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Recombinant tropomyosin from *Penaeus aztecus* (rPen a 1) for measurement of specific immunoglobulin E antibodies relevant in food allergy to crustaceans and other invertebrates

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Immunoglobulin E (IgE)-mediated food allergy to crustaceans and mollusks is relatively common and affected individuals typically react to a range of different species. The only known major allergen of shrimp was first described over 20 years ago and later identified as the muscle protein tropomyosin. This protein may be useful as a defined and relevant diagnostic marker for allergic sensitization to invertebrate foods. In order to generate an assay reagent suitable for this purpose, tropomyosin from the shrimp *Penaeus aztecus* (Pen a 1) was produced as a recombinant protein in *Escherichia coli* and characterized with respect to IgE antibody binding properties in comparison to natural shrimp tropomyosin. Hexahistidine-tagged rPen a 1 accumulated as a predominantly soluble protein in the *E. coli* expression host and a two-step chromatographic procedure provided a high yield of pure and homogeneous protein. rPen a 1 displayed chromatographic and folding characteristics similar to those of purified natural shrimp tropomyosin. Serum preincubation with serial protein dilutions revealed similar capacity of recombinant and natural tropomyosin to compete with immobilized shrimp extract for IgE binding. rPen a 1 was further shown to extensively and specifically compete for IgE binding to extracts of other crustacean species, house dust mite and German cockroach.

Keywords: Allergen / Crustaceae / Food allergy / Shrimp / Tropomyosin

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1 Introduction

Hypersensitivity to crustacean foods, such as shrimp, lobster, crayfish, and crab, is relatively common and symptoms following ingestion can be severe, including both local and systemic reactions [1]. Affected individuals typically display allergic reactivity to multiple crustacean species and and immunoglobulin E (IgE) sensitization shows association to a wide range of invertebrate foods, including mussels, oyster, squid, and octopus [2–4].

Two fractions of allergenic activity were initially identified in shrimp, one heat-labile and one heat-stable, of which the latter showed stronger IgE-binding activity and was found to dominate the allergenicity of the shrimp extract [5]. The causative protein component of this fraction was later identified as tropomyosin, an abundant and highly soluble muscle protein [6, 7].

Shrimp tropomyosin is a slightly acidic protein of 276–284 amino acid residues [8, 9], reported to carry a minor glycan modification [5, 7, 9]. The protein lacks cystein residues, migrates as a 34–38 kDa band in SDS-PAGE, but exists as an extended coiled-coil homodimer in its native state, with an apparent size in gel filtration which greatly exceeds its actual molecular mass [5, 10]. Tropomyosin is highly conserved in amino acid sequence among a wide range of invertebrate organisms [6, 7], as illustrated in Table 1. Direct and indirect evidence has demonstrated IgE antibody cross-reactivity among tropomyosins from different crustacean species and other edible invertebrates [2–4] which is consistent with observed clinical sensitivity patterns [9, 11–15]. Further, sensitization to tropomyosin has been implicated in associations between allergy to house dust mite and seafood, in particular following allergen immunotherapy with mite extract [13]. A recent study suggested that exposure to house dust mite tropomyosin may sensitize against shrimp tropomyosin [16]. In contrast, even though

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Abbreviations: CD, circular dichroism; **IgE**, immunoglobulin E; **MBP**, maltose-binding protein; **rPen a 1**, recombinant Pen a 1

Table 1. Amino acid sequence identity among tropomyosins from different organisms

	Mytilus edulis (Blue mussel)	*Homo sapiens*	*Schistosoma haematobium* (Trematode)	*Mizuhopecten yessoensis* (Scallop)	*Caenorhabditis elegans* (Nematode)	*Drosophila melanogaster* (Fruit fly)	*Dermatophagoides pteronyssinus* (House dust mite)	*Locusta migratoria* (Locust)	*Blatella germanica* (German cockroach)	*Charybdis feriatus* (Crab)	*Homarus americanus* (Lobster)
Penaeus aztecus (Shrimp)	57%	57%	60%	62%	71%	78%	81%	82%	83%	92%	99%
Homarus americanus (Lobster)	57%	57%	60%	62%	71%	79%	81%	82%	83%	92%	
Charybdis feriatus (Crab)	56%	60%	58%	62%	73%	81%	83%	84%	84%		
Blatella germanica (German cockroach)	57%	55%	56%	60%	69%	86%	81%	90%			
Locusta migratoria (Locust)	59%	57%	57%	61%	71%	88%	81%				
Dermatophagoides pteronyssinus (House dust mite)	56%	59%	59%	62%	72%	77%					
Drosophila melanogaster (Fruit fly)	54%	56%	56%	60%	69%						
Caenorhabditis elegans (Nematode)	56%	60%	57%	60%							
Mizuhopecten yessoensis (Scallop)	68%	55%	64%								
Schistosoma haematobium (Trematode)	58%	50%									
Homo sapiens	54%										

Sequences were aligned pairwise using the Bestfit program of the Wisconsin Package and the resulting percentages of residue identity are shown. The species and corresponding sequence accession numbers are: *Penaeus aztecus*, [Reese et al. 1999, *Int. Arch. Allergy Immunol. 119*, 247–258]; *Homarus americanus*, AAC48288; *Charybdis feriatus*, Q9N2R3; *Batella germanica*, AAF72534; *Locusta migratoria*, P31816; *Dermatophagoides pteronyssinus*, AAB69424; *Drosophila melanogaster*, P06754; *Caenorhabditis elegans*, S58921; *Mizuhopecten yessoensis*, BAB17858; *Schistosoma haematobium*, AAA88530; *Homo sapiens*, P06753; *Mytilus edulis*, AAA82259.

mammalian and invertebrate tropmyosins share significant homology, no cross-reactivity has been observed and mammalian tropomyosin appears to be nonallergenic [9].

The purpose of the present work was to develop an efficient method for production of recombinant tropomyosin from *Penaeus aztecus*, rPen a 1, with biochemical and immunological characteristics similar to natural shrimp tropomyosin. rPen a 1 may be useful as a diagnostic tool in the investigation of allergies to foods in which tropomyosin is a major determinant, and in studies of their relation to respiratory allergies to mites, cockroaches, and other arthropods.

2 Materials and methods

2.1 Bioinformatics

Publicly available amino acid sequences of tropomyosins from different species were retrieved from the bioinformatic search engine Entrez at NCBI (www.ncbi.nlm.nih.gov/entrez). DNA and amino acid sequence analyses were performed using programs of the GCG Wisconsin Package (www.accelrys.com). Sequences were aligned pairwise using Bestfit and the percentage identity in each comparison was recorded. Sequences selected for comparison to Pen a 1 [9] and among themselves were: AAC48288

(*Homarus americanus*, lobster), Q9N2R3 (*Charybdis feriatus*, crab), AAF72534 (*Blatella germanica*, German cockroach), P31816 (*Locusta migratoria*, locust), AAB69424 (*Dermatophagoides pteronyssinus*, house dust mite), P06754 (*Drosophila melanogaster*, fruit fly), S58921 (*Caenorhabditis elegans*, nematode), BAB17858 (*Mizuhopecten yessoensis*, scallop), AAA88530 (*Schistosoma haematobium*, trematode), P06753 (*Homo sapiens*), AAA82259 (*Mytilus edulis*, blue mussel).

2.2 Cloning of recombinant Pen a 1

A cDNA encoding Pen a 1 [9] was PCR amplified using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA, USA), forward primer 5′-AGT CAC ATA TGG ACG CCA TCA AGA AGA AGA TGC-3′ and reverse primer 5′-CTC AGA AGC TTA GTG GTG GTG GTG GTG ATG GTA GCC AGA CAG TTC GCT GAA AGT C-3′, such that a hexahistidine tag was added to the *C*-terminal of the intended recombinant protein. The PCR product was digested with *Nde*I and *Hin*dIII, cloned into pET23a (+) (Novagen, Madison, WI, USA) using *Escherichia coli* strain XL1-Blue (Stratagene) and the construct verified by DNA sequencing. DNA restriction and modifying enzymes were obtained from New England Biolabs (Beverly, MA, USA) and oligonucleotides from Invitrogen Life Technologies (Carlsbad, CA, USA). Plasmid DNA was prepared using Wizard Plus Maxipreps DNA Purification System (Promega, Madison, WI, USA). For DNA sequencing, TempliPhi 100 Amplification Kit, Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit and Redivue [α-^{33}P]ddNTPs Terminator Pack were used, all obtained from Amersham Biosciences (Uppsala, Sweden). All reagents were used according to the manufacturers' recommendations.

2.3 Expression and purification of recombinant Pen a 1

For the purpose of expression, the rPen a 1-encoding plasmid construct was transferred to *E. coli* BL21 cells (Novagen) containing plasmid pT7POL23 [17], which served to provide T7 RNA polymerase in a temperature-controlled manner. Recombinant protein was expressed either in shaking culture as previously described [18] or using a 3 L bioreactor (Belach Bioteknik, Solna, Sweden). *E. coli* maltose-binding protein (MBP) was produced as described earlier [18] and used as a negative control in immunoassay experiments. Hexahistidine-tagged rPen a 1 was purified by IMAC and SEC. Following culture harvest by centrifugation, the cell pellet was resuspended in 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 mM PMSF, 5 mM imidazole, and homogenized twice using an EmulsiFlex C5 (Avestin, Ottawa, Canada) set to operate at a mid-pulse pressure of 13 MPa. The homogenate was then clarified by centrifugation (15 000 × *g*, 20 min, 4°C) and finally passed sequentially through 1.2 μm and 0.45 μm mixed cellulose ester filters (Millipore, Billerica, MA, USA). The resulting extract was subjected to IMAC, using Ni^{2+}-charged chelating Sepharose HP packed in a XK50/20 column (Amersham Biosciences). After loading and washing the column with 20 mM imidazole in 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, bound rPen a 1 was eluted with a linear 20–500 mM gradient of imidazole in the same buffer. Fractions containing rPen a 1 were identified by SDS-PAGE, pooled and subjected to preparative SEC, using a Superdex 200 XK50/100 column (Amersham Biosciences), eluted with 20 mM MOPS, pH 7.4, 0.5 M NaCl. The purity of the final preparation was analyzed by SDS-PAGE and the tropomyosin concentration determined from absorbance at 280 nm, using a calculated extinction coefficient of 0.15 per mg/mL.

2.4 Purification of natural shrimp tropomyosin

Natural tropomyosin was purified according to methods described previously [19]. Briefly, shrimps (*Pandalus borelis*) obtained from a local supermarket were dissected and finely chopped tail muscle was boiled for 10 min in 1 mL of distilled water per gram of tissue. After clarification by centrifugation and sequential passage through 1.2 μm and 0.45 μm mixed cellulose ester filters, the resulting extract was subjected to ultrafiltration using a PM-10 membrane (molecular mass cut-off: 10 000 Da; Millipore). The retentate after ultrafiltration was applied to a Q Sepharose FF ion exchange column (Amersham Biosciences), equilibrated with 20 mM Tris-HCl, pH 8.0 and eluted with a linear 0–0.5 M NaCl gradient in the same buffer, while recording absorbance at 215 and 280 nm. A peak of absorbance at 215 nm was analyzed by SDS-PAGE and fractions containing a strong 38 kDa band, assumed to be tropomyosin, were pooled for further purification by SEC as described above. The purity of the final preparation was analyzed by SDS-PAGE and the tropomyosin concentration determined from absorbance at 280 nm, using an extinction coefficient of 0.15 per mg/mL.

2.5 Analytical SEC, SDS-PAGE, and *N*-terminal sequencing

For analytical SEC, 0.2 mL samples were applied to a Superdex 200 HR 10/30 column (Amersham Biosciences) equilibrated with 20 mM MOPS, pH 7.4, 0.5 M NaCl and eluted with the same buffer. Molecular mass calibration of the column was performed using proteins included in LMW and HMW Marker Kits (Amersham Biosciences): ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), and ferritin (440 kDa). As a marker of void volume, blue dextran was used. SDS-PAGE was performed

on reduced samples using 10% NuPAGE precast gels and Mark12 as MW markers (Invitrogen Life Technologies). Protein bands were visualized by staining with Coomassie Brilliant Blue. *N*-terminal sequencing of rPen a 1 and purified natural tropomyosin by Edman degradation was performed using a Hewlett-Packard G1000A instrument (Hewlett-Packard, Palo Alto, CA, USA).

2.6 Circular dichroism (CD) spectroscopy

Natural shrimp tropomyosin and rPen a 1 were dialyzed against 10 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.4 and protein concentrations adjusted to 5.2 μM (0.17 mg/mL). CD spectroscopy was performed on a J-810 S spectropolarimeter (Jasco, Groß Umstadt, Germany) with constant N_2 flushing. The temperature of the optical cell chamber was maintained at 20°C by a circulating water bath and the rectangular cell had a optical path length of 1 mm. For wavelength analysis, natural tropomyosin and rPen a 1 were scanned with step increments of 0.2 nm and a band width of 1 nm. The spectral range was 185 – 255 nm and the scanning speed 50 nm/min. Ten scans were accumulated. Mean residue molar ellipticity (θ_{MRV}) was calculated according to the following equation:

$$\theta_{MRV} = \frac{\theta_{obs} \cdot mrv}{10 \cdot l \cdot c}$$

where θ_{obs} is the observed ellipticity (mdeg), *mrv* is the mean residue molecular mass (Da), *l* is the optical path length of CD cell (cm), and *c* is the protein concentration (mg/mL).

2.7 Patient's sera

Nine sera from sensitized donors were used in this study, selected from an in-house collection at Pharmacia Diagnostics on the basis of specific IgE to shrimp and rPen a 1. A subset of six sera were pooled and used for comparative activity analysis of rPen a 1 and purified natural tropomyosin and for studies of dose-dependent inhibition of IgE-binding to protein extracts of different invertebrate species by soluble rPen a 1. The serum pool was comprised of serum A, B, F, G, H, and I (Fig. 5), composed so as to achieve an equal contribution of rPen a 1-specific IgE from each serum.

2.8 Measurement of specific IgE

Measurements of specific IgE-binding to allergen extracts and purified recombinant and natural tropomyosin were performed using Pharmacia UniCAP (Pharmacia Diagnostics, Uppsala, Sweden), a quantitative solid-phase immunoassay system used in clinical diagnosis of atopic allergy. Regular ImmunoCAP tests were used for analysis of specific IgE to natural allergen extracts of shrimp (*Pandalus borealis*, allergen code f24), lobster (*Homarus gammarus*, f80), crab (*Cancer pagurus*, f23), blue mussel (*Mytilus edulis*, f37), octopus (*Octopus vulgaris*, f59), house dust mite (*Dermatophagoides pteronyssinus*, d1), German cockroach (*Blatella germanica*, i6) and, for control purposes, timothy grass pollen (*Phleum pratense*, g6). Experimental ImmunoCAP tests carrying recombinant or natural tropomyosin were prepared as described [18]. All assays were performed according to the manufacturer's instructions and calculation of assay parameters were carried out by the UniCAP instrument software.

2.9 Analysis of fluid-phase binding of specific IgE

The potency of IgE antibody binding of rPen a 1 and purified natural tropomyosin was assessed as their ability to block IgE-binding to an immobilized shrimp protein extract. Samples of a serum pool were preincubated for 2 h at room temperature with serial dilutions of rPen a 1 or natural shrimp tropomyosin (0.01, 0.05, 0.25, and 25 μg/mL), or serum dilution buffer (Total IgE and Specific IgE Sample Diluent, Pharmacia Diagnostics) alone. All samples were then analyzed for IgE-binding to shrimp extract and the proportion of uncomplexed IgE at each inhibitor concentration calculated, using the value from a buffer-incubated sample for normalization. In a separate experiment, where the ability of rPen a 1 to block antibody binding to cross-reactive determinants present in a variety of invertebrate allergen extracts was examined, the procedure was identical, except that different ImmunoCAP tests were used for IgE measurements following serum preadsorption. To assess subject-to-subject variation in cross-reactivity and relative contribution of tropomyosin-specific IgE, a single-dose IgE inhibition experiment was performed in serum samples from individual donors. All nine sera used in the experiment were preincubated with 25 μg/mL rPen a 1, or with dilution buffer alone, and then analyzed using the set of different ImmunoCAP tests described above. Throughout the study, preincubation with MBP protein at 100 μg/mL was used as a control for non-specific procedural effects. As a control for inhibitor specificity, a serum from a grass pollen-sensitized individual was preincubated with rPen a 1 at 25 μg/mL, or dilution buffer alone, and then tested for IgE-binding to timothy grass pollen extract.

3 Results

3.1 Cloning, expression, and purification of recombinant Pen a 1

A plasmid construct designed for cytoplasmic expression of hexahistidine-tagged recombinant tropomyosin was pre-

pared by inserting a full-length Pen a 1 cDNA into vector pET23a(+), under the transcriptional control of the T7 promoter. The construct was transformed into *E. coli* strain BL21[pT7POL23] which confers temperature-controlled expression of the protein of interest. Following induction at 42°C, a protein product with an apparent MW of about 38 kDa in SDS-PAGE, as expected for rPen a 1, accumulated and could be recovered from the soluble fraction after culture harvest and cell disruption (Fig. 1).

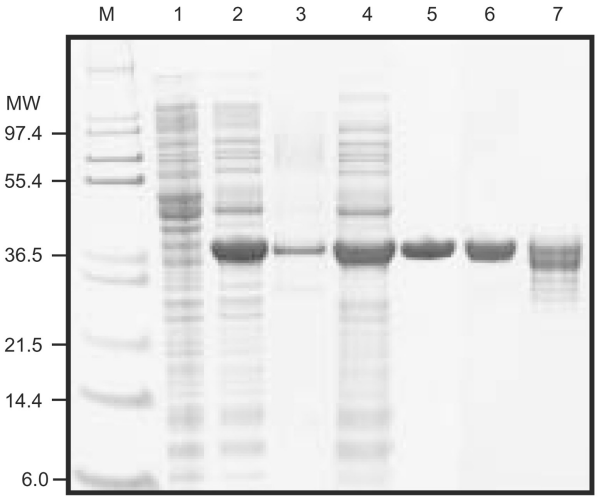

Figure 1. SDS-PAGE analysis of rPen a 1 expression in *E. coli*. Lane 1, preinduction sample of *E. coli* culture; lane 2, culture sample at harvest; lane 3, insoluble cell fraction; lane 4, soluble cell fraction; lane 5, pool of IMAC fractions; lane 6, final rPen a 1 preparation; lane 7, purified natural tropomyosin; lane M, molecular weight markers. All samples were reduced prior to analysis.

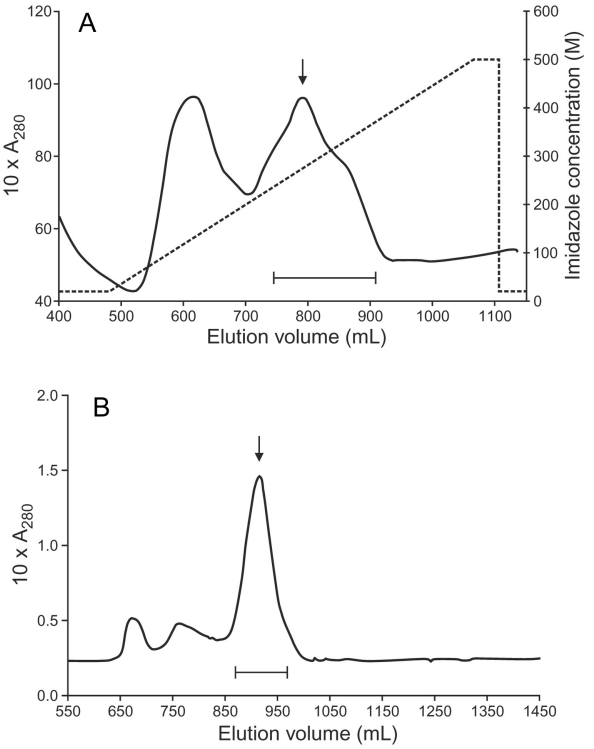

Figure 2. Purification of rPen a 1. (A) IMAC elution profile recorded at 280 nm (solid line) and imidazole gradient (hatched line). A horizontal bar indicates fractions pooled for further purification. (B) Elution profile of preparative SEC of pooled IMAC fractions, recorded at 280 nm. A horizontal bar indicates fractions pooled to form the final rPen a 1 preparation.

3.2 Purification of recombinant Pen a 1

A two-step chromatographic procedure was devised for purification of rPen a 1 from *E. coli* homogenate. After a first step of IMAC (Fig. 2A), a protein product essentially free from host protein was obtained (Fig. 1), which, however, consisted of multiple aggregation forms as revealed by SEC. A second step of preparative SEC using a Superdex 200 column was therefore employed, whereby the dominant, slowest migrating aggregation form of rPen a 1 was isolated (Figs. 2B and Fig. 1). The *N*-terminal part of the protein was confirmed by amino acid sequencing, which also revealed complete removal of the initiator methionine. The yield of this rPen a 1 preparation was 8 mg per gram (fresh weight) of *E. coli* cell pellet, or 0.7 g per liter of reactor culture of the production strain.

3.3 Purification of natural shrimp tropomyosin

Natural tropomyosin was purified from an extract of shrimp tail muscle tissue using cation exchange chromatography and SEC. Taking advantage of the low absorbance at 280 nm and expected abundance of the tropomyosin, a major peak with high A_{215} over A_{280} was identified in the ion exchange elution profile (Fig. 3A). Fractions from this peak were analyzed by SDS-PAGE and found to contain two major protein bands: one of approximately 38 kDa, as expected for tropomyosin, and another of approximately 19 kDa (not shown). Pooled fractions were subjected to preparative SEC, which completely separated the two proteins (Fig. 3B). The yield of purified shrimp tropomyosin was 0.17 mg per gram of tail muscle tissue.

The final tropomyosin preparation gave rise to a single peak in analytical SEC and produced two tightly spaced bands of comparable intensity in SDS-PAGE under reducing conditions (Fig. 1, lane 7). *N*-terminal amino acid sequencing of the lower band, extracted from the polyacrylamide gel, revealed the sequence: Lys-Leu-Glu-Lys-Asp-Asn-Ala-Met-Asp-Arg-Ala-Asp-Thr-Leu-Glu, which matched exactly residues 12 – 26 of rPen a 1 and the corresponding portion of tropomyosins from several other crustaceans, including shrimp, lobster and crab species. No

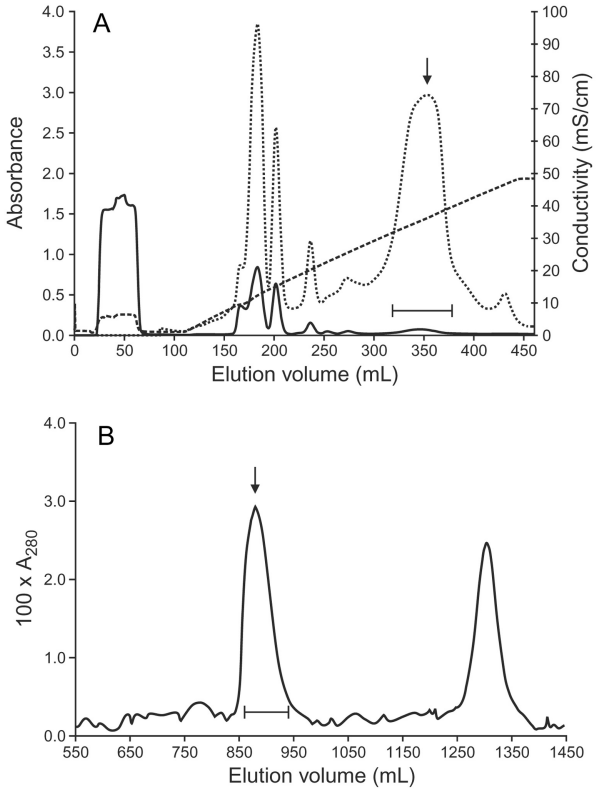

Figure 3. Purification of natural shrimp tropomyosin. (A) Elution profile of cation exchange chromatography of shrimp tail muscle extract, recorded at 280 nm (solid line) and 215 nm (dotted line). Conductivity is shown as a hatched line, reflecting the NaCl gradient applied. The peak containing tropomyosin is indicated by an arrow and pooled fractions are marked by a horizontal bar. (B) Chromatogram of preparative SEC of pooled fractions from the previous purification step using a Superdex 200 column and recording at 280 nm. The peak containing tropomyosin is indicated by an arrow and pooled fraction are marked by a horizontal bar.

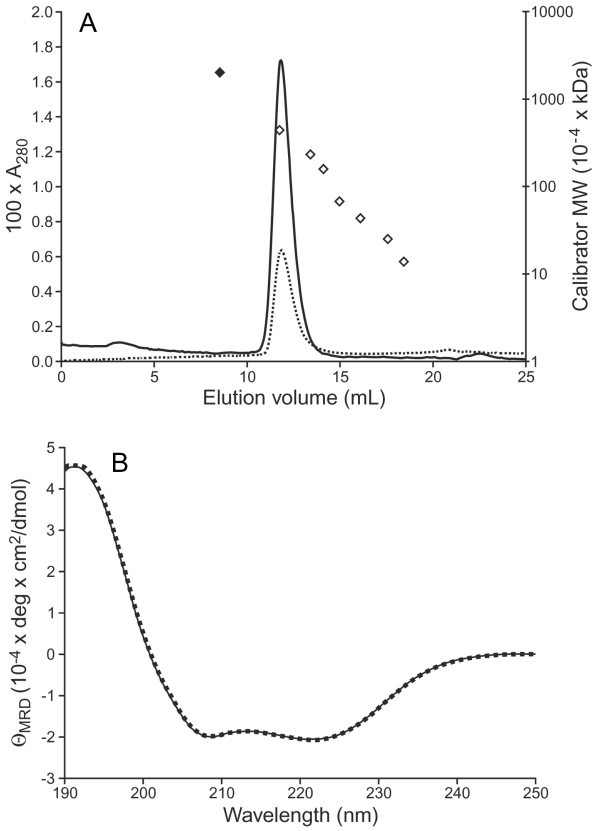

Figure 4. Comparative analysis of folding properties of purified natural shrimp tropomyosin and rPen a 1. (A) Analytical SEC of natural tropomyosin (solid line) and rPen a 1 (dotted line) on a Superdex 200 column. Elution volumes of calibration proteins are marked by open diamond symbols and dextran blue (void volume) by a filled diamond symbol. The void volume of the column was 8.5 mL and the total volume 21.7 mL. (B) CD spectroscopy of natural tropomyosin (solid line) and rPen a 1 (dotted line).

sequence was obtained from the higher band in SDS-PAGE, which we assume to be the full-length protein, consistent with the presence of *N*-terminal blockage [20].

3.4 Comparative biochemical analysis of natural and recombinant shrimp tropomyosin

Recombinant Pen a 1 and purified natural tropomyosin were subjected to a comparative analysis by SEC and CD. In analytical SEC, using a calibrated Superdex 200 column, the recombinant and natural proteins each gave rise to a single dominant peak eluting at a volume corresponding to a molecular mass of 400–500 kDa for a globular protein (Fig. 4 A). This observation suggests a similar conformation of rPen a 1 and natural tropomyosin and is consistent with the extended, coiled-coil dimeric arrangement that has been reported for tropomyosin [10]. Further evidence of similarity or equivalence of rPen a 1 and natural tropomyosin was obtained by CD spectroscopy. The two protein preparations produced distinctive and virtually identical spectra indicative of similar overall folding (Fig. 4 B).

3.5 Specific IgE-binding to pure tropomyosin and allergen extracts

Serum samples from nine shrimp-sensitized subjects were used to study IgE-binding properties of recombinant and natural tropomyosin. In addition to pure tropomyosin, the sera were analyzed for specific IgE-reactivity to extracts of *Pandalus borealis* (shrimp), *Homarus gammarus* (lobster), *Cancer pagurus* (crab), *Mytilus edulis* (blue mussel), *Octopus vulgaris* (octopus), *Dermatophagoides pteronyssinus* (house dust mite), and *Blatella germanica* (German cockroach) (Fig. 5). Six sera exhibited predominant reactivity to

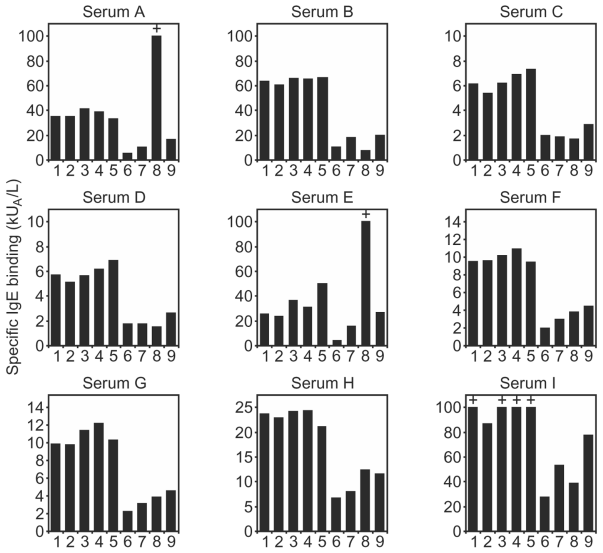

Figure 5. Specific IgE antibody binding to tropomyosin and seven different invertebrate allergen extracts. Serum samples from nine donors were analyzed for specific IgE to purified natural shrimp tropomyosin (1), rPen a 1 (2), shrimp (3), lobster (4), crab (5), blue mussel (6), octopus (7), house dust mite (8), and German cockroach (9). Specific IgE concentrations (*y*-axis) are expressed as kU$_A$/L in all panels. Values exceeding the highest calibration point of the standard curve (100 kU$_A$/L) are marked by a "+" symbol above the bar. Mean values of duplicate determinations are shown.

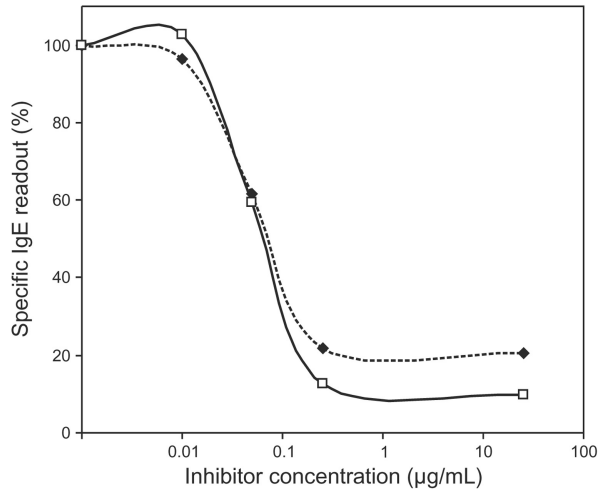

Figure 6. Comparison of fluid-phase IgE-binding potency of natural shrimp tropomyosin and rPen a 1. Analysis of residual IgE-binding to immobilized shrimp protein extract after preincubation of serum pool samples with dilution series (0.01, 0.05, 0.25, and 25 µg/mL) of purified natural tropomyosin (solid line) and rPen a 1 (hatched line). Results are expressed as percentage of a serum pool sample incubated with dilution buffer alone. Mean values of duplicate determinations are shown.

extracts of crustaceans while two displayed stronger IgE-binding to house dust mite extract and one showed a similar level of IgE-binding to all extracts tested. All nine sera showed comparable IgE-binding to rPen a 1 and natural tropomyosin, with a median deviation of 4% between the two sets of measurements. In the six sera that were biased towards the three crustacean species, the concentration of IgE to tropomyosin was on average 94% of the concentration of IgE to the whole crustacean extract, suggesting that tropomyosin accounts for most of the IgE-binding to these foods.

3.6 Comparison of IgE-binding activity of recombinant and natural tropomyosin

In order to compare the IgE-binding activity of rPen a 1 and purified natural tropomyosin, the ability of either protein to compete for antibody-binding to immobilized natural shrimp extract was analyzed. Aliquots of a pool of six tropomyosin-reactive sera were preincubated with serial dilutions of the two proteins and subsequently analyzed for specific IgE using shrimp ImmunoCAP (Fig. 6). Values used for normalization were obtained by preincubation with dilution buffer alone. Both proteins were able to outcompete a significant and almost identical proportion of the IgE-binding to the immobilized shrimp extract at a nominal concentration of 50 ng/mL, demonstrating that dominant tropomyosin epitopes are faithfully represented on rPen a 1. At the highest concentration of inhibitor, a residual of unquenched IgE-binding to the allergen extract remained for both the recombinant and natural tropomyosin, but was higher for rPen a 1, 21% as compared to 10% for the natural allergen.

3.7 Cross-reactivity between rPen a 1 and tropomyosin from other species

The extent of cross-reactivity between rPen a 1 and tropomyosin from a range of invertebrate species was studied in a series of IgE-inhibition experiments. Dose-dependent inhibition was analyzed using aliquots of a pool of six tropomyosin-reactive sera which were preincubated with serial dilutions of rPen a 1, followed by measurement of IgE-binding to extracts of shrimp, lobster, crab, blue mussel, octopus, house dust mite, and German cockroach (Fig. 7A). rPen a 1 was found to cause a dose-dependent inhibition of IgE binding to all extracts tested. The lowest level of inhibition occurred to house dust mite, for which a reduction of only 21% was observed at the highest level of inhibitor. For the other allergen sources tested, inhibition of IgE-binding by 69–92% resulted after serum preincubation with 25 µg/mL of rPen a 1 and interpolation indicated that 50% inhibition would occur at approximately 0.1 µg/mL.

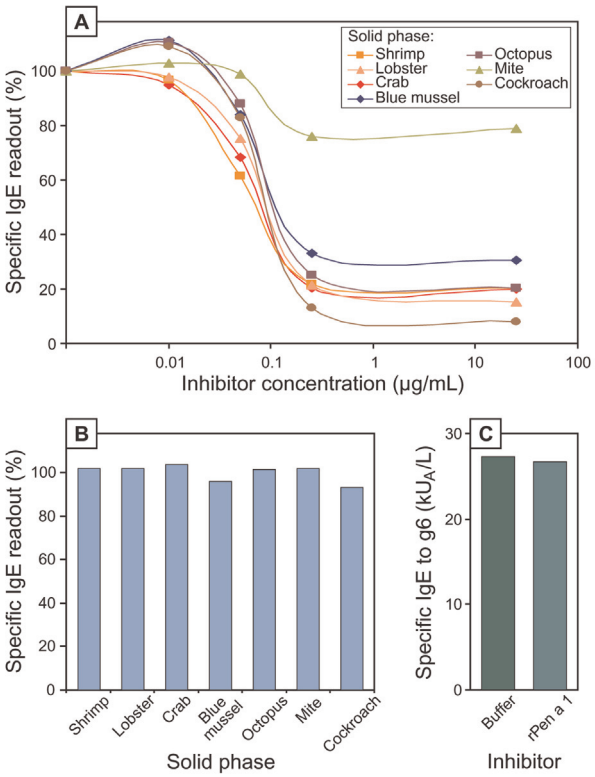

Figure 7. Efficiency of rPen a 1 competition for IgE-binding to different invertebrate allergen extracts. (A) Analysis of residual IgE binding to immobilized protein extracts of seven different invertebrate species after preincubation of serum pool samples with dilution series (0.01, 0.05, 0.25, and 25 µg/mL) of rPen a 1. Testing was performed with ImmunoCAP tests carrying extracts of the following species: shrimp (orange squares), lobster (pink triangles), crab (red diamonds), blue mussel (blue diamonds), octopus (purple squares), house dust mite (green triangles) and German cockroach (brown circles). Results are expressed as percentage of a serum pool sample incubated with dilution buffer alone. (B) Control experiment where serum pool samples were preincubated with MBP protein (100 µg/mL) before IgE analysis as above. Results are expressed as percentage of results from a serum pool sample incubated with dilution buffer alone. (C) Control experiment where samples of a grass pollen-reactive serum were preincubated with rPen a 1 (25 µg/mL) or dilution buffer alone before analyzed of IgE-binding to timothy grass pollen ImmunoCAP (allergen code g6). Specific IgE concentrations are expressed as kU_A/L. (A)–(C) Mean values of duplicate determinations are shown.

Two control experiments revealed little or no inhibition of IgE-binding to the same extracts by preincubation with MBP (Fig. 7B) and virtually no effect of rPen a 1 on IgE-binding to grass pollen extract (Fig. 7C). Thus, the observed inhibition activity of rPen a 1 was neither unspecific, nor a result of procedural effects.

In another experiment, cross-reactivity between rPen a 1 and tropomyosin from other sources was studied in sera of

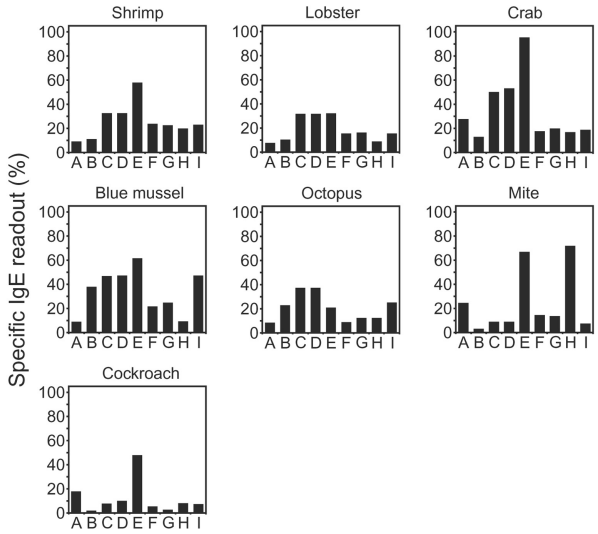

Figure 8. Cross-reactivity of anti-tropomyosin IgE antibodies among individual serum donors. Serum samples from nine individual donors (A–I) were preincubated with rPen a 1 (25 µg/mL) and analyzed using ImmunoCAP tests for shrimp, lobster, crab, blue mussel, octopus, house dust mite, and German cockroach. Results are expressed as percentage of buffer-incubated samples.

nine individual sensitized subjects (Fig. 8). Serum samples were preincubated with either 25 µg/mL of rPen a 1 or dilution buffer alone and subsequently analyzed for IgE-binding to the set of species listed above. Again, extensive inhibition of IgE-binding occurred to all species, however, with considerable variation between the individual serum donors.

4 Discussion

Food allergy to shellfish, such as shrimp, lobster, crayfish, and crab, is relatively common and reactions upon ingestion can be severe [3, 4]. The only known major allergen in these crustaceans is the muscle protein tropomyosin [6, 7, 21, 22], a highly conserved protein characterized by a homodimeric, coiled-coil structure [10]. In this report, we describe the production of recombinant tropomyosin from the shrimp *Peneaus aztecus* (rPen a 1) and demonstrate its close biochemical and immunological similarity to purified natural tropomyosin. We further report a diagnostic test, based on rPen a 1, for quantitative measurement of specific IgE antibodies directed to tropomyosin and examine cross-reactivity between rPen a 1 and tropomyosins from a range of invertebrate species known to cause food and respiratory allergy.

An expression construct encoding Pen a 1 was prepared and an *E. coli* strain established which produced high amounts

of the recombinant allergen as a soluble, intracellular protein. Purification of rPen a 1 by IMAC resulted in mixture of several aggregation forms of the protein, which were separated by preparative SEC. The migration rate of the dominant aggregation form of rPen a 1 coincided closely with that of natural tropomyosin, purified from shrimp tail muscle by ion exchange chromatography and SEC, and was therefore tentatively considered to represent the authentic form of the protein. In addition to their nearly identical migration rate in SEC, the recombinant and natural proteins displayed virtually identical spectra in circular dichroism spectroscopy. These two sets of analysis results comprise compelling evidence towards close resemblance between rPen a 1 and natural tropomyosin with respect to secondary, tertiary and quaternary structure.

The IgE-binding activity of rPen a 1 was assessed both as an immobilized solid-phase reagent and in fluid-phase antibody interaction. In both cases it was found to be fully comparable to natural tropomyosin, except for a slightly lower level of inhibition of IgE-binding to immobilized shrimp extract at the highest inhibitor concentrations. Although the reason for this minor difference was not further examined, it may have been due to an IgE-binding impurity in the natural tropomyosin preparation, present at a level so low that an effect occurred only at the higher inhibitor concentrations. Another conceivable explanation, that the recombinant allergen quantitative lacks some epitope structure, appears less likely since that ought to have affected also the low-concentration part of the inhibition curve. Regardless, the equivalent inhibition activity of the recombinant and natural allergens in the critical low-concentration range indicates authentic formation in rPen a 1 of major epitopes shared by the two proteins.

Consistent with the structural conservation of tropomyosin [10], as well as with previously reported findings [4, 11, 23–25], IgE-inhibition experiments demonstrated extensive cross-reactivity between rPen a 1 and tropomyosin present in protein extracts from a range of different invertebrate species. Further, the powerful inhibition of IgE-binding to the different extracts by soluble rPen a 1, in most of the sera analyzed, illustrates that a large proportion of an IgE response to any of these food allergen sources may be attributable to tropomyosin. Taking these and other authors' data [3, 4, 16] into consideration, the clinical manifestation of food allergy to crustaceans and other invertebrates, with patients typically showing sensitivity to multiple species, clearly reflects the basic immunochemistry of the antibody response involved. The same consideration would also apply to reported and anecdotal evidence of association between allergy to house dust mite and invertebrate foods [11, 14, 16], and in particular the occurrence of such food allergies following specific immunotherapy with house dust mite extract [13, 26, 27].

While tropomyosin is a very dominant allergen in shrimp and other crustaceans, it has a much less prominent role in sensitization to house dust mite or cockroach, whose allergenicity is dominated by other components [28–33]. Two of the sera used in this study (A and E) showed an IgE-reactivity to house dust mite extract which by far exceeded that to pure tropomyosin, indicating sensitization predominantly to mite-specific allergens in those serum donors. Thus, the more limited inhibition of IgE-binding to mite extract by soluble rPen a 1 observed in the serum pool experiment may in part be explained by the presence of non-tropomyosin sensitization in the sera used, and not primarily reflect the level of cross-reactivity between shrimp and mite tropomyosins. A more thorough assessment of cross-reactivity, unaffected by confounding antibody reactivities, would require an experimental system employing pure tropomyosin from the different allergen sources of interest.

In conclusion, we describe a method for high-yield production of recombinant shrimp tropomyosin, essentially identical to natural tropomyosin with respect to several relevant biochemical and immunological characteristics. We further report a quantitative assay employing rPen a 1 as an antibody-capturing reagent and demonstrate its utility for measurement of specific IgE directed to tropomyosin from a range of different invertebrate allergen sources relevant to food and respiratory allergy. In addition to diagnostic applications in cases of suspected food allergy to crustaceans and other invertebrates, the rPen a 1 immunoassay may be useful for monitoring purposes in relation to house dust mite immunotherapy and possible development of tropomyosin sensitization.

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5 References

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