

Structural and Immunological Characterization of Recombinant Pan b 1, a Major Allergen of Northern Shrimp, *Pandalus borealis*

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Key Words

Recombinant allergens • Tropomyosin • Shrimp • Allergens • Food allergy • IgE • Circular dichroism • Mass spectroscopy • Basophil activation test • Skin prick test

Abstract

Background: Shellfish allergy is one of the major causes of life-threatening allergic reactions to food. The shrimp species *Pandalus borealis* is the commercially most important coldwater shrimp species, and its protein extract is commonly used in shrimp allergy diagnostics. However, the DNA sequence of its major allergen, tropomyosin, designated Pan b 1, was not previously described. Our aim was to identify the cDNA sequence of Pan b 1 and to generate a recombinant protein with similar structure and allergenicity as the natural protein. **Methods:** *P. borealis* shrimps were caught in the Oslofjord (Norway). cDNA from Pan b 1 was generated, an N-terminal histidine tag was added, and the protein was expressed in *Escherichia coli*. The recombinant Pan b 1 was characterized by structural and IgE-binding studies and investigated further with basophil activation tests (BATs) and skin prick tests (SPTs) on Norwegian shrimp-allergic individuals. **Results:** The open reading frame encoded 284 amino acids that shared 97–100% identity with other shrimp tropomyosins. Mass spectroscopy of natural Pan b 1 confirmed the

protein's molecular mass and indicated the absence of post-translational modifications. Circular dichroism spectroscopy revealed virtually identical spectra between recombinant and natural Pan b 1, which together with native PAGE and size exclusion chromatography results indicated a similar structure. Furthermore, immunoblot and ELISA studies as well as BATs and SPTs showed equivalent results of recombinant and natural Pan b 1. **Conclusion:** A recombinant tropomyosin from *P. borealis* was generated that can be used in diagnostics and further studies on tropomyosin allergenicity and specific immunotherapy.

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Introduction

Shellfish allergy is a potentially life-threatening disease that is seldom outgrown [1, 2] and, in some parts of the world, the most common food allergy among adults [3]. The shellfish species that most frequently elicit food-allergic reactions belong to the taxonomic class Crustacea, which includes shrimp, crab, crawfish and lobster. Affected individuals usually display allergic reactivity to multiple crustacean species. Molecular and clinical cross-reactivity was reported between crustaceans and other invertebrate foods such as mussels, oyster, squid and oc-

topus, but also invertebrate aeroallergens such as house dust mite and cockroaches (reviewed by Lopata et al. [1]).

The presence of a heat-stable allergen in shellfish was first identified in shrimp by Hoffman et al. [4], and this allergen was later identified as the muscle protein tropomyosin [5–7]. More than 80% of shrimp-allergic individuals were reported to have serum IgE against shrimp tropomyosin [6, 8–10]. Shrimp tropomyosin also inhibited 75% of IgE binding to whole-shrimp extract in radioallergosorbent tests [6]. The amino acid sequence of invertebrate tropomyosins is highly conserved, with 95% identity between shrimp and storage mite (*Tyrophagus putrescentiae*). Tropomyosin was found to play an important role in the cross-reactivity seen between the different invertebrate species, suggesting tropomyosin to be an invertebrate pan-allergen [1].

Tropomyosin constitutes 20% of the water-soluble protein of shrimp meat [11] and plays a role in the contractile activity of muscle cells [12]. It is present as a homodimer of two α -helical molecules that are generally 284 amino acids long and wound around each other to form a coiled-coil structure of approximately 40 nm in length [12]. Shrimp tropomyosin migrates as a 34- to 39-kDa band in SDS-PAGE [1], although the calculated molecular mass of the amino acids is 33 kDa. Muscle tropomyosin has been described as a water-soluble protein containing an N-terminal acetylation [12] that might be critical for the polymerization and function of tropomyosin [13]. Shrimp tropomyosin was also reported to contain a small percentage of carbohydrates [4, 6], and a recent study suggested that mature shrimp tropomyosins are formed by the elimination of a leader peptide of 9 amino acids [14].

Shrimp tropomyosin was first cloned from the sand shrimp *Metapenaeus ensis* (Met e 1) by Leung et al. [15] and afterwards from several other shrimp species such as the brown shrimp *Farfantepenaeus aztecus* (Pen a 1) [16] and the Alaskan pink shrimp *Pandalus eous* [17]. Recombinant allergens are considered useful for allergen standardization, improvement of diagnostics and the development of novel strategies of allergen-specific immunotherapy (reviewed by Valenta et al. [18]). The Northern Atlantic shrimp *Pandalus borealis* is the commercially most important coldwater shrimp species [19] and the shrimp species most commonly eaten in Norway. Furthermore, it is frequently used in shrimp extracts for shrimp allergy diagnostics [Phadia ImmunoCap and ALK-Abelló skin prick test (SPT) extract]. However, the sequence of tropomyosin from *P. borealis* has not yet been described. The aim of the present study was therefore to

identify the cDNA sequence of tropomyosin from *P. borealis* and to generate a recombinant form of the protein that can be used in diagnostics and further studies on tropomyosin allergenicity and specific immunotherapy.

Materials and Methods

Bioinformatics

Amino acid alignment and sequence identities were obtained using ClustalW and Boxshade (<http://mobyle.pasteur.fr/cgi-bin/portal.py?forms::boxshade>) with data from GenBank.

cDNA Synthesis and Cloning

P. borealis shrimps were caught in the Oslofjord (Norway). The tips of the tails from live *P. borealis* were cut off and stored at 4°C in RNAlater Stabilization Reagent (Qiagen, Hilden, Germany) until homogenization (MagNALyzer, Roche Diagnostics, Mannheim, Germany) and isolation of total RNA (RNeasy Fibrous Tissue Mini Kit, Qiagen). cDNA was obtained by RT-PCR with random hexamer primers (SuperScript™ III First-Strand Synthesis System for RT-PCR, Invitrogen, Carlsbad, Calif., USA). Tropomyosin cDNA was amplified with gene-specific primers deduced from the untranslated regions of *P. eous* tropomyosin mRNA (GenBank accession No. AB270631; forward primer, 5'-CTTTCTCAGTTCTTTTGTGG-3' and reverse primer, 5'-AAAGTGAATAATGAGATAAATAAGC-3'). The PCR parameters were as follows: 95°C for 1 min, 40 cycles of 95°C for 20 s, 50°C for 20 s and 72°C for 32 s, and finally 72°C for 3 min. The PCR product was sequenced by GATC Biotech (Konstanz, Germany) with the same primers. The identified isoform of *P. borealis* tropomyosin was designated Pan b 1.0101.

The open reading frame of the cDNA was amplified and cloned into the vector pET19b (Novagen, Darmstadt, Germany) using In-Fusion cloning (Clontech, Saint-Germain-en-Laye, France) with 15 overlapping base pairs from the vector, according to the instructions from the manufacturer. The following primers were used: forward primer, 5'-GACGACGACGACAAGATGGA CGCCATCAAGAAGAAG-3' and reverse primer, 5'-GCTTTGT TAGCAGCCTTAGTAGCCAGACAGTTCGCTGA-3' (the overlapping base pairs are underlined). The cDNA clone was expanded in XL10-Gold cells (Stratagene, San Diego, Calif., USA), plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen) and the insert was sequenced by GATC Biotech. The pET19b vector encoding Pan b 1 connected to an N-terminal decahistidine tag and an enterokinase cleavage site (MGHHHHHHHHH-HHSSGHIDDDDK) was then transformed into *Escherichia coli* Rosetta™ 2(DE3) competent cells (Novagen) for expression. The recombinant His-tagged form of Pan b 1.0101 is referred to as rPan b 1 in the text of this article. All primers were synthesized at Eurofins MWG (Ebersberg, Germany).

Expression and Purification of rPan b 1

Expression of rPan b 1 was performed using the Overnight Express™ Autoinduction System 1 (Novagen). Cells were harvested by centrifugation (1 h, 5,500 g, 4°C) and stored at –80°C overnight. Protein was extracted using 5 ml/g of pellet of a denaturing extraction buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imid-

azole, 8 M urea, pH 7.4). rPan b 1 was purified by immobilized metal affinity chromatography (IMAC) using HisPur Cobalt Spin Columns according to the manufacturer's instructions (Pierce Biotechnology, Rockford, Ill., USA). Final purification and buffer exchange to 3-(N-morpholino)propanesulfonic acid (MOPS; 20 mM, 500 mM NaCl, pH 7.4) was performed by size exclusion chromatography [SEC; Superdex 75 pg (16/60), GE Healthcare, UK]. IMAC-purified rPan b 1 was used in the protein identification by mass spectroscopy (MS), while for all the other studies the samples were also SEC-purified.

Protein concentrations were determined according to the Pierce 660 method (Pierce Biotechnology) for samples containing 8 M urea and according to the Lowry method for all other samples (DC protein assay, Bio-Rad, Hercules, Calif., USA) using bovine serum albumin (BSA) as a standard.

His-tag Removal

The decahistidine tag and the additional amino acids of the enterokinase cleavage site were removed by enterokinase cleavage using 1 U per 40 µg of protein for 16 h at room temperature (Enterokinase Cleavage Capture Kit, Novagen).

Identification of rPan b 1 by MS

rPan b 1 was purified with IMAC and SDS-PAGE, and the 39-kDa protein was excised from the Coomassie Blue-stained gel. The protein was digested by 3 µl of trypsin (porcine trypsin, Promega, USA; 100 ng/µl dissolved in 50 mM acetic acid) at a pH of 8 for 16 h at 37°C. The peptides were analyzed on an Ultraflex III matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF)/TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) operated in reflector mode and used at an accelerating potential of 25 kV (at the Proteomics Unit at the University of Bergen, Norway). A number of MS spectra, each generated with 200 shots, were accumulated. The peptide mapping data of the protein were analyzed using the Mascot search engine (<http://www.matrixscience.com/>), with NCBI as database and taxonomy metazoa chosen as parameters.

Whole-Shrimp Extract and Natural Pan b 1

Whole-shrimp extract and natural Pan b 1 were obtained from frozen, peeled, boiled *P. borealis* caught in the Oslofjord (Norway). Whole-shrimp extract was prepared essentially according to the methods reported in the literature with some modifications [20, 21]. Whole shrimp bodies were blended for 3 min in 0.01 M (1:10 w/v) phosphate-buffered saline (PBS) and boiled for 15 min. After stirring at 4°C for 5 h, the proteins were dialyzed for 48 h against several changes of bidistilled water using a 10,000 molecular weight cutoff membrane (Spectrum Laboratories Inc., Rancho Dominguez, Calif., USA) and freeze dried.

Natural Pan b 1 was extracted as previously described [22], followed by SEC purification as described for rPan b 1. The protein concentration of whole-shrimp extract and natural Pan b 1 was determined by the Lowry method.

Molecular Mass Determination of Recombinant and Natural Pan b 1 by MS

Recombinant and natural Pan b 1 were analyzed in the linear TOF mode of an Ultraflex II (Bruker Daltonics) MALDI-TOF/TOF mass spectrometer (at the Biotechnology Centre of Oslo, Norway). The protein samples were purified using a ZipTipC4

(Millipore, Billerica, Mass., USA) and directly eluted onto a stainless steel sample holder with 0.6 µl of matrix solution [20 mg/ml α-cyano-4-hydroxycinnamic acid in 0.3% aqueous trifluoroacetic acid/acetonitrile (2:1)]. After recording the MS spectra, 0.4 µl of horse cytochrome C (1 pmol/µl) was added to the rPan b 1 sample and 0.4 µl of BSA (1 pmol/µl) was added to the natural Pan b 1 sample for internal calibration. MS spectra were transformed into peak lists using the software FlexAnalysis version 2.4 (Bruker Daltonics). The basic settings of the MALDI-TOF/TOF instrument (Ultraflex II, Bruker Daltonics) were as follows: ion source 1, 25 kV; ion source 2, 23.8 kV; lens, 6.80 kV; gating mode with high gating strength; pulsed ion extraction, 140 ns; polarity positive, and suppression up to 7,000 Da.

Circular Dichroism Spectroscopy

Recombinant and natural Pan b 1 were dialyzed against 10 mM KH₂PO₄/K₂HPO₄ buffer (pH 7.4), and the protein concentrations were adjusted to approximately 125 µg/ml. Circular dichroism spectra were recorded and analyzed by Dr. Kristin Gunnarsen (Centre for Immune Regulation, University of Oslo, Norway) as previously described [23].

Study Subjects

Six individuals with positive SPTs to shrimp extract (*P. borealis*, ALK-Abelló A/S, Hørsholm, Denmark) were recruited at the Haukeland University Hospital (Bergen, Norway). For control experiments, 6 individuals with negative SPTs to shrimp extract were included for the basophil activation tests (BATs), and 3 non-atopic individuals were included for the SPTs. Serum samples were stored at -20°C until further analyses. The clinical and laboratory features of the shrimp-allergic individuals are listed in table 1. Approval of the studies involving human subjects was obtained from the Norwegian National Ethical Board.

SDS-PAGE and Immunoblotting

Proteins were separated by SDS-PAGE under reducing conditions using 4–12% Bis-Tris precast gels (Invitrogen) and detected by SimplyBlue SafeStain (Invitrogen). For immunoblot analysis, proteins were electrophoretically transferred from the gels to nitrocellulose membranes (pore size 0.45 µm, Bio-Rad). Membranes were blocked with PBS containing 0.05% Tween-20 (PBST) and 3% horse serum for 1 h, following incubation with patient serum (diluted 1:30) overnight at 4°C in blocking buffer. For IgE detection, membranes were first incubated with rabbit antihuman IgE (1:6,000; DakoCytomation, Glostrup, Denmark) and then with goat antirabbit IgG horseradish peroxidase conjugate (1:5,000; Zymed, San Francisco, Calif., USA), each for 1 h. IgE binding was revealed with 3,3',5,5'-tetramethylbenzidine substrate (Single solution, Zymed). In between each incubation, blots were washed three times with PBST for 15 min. All incubation and washing steps were performed at room temperature with gentle shaking. Novex® Sharp Pre-Stained Protein Standard (Invitrogen) was used as the protein size marker.

Native PAGE

SEC-purified proteins and protein extracts were separated by SDS-PAGE under nonreducing conditions using native 10% Tris-Glycine precast gels (Invitrogen) and were detected by SimplyBlue SafeStain (Invitrogen). NativeMark Unstained Protein Standard (Invitrogen) was used as the protein size marker.

Table 1. Clinical and laboratory features of shrimp-allergic individuals

Donor	Age years	Sex	Dominating symptoms	Total IgE kU/l	sIgE shrimp kU _A /l	sIgE rPen a 1 kU _A /l	SPT shrimp mm	SPT nPan b 1 mm	Positive SPT to other invertebrates
A	27	M	anaphylaxis	328	35.7	39.9	5.0	8.7	HDM
B	49	M	urticaria	71	5.52	4.02	6.4	6.8	<i>Anisakis</i> , <i>Artemia</i> , HDM
C	37	F	anaphylaxis	780	6.26	5.07	7.0	7.1	n.f.
D	38	F	unknown	51	<0.35	<0.35	4.0	7.0	n.f.
E	33	M	anaphylaxis	124	24.5	18.1	13.3	10.4	<i>Anisakis</i> , HDM
F	37	M	urticaria, dyspnea	242	56.3	38.9	9.5	6.2	n.f.

sIgE = Specific IgE; U_A = allergen specific unit; nPan b 1 = natural Pan b 1; HDM = house dust mite; n.f. = none found.

ELISA

Tropomyosin and whole-shrimp extract (1.25 µg/ml in 0.05 M carbonate-bicarbonate buffer, pH 9.6, Sigma-Aldrich) were coated on high-binding, flat-bottomed, 96-well microplates (Corning Inc., Corning, N.Y., USA) at 37°C for 1 h (100 µl/well). Plates were washed with PBST and blocked with 1% w/v BSA in PBST (200 µl/well). Plates were incubated with sera from shrimp-allergic individuals (diluted 1:20) in blocking buffer (100 µl/well) for 1 h at room temperature under gentle shaking. For IgE detection, wells were incubated with rabbit antihuman IgE (1:15,000, 100 µl/well; DakoCytomation), followed by incubation with goat antirabbit IgG horseradish peroxidase conjugate (1:10,000, 100 µl/well; Zymed). Binding was revealed with K-Blue 3,3',5,5'-tetramethylbenzidine substrate solution (75 µl/well; Neogen, Lexington, Ky., USA). The reaction was stopped with 2 M H₂SO₄ (50 µl/well), and the optical density was read at 450 nm.

Basophil Activation Test

The Flow2 CAST was performed according to the manufacturer's instructions (Bühlmann Laboratories, Allschwil, Switzerland) and has been fully described elsewhere [24]. Flow cytometric analysis was performed using an Accuri C6 flow cytometer (Accuri Cytometers Ltd., UK) with CFlow Plus software. In each assay, at least 500 basophils were assessed. The upregulation of the activation marker CD63 was calculated by the percentage of CD63-positive cells compared with the total number of identified basophilic cells.

Skin Prick Test

SPTs were performed with a commercial extract of *P. borealis* (1 mg/ml; ALK-Abelló) and 0.5 mg/ml recombinant and natural tropomyosin diluted in MOPS buffer (20 mM, 500 mM NaCl, pH 7.4). MOPS buffer and histamine hydrochloride (10 mg/ml; ALK-Abelló) were used as negative and positive controls, respectively. Twenty-microliter aliquots of the test solutions were placed on the patients' forearms and pricked in double with a minimum distance of 3 cm between individual application points. Reactions were recorded after 15 min. A positive SPT result to an allergen was defined as a mean duplicate reaction diameter equal to or greater than 3 mm larger than that of the negative control.

Results

Sequence of Pan b 1

The open reading frame of the cDNA identified for *P. borealis* tropomyosin (GenBank accession No. FR728681) was 855 bp long. The encoded protein was assigned the designation Pan b 1.0101 by the International Union of Immunological Societies allergen nomenclature subcommittee. The deduced amino acid sequence of 284 residues (fig. 1) had a calculated molecular mass of 32,753 Da and a theoretical isoelectric point of 4.70. In line with this, MS analysis of natural Pan b 1 revealed a mass of 32,756 Da, indicating the absence of posttranslational modifications. The difference of 3 Da was within the uncertainty of this MS analysis. Alignment of Pan b 1.0101 with other tropomyosins showed a sequence identity of 97–100% for other shrimp species and 81% for house dust mite (fig. 2).

High-Level Expression and Purification of rPan b 1

rPan b 1 was expressed in *E. coli* including an N-terminal purification tag (rPan b 1.0101-His₁₀Ek). Protein expression yielded 10–16 g of wet-weight *E. coli* per liter of medium. Up to 37 mg of rPan b 1 per gram of *E. coli*, equivalent to 407 mg/l of medium, was obtained from one round of IMAC purification. Reapplying the flow-through to a new IMAC column yielded another 18% compared to the yield from the first column.

The molecular mass of rPan b 1 was calculated to be 35,384 Da and confirmed by MS analysis, which revealed 2 major peaks at 35,303 and 35,347 Da. The difference from the calculated mass corresponds to a loss of the initial methionine and a partial acetylation. In SDS-PAGE analysis, rPan b 1 appeared as a 39-kDa protein and natural Pan b 1 as a 37-kDa protein (fig. 3a).

Fig. 1. cDNA sequence and deduced amino acid sequence of Pan b 1.0101 (GenBank accession No. FR728681, UniProt accession No. E5BBS3). Pan b 1.0101 is 284 amino acids long and has a theoretical isoelectric point of 4.70 and a calculated molecular mass of 32,753 Da.

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ATG GAC GCC ATC AAG AAG AAG ATG CAG GCT ATG AAG CTC GAG AAG GAC AAC GCC ATG GAC
M D A I K K K M Q A M K L E K D N A M D

AGG GCG GAT ACT CTC GAG CAG CAG AAC AAG GAG GCC AAC AAC AGG GCT GAG AAG TCC GAG
R A D T L E Q Q N K E A N N R A E K S E

GAG GAG GTT TTC GGC CTT CAG AAG AAG CTG CAG CAG CTT GAG AAC GAC CTC GAC AGT GTA
E E V F G L Q K K L Q Q L E N D L D S V

CAG GAA GCT CTG CTG AAG GCT AAC CAA CAC CTT GAG GAG AAG GAC AAG GCT CTC TCT AAC
Q E A L L K A N Q H L E E K D K A L S N

GCT GAG GGT GAG GTT GCC GCT CTT AAC CGT CGC ATC CAG CTT CTA GAG GAG GAC CTC GAG
A E G E V A A L N R R I Q L L E E D L E

AGG TCT GAG GAG CGA CTC AAC ACT GCC ACC ACC AAG TTG GCC GAG GCT TCC CAG GCA GCC
R S E E R L N T A T T K L A E A S Q A A

GAC GAG TCC GAG CGT ATG CGC AAG GTG CTC GAG AAT CGT TCC CTC TCC GAC GAG GAG CGC
D E S E R M R K V L E N R S L S D E E R

ATG GAC GCC CTC GAG AAC CAA CTC AAG GAA GCC CGA TTC CTG GCT GAA GAA GCC GAC AGG
M D A L E N Q L K E A R F L A E E A D R

AAA TAC GAC GAG GTC GCC CGT AAG CTG GCC ATG GTT GAA GCT GAT CTT GAG CGA GCA GAG
K Y D E V A R K L A M V E A D L E R A E

GAG CGC GCC GAG ACC GGT GAA TCA AAG ATC GTT GAG CTT GAG GAG GAG CTC CGC GTC GTT
E R A E T G E S K I V E L E E E L R V V

GGC AAC AAC CTG AAG TCT CTC GAA GTG TCC GAG GAG AAG GCC AAC CAG CGT GAA GAA GCC
G N N L K S L E V S E E K A N Q R E E A

TAC AAG GAA CAG ATT AAG ACT CTC ACC AAC AAG CTG AAG GCG GCT GAG GCC CGC GCT GAG
Y K E Q I K T L T N K L K A A R A E

TTC GCT GAG AGA TCT GTG CAG AAG CTC CAG AAG GAG GTC GAC AGG CTC GAA GAC GAA CTG
F A E R S V Q K L Q K E V D R L E D E L

GTT AAC GAA AAG GAG AAG TAC AAG TCA ATT ACC GAC GAG CTC GAC CAG ACT TTC AGC GAA
V N E K E K Y K S I T D E L D Q T F S E

CTG TCT GGC TAC TAA
L S G Y Stop

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Native PAGE indicated that both proteins were dimerized (fig. 3b). rPan b 1 also appeared as a dimer on native PAGE when applied directly onto the gel in denaturing buffer (8 M urea; data not shown).

Peptide mass fingerprint analysis of rPan b 1 provided sequences of 31 peptides that covered 173 amino acids in total, representing a sequence coverage of 57% of the complete rPan b 1 amino acid sequence (data not shown).

Circular dichroism analysis revealed virtually identical spectra between both natural and recombinant Pan b 1, with maxima at 192 nm and minima at 208 and 222 nm, typical for α -helical proteins such as tropomyosins [25–29] (fig. 4). The ratio of molar ellipticity ($[\theta]_{222}/[\theta]_{208}$) was 1.037 and 1.068 for natural and recombinant Pan b 1, respectively.

Strong IgE Binding to rPan b 1 in Immunoblots

All 6 sera showed strong binding to rPan b 1 and weaker binding to natural Pan b 1 and whole-shrimp extract

in immunoblots (fig. 5). No binding to the control protein human albumin was detected (data not shown). SDS-PAGE analyses of enterokinase-treated Pan b 1 showed that approximately half the protein present was cleaved during a 16-hour incubation period (fig. 6a), visible as a distinct band of approximately 37 kDa below the uncleaved protein of approximately 39 kDa and a fragment of approximately 3.5 kDa. In addition, enterokinase cleavage resulted in the appearance of several other fragments, including a strong representation of a fragment of approximately 19 kDa. Immunoblot analysis showed strong IgE binding to both cleaved and uncleaved rPan b 1 and the fragment of 19 kDa, while no IgE binding to the small fragment of 3.5 kDa was observed (fig. 6b). A control cleavage of natural Pan b 1 with enterokinase resulted in two clear fragments in SDS-PAGE of approximately 17 and 19 kDa and several truncated versions of whole Pan b 1 and the 17- and 19-kDa fragments, but no fragment of 3.5 kDa (data not shown).

	10	20	30	40	50	60
Northern shrimp	MDAIKKKQAMKLEKDNAMD	RADTLEQQNKEANNRAEKSEEEV	FLGQKKLQQL	ENLDLDSV		
Alaskan pink shrimp	MDAIKKKQAMKLEKDNAMD	RADTLEQQNKEANNRAEKSEEEV	FLGQKKLQQL	ENLDLDSV		
North Sea shrimp	MDAIKKKQAMKLEKDNAMD	RADTLEQQNKEANNRAEKSEEEV	FLGQKKLQQL	ENLDLDSV		
Brown shrimp	MDAIKKKQAMKLEKDNAMD	RADTLEQQNKEANNRAEKSEEEV	FLGQKKLQQL	ENLDLDSV		
Whiteleg shrimp	MDAIKKKQAMKLEKDNAMD	RADTLEQQNKEANNRAEKSEEEV	FLGQKKLQQL	ENLDLDSV		
Black tiger shrimp	MDAIKKKQAMKLEKDNAMD	RADTLEQQNKEANNRAEKSEEEV	FLGQKKLQQL	ENLDLDSV		
Sand shrimp	-----MKLEKDNAMD	RADTLEQQNKEANNRAEKSEEEV	FLGQKKLQQL	ENLDLDSV		
American lobster	MDAIKKKQAMKLEKDNAMD	RADTLEQQNKEANNRAEKSEEEV	FLGQKKLQQL	ENLDLDSV		
King crab	MDAIKKKQAMKLEKDNAMD	RADTLEQQNKEANNRAEKSEEEV	FLGQKKLQQL	ENLDLDSV		
Storage mite	MDAIKKKQAMKLEKDNAMD	RADTLEQQNKEANNRAEKSEEEV	FLGQKKLQQL	ENLDLDSV		
German cockroach	MDAIKKKQAMKLEKDNAMD	RADTLEQQNKEANNRAEKSEEEV	FLGQKKLQQL	ENLDLDSV		
House dust mite	MDAIKKKQAMKLEKDNAMD	RADTLEQQNKEANNRAEKSEEEV	FLGQKKLQQL	ENLDLDSV		
Human	MDAIKKKQAMKLEKDNAMD	RADTLEQQNKEANNRAEKSEEEV	FLGQKKLQQL	ENLDLDSV		
	70	80	90	100	110	120
Northern shrimp	QEALLKANQHLEEKDKALSNAE	GEVAALNRRIQLEEDLSEER	LNNTATTTKLAEASQAA			
Alaskan pink shrimp	QEALLKANQHLEEKDKALSNAE	GEVAALNRRIQLEEDLSEER	LNNTATTTKLAEASQAA			
North Sea shrimp	QEALLKANQHLEEKDKALSNAE	GEVAALNRRIQLEEDLSEER	LNNTATTTKLAEASQAA			
Brown shrimp	QEALLKANQHLEEKDKALSNAE	GEVAALNRRIQLEEDLSEER	LNNTATTTKLAEASQAA			
Whiteleg shrimp	QEALLKANQHLEEKDKALSNAE	GEVAALNRRIQLEEDLSEER	LNNTATTTKLAEASQAA			
Black tiger shrimp	QEALLKANQHLEEKDKALSNAE	GEVAALNRRIQLEEDLSEER	LNNTATTTKLAEASQAA			
Sand shrimp	QEALLKANQHLEEKDKALSNAE	GEVAALNRRIQLEEDLSEER	LNNTATTTKLAEASQAA			
American lobster	QEALLKANQHLEEKDKALSNAE	GEVAALNRRIQLEEDLSEER	LNNTATTTKLAEASQAA			
King crab	QEALLKANQHLEEKDKALSNAE	GEVAALNRRIQLEEDLSEER	LNNTATTTKLAEASQAA			
Storage mite	QEALLKANQHLEEKDKALSNAE	GEVAALNRRIQLEEDLSEER	LNNTATTTKLAEASQAA			
German cockroach	MEQLMQVNAKDEKDKALNAE	GEVAALNRRIQLEEDLSEER	LNNTATTTKLAEASQAA			
House dust mite	MEQLMQVNAKDEKDKALNAE	GEVAALNRRIQLEEDLSEER	LNNTATTTKLAEASQAA			
Human	SEALKDAQEKLEAEKATDAE	ALVAALNRRIQLEEDLSEER	LNNTATTTKLAEASQAA			
	130	140	150	160	170	180
Northern shrimp	DESERMRKVLNRSLSDEERMD	ALLENQLEAEFLAEADRK	YDEVARKLAMVEADLERAE			
Alaskan pink shrimp	DESERMRKVLNRSLSDEERMD	ALLENQLEAEFLAEADRK	YDEVARKLAMVEADLERAE			
North Sea shrimp	DESERMRKVLNRSLSDEERMD	ALLENQLEAEFLAEADRK	YDEVARKLAMVEADLERAE			
Brown shrimp	DESERMRKVLNRSLSDEERMD	ALLENQLEAEFLAEADRK	YDEVARKLAMVEADLERAE			
Whiteleg shrimp	DESERMRKVLNRSLSDEERMD	ALLENQLEAEFLAEADRK	YDEVARKLAMVEADLERAE			
Black tiger shrimp	DESERMRKVLNRSLSDEERMD	ALLENQLEAEFLAEADRK	YDEVARKLAMVEADLERAE			
Sand shrimp	DESERMRKVLNRSLSDEERMD	ALLENQLEAEFLAEADRK	YDEVARKLAMVEADLERAE			
American lobster	DESERMRKVLNRSLSDEERMD	ALLENQLEAEFLAEADRK	YDEVARKLAMVEADLERAE			
King crab	DESERMRKVLNRSLSDEERMD	ALLENQLEAEFLAEADRK	YDEVARKLAMVEADLERAE			
Storage mite	DESERMRKVLNRSLSDEERMD	ALLENQLEAEFLAEADRK	YDEVARKLAMVEADLERAE			
German cockroach	DESERMRKVLNRSLSDEERMD	ALLENQLEAEFLAEADRK	YDEVARKLAMVEADLERAE			
House dust mite	DESERMRKVLNRSLSDEERMD	ALLENQLEAEFLAEADRK	YDEVARKLAMVEADLERAE			
Human	DESERMRKVLNRSLSDEERMD	ALLENQLEAEFLAEADRK	YDEVARKLAMVEADLERAE			
	190	200	210	220	230	240
Northern shrimp	ERAETGESKIVELEELRVVGN	NLKSLEVSEKANQREEAYKE	QIKITLTKNLKAAEAREAE			
Alaskan pink shrimp	ERAETGESKIVELEELRVVGN	NLKSLEVSEKANQREEAYKE	QIKITLTKNLKAAEAREAE			
North Sea shrimp	ERAETGESKIVELEELRVVGN	NLKSLEVSEKANQREEAYKE	QIKITLTKNLKAAEAREAE			
Brown shrimp	ERAETGESKIVELEELRVVGN	NLKSLEVSEKANQREEAYKE	QIKITLTKNLKAAEAREAE			
Whiteleg shrimp	ERAETGESKIVELEELRVVGN	NLKSLEVSEKANQREEAYKE	QIKITLTKNLKAAEAREAE			
Black tiger shrimp	ERAETGESKIVELEELRVVGN	NLKSLEVSEKANQREEAYKE	QIKITLTKNLKAAEAREAE			
Sand shrimp	ERAETGESKIVELEELRVVGN	NLKSLEVSEKANQREEAYKE	QIKITLTKNLKAAEAREAE			
American lobster	ERAETGESKIVELEELRVVGN	NLKSLEVSEKANQREEAYKE	QIKITLTKNLKAAEAREAE			
King crab	ERAETGESKIVELEELRVVGN	NLKSLEVSEKANQREEAYKE	QIKITLTKNLKAAEAREAE			
Storage mite	ERAETGESKIVELEELRVVGN	NLKSLEVSEKANQREEAYKE	QIKITLTKNLKAAEAREAE			
German cockroach	ERAETGESKIVELEELRVVGN	NLKSLEVSEKANQREEAYKE	QIKITLTKNLKAAEAREAE			
House dust mite	ERAETGESKIVELEELRVVGN	NLKSLEVSEKANQREEAYKE	QIKITLTKNLKAAEAREAE			
Human	ERAETGESKIVELEELRVVGN	NLKSLEVSEKANQREEAYKE	QIKITLTKNLKAAEAREAE			
	250	260	270	280	Identity	
Northern shrimp	FAERSVQKLQKEVDRL	DELVNEKEKYKSITDEL	QTFSELSGY			
Alaskan pink shrimp	FAERSVQKLQKEVDRL	DELVNEKEKYKSITDEL	QTFSELSGY			100.0%
North Sea shrimp	FAERSVQKLQKEVDRL	DELVNEKEKYKSITDEL	QTFSELSGY			98.6%
Brown shrimp	FAERSVQKLQKEVDRL	DELVNEKEKYKSITDEL	QTFSELSGY			96.8%
Whiteleg shrimp	FAERSVQKLQKEVDRL	DELVNEKEKYKSITDEL	QTFSELSGY			96.8%
Black tiger shrimp	FAERSVQKLQKEVDRL	DELVNEKEKYKSITDEL	QTFSELSGY			96.8%
Sand shrimp	FAERSVQKLQKEVDRL	DELVNEKEKYKSITDEL	QTFSELSGY			96.7%
American lobster	FAERSVQKLQKEVDRL	DELVNEKEKYKSITDEL	QTFSELSGY			96.5%
King crab	FAERSVQKLQKEVDRL	DELVNEKEKYKSITDEL	QTFSELSGY			96.1%
Storage mite	FAERSVQKLQKEVDRL	DELVNEKEKYKSITDEL	QTFSELSGY			94.7%
German cockroach	FAERSVQKLQKEVDRL	DELVNEKEKYKSITDEL	QTFSELSGY			82.4%
House dust mite	FAERSVQKLQKEVDRL	DELVNEKEKYKSITDEL	QTFSELSGY			80.6%
Human	FAERSVQKLQKEVDRL	DELVNEKEKYKSITDEL	QTFSELSGY			57.4%

Fig. 2. Amino acid sequence alignment and percentage identity of Pan b 1.0101 with other allergenic invertebrate tropomyosins and the vertebrate human α -tropomyosin. Crustacea: Northern shrimp (*Pandalus borealis*, Pan b 1.0101, CBY17558), Alaskan pink shrimp (*Pandalus eous*, BAF47264), North Sea shrimp (*Crangon crangon*, FJ457621), brown shrimp (*Farfantepenaeus aztecus*, Pen a 1.0101, AAZ76743), whiteleg shrimp (*Litopenaeus vannamei*, Lit v 1.0101, ACB38288), black tiger shrimp (*Penaeus monodon*, Pen m 1.0101, AAX37288), sand shrimp (*Metapenaeus*

ensis, Met e 1.0101, AAA60330), American lobster (*Homarus americanus*, Hom a 1.0102, AAC48288) and king crab (*Paralithodes camtschaticus*, Par c 1, BAF47265). Insecta: German cockroach (*Blattella germanica*, Bla g 7.0101, AAF72534). Arachnida: storage mite (*Tyrophagus putrescentiae*, Tyr p 10.0101, ABQ96644) and house dust mite (*Dermatophagoides pteronyssinus*, Der p 10.0101, ACI32128). Vertebrata: human (*Homo sapiens*, tropomyosin α -1 chain isoform 1, NP_001018005).

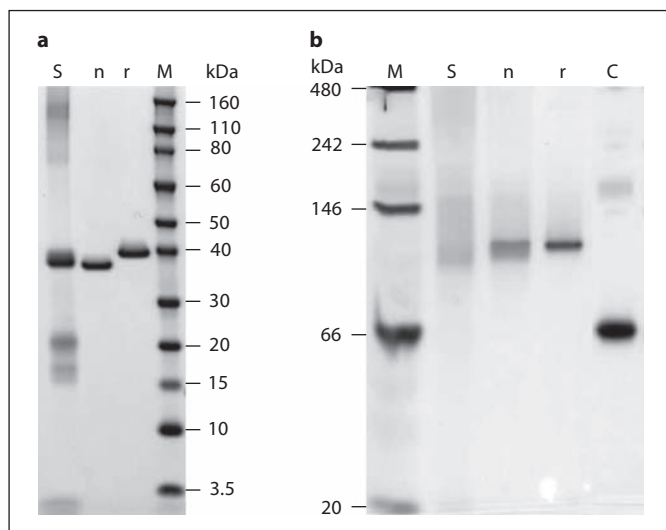


Fig. 3. A comparison of recombinant and natural Pan b 1 by SDS-PAGE (**a**) and native PAGE (**b**). S = Shrimp extract (6 μ g in **a**, 3.4 μ g in **b**); n = natural Pan b 1 (1.2 μ g); r = rPan b 1 (1.2 μ g); C = control protein human albumin (12 μ g); M = marker.

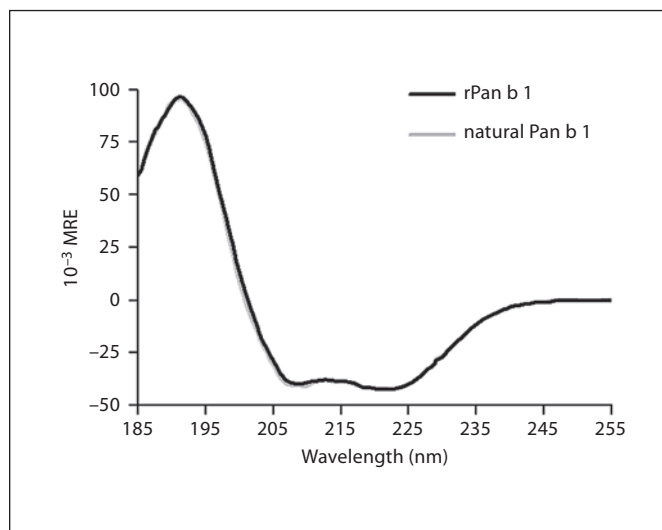


Fig. 4. Circular dichroism spectra of recombinant and natural Pan b 1. The two spectra are almost identical, showing minima at approximately 208 and 222 nm, characteristic for α -helical proteins. MRE = Mean molar ellipticity per residue.

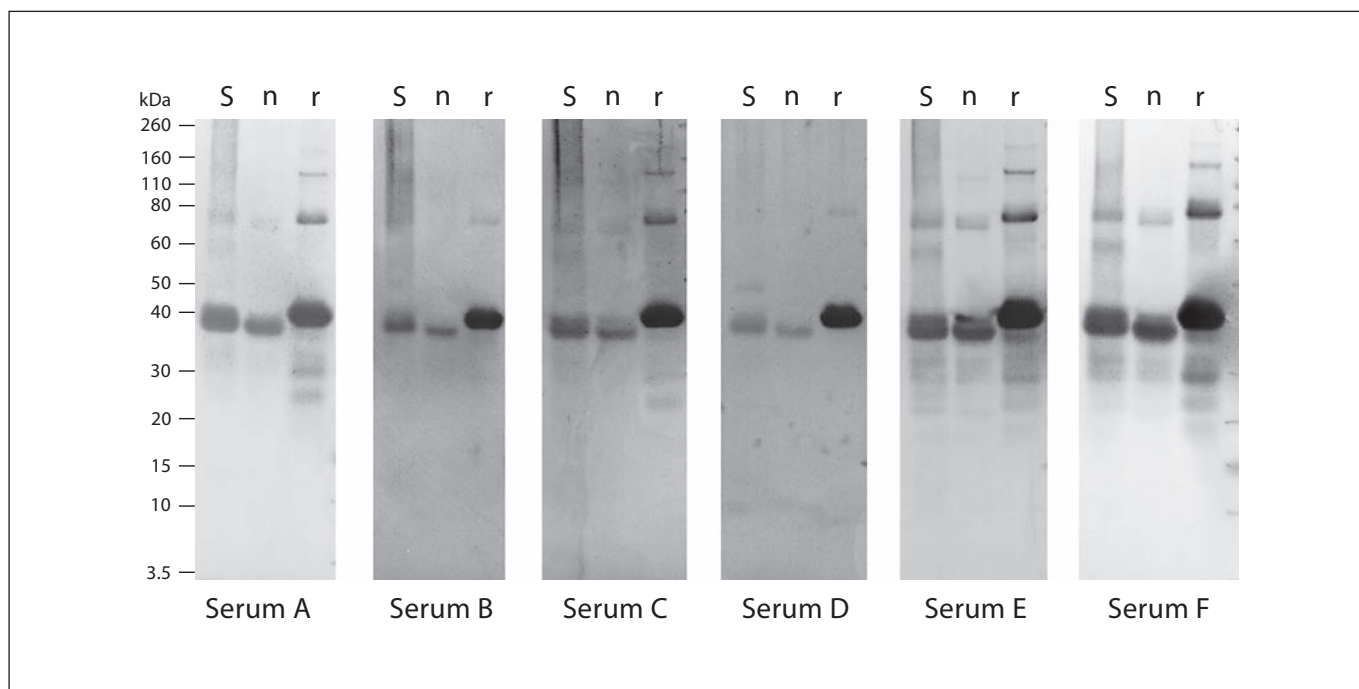


Fig. 5. Immunoblot comparison of human IgE binding to recombinant and natural Pan b 1. S = Shrimp extract (6 μ g); n = natural Pan b 1 (1.2 μ g); r = rPan b 1 (1.2 μ g).

Comparable IgE Binding to Recombinant and Natural Pan b 1 in ELISA

Similar IgE binding to both recombinant and natural Pan b 1 and also whole-shrimp extract was observed in ELISA with all 6 sera (fig. 7a). Serial dilution studies showed that the similarity in binding to the proteins was not dependent on the serum concentration (fig. 7b).

BATs and SPTs

rPan b 1 induced a similar percentage of activation of basophils to natural Pan b 1 (fig. 8a). The whole-shrimp extract induced a comparable percentage of activation of basophils to recombinant and natural Pan b 1 in the blood of 4 patients, whereas basophils of patients D and E were activated more strongly by the whole-shrimp extract. All 6 controls were negative to whole-shrimp extract, natural Pan b 1 and rPan b 1 in the BAT (data not shown).

In SPTs, rPan b 1 induced positive wheal sizes that were equivalent to or larger than those induced by natural tropomyosin in 5 out of 6 patients (fig. 8b). The 3 controls were negative to all allergens tested (data not shown).

Discussion

In the present study, cloning, expression and characterization of the important allergen of *P. borealis*, tropomyosin, was described, resulting in a recombinant protein of similar structural and immunological properties as the natural protein. The recombinant protein will be used for the development of novel strategies of allergen-specific immunotherapy by our research group, which involves Norwegian shrimp-allergic individuals who are likely to have a high reactivity towards Northern shrimp, *P. borealis*.

MS analysis of natural Pan b 1 revealed the molecular mass of the 284-amino acid protein encoded by the open reading frame, without any posttranslational modifications. This is in contrast to earlier reports of N-terminal acetylation [12], carbohydrate modifications [4, 6] and cleavage of a 9-amino acid leader peptide for other shrimp tropomyosins [14]. Although this N-terminal acetylation has been stated to be critical for the function of muscle tropomyosin [13], it was not detected for snow crab tropomyosin [30], which together with the current study suggests that a negatively charged N terminus might not be a requirement for the polymerization and function of tropomyosin.

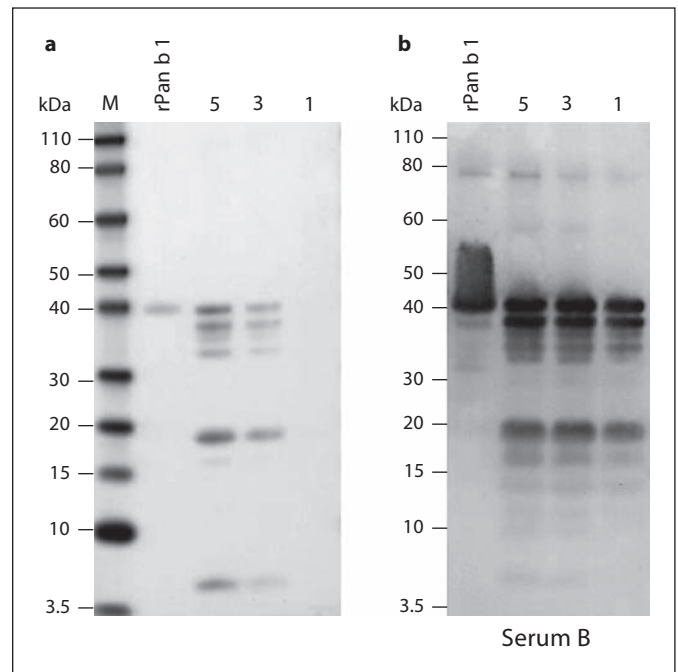


Fig. 6. IgE binding to enterokinase-treated rPan b 1. **a** SDS-PAGE of rPan b 1 treated with enterokinase for 16 h. **b** Immunoblot analysis of IgE binding to enterokinase-treated rPan b 1 by serum from a shrimp-allergic individual (serum B). The numbers 5, 3 and 1 represent 5, 3 and 1 µg of enterokinase-treated denatured rPan b 1 per lane, respectively. One microgram of untreated denatured rPan b 1 was added. M = Marker.

Our protocol provided a yield of His-tag-purified recombinant shrimp tropomyosin 80 times higher than that reported in the expression and purification protocol for rPen a 1 (5 mg/l medium) by Albrecht et al. [25]. The choice of expression host and autoinduction of protein expression might have favored the high yield. Both protocols employed IMAC columns loaded with cobalt. DeWitt et al. [27] reported an even higher yield per liter of medium (rPen a 1, 700 mg/l medium), despite a lower yield per gram of *E. coli* (8 vs. 37 mg/g), which can be explained by the use of a bioreactor optimized for high-density *E. coli* fermentation in that study (pers. commun., Research Manager Jonas Lidholm, Phadia AB).

Immunoblots of enterokinase-treated rPan b 1 showed that the 22-amino acid tag did not influence the IgE binding to rPan b 1. In addition, a strong unspecific cleavage was found for both natural and recombinant Pan b 1. The presence of an aspartic acid in position 137 of Pan b 1 might facilitate cleavage by enterokinase after Arg133, as seen for other proteases [12, 31], which would result in the

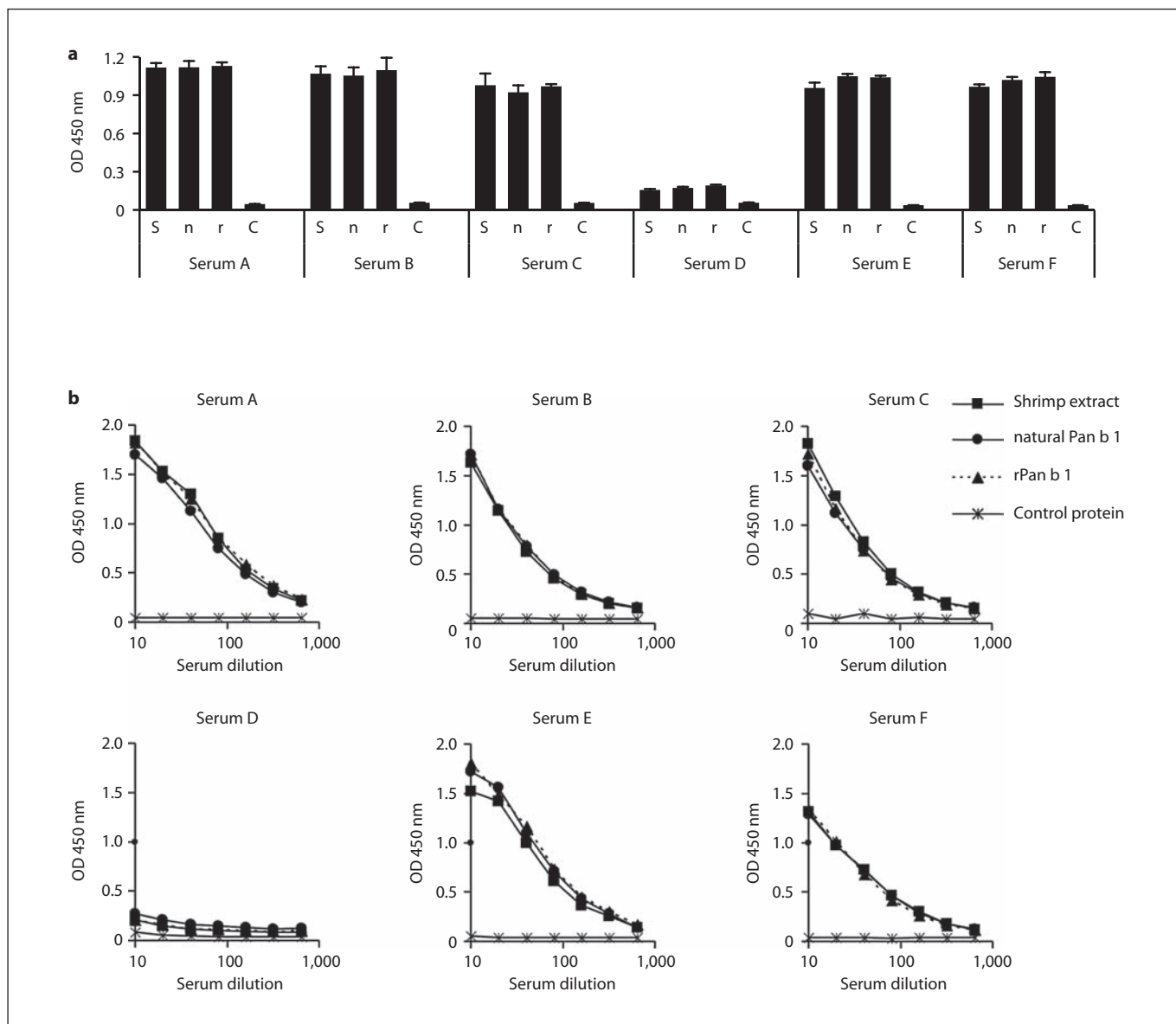


Fig. 7. Comparison of human IgE binding to recombinant and natural Pan b 1 by ELISA. **a** ELISA with 1.25 $\mu\text{g/ml}$ immobilized antigen and a 1:20 serum dilution. **b** ELISA with 1.25 $\mu\text{g/ml}$ immobilized antigen and a serum dilution series. S = Shrimp extract; n = natural Pan b 1; r = rPan b 1; C = control protein human albumin; OD = optical density.

observed fragment sizes. Asp137 is a negatively charged core residue conserved among all muscle tropomyosins, causing a destabilized region in the middle of tropomyosin. This region is responsible for the functionally necessary flexibility of the protein, but also creates a 'weak spot' of unfolded tropomyosin that might be accessible for proteolytic enzymes. Based on our current results, we would not recommend the use of enterokinase for large-scale tag removal from recombinant tropomyosin.

The Alaskan pink shrimp, *P. eous*, was until recently regarded as a variety of *P. borealis*, but after Squires' (1992) findings on differences in adult morphology and larval sizes they are now officially regarded as two separate species [32]. The cDNA sequence for *P. borealis* tropomyosin described here was found to be identical to the tropomyosin sequence of *P. eous*, which demonstrates their phylogenetical proximity. Compared to tropomyosins from the more widely studied shrimp species *F. az-*

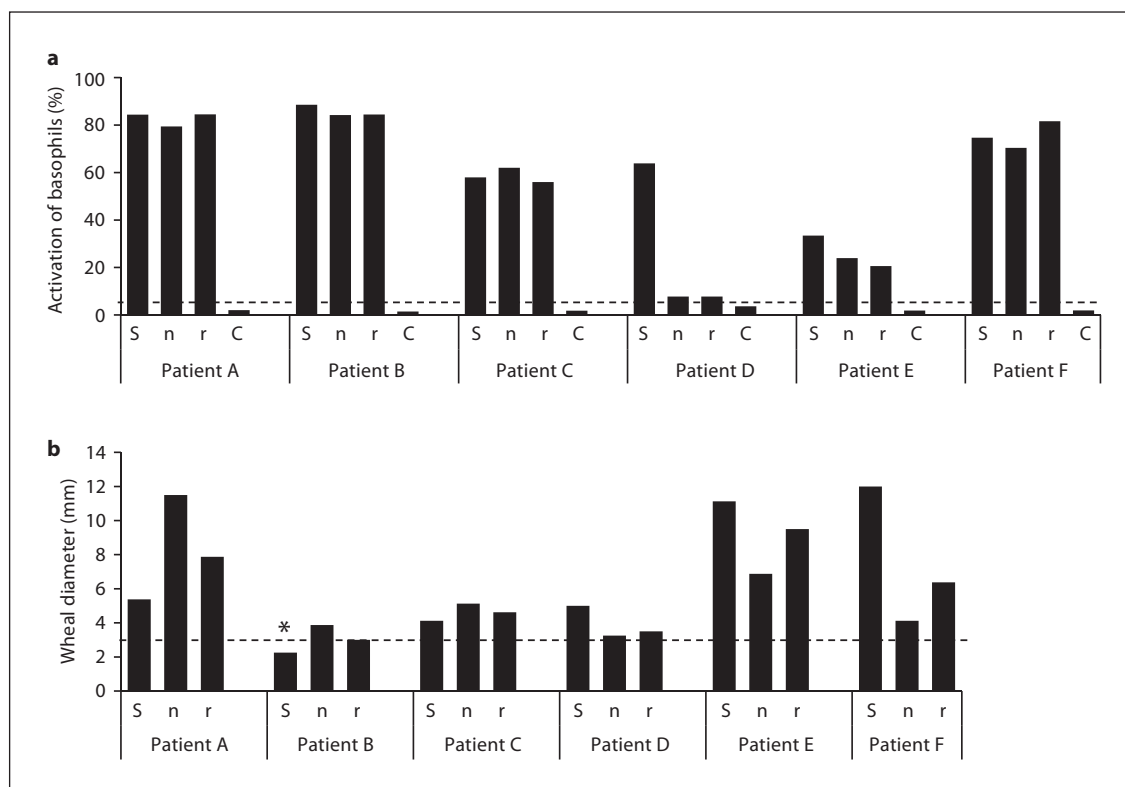


Fig. 8. BATs and SPTs with shrimp extract and natural and recombinant Pan b 1. **a** BATs with 1 µg/ml of each allergen. S = Shrimp extract; n = natural Pan b 1; r = rPan b 1; C = MOPS buffer control. **b** SPTs. S = Shrimp extract (928 µg/ml); n = natural Pan b 1 (500 µg/ml = 15,265 µM); r = rPan b 1 (540 µg/ml = 15,265 µM). * Patient B had a positive SPT for *P. borealis* extract (7 mm) in the initial screening (table 1).

tecus (brown shrimp), *Litopenaeus vannamei* (whiteleg shrimp), *Penaeus monodon* (black tiger shrimp) and *M. ensis* (sand shrimp), Pan b 1.0101 differs in 9 amino acids that are localized between amino acid number 44 and 72. Four of these 9 amino acid substitutions are inside the region previously identified as major IgE-binding region 1 out of 5 by Ayuso et al. [33].

Six different allergens have so far been identified from shrimp (tropomyosin, arginine kinase, sarcoplasmic calcium-binding protein, myosin light chain, troponin C and triose phosphate isomerase). However, tropomyosin is still considered the major allergen in shrimp [1, 17, 34]. This was recently confirmed for the coldwater shrimp species *Crangon crangon* (North Sea shrimp) [34], a species whose tropomyosin differs from *P. borealis* tropomyosin in only 1 amino acid. In addition, the recruitment of shrimp-allergic individuals for the current study acquired 9 patients who were SPT positive to *P. borealis* extract. Of these 9 individuals, 8 were clearly positive to

Pan b 1 in SPTs and/or BATs. This indicates that, at least for Norwegian shrimp-allergic individuals, Pan b 1 is a major allergen in *P. borealis*.

Throughout all our experiments, we observed stronger human IgE binding to rPan b 1 than natural Pan b 1 in immunoblots, which cannot be explained by differences in the amounts of protein loaded. Natural Pan b 1 was extracted from boiled shrimps, but this was not associated with reduced IgE binding to tropomyosin in previous studies [21, 35]. The reason for the differences in IgE binding in immunoblots therefore remains elusive.

In conclusion, in the current study, the cDNA sequence, structure, IgE binding and diagnostic usefulness of recombinant tropomyosin from *P. borealis* are described for the first time. In addition, the study contributes to the knowledge of tropomyosin stability and post-translational modifications and provides a time-saving procedure for the cloning, expression and purification of

recombinant tropomyosin in *E. coli* with high protein yields. The tropomyosin generated in the current study has already been proven useful in shrimp allergy diagnostics (SPTs and BATs) and will be further investigated for this purpose and for the improvement of detection of allergens in food. The recombinant protein will also be employed in the development of novel strategies of allergen-specific immunotherapy.

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