

Short communication

Cloning and high level expression in *Escherichia coli* of an *Anisakis simplex* tropomyosin isoform[☆]

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Anisakis simplex is a nematode of the Ascaridata order which parasitizes, in its third larval stage (L₃), crustaceans, cephalopods and seafish. Humans are accidental hosts and acquire the disease known as anisakiasis when they eat infected raw or undercooked fish. Symptoms of anisakiasis include nausea, intense abdominal pain and vomiting [1]. This parasite can also induce hypersensitivity IgE-mediated reactions with several clinical manifestations ranging from urticaria/angioedema to anaphylaxis [2], and it has been suggested that tropomyosin could be involved in this IgE response [3,4]. Tropomyosins are a family of closely related proteins present in muscle and non-muscle cells and mediate the interaction between the tro-

ponin complex in the actin-linked calcium regulatory systems in striated muscle [5]. In muscle, two parallel α -helical tropomyosin molecules are wound around each other forming a coiled-coil structure [6]. Tropomyosin has been suggested as a cross-reacting allergen among invertebrates and may elicit hypersensitivity reactions of different severity in atopic individuals [4,7,8]. In the present work, we cloned, sequenced, and obtained recombinant tropomyosin from *A. simplex* in order to obtain a well-characterized tropomyosin to be used in diagnostic screening of *A. simplex*-allergic patients.

cDNA encoding for an *A. simplex* L₃ tropomyosin isoform (*As*-TPM) was obtained by RT-PCR using primers designed according to described nucleotide sequences for invertebrate tropomyosins. The DNA fragment was subcloned in pKN172 [9] and sequenced using fluorescence-labeled dideoxynucleotides. The insert has a length of 855 bp, encoding 284 amino acids with a predicted molecular mass of 33 203 Da and an isoelectric point value of 4.68. The tropomyosin

Abbreviations: *As*-TPM, *Anisakis simplex* L₃ tropomyosin isoform; L₃, third larval stage.

[☆] **Note:** Nucleotide sequence data reported in this paper are available in the EMBL, GenBankTM and DDJB databases under the accession number Y19221.

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signature, L-K-E-A-E-x-R-A-E, present in all sequenced tropomyosins is also conserved in the C-terminal region of *As*-TPM (Fig. 1A). Traditionally, coiled-coil tropomyosins have been identified by the occurrence of a heptad repeat in which generally hydrophobic non-polar residues

occur at positions *a* and *d* and are responsible for the interaction between two α -helices in a coiled-coil structure. At the same time, charge–charge interaction between acidic residues found in position *e*, and basic residues in positions *g* help also to stabilize the coiled-coil. This periodic hept-

A

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gcgtgtattctcgcggttgattcccaatctatcaaccaattcaaccagg
accATTGACGCAATCAAGAAGAAGATCGAGGCATGAAATCGAGAAGGAC
  D A I K K K Q A M K I E K D 16
AATGCCCTCGATCGAGCCGATGCCGCCGAAGAGAAATCCGACAGATGACC
  N A L D R A D A A E E K V R Q M T 33
GACAAGCTGGAGAGAATCGAAGAGAACTCCGAGACACACAAAGAAGATG
  D K E R I E E E R D T Q K K 50
ATGCAAACTGAGAATGATTGGACAAAGCTCAAGAAGACCTTTCAACGGCC
  M Q T E N D D K A Q E D S T A 67
AACTCGAATCTCGAAGAGAAGGAAAGAAAGCTCAAGAAGCTGAGGCAGAA
  N S N E E E K E K A E 84
GTAGCTGCTTTGAACCGTCTGATGACTGCTCGAAGAGGAAGCTCGAAAG
  V A A L N R R T L L E E E E R 101
GCTGAAGAACGCTGAAATGGCAACGGCCAACTTGAAGAGGCACACAT
  A A E R K L A T A K E A T H 118
ACAGCTGATGAATCTGAGCGTGTGCGCAAGTGATGGAGAACCCTCATTC
  T D E S E R V R K V M E N R S F 135
CAAGATGAAGCGTGCAGTTCGGAATGAGTCAACTCAAGGAAGCGCAA
  Q D G A E R A N T V A S Q K E A Q 152
ATGCTTGCCGAAGAGGCTGATCGCAATACGATGAGGTGCCCGTAAATG
  M L E E A D R K Y D E V A R K L 169
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ACCATGGTTGAAGCGGATTTAGAAAGAGCTGAAGAGCGTGCAGAGACCGGA 612
  T M V E A D E R A E E R A E T G 186
GAAAATAAGATCGTTGAATTTGAAGAGGAATTCGCGATCGTTGGTAACAAC
  E N K V E L E E E R V G G N N 203
TTGAAATCGTTGAAGTTTCCGAAGAGAAGGCTTTGCAACGAGAGGATTCA
  L K S L E V S E E K A L Q R E D S 220
TAGCAAGAACAGATCCGTACCGTTTCTGCACGCTCAAGAGGCGCGAAACT
  Y E E Q T R T V S A R L K E A E T 237
CGTGCCGAATTTGAGAAAGATCCGTTTCAGAACTACAGAAGGAGGTTGAC
  R A E F A E R S V Q K L Q K E V D 254
AGACTCGAAGACGAAGCTCGTCCACGAAAAAGACGATACAGAGCATTTC
  R L E D E V H E K E R Y K S I S 271
GAGGAACCTCGATCAAACTTCCAAGAGCTGTCTGGATATTAAgatatag
  E E D Q T F Q E L S G Y * 284
ctgtttaacccaacaaaaaaaccagttctgtgctgttttaactcgaaata
ccctatttatctgctcacactatctgcttcttaactactatgttatt 1020
ctcgagaagcatcatcttactactactactactactactactattatt
gcatcatctctgctcatatgtgtataacttaggaacatttaactttgtt
accgaacaatgtcgaaatgtcattcagtgaccatcatcagcagcattacga
atctaataaatggaacatcttaaaaaaaaaaaaaaaaaaaaaaaaaaa 1224
actctgctgtgttaccactgcttgccctatagttagtgatgtattag 1269
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B

	1	50	100
As-TPM	MDAIIKKKQAMKIEKDNDALDRADAEEKVRQMTDKLERIEEELRDQTKKMMQTFENDLKAQEDLSANSLNLEEKKVQEAEEVAALNRRMTLLEEL		
Trichostrongylus	MDAIIKKKQAMKIEKDNDALDRADAEEKVRQMTDKLERIEEELRDQTKKMMQTFENDLKAQEDLSANSLNLEEKKVQEAEEVAALNRRMTLLEEL		
Caenorhabditis	MDAIIKKKQAMKIEKDNDALDRADAEEKVRQMTDKLERIEEELRDQTKKMMQTFENDLKAQEDLSANSLNLEEKKVQEAEEVAALNRRMTLLEEL		
Onchocerca	MDAIIKKKQAMKIEKDNDALDRADAEEKVRQMTDKLERIEEELRDQTKKMMQTFENDLKAQEDLSANSLNLEEKKVQEAEEVAALNRRMTLLEEL		
Mite	MDAIIKKKQAMKIEKDNDALDRADAEEKVRQMTDKLERIEEELRDQTKKMMQTFENDLKAQEDLSANSLNLEEKKVQEAEEVAALNRRMTLLEEL		
Shrimp	MDAIIKKKQAMKIEKDNDALDRADAEEKVRQMTDKLERIEEELRDQTKKMMQTFENDLKAQEDLSANSLNLEEKKVQEAEEVAALNRRMTLLEEL		
Cockroach	MDAIIKKKQAMKIEKDNDALDRADAEEKVRQMTDKLERIEEELRDQTKKMMQTFENDLKAQEDLSANSLNLEEKKVQEAEEVAALNRRMTLLEEL		
Snail	MDAIIKKKQAMKIEKDNDALDRADAEEKVRQMTDKLERIEEELRDQTKKMMQTFENDLKAQEDLSANSLNLEEKKVQEAEEVAALNRRMTLLEEL		
Human	MDAIIKKKQAMKIEKDNDALDRADAEEKVRQMTDKLERIEEELRDQTKKMMQTFENDLKAQEDLSANSLNLEEKKVQEAEEVAALNRRMTLLEEL		
Schistosoma	MDAIIKKKQAMKIEKDNDALDRADAEEKVRQMTDKLERIEEELRDQTKKMMQTFENDLKAQEDLSANSLNLEEKKVQEAEEVAALNRRMTLLEEL		
	111	150	200
As-TPM	ERAERLKTATEKLEATHNDESERVVKVMENRSFQDEERANTVSQLKEAQLAEADRKYDEVARKLAMVEADLRAEERAETGENKIVELEELRV		
Trichostrongylus	ERAERLKTATEKLEATHNDESERVVKVMENRSFQDEERANTVSQLKEAQLAEADRKYDEVARKLAMVEADLRAEERAETGENKIVELEELRV		
Caenorhabditis	ERAERLKTATEKLEATHNDESERVVKVMENRSFQDEERANTVSQLKEAQLAEADRKYDEVARKLAMVEADLRAEERAETGENKIVELEELRV		
Onchocerca	ERAERLKTATEKLEATHNDESERVVKVMENRSFQDEERANTVSQLKEAQLAEADRKYDEVARKLAMVEADLRAEERAETGENKIVELEELRV		
Mite	ERAERLKTATEKLEATHNDESERVVKVMENRSFQDEERANTVSQLKEAQLAEADRKYDEVARKLAMVEADLRAEERAETGENKIVELEELRV		
Shrimp	ERAERLKTATEKLEATHNDESERVVKVMENRSFQDEERANTVSQLKEAQLAEADRKYDEVARKLAMVEADLRAEERAETGENKIVELEELRV		
Cockroach	ERAERLKTATEKLEATHNDESERVVKVMENRSFQDEERANTVSQLKEAQLAEADRKYDEVARKLAMVEADLRAEERAETGENKIVELEELRV		
Snail	ERAERLKTATEKLEATHNDESERVVKVMENRSFQDEERANTVSQLKEAQLAEADRKYDEVARKLAMVEADLRAEERAETGENKIVELEELRV		
Human	ERAERLKTATEKLEATHNDESERVVKVMENRSFQDEERANTVSQLKEAQLAEADRKYDEVARKLAMVEADLRAEERAETGENKIVELEELRV		
Schistosoma	ERAERLKTATEKLEATHNDESERVVKVMENRSFQDEERANTVSQLKEAQLAEADRKYDEVARKLAMVEADLRAEERAETGENKIVELEELRV		
	201	250	286
As-TPM	VGNLKLSEVSEKALQREDSYEEQIRTVSRRLKEAETRAEFAERSVQKLQKEVDRLDELVHEKERYKISSELDQTFQELSGY*		
Trichostrongylus	VGNLKLSEVSEKALQREDSYEEQIRTVSRRLKEAETRAEFAERSVQKLQKEVDRLDELVHEKERYKISSELDQTFQELSGY*		
Caenorhabditis	VGNLKLSEVSEKALQREDSYEEQIRTVSRRLKEAETRAEFAERSVQKLQKEVDRLDELVHEKERYKISSELDQTFQELSGY*		
Onchocerca	VGNLKLSEVSEKALQREDSYEEQIRTVSRRLKEAETRAEFAERSVQKLQKEVDRLDELVHEKERYKISSELDQTFQELSGY*		
Mite	VGNLKLSEVSEKALQREDSYEEQIRTVSRRLKEAETRAEFAERSVQKLQKEVDRLDELVHEKERYKISSELDQTFQELSGY*		
Shrimp	VGNLKLSEVSEKALQREDSYEEQIRTVSRRLKEAETRAEFAERSVQKLQKEVDRLDELVHEKERYKISSELDQTFQELSGY*		
Cockroach	VGNLKLSEVSEKALQREDSYEEQIRTVSRRLKEAETRAEFAERSVQKLQKEVDRLDELVHEKERYKISSELDQTFQELSGY*		
Snail	VGNLKLSEVSEKALQREDSYEEQIRTVSRRLKEAETRAEFAERSVQKLQKEVDRLDELVHEKERYKISSELDQTFQELSGY*		
Human	VGNLKLSEVSEKALQREDSYEEQIRTVSRRLKEAETRAEFAERSVQKLQKEVDRLDELVHEKERYKISSELDQTFQELSGY*		
Schistosoma	VGNLKLSEVSEKALQREDSYEEQIRTVSRRLKEAETRAEFAERSVQKLQKEVDRLDELVHEKERYKISSELDQTFQELSGY*		

Fig. 1.

tameric sequence occurs throughout the *As*-TPM sequence.

Sequence similarity searches using the BLAST program revealed that *As*-TPM has the highest homology to tropomyosins from other nematodes such *Trichostrongylus colubriformis* (93% of identical residues), the free-living nematode *Caenorhabditis elegans* (92%), and the filarial parasite *Onchocerca volvulus* (91%). Homology decreased among other invertebrates, crustaceans (70%), insects (69%), and mollusks (63–56%). Tropomyosins from vertebrates (human, chicken, mouse) showed less than 58% identity, although the high similarity among tropomyosins reflects a similar three-dimensional structure. One of the lowest homologies was to the tropomyosin from the trematode *Schistosoma mansoni* with only 57% identical amino acids. A recent work with a well-known allergen, shrimp tropomyosin, described six IgE binding sites (reviewed in [7]) which are quite homologous to *As*-TPM (Fig. 1B). The high homology of amino acid sequence and IgE-binding epitomes between *As*-TPM and invertebrate tropomyosins could explain the high cross-reactivity found among invertebrates [7], and supports the idea of tropomyosin as a pan-allergen. Human tropomyosin also contains some of these IgE-binding epitomes and it is possible that human

tropomyosin acts as a booster in tropomyosin-reacting patients when the allergen is not present, as has been suggested in the case of the plant pan-allergen, profilin [10]. However, the clinical and allergological implications of the high homology found among tropomyosins remain to be investigated.

The entire coding-region of *As*-TPM was cloned into the expression vector pKN172 and a non-fusion form of the recombinant protein was expressed in *Escherichia coli* JM109 (DE3) [11]. The yield of recombinant tropomyosin was 15 mg l⁻¹ of bacterial culture, which represented approximately 23% of total *E. coli* proteins. Expressed protein was detected both in the soluble and insoluble fractions (data not shown), but only the soluble fraction was used for purification. Purification of recombinant *As*-TPM by isoelectric precipitation gave a 99% pure preparation of a 40.9 kDa band, as determined by SDS-PAGE (Fig. 2A). The size is larger than the one calculated from the nucleotide sequence, but similar to that of the natural *As*-TPM as detected by immunoblotting with antibodies against shrimp tropomyosin (Fig. 2B). In addition, in presence of 6 M, the purified *As*-TPM had an apparent molecular mass of 51 kDa, whereas control proteins migrated at the expected positions (Fig. 2C). The

Fig. 1. (A) Nucleotide and deduced amino acid sequence of cDNA encoding *As*-TPM. A putative polyadenylation signal (AATAAA) is italicized. The tropomyosin signature (L-K-E-A-E-x-R-A-E) is underlined. Non-polar residues at positions *a* of the heptad repeats are marked. Poly(A⁺)-mRNA was isolated from 100 mg of *A. simplex* L₃ larvae (manually extracted from *Micromesistius poutassou*) using the Quick Prep MicroRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden). First strand cDNA was synthesized from 1 µg poly(A⁺)-enriched mRNA using a first-strand synthesis kit (Pharmacia Biotech). Degenerate oligodeoxynucleotide primers (Genosys Biotechnologies, Pampisford, UK) used for cDNA amplification of the coding region were TRAF, 5'-CCGGATCCCATATGGA(C/T)GC(C/G)ATCAAGAA(A/G)AAGATG (sense); and TRAR, 5'-CCGAATTC AAGCTTT(T/C)AATA(G/T)CC(A/G)GA(A/G)AGTTC(C/T)TG (antisense). *Bam*HI, *Nde*I, *Eco*RI and *Hind*III restriction sites are underlined. The single-stranded cDNA (5 µl) were mixed with the described degenerate primers in standard PCR mixture, and subjected to amplification with Taq DNA polymerase (Bioline, London, UK). A predominant band of ca. 850 bp was isolated from 1% agarose gels (GeneClean, Bio101, La Jolla, CA) and cloned into the expression vector pKN172 digested with *Nde*I–*Hind*III. cDNA (5' and 3') ends were obtained using SMART RACE cDNA Amplification kit (Clontech Laboratories, Palo Alto, CA) as recommended by the manufacturer. Both strands were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready reaction kit and run on an ABI 373A DNA sequencer (both, Perkin-Elmer, Foster City, CA). (B) Alignment of the predicted protein encoded by *As*-TPM with other known tropomyosins, *Trichostrongylus* (*T. colubriformis*, M94502), *Caenorhabditis* (*C. elegans*, GenBank D38540), *Onchocerca* (*O. volvulus*, GenBank L41633), Mite (*Dermatophagoides pteronyssinus*, GenBank Y14906), Shrimp (*Metapenaeus ensis*, GenBank U08008), Cockroach (*Periplaneta americana*, GenBank Y14854), Snail (*Helix aspersa*, GenBank Y14855), Human (skeletal, GenBank X04201), and *Schistosoma* (*S. mansoni*, SwissProt P42638). Amino acids common to *As*-TPM and at least one other sequence are contained in black boxes. IgE-binding regions on invertebrate TPM [7] are double underlined. Database searches were performed using the BLAST server [12] and sequence alignments were carried out using the GCG PILEUP program (Genetics Computer Group, Madison, WI, USA) and the Boxshade server.

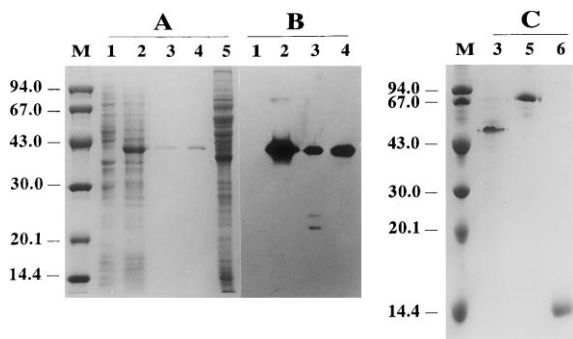


Fig. 2. Analysis of recombinant *As*-TPM. (A) Coomassie-stained 12.5% SDS-PAGE. (B) A Western blot incubated with shrimp tropomyosin antiserum experimentally obtained after immunization of white New Zealand rabbits with purified shrimp tropomyosin. Lane M, molecular mass markers; lanes 1 and 2, total cell lysate from *E. coli* JM109 (DE3) containing pKN172 alone and pKN172 with *As*-TPM-encoding cDNA, respectively; lanes 3 and 4, recombinant and natural tropomyosins after isoelectric precipitation, respectively; lane 5, total extract from *A. simplex* larvae. (C) SDS-PAGE (12.5%) containing 6 M urea. Lane M, molecular mass markers; lane 3, *As*-TPM; lane 5, BSA; lane 6, cytochrome *c*. Purification of *As*-TPM expressed in *E. coli* JM109 (DE3) was performed as follows, cells were grown to an OD₆₀₀ of 0.6 in Luria-Bertani medium containing 200 µg ml⁻¹ ampicillin and, after addition of 0.6 mM isopropyl-thio-β-galactoside, incubation was continued for another 3 h. Cells were harvested by centrifugation, and the pellet was resuspended in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 1 mM EDTA, containing 100 µg ml⁻¹ lysozyme, and incubated for 15 min at 37°C. After mild sonication (six pulses of 10 s each), tropomyosin (natural or recombinant) was purified by the isoelectric precipitation method of Smillie [5] based on successive precipitations at pH 4.6 and a final precipitation with 35–60% ammonium sulfate.

isoelectric behavior and the change in migration on electrophoresis in urea gels are characteristics of tropomyosins [5]. The yield of the purified natural *As*-TPM was 0.36 mg g⁻¹ of *Anisakis* larvae, while the final yield of the purified recombinant protein was about 6.6 mg l⁻¹ of *E. coli* culture. Experiments which could elucidate the importance of *As*-TPM as an allergen are now in progress by using this recombinant protein in in vitro and in vivo tests among *Anisakis*-allergic patients.

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