
E L E T E L A Q A D E V L D E V T K E L D 4260
CATGCACGAGATGAAGTGCAGAACTGCGCATTAACAGATGCGGCAAGTGCAGTGAAGAG 4320
H A A D E T A N D A D A V Q E 4380
TTGCACGAGGAACAGAGCACTCAATGAAGATCGATGCTCTTCTGTAAGATCTTCAAGAG 4440
L H E B E Q E H S M K I D A L R K S L E B E 4500
CAAGTGAACAACTTCAAGTACAAATTCAGAAAGCTGAAGCAGCGGCACTTCGGCGGT 4560
Q V K T C Q V Q I Q E A E A A L L Q G 4620
AAACGCTTTATCGCTAAGCTCGAAACACGCACTTGTGACTGGAAGTGGCATTTGGACGAG 4680
K R I A K L A E C R I R D L E V A L D R 4740
GAGCTCGACGACACAAAGLACACAGAGCGCATTTGCGCAGAAAGATCGTCTGATCAAG 4800
T E R H E T S A L R E R I K 4860
GAAGTGCATAATGACATCGATGAAGAGCACAAGATGTTCTGATGAGCAACAGATACCGCT 4920
E V Q M Q I D E H K M Q F V M A Q D T A 4980
GATCGTATGCTCGAGAAAGTTGAATATCCAGAAACGTCAATTTGGGTGAAGCCGAAGCGATG 5040
D R M L L Q R V R Y Q R E L E D A E 5100
ACCATGCAAAATCTGCAACGAGTTGCGCGATACCAACGTTGAGTTGGAAGGATGCCGAAGGT 5160
T T A C C A T T T G C T T T T T T T T T T T T T T T T T T T T T T T T T T T T T T T T 5220
CGTGGGATCAAGCCGAAAGTTGTTGAAATTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG 5280
R A D Q A E S S L N L I R A K H R Q T V 5340
CGGGTTGTAAGCTACCGATGTTTATGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG 5400
A V G K A T D V Y V E B D + 5460
T A A C A C C C G A A T A T G A T C G A T T T T T T T T T T T T T T T T T T T T T T 5520
ACACAAAAATTTGTTCTAATCGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG 5580
TTTACCATCTTTGTTTTCGATTACCATTTTTCAGTTTTCAGTTTTCAGTTTTCAGTTTTCAG 5640
TTAAGCCCTAAGTTTGTGCTTATTCGATTTCATCTTATTCGATTTCGATTTCGATTTCGATT 5700
TGACTGTGAATTTATTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG 5760
GATATTGAATCTGC 5820

Fig. 1. Nucleotide and deduced amino acid sequence (in bold) of *A. simplex* r-paramyosin, clone 1. The start and stop codons are shown in bold and underlined. The putative Asn glycosylation (NLTA) site is underlined. The DNA sequences indicated in bold show the oligonucleotides matching regions used for PCR. The DNA sequence is available from the GenBank under accession No. AF173004.

The partial nucleotide and amino acid sequence of the cDNA of the second form of the isolated paramyosin is shown in figure 3 (GenBank accession No. AF208981). The sequence is 2,076 bp in length. It has an ORF of 473 amino acids. This sequence also has a potential Asn glyco-

sylation site (NLTT) at amino acid 249 and several phosphorylation sites. The amino acid identity between the two *A. simplex* paramyosins is 86%. In the coding region the identity at the nucleotide level is 75%. Both genes are highly divergent in the non-coding region.

As1	1	M-----SDTLYRSPSMAIRSSSTADMGALTSMVSADLGSLTRLEDKIRL
Ov	1	M-----SGSLYRSPSAAALYKSPSMS--AFSCLPAAFGSMVSADLGSLTRLEDKIRL
Ce	1	MERLESLEPLELMERMELQELLIVSLKSPSQAAFGAPFGSMVSADLGSLTRLEDKIRL
Dm	1	M-----SSSQAVRSKSYRATSTG-PGTADVNIET-----IQDLSSLSRLLEDKIRL
Sj	1	M-----MNH-DTESHVKISRTIYRGVSPS-----TTLLESVRKE
As1	44	LQDDFESERELNRIERERADLSVQLIALTDRLQDAECATDSQIESNRKREVELSKLRKL
Ov	50	LQEDLESERELNRIERERADLSVQLIALTDRLQDAEGTTDSQIESNRKREVELSKLRKL
Ce	61	LQEDLESERELNRIERERADLSVQLIALTDRLQDAEGTTDSQIESNRKREVELSKLRKL
Dm	47	LQDVLVERELRQIERERADLSVQVIQMSERLEEAEGGAHQFQIEANRKRDAEALLKLRKL
Sj	34	LEDILDLERDARVRAERHAADLSYQVDALSERLDEAGGSTTQTELLKRRMEINKLRKD
As1	104	LEESQLENEDAMNVLRRKHQDVCCLDYTEQIEQLQKKNSKIDRERQRLQHEVIELTATIDQ
Ov	110	LEESQLENEDAMNVLRRKHQDACLDDYTEQIEQLQKKNSKIDRERQRLQHEVIELTAAIDQ
Ce	121	LEESQLESEDAMNVLRRKHQDSCLDYQDQIEQLQKKNAKIDRERQRLQHEVIELTATIDQ
Dm	107	LEDVHLESEETLLKKKHNEIITDFQEQVEILTNNKARAEDKAKFQTEVYVLLSQIES
Sj	94	LENANASLELAETSMRRRHQTALNELSLEVENLQKQKGAEDKSHLIMEVDNLVGLQDQ
As1	164	LQKDKHVAEKMAQKFEQQTIELSNKVEDLNKHVNDLAQQRQRLQAENSDDLAEIHDKQVK
Ov	170	LQKDKHLAEKAAERFPAQTIELSNKVEDLNKHVNDLAQQRQRLQAENSDDLAEIHDKQVK
Ce	181	LQKDKHTAEKAAERFPAQANELANKVEDLNKHVNDLAQQRQRLQAENSDDLAEIHDKQVK
Dm	167	YNKEKIVSEKHISKLEVISISELNVKIEELNRTVIDISSHRSLSQENIELTKDVQDLKVK
Sj	154	ALKAKQSAESKLEGLDLSQLNRLKTLTDDLQRLTELNNAKSLTSENFEHLHINQDVEAQ
As1	224	LDNLQHVKYQLAQQLLEESRRRLLEDAERERSQMQAQLHQVQLELDSVRVALDEESAARVEA
Ov	230	LDNLQHVKYQLAQQLLEESRRRLLEDAERERSQMQAQLHQVQLELDSVRVALDEESAARVEA
Ce	241	LDNLQHVKYQLAQQLLEESRRRLLEDAERERSQMQAQLHQVQLELDSVRVALDEESAARVEA
Dm	227	LDTVSFSKQVLSQLEDAARRLEDEDRRRLSLESSLHQVEILDVSRNQLEESARIDOL
Sj	214	ILNYSKAKSSLESQVDDLRSLDDESRRNRLNQAQLTSLQMDVDNLQAKYDEESEASNL
As1	284	EHLKSLANTEITQWKSKEFAEVALHHEEVEDLRKKMMQKQAEYEEQIEIMLQKVSQLEKA
Ov	290	EHLKSLANTEITQWKSKEFAEVALHHEEVEDLRKKMMQKQAEYEEQIEIMLQKVSQLEKA
Ce	301	EHLKSLANTEITQWKSKEFAEVALHHEEVEDLRKKMMQKQAEYEEQIEIMLQKVSQLEKA
Dm	287	ERQLVKANADATSWQNKWNSEVAARAEVEEIRKRYQVRIITELESHIESLTVKVNLEKM
Sj	274	RNQVSKFNADIAALKSKFERELMSKTEFEEMKRKLTMRIITELEDVAERERLKAVALLEKL
As1	344	KARLQSEVEVLIVDLKAKQNTIAILERAKEQLEKQVLEMKSRIDELVLEEAQAQREARAA
Ov	350	KARLQSEVEVLIVDLKAKQNTIAILERAKEQLEKQVLEMKSRIDELVLEEAQAQREARAA
Ce	361	KARLQSEVEVLIVDLKAKQNTIAILERAKEQLEKQVLEMKSRIDELVLEEAQAQREARAA
Dm	347	KTRLASEVEVLIVDLKAKQNTIAILERAKEQLEKQVLEMKSRIDELVLEEAQAQREARAA
Sj	334	KTKLTLEIKDLQSEIESLSLENGELIRRAKSAESLASELQRRVDELTEIVNTLTSSQNNQL
As1	404	LAEQLKMKQLYEKAVEQKEALARENKKLQDDLHEANEALADANRKLHELDLENARLAGEI
Ov	410	LAEQLKMKQLYEKAVEQKEALARENKKLQDDLHEANEALADANRKLHELDLENARLAGEI
Ce	421	LAEQLKMKQLYEKAVEQKEALARENKKLQDDLHEANEALADANRKLHELDLENARLAGEI
Dm	407	HADLVRTVHELQKVDNNQLTRENKKLQDDLHEANEALADANRKLHELDLENARLAGEI
Sj	394	ESENMRLLKSLVNDLTDKNNALARENQMNQVKEKSSLRDANRRRLTDLEALRSQLEAER
As1	464	RDLQVALKESEAAARRDAEARAQALAEQLQVRIEMERRLQEKKEEEMALRKSMMQFEIDRL
Ov	470	RELQALKESEAAARRDAEARAQALAEQLQVRIEMERRLQEKKEEEMALRKSMMQFEIDRL
Ce	481	RELQALKESEAAARRDAEARAQALAEQLQVRIEMERRLQEKKEEEMALRKSMMQFEIDRL
Dm	467	DELTAAYKEAEAGRAEAGQGRQALADFNQYRHAERRLAEKDEEIEAIRKQTSIEIEQL
Sj	454	DNLASALHDAEALRDMDQKYQASQAALNHLKSEMEQRLRDEELESRRKSTTRTIEEL
As1	524	TAALADAEARMKAEIARLRKKYQAEIAELEMTVDNLNRRANLEAQKTIKKQSEQIIQLQAN
Ov	530	TAALADAEARMKAEIARLRKKYQAEIAELEMTVDNLNRRANLEAQKTIKKQSEQIIQLQAN
Ce	541	IAALADAEARMKSEISRLKKKYQAEIAELEMTVDNLNRRANLEAQKTIKKQSEQIIQLQAN
Dm	527	NARVIEAEATRLKTEVTRIKKKLQIQITELEMSLDVANKTNIDLQKVKKKQLQLTELQAH
Sj	514	TVTITHEVVKYKSLKKRYESSIADLEIQLDATNKANANLMEKNANLADLELRQEQENY
As1	584	LEDTRQLQQLTLDQYALARKKISALSAALEECKTALDANIRARKQAEADLEEAHVRIIDL
Ov	590	LEDTRQLQQLTLDQYALARKKISALSAALEECKTALDANIRARKQAEADLEEAHVRIIDL
Ce	601	LEDTRQLQQLTLDQYALARKKISALSAALEECKTALDANIRARKQAEADLEEAHVRIIDL
Dm	587	YEDVQRLQATLDQYALARKKISALSAALEECKTALDANIRARKQAEADLEEAHVRIIDL
Sj	574	LDDERRLRSAEENNLQITEHKRIQLANEVEELRSAMENLERLKKHAELEESTQSRVSEL
As1	644	TSINSNLTATIKNKLETELSTAQADLDEVTKELHAADERANRALADAARAVQELHHEEQEHS
Ov	650	VSINNLTATIKNKLETELSTAQADLDEVTKELHAADERANRALADAARAVQELHHEEQEHS
Ce	661	ISINNLTATIKNKLETELSTAQADLDEVTKELHAADERANRALADAARAVQELHHEEQEHS
Dm	647	TTANVSLVSIKSKLEQELSVVASDYEVSKELEISDERYQKQVQELKHVVEQVHHEEQERI
Sj	634	TIQVNTLSNDKRRLEGGVMDMDDAINAKQAEDRATRLNNEVRLALADELRQEQENY
As1	704	MKIDALRKSLSEEVKQLQVQIQEAEAAALLGGKRVIAKLETRIRDLEVALDEETRRHKET
Ov	710	MKIDALRKSLSEEVKQLQVQIQEAEAAALLGGKRVIAKLETRIRDLEVALDEETRRHKET
Ce	721	MKIDALRKSLSEEVKQLQVQIQEAEAAALLGGKRVIAKLETRIRDLEVALDEETRRHKET
Dm	707	VKLETIKKSLSEEVKQLQVQIQEAEAAALLGGKRVIAKLETRIRDLEVALDEETRRHKET
Sj	694	KHAEALRKQLEIEIREITYKLEAEAFATREGRRMVQKLQARVRELEAEFPGESRCKDA
As1	764	QSALRKDDRIKEVQMQIDEEHKMFVMAQDTADRLLEKLNIOKRLQGEAEAMTMQNLQRV
Ov	770	QNALRKDDRIKEVQMQIDEEHKMFVMAQDTADRLLEKLNIOKRLQGEAEAMTMQNLQRV
Ce	781	QNALRKDDRIKEVQMQIDEEHKMFVMAQDTADRLLEKLNIOKRLQGEAEAMTMQNLQRV
Dm	767	IKILRKKEKRTYKEVLVQCEEDQKNLILLQDALDKSTAKINIYRRLQSEQEGVSQQTTRV
Sj	754	LAQARKFERQYKELQTAEDDRRMVLELQDLDDKTMQMKAYKRLQLEMEEVVSQITMKNY
As1	824	RRYQRELEDAEGRADQAESSLNLIIRAKHRRG-TVAVGKATD---VYVVEED----
Ov	830	RRYQRELEDAEGRADQAESSLNLIIRAKHRRG-TVAVGKATD---VYVVEED----
Ce	841	RRYQRELEDAEGRADQAESSLNLIIRAKHRRG-SVVTGKSSS---KVF-----
Dm	827	RRYQRELEDAEGRADQAESSLNLIIRAKHRRG-FVTTSTVPGSQVYIQTETTTITE
Sj	814	RKAQQQIEAEHRAHMAERTVTVRRVGPGRVAVSARELSVTSNRRGMRATSM--

Fig. 2. Comparison of *A. simplex* paramyosin, clone 1, with different paramyosins: *O. volvulus* (Swiss-Prot Q02171), *C. elegans* (Swiss-Prot P10567), *D. melanogaster* (Swiss-Prot P35415) and *S. japonicum* (Swiss-Prot Q05870). The letters in bold mean identical amino acids. The Asn glycosylation site is boxed and shaded.

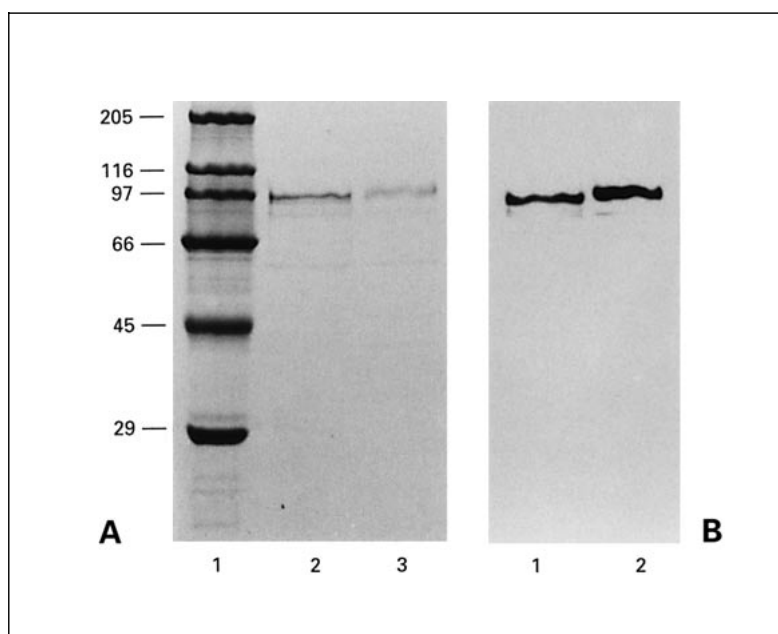


Fig. 5. SDS-PAGE (10% acrylamide) and Western blots of r- and n-paramyosins. **A** Lanes: (1) MW markers in kD; (2) r-paramyosin; (3) n-paramyosin. **B** Western blot of r- and n-paramyosin. Lanes: (1) r-paramyosin; (2) n-paramyosin.

factor and selective acid precipitation, the soluble r-paramyosin was obtained (lane 9). The yield of r-paramyosin was 13 mg/l of culture.

Purification of Native Paramyosin

The n-paramyosin was electrophoresed in an SDS-PAGE (10% acrylamide) in parallel with the r-paramyosin (fig. 5A). Both paramyosins showed the same electrophoretic mobility. The yield of the n-paramyosin was 0.2 mg/g of nematodes. The nature of the purified n-paramyosin was confirmed by Western blot using antibodies against r-paramyosin (fig. 5B).

IgE Binding to Recombinant and Native Paramyosin

The number of human sera containing specific IgE antibodies that recognize the n- and r-paramyosin was determined by ELISA. Values higher than the mean of the control sera plus 5 σ_n were considered positive. The data show that 6 of the 26 sera tested (23%) can be considered as positives to the recombinant protein and 23 (88%) were positive to the native protein (fig. 6A, B).

Inhibition of Specific IgE Binding to a Total A. simplex Extract

At an inhibitor concentration of 2.5 μ g of protein/well, the *A. simplex* extract inhibited 84% of the specific IgE binding to the total extract, while both paramyosins (native and recombinant) were only able to inhibit 11%.

Discussion

Paramyosins are highly conserved proteins found in the muscle of invertebrates. It has been suggested that paramyosin has an α helical-coiled coil structure as indicated for *C. elegans* [32] and that it behaves as a strong immunogen in *Schistosoma*, *Dirofilaria*, *Onchocerca* and *Taenia* infections [38–42]. It has been shown that paramyosin protects mice from infection with *Schistosoma* [43, 44]; its use as a potential vaccine candidate has been postulated in murine experimental models [38, 39, 43, 44]. In the present paper we describe the cloning of two *A. simplex* genes which codify allergens with sequence homology with other nematode paramyosins. The complete coding sequence of one of these genes was obtained. The analysis of the amino acid and nucleotide sequences revealed a high degree of homology with filarial paramyosins. The *A. simplex* paramyosin shows 89% identity at the amino acid level with *O. volvulus* paramyosin [33, 34]. The lower sequence identity of *A. simplex* paramyosin with that of free-living soil nematodes such as *C. elegans* is in agreement with the close evolutionary relationship between *A. simplex* (Ascaridida) and *O. volvulus* (Spirurida) which is higher than with *C. elegans* (Rhabditida) [45].

To date, only one gene copy of paramyosin has been found in nematodes, although the existence of two isoforms of paramyosin has been proposed in *C. elegans*.

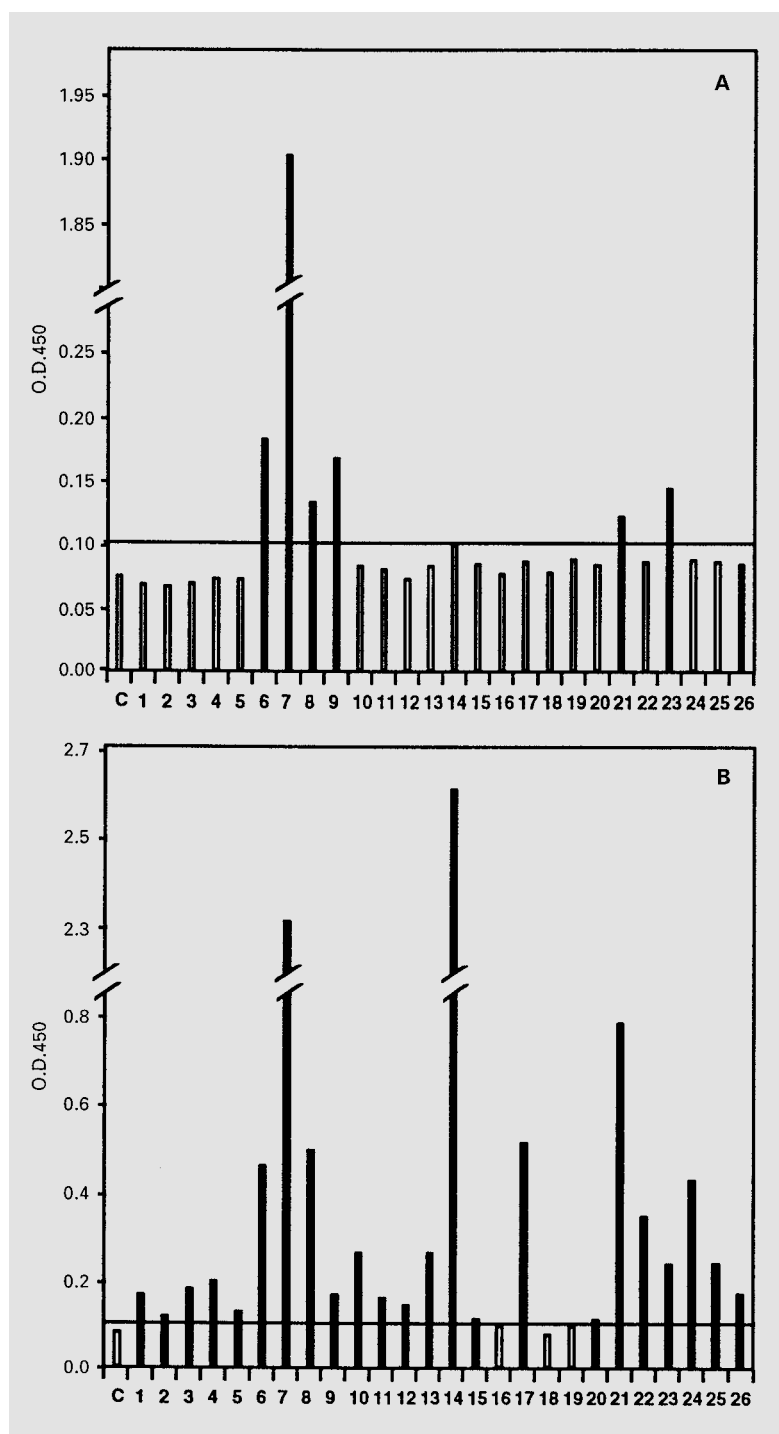


Fig. 6. IgE reactivity of human sera against r-paramyosin (**A**) and n-paramyosin (**B**). Each well was coated with 500 ng of protein. The sera were diluted 1:5. C = Mean OD of the control sera. The horizontal line indicates the value above which the results were considered positive.

The presence of these isoforms may be explained by post-translational modifications [46, 47]. The finding of two genes coding for paramyosin in *A. simplex* may have two possible explanations. First, each of these genes belongs to different populations of phenotypically identical *A. sim-*

plex [48, 49] and, thus, the two identified genes are alleles of the same *locus*. This possibility has been described for the allergen Der p 1 of *Dermatophagoides pteronyssinus*, where mites from different geographical origins present cDNA sequence polymorphisms [50]. The second possi-

bility is that *A. simplex* has two gene copies codifying for paramyosin. This possibility has been described for the parasitic trematode *S. japonicum*, where two genes have been found [51]. In *Taenia solium* (Cestoda), a secreted form of paramyosin has also been identified [41]. This form of paramyosin is not implicated in the muscular function but it may act as a modulator of the immune response of the host due to its complement-inhibition capacity via C1 [27].

Several possibilities may exist to explain the differences in the frequency of specific IgE binding to the recombinant (23%) and the n-paramyosin (88%). One possibility is that there are conformational epitopes in the n-paramyosin which are not present in the r-paramyosin due to the lack of proper folding. Another possibility is that the native protein may contain specific epitopes due to posttranslational modifications such as glycosylations or other covalent modifications. We favor that the effect of glycosylation may account for the difference in the percentage of recognition since paramyosin is glycosylated in *Dermatophagoides farinae* [52] and in *T. solium* [41]. Furthermore, identical glycosylation sites were found in the 3 nematode paramyosins examined (fig. 2). We suggest that the higher prevalence of specific IgE to native paramyosin could be due to carbohydrate epitopes present in the native molecule, as suggested by other authors for other high molecular weight *A. simplex* antigens [53]. Our data demonstrate significant differences in IgE binding to the r- and n-paramyosins by the sera of the analyzed individu-

als. The differences could be due to the intensity of the contact of a particular individual with the allergen and/or the parasite, to higher total and specific IgE levels, or to a genetic predisposition to an exacerbated response. It is noteworthy that all positive sera to r-paramyosin were also positive against the native form. Specific IgE inhibition experiments demonstrated that both n- and r-paramyosins have the same inhibition capacity. These assays demonstrated that paramyosin does not contribute substantially to the total allergenicity of the *A. simplex* extract, although its native form is widely recognized by allergic individuals.

To date, only a partial sequence of a paramyosin protein belonging to the mite *D. farinae* has been described as an allergen [52]. Its native form is recognized by 80% of the sera from mite-allergic subjects and the reported sequence shows 50% amino acid homology with other known paramyosins. The reported frequency of IgE binding to this allergen is very similar to the percentage of sera of *A. simplex*-sensitive individuals that recognize the n-paramyosin of the parasite.

The study of the cross-reactivity between paramyosins of different origin is warranted to verify if these proteins act as panallergens, as it has been demonstrated for other structural proteins like tropomyosin, which also have an α helix structure [54]. Recombinant proteins of *A. simplex* may be useful tools in elucidating the intrinsic mechanisms involved in the humoral and cellular responses to *A. simplex* antigens.

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