

Cloning, Isolation, and IgE-Binding Properties of *Helix aspersa* (Brown Garden Snail) Tropomyosin

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Key Words

Tropomyosin · Gene expression · Sequence amplification · Mollusks · Cross-reactivity

Abstract

Background: Gastropod consumption is quite frequent in the Mediterranean countries and cross-reactivities with crustaceans have been described, but the mechanism of this allergenic cross-reactivity has not been studied in detail. This study aimed to produce recombinant *Helix aspersa* (brown garden snail) tropomyosin and investigate its implication for cross-reactivity among invertebrates. **Methods:** A tropomyosin-specific cDNA encoding *H. aspersa* tropomyosin was synthesized, and recombinant allergen was overexpressed in *Escherichia coli* as nonfusion protein. IgE-binding reactivity was studied by immunoblotting and immunoblot inhibition experiments with sera from snail-allergic patients. **Results:** Cloned brown garden snail tropomyosin shares high homology with other edible mollusk tropomyosins (84–69% identity) as well as with those from arthropods (65–62%), and less homology with vertebrate ones (56% identity). Tropomyosin reacted with 18% of the sera from patients with snail allergy. Inhibition experiments, using

natural and recombinant tropomyosins, showed different degrees of cross-reactivity between invertebrate tropomyosins. Sera from snail-allergic subjects recognized tropomyosins in both mollusks and crustacean extracts. **Conclusions:** Tropomyosin represents a minor allergen in snail extracts, but it is clearly involved in invertebrate cross-reactivity.

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Introduction

Clinical manifestations of food allergy are generally the results of a IgE-mediated type I hypersensitivity and affect 13–18% of the population [1]. Shellfish, peanuts, fish and nuts account for most food hypersensitivity reactions in adults, although the prevalence of reactions to specific food depends in part on the eating habits of a given population. The term shellfish is an artificial nontaxonomic grouping of edible Arthropoda (mainly Decapoda) and Mollusks [2]. Although crustacean allergens, particularly tropomyosin, have been described in detail, mollusk allergens have not been well studied. Recent reports, however, suggest that mollusks are significant sources of food allergens in exposed populations and that these reac-

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tions are IgE mediated [3–6]. Several reports, especially from Spain, Portugal, France and Italy, where snails are a frequent part of the diet, have described cases of snail allergy [6–10]. Apparent molecular masses of snail allergens ranged from 22 to 200, with important IgE-binding bands at 24, 32–33, 49–57, 69–77 and >95 kD [8, 10].

Tropomyosins are a family of closely related proteins present in muscle and non-muscle cells, and in association with the troponin complex play a central role in the actin-linked calcium regulatory system of muscle contraction [11]. Tropomyosins have been described as relevant allergens in extracts from crustaceans, mites and insects [12–15] and could be involved in the high cross-reactivity found among these organisms [16–18].

We report here the cloning of the tropomyosin from *Helix aspersa* and its high-level expression in *Escherichia coli* as a non-fusion protein. Antibody-binding reactivity of the recombinant allergen was compared with the natural counterpart, and recombinant allergen was used for studying the potential panallergen behavior of tropomyosins.

According to the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Subcommittee, the allergen tropomyosin from brown garden snail (*H. aspersa*) was named Hel as 1.

Material and Methods

Purification of Tropomyosin

Brown garden snails (*H. aspersa*) were purchased from a local market. Foot muscles were snap-frozen in liquid nitrogen, ground in a mortar and stored at –20°C until used. Preparations of acetone powder from snails was performed as described by Smillie [11]. Purification of tropomyosin from acetone powder was carried out by a modification of the isoelectric precipitation method as previously described [11]. Further purification was obtained by exchange chromatography on a Sepharose Q column equilibrated with 20 mM phosphate buffer pH 7.0 on an ÄKTA-prime System™ (Amersham Pharmacia Biotech, Uppsala, Sweden).

Cloning of Tropomyosin cDNA by PCR Amplification and Nucleotide Sequencing

Poly(A⁺)-enriched mRNA, isolated from foot muscle of *H. aspersa*, was used for first-strand synthesis (First-strand cDNA Synthesis Kit; Amersham Pharmacia Biotech). Degenerate primers (Cruachem Ltd., Glasgow, UK) for cDNA amplification were TROF: 5'-CCG-GGATCCCATATGGA(G/T)GC(C/T)ATCAAGAA(A/G)AA (sense) and TROR: 5'-CCGAATTCT(T/C)A(A/G)TA(G/A/T)ACCAG-(T/A/C)(A/C)A(G/A)(T/C)TCGG (antisense). *Bam*HI, *Nde*I, and *Eco*RI restriction sites are underlined. Five microliters of the single-stranded cDNA were mixed with the described degenerate primers in standard PCR mixture, and amplified as previously described [15].

The fragment was isolated from the gel and ligated to the pGEM-T vector (Promega, Madison, Wisc., USA). Nucleotide sequences were determined on an ABI 373A DNA sequencer (Perkin-Elmer, Foster City, Calif., USA).

Expression and Purification of Recombinant Tropomyosin

Tropomyosin-encoding cDNA was cloned into the expression vector pKN172 [19] as an *Nde*I-*Eco*RI fragment and used to transform competent *E. coli* JM109 (DE3) cells (Promega). For expression, cells were grown to an OD₆₀₀ of 0.6 in LB-containing 200 µg/ml ampicillin and after addition of 0.6 mM isopropyl-thio-β-galactoside incubation was continued for another 3 h. Cells were harvested and the pellet was resuspended in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 1 mM EDTA, containing 100 µg/ml lysozyme, and incubated for 15 min at 37°C. After mild sonication (6 pulses of 10 s each), tropomyosin was purified by isoelectric precipitation as mentioned above.

Human and Experimental Sera

Sera from 22 patients reporting at least two or more symptoms, including asthma, pruritus, facial edema and gastrointestinal disorders after ingestion of brown garden snail and a positive skin prick test were selected. All sera showed class >2 scores of specific IgE to brown garden snail measured by the Hy-Tec specific IgE EIA assay (Hycor Biomedical, Irvine, Calif., USA). Rabbit specific shrimp antisera were obtained as previously described [15].

Electrophoresis and Immunoblotting

Proteins were analyzed by SDS-PAGE under reducing conditions [20], visualized by Coomassie Brilliant Blue R-250 and evaluated by image analysis in a GS-710 Image Analyser (Bio-Rad, Richmond, Calif., USA). For immunoblotting experiments, proteins after SDS-PAGE were electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp., Bedford, Mass., USA) [21]. Membranes were incubated overnight at 4°C with allergic patient sera (diluted 1:4 or 1:10), or at 37°C for 60 min with polyclonal serum (diluted 1:1,000). Immunodetection was performed as previously described using anti-rabbit IgG (Sigma) or anti-human IgE (Dako, Glostrup, Denmark) antibodies linked to horseradish peroxidase [22]. For immunoblot-inhibition studies, sera from patients allergic to brown garden snails were incubated overnight at 4°C with 20 µg of purified inhibitor or BSA (negative control) per milliliter of sera. The preadsorbed sera were then used for immunoblot experiments.

Results

PCR-Based Cloning of Tropomyosin cDNA

cDNA-encoding for an *H. aspersa* tropomyosin (Hel as 1) obtained by RT-PCR was cloned and sequenced. The sequence encoded a polypeptide of 284 amino acids with a calculated molecular mass of 32,637 D, and an isoelectric point of 4.58. The L-K-E-A-E-x-R-A-E tropomyosin signature and the periodic heptad repeat characteristic of coiled-coil tropomyosin are present in the Hel as 1 sequence (fig. 1a).

Fig. 1. a Nucleotide and deduced amino acid (in bold) sequences of Hel as 1. Primers used for cDNA amplification are underlined. Nonpolar residues at positions of the heptad repeats are marked. These sequence data are available from the GenBank under accession No. Y14855. b Comparison of Hel as 1 with *Haliotis diversicolor* tropomyosin (abalone, GenBank AF216587), Cra g 1.03 (*Crassostrea gigas*, oyster, AF239173), *Chlamys nobilis* tropomyosin (scallop, GenBank AF216520), Der p 10 (*Dermatophagoides pteronyssinus*, GenBank Y14906), Met e 1 (*Metapenaeus ensis*, GenBank U08008), Per a 7 (*Periplaneta americana*, GenBank Y14854), Ani s 3 (*Anisakis simplex*, GenBank Y19221) and *Homo sapiens* tropomyosin (skeletal, GenBank X04201). Amino acids common to Hel as 1 are marked by points.

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1  ATGGATGCTATCAAGAAGAAGATGTTGGCCATGAAATGGAGAAGGAAATGCCTTGGAT 60
1  M D A I K K K M L A M K M E K E N A L D 20

61  AGGCGAGAACAGGTGGAGCAGAAGCTGCGAGACTGCGAGTGTATAAGAACAAGTGGAA 120
21  R A E Q V E Q K L R D C E C N K N K V E 40

121  GAAGATCTGAACAACCTACAAAAGAAATTCGCCATCTTGGAGAAGCACTTCGACAGCATC 180
41  E D L N N L Q K K F A I L E N D F D S I 60

181  AACGAGCAGTGTGCTTGTATGCCAACACCAAACTTGAGGCATCCGAGAAGAAGAATGCAGAG 240
61  N E Q L L D A N T K L E A S E K K N A E 80

241  ATTGAAAGTGAGACAGCAGGTCTGCAAAAGACGTATCCAGCTGTGGAGGAGGACTTGGAG 300
81  I E S E T A G L Q R R L Q L L E E D L E 100

301  AGGTCAGAAGAGCGTCTTCAGAGTGCAACAGAGAAGTTGGAAGAGGCATCTAAAGTGCT 360
101  R S E E R L Q S A T E K L E E A S K A A 120

361  GATGAAAGTGAAAGAGGTCGTAAGGTGCTTGAGAGCAGGAGCTTGGCTGTATGTAACGA 420
121  D E S E R G R K V L E S R S L A D D E R 140

421  TTGGATGGTCTTGAAGCTCAGTTGAAGAAGCTAAATACATCGCCGAGGATGTGAGCGC 480
141  L D G G L E A Q L K E A K Y I A E D A E R 160

481  AAATTCGATGAGGCTGCTCGTAAACTGGCCATCACAGAGGTGGATCTCGAGAGAGCTGAG 540
161  K F D E A A R K L A I T E V D L E R A E 180

541  GCCCGTTTGGAGGCTGCTGAGGCGAAGATCCTTGAGTTGAGAGGAGGAGTTGAAGGTCGTC 600
181  A R L E A A E A K L L E L E E E L K V V 200

601  GGCAACAATATGAAATCTTTGGAATCAGTGAACAAGAGGCATCCAGAGAGAGGATAGC 660
201  G N N M K S L E I S E Q E A S Q R E D S 220

661  TACGAAGAGACGATCCGTGACTTGACCCAGAGGCTCAAGGACGCCGAGAATCGTGCCAGC 720
221  Y E E T L R D L T Q R L K D A E N R A S 240

721  GAGGCTGAGCGCACAGTGTCCAACTACAGAAAGAGGTGGACAGATTGGAAGACGAATTG 780
241  E A E R T V S K L Q K E V D R L E D E L 260

781  CTGGCAGAGAAAGAGAGATACAAGCTACTTCAGACGAATGGATAGTACATTGCCGAA 840
261  L A E K E R Y K A T S D E L D S T F A E 280

841  CTGGCTGGCTACTAA 855
281  L A G Y * 285

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a

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1 50 100
Hel as 1 MDAIKKKMLAMKMEKENALDRAEQVEQKLRDCECNKNKVEEDLNNLQKKFAILENDFDSINEQLLDANTKLEASEKKNAEIESETAGLQRRRIQLLEEDL
H. diversicolor .....V.....N.....T.EQ.A.I.....C.N.....V...VQV.MA...T...RVT.M.Q.VS.TT.K.T.....
Cra g 1.03 .....NSARG...TV...KYQECQ...M.EA...TAS.A.Q.IQS.N.....M
C. nobilis .....Q...VDR...Q.L...M.....T.TA.A.L.EF.E...LTAT...NY.TV...QE.....N...QITQL.DVG...LT...Y
Der p 10 .E...N.Q...L.D.I...IA...A.ANLRAE.S.EVRA...IQOI...EL.QVQ...SA...EK...ALQTA.GDV.A.N...I...
Met e 1 Q...L.D.M...DTL.QNKEANNRAE.S.EVH...RMQQL...L.QVQ...S.LKANQ.VEKD.ALSNA.G.V.A.N.....
Per a 7 .....Q...L.D.M.C.LLC.QA.ANLRAE.A.EARSL...IQOI...L.QTM...MQV.A.DEKD.ALQNA...V.A.N.....
Ani s 3 .....Q...I.D.....DAA.E.V.QMTRKLERI..E.RDT...MMQT...L.KAQ.D.ST..SN..EK...VQ.A.A.V.A.N..MT...E.
H. sapiens M.E.....QML.LD.....A.AEQKQA.ERSKQL.DE.AAM...LKGT.DEL.KYS.A.K..QE...LA...A.DA.A.V.S.N...V..E.

101 150 200
Hel as 1 ERSEERLQSATEKLEEASKAADESERGRKVLERSLADDERLDGLEAQLKEAKYIAEDAERKFDEAARKLAITEVDLERAERAEARLEAAEAKILELEELKV
H. diversicolor ..N...T...R.....LP.....A.....I.Q.....Y.....PK.....
Cra g 1.03 .....T.....N.....NLNN.SE..T.V...L...L...E.D..Y.....V.....
C. nobilis .....K.N.T.....N.....G..NSYE..I.E.K..ET..NV.T.DH.....T...D..V...T.
Der p 10 .....KI..A.....QS...M..M..H..IT.E.MEG..N...RMM...D..Y..V...MV.....E.A.TG.S..V...R.
Met e 1 .....NT..T..A...Q.....M.....N...S.E..M.A.N...RFL..E.D..Y..V...MV.....E.A.TG.S..V...R.
Per a 7 .....AT..A..A...Q.V...A..I...KG...E..M.A.N...RFM..E.DK.Y..V...MV.....E.A.SG.S..V...R.
Ani s 3 ..A...KL..A.....THT.....V..M.N..F..E..ANTV.S...QML..E.D..Y..V...TMV.....E.A.TG.N..V...R.
H. sapiens D.AQ...AT.LQ.....E.....M..I.N.A.K.E.KMELQ.I.....E.D..YE.V...I.G.....T.E.A.L.S.CS.....N

201 250 286
Hel as 1 VGNMMSLEISEQEASQREDSYEETIRDLTQRLKDAENRASEAERTVSKLQKEVDRLDELLEAEKERYKATSDELSTFAELAGY*
H. diversicolor ...T.....T.....K..I...Q.....*
Cra g 1.03 .....T.....I...Q.....*
C. nobilis ...I.T.QVQNDQ.....KS.....T...Q.V.....I..D..Q...I...*
Der p 10 ...L...V..EK.Q...EAH.QQ..IMTTK..E..A..EF...S.Q.....G...VH...K..SI...Q...T...*
Met e 1 ...L...V..EK.N...EA.K.Q.K...NK.A.A..EF...S.Q.....VN...K..SIT...Q..S.S.*
Per 7 ...L...V..EK.NL..EE.KQQ.K...T...E..A..EF...S.Q.....VH...K..FIC.D.M.T...*
Ani s 3 ...L...V..EK.L...Q...VSA...E..T..EF...S.Q.....VH...SI..E...Q..Q..S.*
H. sapiens .T..L...AQAEKY..K..K...E.KI..DK..E..T..EF...S.A..E.TI.D...Y.Q.LK..AI.E...HALNDMTSI*

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b

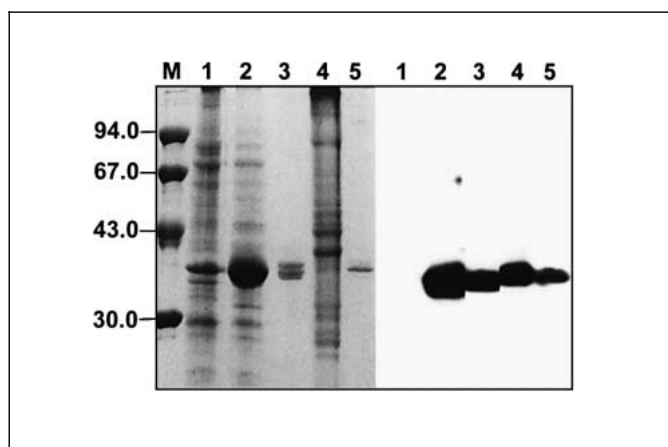


Fig. 2. Purification of natural and recombinant brown garden snail tropomyosins. Coomassie-stained 12.5% SDS-PAGE and Western blot incubated with shrimp tropomyosin antiserum. Lane M, molecular mass markers; lanes 1 and 2, total cell lysate from *E. coli* containing pKN172 alone and pKN172 with tropomyosin-encoding cDNA, respectively; lane 3, purified rHel as 1; lane 4, brown garden snail extract; lane 5, purified nHel as 1.

Genbank data research of Hel as 1 revealed that the protein shares 84% of identical amino acids with abalone (*Haliotis diversicolor*) tropomyosin and 90% with the snail *Biomphalaria glabrata*. Bivalve tropomyosins from scallop (*Chlamys nobilis*, 72%) and mussel (*Mytilus edulis*, 70%) show less homology. The homology to other invertebrate tropomyosins decreased outside the phylum Mollusca, i.e. nematodes (66%), mites (65%), crustaceans (64%) or insects (63%). Significant but lower homology was also found with vertebrate tropomyosins, including human (56% identity, 75% similarity) (fig. 1b).

Purification of Natural Snail Tropomyosin and Purification of Recombinant Protein

Natural Hel as 1, purified from snail foot muscle, gave a single homogeneous band on Coomassie blue-stained SDS-PAGE, with an apparent molecular mass of 36 kD (fig. 2a). In a typical preparation, 33 mg of purified tropomyosin (0.075% yield) were obtained from 44 g of snail feet. Recombinant Hel as 1 was expressed as a non-fusion protein in *E. coli*, using the pKN172 vector system. The yield of recombinant tropomyosin was 5 mg/l of bacterial culture, which represented approximately 6% of total *E. coli* proteins (estimated by densitometry of SDS-PAGE gels). The presence of an endogenous bacterial protein with similar molecular mass in control extract (fig. 2, lane 1) masked the exact yield of expressed protein. Recombi-

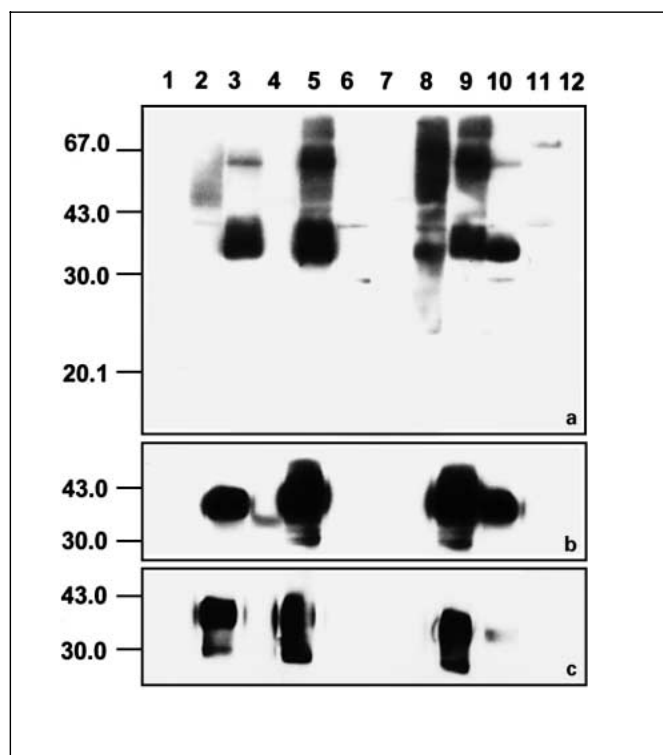


Fig. 3. Representative Western blot of brown garden snail extract (a), natural (b) and recombinant (c) Hel as 1 incubated with sera from snail-allergic patients (lanes 2–12) and from pooled sera from healthy individuals (lane 1).

nant 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 Hel as 1 gave a 98% pure preparation, showing a double band with an apparent molecular mass of 35–36 kD, as determined by SDS-PAGE (fig. 2), which is larger than the expected molecular mass deduced from the nucleotide sequence. This apparent molecular mass is similar to that of the natural Hel as 1 as detected by immunoblotting probed with antibodies against shrimp tropomyosin (fig. 2).

Comparison of Antibody Binding to Natural and Recombinant Brown Garden Snail Tropomyosins

The frequency of IgE binding to tropomyosin from the extract and the purified natural and recombinant proteins was compared by SDS-PAGE immunoblotting (fig. 3). A 36-kD protein in the *H. aspersa* foot extract and the natural and recombinant purified tropomyosins were recognized by IgE antibodies in 4 of 22 sera tested (18%). Nevertheless, in serum No. 10 IgE antibody binding to the

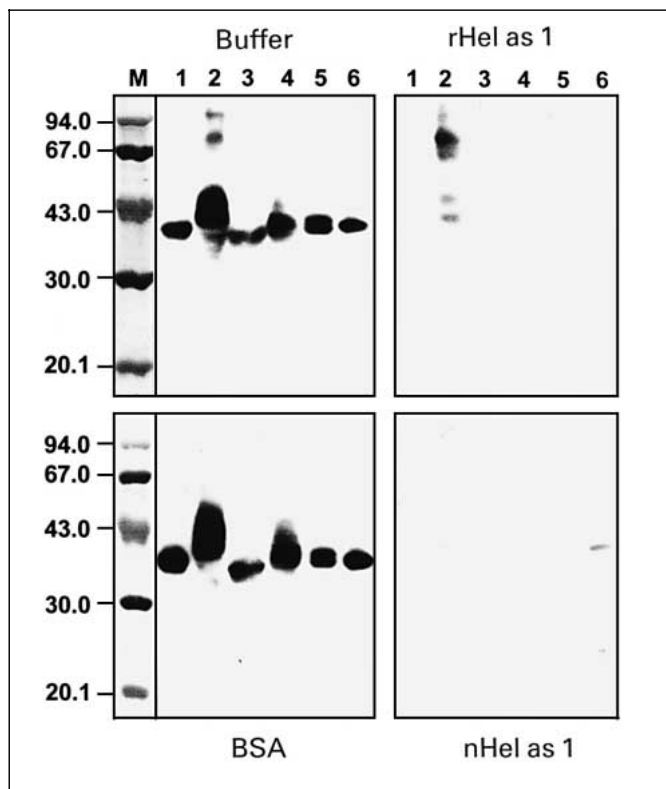


Fig. 4. Western blot inhibition of different mollusk and crustacean extracts (20 μ g, except shrimp, 2 μ g) with serum pool from snail-allergic patients (diluted 1:10) preincubated with buffer alone, BSA, rHel as 1, and nHel as 1 (10 μ g/ml). Lane 1: brown garden snail; lane 2: cuttlefish; lane 3: clam; lane 4: octopus; lane 5: sea snail; lane 6: shrimp. Molecular mass markers (lane M) were stained with Amido Black B.

recombinant Hel as 1 was weaker than to the natural one. Other allergens recognized in the snail foot extract were 74-, 67-, 65-, 45-, 40- and 29-kD proteins. The 34-kD band detected in serum No. 8 was not tropomyosin as it is not detected in purified tropomyosin blots (fig. 3b, c). Most patients showed weak activity because in the snail foot extract used for immunoblotting some allergens (i.e. hemocyanin) could have been lost.

Cross-Reactive Tropomyosin Molecules in Other Invertebrates

Muscle extracts from five common edible mollusks (brown garden snail, cuttlefish, clam, sea snail and octopus) and shrimp were subjected to SDS-PAGE immunoblotting inhibition against a pool of sera from the four Hel as 1-reacting patients. A 36-kD band was detected in shrimp and most of the mollusks, except in cuttlefish

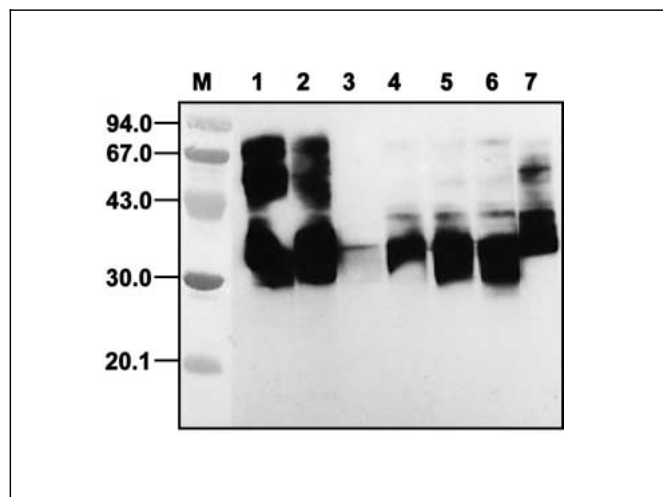


Fig. 5. IgE-Western blot inhibition of snail extract with serum pool of Hel as 1-reacting sera from snail-allergic patients preincubated with buffer alone (lane 1), BSA (lane 2), purified natural and recombinant tropomyosins (10 μ g/ml): nHel as 1 (lane 3), rHel as 1 (lane 4), rPer a 7 (lane 5), nMet e 1 (lane 6), rDer p 10 (lane 7). Molecular mass markers (lane M) were stained with Amido Black B.

where IgE bound to a 41-kD protein (fig. 4, lane 2). Both natural and recombinant Hel as 1 inhibited IgE-binding to these bands almost completely. Sera from healthy individuals did not react with any of the extracts tested (data not shown).

Pooled sera from Hel as 1-reacting patients lost IgE reactivity to proteins of 70–34 kD from snail extract when they were preincubated with natural snail tropomyosin (fig. 5). In contrast, rHel as 1 and tropomyosin from other invertebrates partially inhibited an IgE binding 36-kD protein and produced a significant decay of IgE binding to proteins of higher molecular masses (74 and 65 kD).

Discussion

The description of allergen sequences and the production of recombinant allergens provide the basis for detailed studies of the immune response to these allergens, overcoming the problems related to variability of allergenic extracts. Tropomyosins are a good model to study the contribution of the primary structure to the allergenicity of proteins because in spite of the high degree of homology among tropomyosin, both allergenic and nonallergenic tropomyosin can be found [23]. A simple and efficient method has been employed to clone cDNA mole-

cles encoding the translated region of tropomyosin from *H. aspersa*, and to express this recombinant allergen in *E. coli* as a non-fusion protein. The differences between apparent and expected molecular masses of tropomyosins observed in Hel as 1 and others (Met e 1, Per a 7, Der f 10, Pen i 1) [14, 15, 24, 25] could be due to the particular structure of these proteins.

In Western blots of some patient sera, the IgE-binding capacity of rHel as 1 was weaker than that of its natural counterpart, showing that some epitopes from the natural tropomyosin could be missing in its recombinant counterpart. This fact could be due to improper folding of the recombinant protein produced in *E. coli* or to the existence of various isoforms of tropomyosin involved in the allergenic response. Although the first possibility should be tested by producing the recombinant protein in another host-vector system, the existence of distinct tropomyosin isoforms is well known [26].

Preabsorption with tropomyosin of different origins reduced IgE reactivity to tropomyosin and other IgE-reactive bands of higher molecular masses from snail extract. This fact, reported previously in mollusk and crustacean extracts, could be due to the specific inhibitory effect to other tropomyosin isoforms or tropomyosin aggregates [27]. IgE reactivity to tropomyosin has been found in extracts from organisms such as crustaceans, insects and mollusks, but not in vertebrates [16, 17, 27, 28]. The presence of cross-reactivity among these species, some of which are important food sources in many parts of the world, may be explained by the close phylogenetic relationship between these phyla, although different epitopes must exist among their tropomyosins because no completely IgE-binding inhibition could be observed. The clinical relevance of the presence of conserved tropomyosin IgE-reactive epitopes among invertebrates is still unclear as serological IgE reactivity does not always result in a clinical hypersensitivity reaction. Double-blind placebo

controlled oral challenge tests in patients testing positive for tropomyosin should be performed to identify the initial sensitization routes. Eight IgE epitopes quite homologous to Hel as 1 have been described in shrimp tropomyosin [18]. The high homology among invertebrate tropomyosins on IgE-binding epitope sequences could explain the high cross-reactivity found among invertebrates, and supports the hypothesis of tropomyosin as an invertebrate panallergen. Vertebrate tropomyosins are not described as important allergens although they share an average of 56% of identical amino acids with invertebrate tropomyosins [23]. Changes on critical residues of IgE epitopes could be responsible for this lack of IgE reactivity of vertebrate tropomyosin, as has recently been demonstrated by mutational analysis for shrimp tropomyosin [29]. However, the clinical and allergenic implications of the high homology found among tropomyosins remain to be investigated. Recombinant tropomyosins and IgE epitope mapping data from tropomyosins from different allergenic sources will be necessary to resolve the issues of cross-reactivity among invertebrates. The isolation and expression of allergenic tropomyosin from unreported sources, such as snail, offers the possibility of obtaining high amounts of well-defined and purified rHel as 1 for these purposes.

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