

Mapping of the Immunodominant Regions of Shrimp Tropomyosin Pan b 1 by Human IgE-Binding and IgE Receptor Crosslinking Studies

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Key Words

IgE receptor crosslinking · Epitope mapping · IgE epitope · Basophil activation test · Bis(sulfosuccinimidyl) suberate · Immunoblot · Native PAGE · Mass spectroscopy · Recombinant allergens · Food allergy · Circular dichroism

Abstract

Background: Epitope mapping of an allergen is generally done by IgE-binding assays with short synthetic peptides, but this provides little information about which domains are responsible for IgE receptor crosslinking on effector cells. Our aim was to map the immunodominant regions of shrimp tropomyosin by both IgE-binding and IgE-receptor crosslinking studies. **Methods:** Five overlapping fragments covering *Pandalus borealis* tropomyosin were cloned, expressed in *Escherichia coli* and characterized by circular dichroism spectroscopy, native PAGE and bis(sulfosuccinimidyl) suberate-crosslinking. IgE binding was detected by Western blot, indirect ELISA and inhibition ELISA, and IgE receptor crosslinking was investigated by basophil activation test and skin prick test with Norwegian shrimp allergic adults. **Results:** The N- and C-terminal fragments of tropomyosin showed the highest amount of secondary structure. Western blot studies showed preferential binding to the terminal fragments, while indirect and inhibition ELISA studies showed

binding to all fragments, but with individual variations. Basophil CD63 expression was upregulated by all fragments at high concentrations (1 µg/ml) and showed individual variations comparable to ELISA results. A mixture of the fragments with equal molar ratios induced comparably strong CD63 activation as for tropomyosin. Skin prick test studies showed positive responses to the terminal and middle fragments and increased responses to the fragment mixture compared to whole tropomyosin. **Conclusions:** The terminal and middle fragments of tropomyosin had the highest IgE reactivity, but overall no clear immunodominant region was observed in this study. These results correlated well with previous studies with short peptides. Dividing shrimp tropomyosin into five fragments did not reduce the allergenicity of the protein.

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Introduction

Food allergy, and in particular IgE-mediated food allergy, is a growing health problem worldwide [1, 2]. Still, there is no treatment available. Identification of the most important allergens and mapping of their major IgE-binding epitopes are useful for better diagnostics and development of safe food allergy vaccines [3].

An IgE antibody might bind specifically to amino acids adjacent to each other (linear or sequential epitopes) or to amino acids from different parts of a polypeptide chain or different polypeptide chains (structural or conformational epitopes). In the latter case, a correct folding of the protein is necessary for recognition. Most airway allergens contain primarily conformational IgE-binding epitopes. Sequential epitopes play a more important role in food allergens [4–6]. These allergens are often partly unfolded and fragmented by heat treatment and digestive enzymes and thereby display more sequential epitopes to the immune system [7]. At the same time, a common feature of food allergens is their resistance to degradation by heat, acid and enzymes, leading to the uptake of large immunogenic protein fragments [8, 9].

The immunodominant epitope/region of a protein is the epitope/region that provokes the most intense immune response. Mapping of immunodominant IgE-binding epitopes/regions is generally performed by IgE binding to short (<16 amino acid) overlapping synthetic peptides [4, 10–16]. These peptides are too short to display conformational epitopes. They have the potential to be recognized by low-affinity IgE binding but may lack the stable structure of the full epitope that might be necessary for IgE receptor crosslinking and effector cell activation [17].

The heat-stable muscle protein tropomyosin is an invertebrate pan-allergen and the major allergen in shellfish [18]. The protein is composed of two identical α -helices of 33 kDa, wound around each other forming a coiled coil [19]. The rod-like structure suggests that its allergenicity is mainly constituted by sequential epitopes. Previously, five major IgE-binding regions, containing eight sequential IgE-binding epitopes, were identified for shrimp tropomyosin (Pen a 1, *Farfantepenaeus aztecus*) by SPOTS analysis with short synthetic peptides [20, 21]. However, a more recent study found that IgE binding to soluble short tropomyosin peptides identified by SPOTS analysis contributed minimally to the total IgE binding to whole recombinant tropomyosin [17]. Common to these previous tropomyosin studies is the measurement of IgE binding to short peptides [17, 20–24], while the ability to crosslink receptor-bound IgE on effector cells was not studied.

The aim of our study was to investigate the presence of immunodominant regions of shrimp tropomyosin, as measured by effector cell activation, and whether they can be correctly predicted by IgE-binding assays. For this, we generated five overlapping recombinant fragments spanning the entire sequence of tropomyosin from the North-

ern Atlantic shrimp species *Pandalus borealis* (Pan b 1). The amino acid sequence of Pan b 1 was recently identified by our group [25].

Materials and Methods

Cloning

A cDNA clone with Pan b 1.0101 was obtained as described in Myrset et al. [25]. Five overlapping fragments of Pan b 1.0101 cDNA, spanning the entire open reading frame, were amplified and cloned into the vector pET19b (Novagen, Darmstadt, Germany) using In-Fusion cloning (Clontech, Saint-Germain-en-Laye, France) according to the manufacturer's instructions (F1–F5):

F1 forward primer (fp): 5'-GACGACGACGACAAGATGGA CGCCATCAAGAAGAAGATG-3';

F1 reverse primer (rp): 5'-GCTTTGTTAGCAGCCTTAGAG AGCCTTGTCTTCTCTCAAG-3';

F2 fp: 5'-GACGACGACGACAAGGAAGCTCTGCTGAAGG CTAAC-3';

F2 rp: 5'-GCTTTGTTAGCAGCCTTACTCGAGCACCTTGC GCATAC-3';

F3 fp: 5'-GACGACGACGACAAGGACGAGTCCGAGCGTA TG-3';

F3 rp: 5'-GCTTTGTTAGCAGCCTTACTCCTCTGCTCGCT CAAG-3';

F4 fp: 5'-GACGACGACGACAAGGAAGCTGATCTTGAGC GAGCAGAG-3';

F4 rp: 5'-GCTTTGTTAGCAGCCTTACTCAGCCGCCTTCA GCTTGT-3';

F5 fp: 5'-GACGACGACGACAAGAAGACTCTCACCAACA AGCTGAAG-3';

F5 rp: 5'-GCTTTGTTAGCAGCCTTAGTAGCCAGACAGT TCGCTGA-3'

(the base pairs from the vector are underlined). The primers were synthesized at Eurofins MWG (Ebersberg, Germany). The five constructs were expanded in XL10-Gold cells (Stratagene, San Diego, Calif., USA), plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and the inserts were sequenced by GATC Biotech (Konstanz, Germany). The pET19b vectors encoding the five Pan b 1 fragments linked to an N-terminal decahistidine tag and an enterokinase cleavage site (MGHHH-HHHHHHHSSGHIDDDDK) were then transformed into *Escherichia coli* Rosetta™ 2(DE3) competent cells (Novagen) for expression.

Protein Expression and Purification

Protein expression 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 imidazole, 8 M urea, pH 7.4). Proteins were 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 chro-

matography [SEC; Superdex 75 pg (16/60), GE Healthcare, UK]. Protein concentrations were determined according to the Pierce 660 method (Pierce Biotechnology) for samples containing 8 M urea and for the circular dichroism (CD) spectroscopy, and according to the Lowry method for all other samples (DC protein assay, Bio-Rad, Hercules, Calif., USA) using bovine serum albumin (BSA) as standard.

Protein Identification by Mass Spectroscopy (Nano-LC ESI-Orbitrap-MS/MS)

IMAC and SDS-PAGE purified proteins were excised from the Coomassie blue-stained gel. The proteins were reduced, alkylated, digested in gel with trypsin (Porcine trypsin; Promega, USA) overnight at 37°C and extracted [26]. Reverse-phase (C18) nano online liquid chromatographic mass spectroscopy (MS)/MS analysis of tryptic peptides was performed using a system consisting of two Agilent 1200 HPLC binary pumps (nano and capillary) with autosampler, column heater and integrated switching valve (Agilent, Waldbronn, Germany). This LC system was coupled via a nano-electrospray ion source to a LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptide solutions (4 µl) were injected onto the 5 × 0.3-mm extraction column filled with Zorbax 300 SB-C18 of 5 µm particle size (Agilent). Samples were washed with a mobile phase consisting of 97% 0.1% formic acid and 3% acetonitrile. The flow rate was 4 µl/min provided by the capillary pump. After 7 min, the integrated switching valve was activated and peptides were eluted in the back-flush mode from the extraction column onto a 150 × 0.075-mm C18, 3-µm resin column (GlycproSIL C18-80Å; Glycpromass, Stove, Germany). Chromatographic separation was achieved using an acetonitrile/water (0.1% formic acid) binary gradient from 5 to 55% acetonitrile in 70 min and a flow rate of 0.2 µl/min provided by the nanoflow pump. Mass spectra were acquired and data analyzed using the Proteome Discoverer 1.0 software (Thermo Fisher Scientific) and the Mascot search engine (Matrix Science Inc., Boston, Mass., USA) [27].

Natural Pan b 1

nPan b 1 was obtained from frozen, peeled, boiled *P. borealis* caught in the Oslo Fjord (Norway). The protein was extracted as previously described [22], followed by SEC purification as described for the Pan b 1 fragments. The protein concentration was determined by the Lowry method.

Molecular Mass Determination by MS

SEC-purified proteins were analyzed in the linear TOF mode of an Ultraflex II (Bruker Daltonics) MALDI-TOF/TOF mass spectrometer (The Biotechnology Centre of Oslo, Oslo, Norway). The protein samples were purified using a ZipTip C4 (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 samples 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

IMAC-purified proteins were dialyzed against phosphate-buffered saline (PBS; pH 7.4) or 50 mM K₂HPO₄ buffer (pH 10.5) (Slide-A-Lyzer® Dialysis Cassette G2, 3,500 MWCO; Thermo Scientific) and the protein concentrations were adjusted to approximately 160 µg/ml, according to the Pierce 660 method (Pierce Biotechnology). CD spectra were recorded using a Jasco J-810 spectropolarimeter (Jasco International Co., Ltd., Tokyo, Japan). Measurements were performed at 20°C using a quartz cuvette (Starna, Essex, UK) with a path length of 0.1 cm. Samples were scanned at 50 nm/min, using a band width of 1 nm. The response time was 2 s, and the wave length range was 185–245 nm. Data of five readings were averaged, and the spectrum of a sample-free control was subtracted. The temperature was controlled with a TPC-423S/L system (Jasco International Co.). In addition, the Pan b 1 fragments in K₂HPO₄ buffer were diluted 1:1 (v/v) with tetrafluoroethylene (TFE) and rerun. The α-helical content of the Pan b 1 fragments was determined from the mean residue ellipticity at 222 nm $[\theta]_{MR,222}$ according to [28]: $f_H = 100 \cdot [\theta]_{MR,222} / [-40,000 \cdot (1 - 2.5/N)]$ where N is the number of residues in the molecule. The 22 residues from the tags were not included in the calculations.

Study Subjects

Five individuals with a clear clinical history of shellfish allergy and positive skin prick tests (SPTs) to both shrimp extract (*P. borealis*, ALK-Abelló A/S, Hørsholm, Denmark) and shrimp tropomyosin (nPan b 1) were recruited at Haukeland University Hospital (Bergen, Norway). These patients were part of a previous related study including 6 patients [25], and the designation of the patients is therefore continued in the current study (patient A, B, C, E and F). For control experiments, 3 individuals with negative SPTs to shrimp extract were included for the basophil activation tests (BAT), 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. All participants signed a written consent before inclusion in the study.

SDS-PAGE and Immunoblotting

SEC-purified proteins (0.5 µg) were separated by SDS-PAGE under reducing conditions using 4–12% Bis-Tris precast gels (Invitrogen, Carlsbad, Calif., USA) 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 anti-human IgE (1:6,000; DakoCytomation, Glostrup, Denmark) and then with goat anti-rabbit 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). Between the different incubation steps, 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.

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

Donor	Age	Sex	Main symptoms of reaction to ingestion	Reactions n	Years since last reaction	Approximate amount causing reaction	Total IgE kU/l	sIgE shrimp kU _A /l	sIgE rPen a 1 kU _A /l	SPT shrimp mm	SPT Pan b 1 mm
A	27	M	Anaphylaxis	2	14	4 shrimp	328	35.70	39.90	5.0	8.7
B	49	M	Urticaria	>3	20	<1 shrimp	71	5.52	4.02	6.4	6.8
C	37	F	Anaphylaxis	3	30	<1 shrimp	780	6.26	5.07	7.0	7.1
E	33	M	Anaphylaxis	>3	2	<1 shrimp	124	24.50	18.10	13.3	10.4
F	37	M	Urticaria, dyspnea	1	20	2 shrimp	242	56.30	38.90	9.5	6.2

sIgE = Specific IgE; SPT = skin prick test mean duplicate wheal diameter.

Native PAGE and Immunoblotting

SEC-purified proteins (0.5 µg) were separated under non-reducing conditions using native 10% Tris-Glycine pre-cast gels and running buffer with zero salt content (Invitrogen) and were detected by SimplyBlue SafeStain (Invitrogen). Immunoblot analysis was performed as described for SDS-PAGE gels except that the blot was incubated with a pool of the five patient sera. NativeMark™ Unstained Protein Standard (Invitrogen) was used as the protein size marker.

Bis(sulfosuccinimidyl) Suberate Crosslinking

Twenty-five microliter 15,265 pmol/ml of each SEC-purified protein in MOPS buffer were subjected to buffer exchange to PBST using 3-kDa MWCO spin columns (Vivaspin 500; GE Healthcare) adding 2× 475 µl PBST and spinning down to 25–35 µl. Ten microliter of each protein was treated with ~50:1 moles with BS³ [Bis(sulfosuccinimidyl) suberate] (1 µl, 7,633 pmol; Pierce Biotechnology) for 30 min. The reaction was stopped by 1 µl Tris-HCl (0.5 M, pH 7.5) for 15 min at room temperature. Approximately 80 pmol of each protein was separated by SDS-PAGE under reducing conditions using 4–12% Bis-Tris pre-cast gels (Invitrogen) and detected by SimplyBlue SafeStain (Invitrogen).

ELISA with Human Sera

Proteins (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 anti-human IgE (1:15,000, 100 µl/well; DakoCytomation) followed by incubation with goat anti-rabbit 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.

For the inhibition ELISAs, the plates were coated with nPan b 1 and the diluted sera were pre-incubated with a serial dilution of proteins for 2 h at room temperature under gentle shaking. For the

ELISA with single Pan b 1 fragments, the final concentrations were 5, 1.67, 0.56, 0.19, 0.06 and 0.02 µg/ml. For the ELISA with Pan b 1 fragment mixture, final concentrations for each protein were 153, 50.9, 17.0, 5.65, 1.88 and 0.628 pmol/ml, corresponding to 5, 1.67, 0.56, 0.19, 0.06 and 0.02 µg/ml nPan b 1.

Basophil Activation Test

The Flow2 CAST was performed according to the manufacturer's instructions (Bühlmann Laboratories, Allschwil, Switzerland) and has been fully described elsewhere [29]. 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 to the total number of identified basophilic cells.

For the dose-response assay, 305, 30.5, 3.05 and 0.305 pmol/ml of each allergen were analyzed, equivalent to 10, 1, 0.1 and 0.01 µg/ml of nPan b 1.

Skin Prick Test

SPTs were performed with 15,265 pmol/ml of each protein, corresponding to 0.5 mg/ml nPan b 1, diluted in MOPS buffer. Histamine hydrochloride (10 mg/ml; ALK-Abelló) and MOPS buffer were used as positive and negative 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 ≥3 mm larger than that of the negative control.

Results

Design of Five Overlapping Pan b 1 Fragments

Five overlapping Pan b 1 fragments (F1–F5) were designed to each include one major sequential IgE-binding region as identified by Ayuso et al. [20] for Pen a 1 (fig. 1). The five fragments were 59–78 amino acids long and

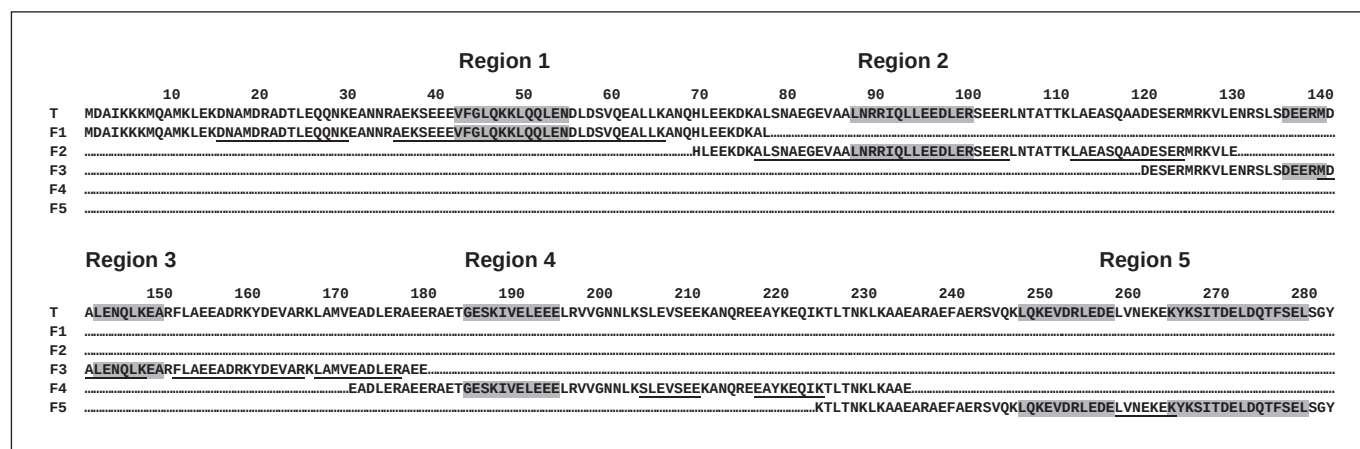


Fig. 1. Amino acid sequence and position of Pan b 1 fragments. Amino acids identified by MS analysis are underlined. IgE-binding regions earlier identified for *F. aztecus* tropomyosin (Pen a 1) [20] are marked with shading. Pan b 1 differs from Pen a 1 in nine amino acids localized between amino acid number 44 and 72 [25]. T =

Pan b 1.0101 (GenBank accession No. FR728681); F1–F5 = Pan b 1 fragments 1–5. The five fragments and whole rPan b 1 contain a 22 amino acid N-terminal His-tag (MGHHHHHHHHHHSSG-HIDDDDK; not shown in the figure).

linked to a 22 amino acid His-tag. With 9–11 amino acids overlap, the fragments covered the entire sequence of Pan b 1 (F1^{1–78}, F2^{70–131}, F3^{121–181}, F4^{173–236} and F5^{226–284}).

Identification

The Pan b 1 fragments were expressed in *E. coli* and identified by MS, obtaining 15–68% amino acid recognition (underlined in fig. 1). The fragments had calculated molecular masses of 9.5–11.7 kDa and appeared as 10- to 14-kDa proteins in SDS-PAGE (fig. 2a). MS analysis revealed 2 major peaks for each fragment and whole rPan b 1, corresponding to their calculated molecular masses with loss of the initial methionine and partial acetylation (fig. 2b).

Secondary Structure

It was previously shown by CD spectroscopy that nPan b 1 has a predominantly α -helical structure with the characteristic minima at 208 and 222 nm and maximum at 192 nm [25, 30] and that the 22 amino acid His-tag added to the Pan b 1 fragments did not interfere with the folding of rPan b 1 [25]. CD spectroscopy of the Pan b 1 fragments in buffer with physiological salt content (PBS; fig. 2c) showed CD spectra typical for α -helical structure for F1 and F5. F2 and F4 presented spectra characteristic for random coils, with a minimum at 200 and low signal at 220 nm [31], whereas the spectrum obtained from F3 was somewhere in between. In buffer with low salt content (50 mM K₂PO₄), the CD spectra for all the Pan b 1

fragments, and especially F1 and F5, shifted towards more random coil (online suppl. fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000350791). Adding the α -helix inducing solvent TFE showed that all the five Pan b 1 fragments were able to form more than 50% α -helical secondary structure (online suppl. fig. S1).

Oligomerization

Muscle tropomyosin is a dimer in nature, and both natural and recombinant Pan b 1 existed entirely in the dimeric form in native PAGE [25]. Based on the length of the Pan b 1 fragments compared to previous structural studies of tropomyosin-based peptides [32, 33] (21–35 amino acids), the fragments were expected to form dimers in native buffers. According to BS³ crosslinking studies with PBST buffer, all fragments except F5 were primarily monomers in native buffer at physiological salt concentration (fig. 2d). However, all fragments showed an ability to form dimers, as seen by the weaker bands above the bands representing the monomers. In native PAGE (zero salt in running buffer), only F2 and F4 appeared as dimers, F3 was monomeric, while F1 and F5 were tetramers (fig. 2e).

IgE Binding

Mapping of IgE binding to the five Pan b 1 fragments was investigated by Western blot and ELISA with serum from five Norwegian shrimp-allergic individuals.

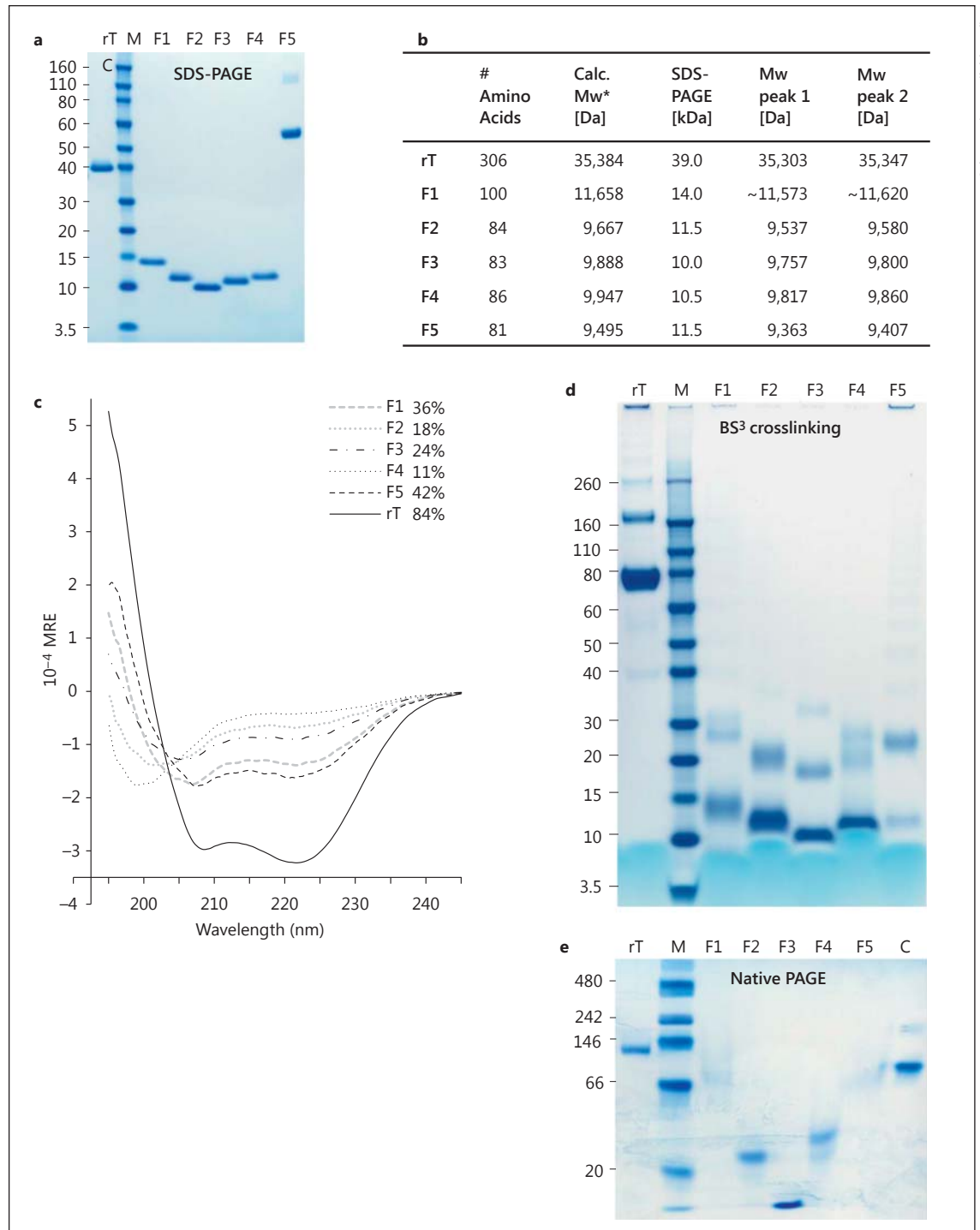


Fig. 2. Structural analyses of Pan b 1 fragments. **a** SDS-PAGE. 0.5 μ g protein was loaded per lane. **b** MS molecular mass analysis. The 22 amino acid tag was included in the number of amino acids and the molecular mass calculations. The difference between calculated and measured molecular masses corresponded to a loss of the initial methionine and partial acetylation. * Calculated average molecular mass. Mw = Molecular mass. **c** CD spectroscopy in PBS. rPan b 1 showed a typical spectrum for α -helices with minima at 208 and 222.

The percentages of α -helix, calculated from the mean residue ellipticity at 222 nm according to [28], are given in the figure. In the determination of α -helix content, the 22 amino acid tag was assumed not to contribute to any signal at 222 nm. **d** SDS-PAGE after BS³ fixation in PBS-Tween. Approximately 80 pmol protein was loaded per lane. **e** Native PAGE with zero salt content in running buffer. 0.5 μ g protein was loaded per lane. rT = rPan b 1; M = marker; F1–F5 = Pan b 1 fragments 1–5; C = control protein human albumin.

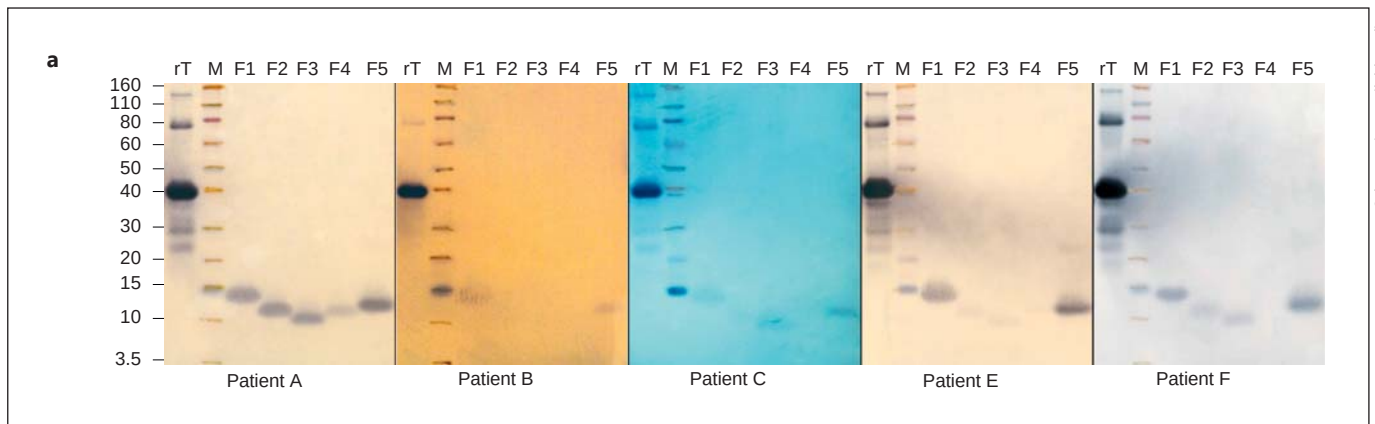
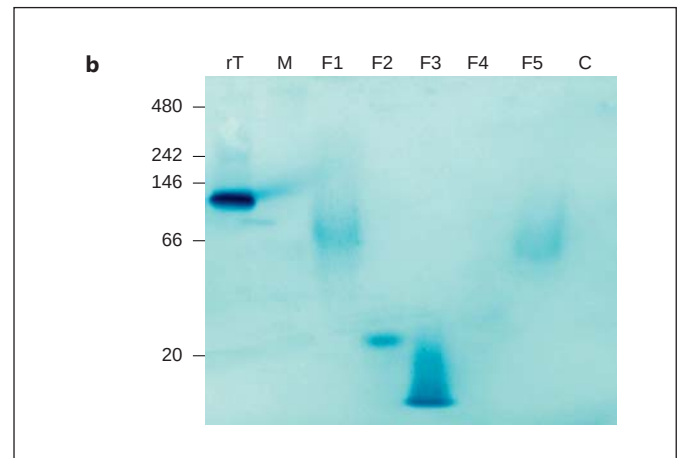


Fig. 3. Immunoblot analysis of IgE binding to Pan b 1 fragments with serum from shrimp allergic individuals. **a** Western blot with individual sera. **b** Native immunoblot with pooled sera from the five patients. 0.5 μ g protein was loaded per lane. rT = rPan b 1; F1–F5 = Pan b 1 fragments 1–5; C = control protein human albumin.



Western Blot

In Western blot, IgE binding to unfolded immobilized Pan b 1 fragments was observed (fig. 3a). All patient sera had the highest binding to F1 and F5. Four patient sera recognized F3, three recognized F2 and one patient serum recognized F4. Whole rPan b 1 showed a higher IgE binding than the five fragments.

Native Immunoblot

A serum pool from the five patients was used to study a blot made from native PAGE. This blot confirmed the low binding to F4 and revealed high binding to F3 (fig. 3b). The tail from the F3 band indicated some association between the F3 monomers.

Indirect ELISA

A less clear pattern of IgE binding was seen in indirect ELISA with immobilized native Pan b 1 fragments (fig. 4). The IgE-binding reactivity to the individual fragments varied among the five shrimp-allergic patients. Four of

the sera had the lowest binding to F4. Two sera had the highest binding to F1 and two other sera to F3.

Inhibition ELISA

The ability of native Pan b 1 fragments in solution to inhibit serum IgE from binding to immobilized native nPan b 1 was investigated by inhibition ELISA. Some of the patterns from the indirect ELISA were similar to the results observed with inhibition ELISA (fig. 5a). F1 and F5 inhibited IgE binding of the sera B and E with up to 42% inhibition at the highest concentration (5 μ g/ml). F3 inhibited IgE binding of the sera A and C to a maximum of 54% inhibition at the highest concentration. For comparison, nPan b 1 inhibited approximately 80% of the binding at this concentration, closely followed by rPan b 1.

Combining the five Pan b 1 fragments in a mixture, each at the same molarity as whole rPan b 1, resulted in an equivalent capacity to inhibit IgE binding compared to whole rPan b 1 in sera from three of the patients and an

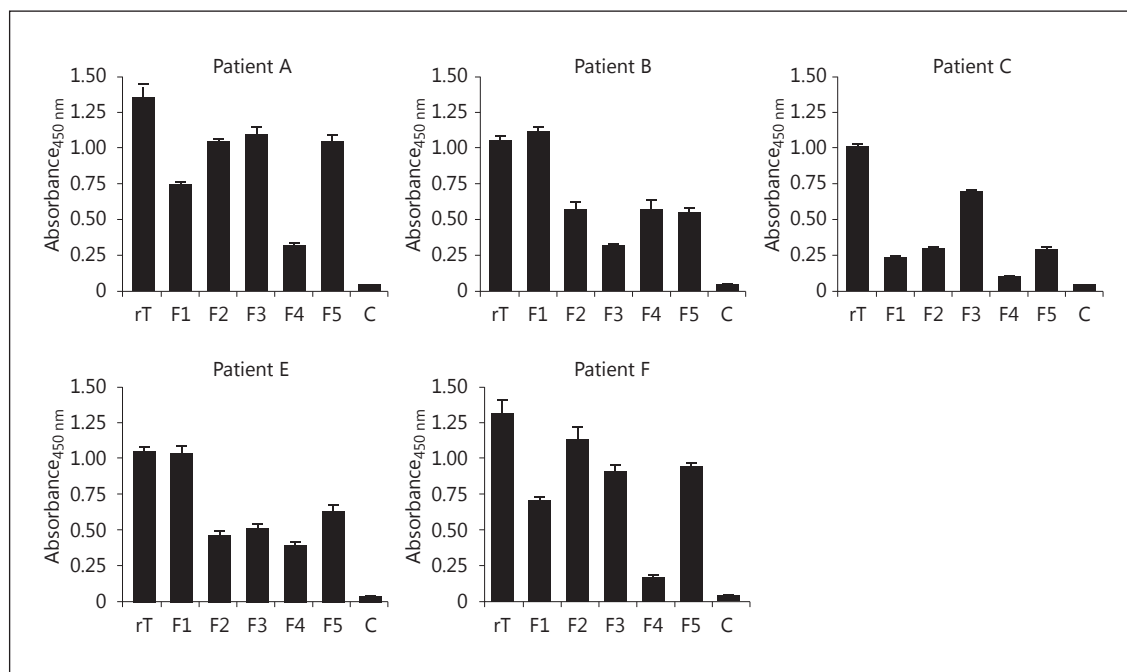


Fig. 4. Indirect ELISA of specific IgE reactivity to Pan b 1 fragments with serum from shrimp-allergic individuals. rT = rPan b 1; F1–F5 = Pan b 1 fragments 1–5; C = control protein human albumin. The mean values of triplicates are shown, and the standard error of the mean is indicated by error bars.

11–20% reduced inhibition capacity in the last two (at the highest concentration; fig. 5b).

IgE Receptor Crosslinking

Mapping of the Pan b 1 fragments' abilities to induce IgE receptor crosslinking and activate effector cells was performed by BAT and SPT. The mixture of the five fragments, each at the same number of micrograms or molarity, was included.

Basophil Activation Test

Blood was incubated with native Pan b 1 fragments and the portion of activated basophils, determined by CD63 upregulation, was detected by flow cytometry. When 1 µg of each protein was compared, the fragments activated equivalent or higher numbers of basophils as whole rPan b 1 and the fragment mixture for four patients (fig. 6). A dose response assay was performed to further compare the fragments for each patient.

The activation of basophils by Pan b 1 fragments was dose dependent (fig. 7). F4 lost the ability to induce CD63 upregulation at higher concentrations than the other fragments. As opposed to the fragments, rPan b 1 retained the high activation potential also at lower concen-

trations. Responses induced by the fragment mixture were comparable to those induced by rPan b 1. Control individuals were negative to rPan b 1 and the Pan b 1 fragments in BAT.

Skin Prick Test

An in vivo investigation of the ability to induce an allergic reaction by the Pan b 1 fragments was assessed by SPT. The fragment mixture induced a larger wheal than whole rPan b 1 in all patients (fig. 8). Compared to the other fragments, F3 had the highest response in patients A, C and F, while patient E responded strongest to F1. F2 and F4 both had two negative responses. The non-atopic control individuals were negative to both rPan b 1 and the Pan b 1 fragments in SPT.

Discussion

IgE epitope mapping is valuable for the improvement of diagnostics, treatment and prediction of progress and severity of allergic disease, especially in food allergy [4–6, 23]. However, the clinical relevance of IgE binding to short synthetic peptides, like the ones previously used in

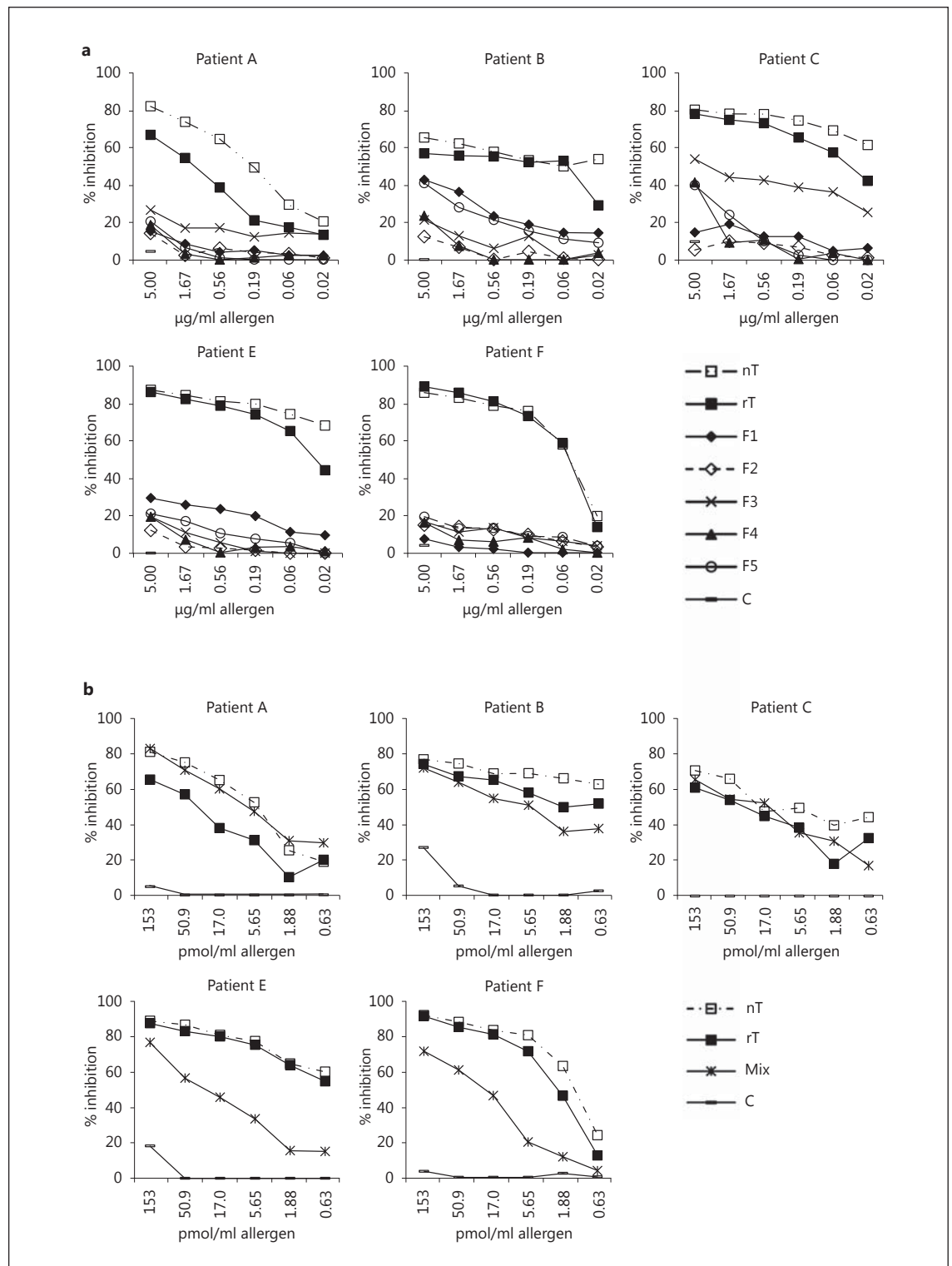


Fig. 5. Inhibition of IgE binding to nPan b 1 by Pan b 1 fragments in ELISA. Serum from shrimp-allergic individuals was preincubated with Pan b 1 proteins for 2 h before addition to immobilized nPan b 1. **a** Inhibition of IgE binding induced by the Pan b 1 fragments with concentrations in $\mu\text{g/ml}$. **b** Inhibition of IgE binding induced by a mixture of the Pan b 1 fragments in equal molar ratios. nT = nPan b 1; rT = rPan b 1; F1–F5 = Pan b 1 fragments 1–5; Mix = mixture of the five Pan b 1 fragments in equal molar ratios; C = control protein human albumin.

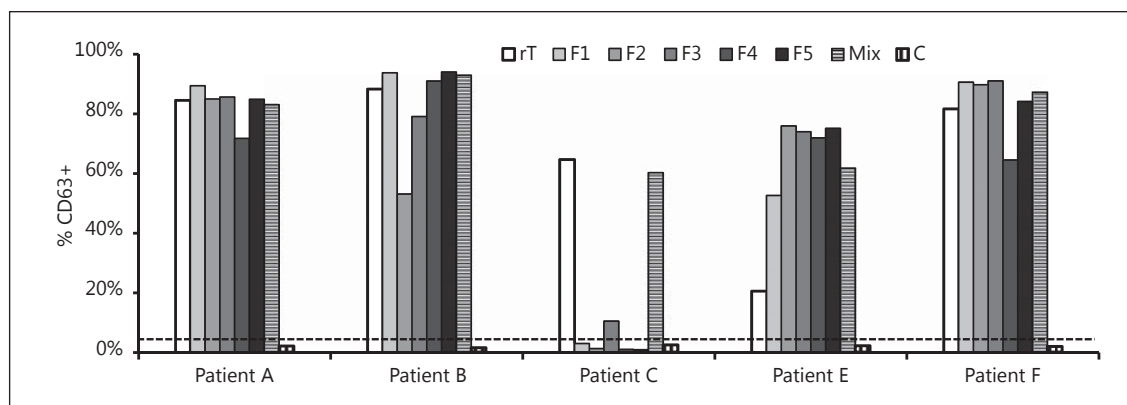


Fig. 6. Basophil CD63 upregulation induced by Pan b 1 fragments (1 μ g/ml). Responses above the dotted line at 5% were regarded as positive. rT = rPan b 1; F1–F5 = Pan b 1 fragments 1–5; Mix = mixture of the five Pan b 1 fragments at equal molar ratios; C = MOPS buffer control.

the epitope mapping of shrimp tropomyosin, is debated [17, 21–23, 34]. The advantages of the current study are the use of longer protein fragments and the mapping of immunodominant regions by effector cell activation, in addition to IgE binding.

According to the five Pan b 1 fragments' abilities to activate effector cells, the patients could roughly be divided into two groups. One group was predominantly sensitized to the middle of the protein (F3; patients A, C and F), and the other group was predominantly sensitized to the N- and C-terminus of the protein (F1 and F5; patients B and E). Overall, the results from the IgE receptor crosslinking studies (BAT and SPT) correlated well with the native fragments' abilities to bind to IgE from the different patients (ELISA and native blot studies). In contrast, IgE binding to unfolded fragments, as done in a Western blot made from SDS-PAGE (current study and Kunimoto et al. [24]), was not successful in predicting the fragments' ability to activate effector cells. Considering all five patients together, the IgE-reactive regions found in the current study agreed well with previous results obtained by microarray with short synthetic peptides [22, 23]. In those studies, short synthetic peptides corresponding to F3 were recognized with the highest frequency, peptides corresponding to F1 ranked second in binding activity and peptides corresponding to F5 ranked third. Synthetic peptides corresponding to F4 had the lowest binding activity. Altogether, the recognition of the regions corresponding to F1, F3 and F5, and the low binding to F4, is in line with the current results obtained with BAT and SPT. Thus, this indicates that studies with short synthetic peptides might have a predictive value for iden-

tifying regions inducing IgE receptor crosslinking on effector cells.

Muscle tropomyosin is a homodimer of two α -helices in vivo, forming a head-to-tail polymer with 9 amino acids overlap [35]. In vitro, the protein easily forms crystals and precipitates (own observations). A recent study using rabbit muscle tropomyosin revealed that at physiological salt concentration, in the absence of actin, the protein was predominantly in the state of a tetramer. At lower salt concentrations, even larger oligomers dominated [36]. In contrast, we found Pan b 1 predominantly present as a dimer both at physiological salt concentration (BS³ studies) and zero salt concentration (native PAGE). This indicates that, in a soluble state, invertebrate tropomyosins are present as dimers, also in the absence of actin.

The structure of the Pan b 1 fragments, on the other hand, was markedly influenced by salt concentration, each reacting in a different way. Fragments fixed with the crosslinker BS³ in the presence of a high salt concentration (0.5 M NaCl; data not shown) or at physiological salt concentration (PBS) were primarily monomeric, but also partially present as dimers. At zero salt concentration (native PAGE), the fragments were either monomeric, dimeric, dimeric plus trimeric or tetrameric. The fact that all the fragments were able to form a dimeric or multimeric state implies the presence of a secondary structure. CD analyses indicated a large variation in the amount of secondary structure between the fragments. As expected, it was found that all the five fragments obtained more than 50% α -helix after addition of TFE, confirming that the protein sequence present in the fragments allows formation of α -helix. At physiological salt concentration, the

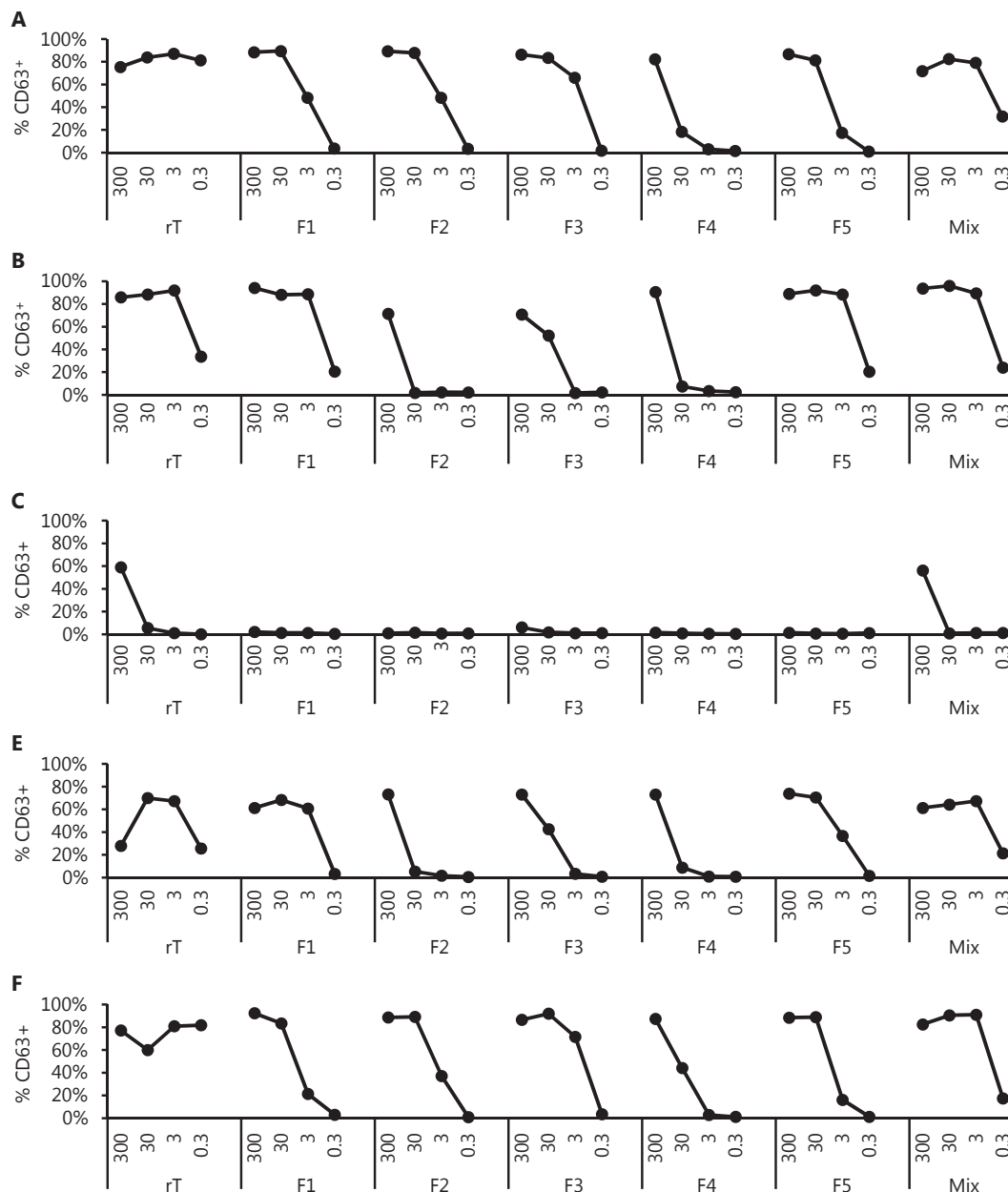


Fig. 7. Dose response of Pan b 1 fragments on CD63 upregulation. The final concentrations of the Pan b 1 fragments and rPan b 1 are given in pmol/ml. A–C, E and F represent experiments with blood samples from patients A–C, E and F, respectively. rT = rPan b 1; F1–F5 = Pan b 1 fragments 1–5; Mix = mixture of the five Pan b 1 fragments at equal molar ratios.

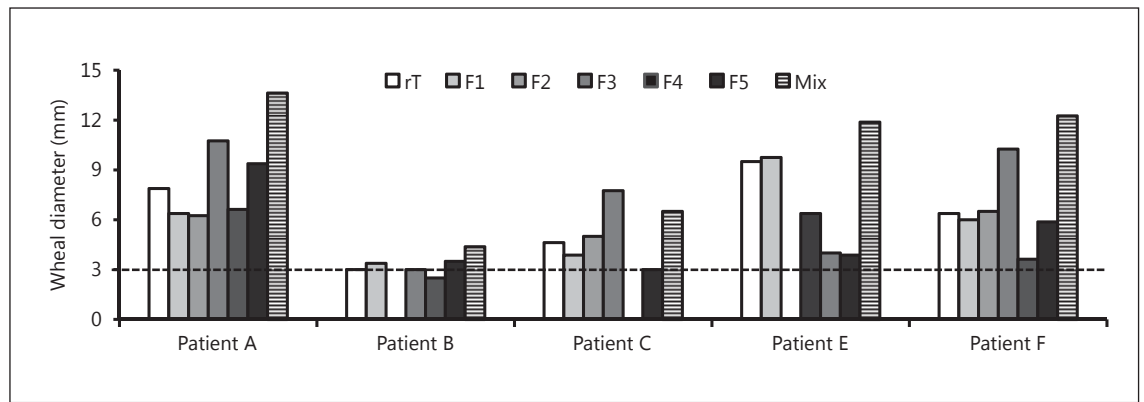


Fig. 8. SPT with Pan b 1 fragments. Mean wheal diameter induced by the Pan b 1 fragments and rPan b 1 at the same molarity of 15 nmol/ml (equivalent to 0.5 mg/ml of nPan b 1). The value of the negative control was subtracted from the diameter. Responses above the dotted line at 3 mm were regarded as positive. rT = rPan b 1; F1–F5 = Pan b 1 fragments 1–5; Mix = mixture of the five Pan b 1 fragments at equal molar ratios.

two Pan b 1 fragments with the highest tendency to oligomerize in native PAGE (F1 and F5) showed the highest amount of α -helix in CD analyses. In contrast, F2 and F4 were observed as multimers in native PAGE despite the low amount of secondary structure found in CD analyses. This indicates that even a low amount of α -helix such as present in F2 and F4 can still induce oligomerization. The middle fragment, F3, ranked third in α -helix content, but appeared as a monomer in native PAGE. One explanation for the low oligomerization tendency of F3 is the presence of the highly conserved negatively charged core residue Asp137 that is known to cause a destabilized region in the middle of tropomyosin, responsible for the necessary flexibility of the protein [37]. In addition, *in silico* secondary structure calculations [38] of the Pan b 1 fragments predicted an area of random coil structure around this residue for F3 not seen for any of the other fragments.

The length of the fragments suggests presence of several IgE epitopes, which allows IgE receptor crosslinking in the biological studies. Dimerization of the fragments would substantially facilitate biological activity, since this allows induction of IgE receptor crosslinking by monospecific IgE. The two fragments with the highest proportion of secondary structure (α -helix) and highest tendency to oligomerize (F1 and F5) were also two of the three fragments with the highest biological activity. This is in contrast to the third and maybe most important fragment, F3, which appeared as a monomer in our native PAGE studies. It is therefore likely that F3 contains more than one major IgE-binding epitope on its protein sequence, as previously suggested by Ayuso et al. [20].

A mixture containing the fragments in equal molar ratios as whole rPan b 1 inhibited the same level of IgE binding to nPan b 1 and whole rPan b 1 for three of the five patients, indicating that most IgE-binding epitopes (both conformational and sequential) were present in the fragment mixture. For comparison, Albrecht et al. [17] obtained only marginal inhibition to shrimp tropomyosin by the 15 short peptides identified as IgE reactive by SPOTS analysis. The fragment mixture showed the same potential as rPan b 1 to activate basophils. In skin prick testing, the molarity of the fragment solution seemed to be more important than the number of IgE-binding sites, since the application of the mixed fragments resulted in larger wheal sizes than that produced by rPan b 1. This suggests that processing or partial digestion of shrimp tropomyosin might provoke an even stronger allergic reaction than a complete protein.

For the development of allergy vaccines, dividing an allergen into a few long fragments can be a successful approach to reduce IgE binding while retaining all potential T-cell epitopes, at least for globular proteins [39, 40]. For fibrous allergens like tropomyosin, however, neither conformational nor sequential IgE-binding epitopes are necessarily eliminated by dividing the allergen into long fragments, as shown in the current study. Thus, this indicates that dividing tropomyosin into five fragments does not result in a hypoallergenic vaccine candidate.

In the development of a chimeric allergy vaccine by our research group, we have chosen the promising approach of including all potential IgE-binding epitopes, to ensure tolerance induction to the entire allergen. Tropo-

myosin is a relatively long protein, and for the production and administration of the vaccine, a smaller molecule could be favorable. In preliminary studies of the chimeric vaccine, including one of the fragments instead of whole Pan b 1 has given promising results (unpublished results). The fragments generated in the current study are therefore now investigated for inclusion into the chimeric vaccine.

In conclusion, for the first time, the immunodominant regions of shrimp tropomyosin were mapped by IgE receptor crosslinking studies with long protein fragments. In the IgE receptor crosslinking studies, no specific part of tropomyosin was found to be immunodominant for all patients tested; the patients were either predominantly sensitized to the middle fragment or to the two terminal fragments of the protein. These results correlated well with IgE-binding results of native fragments and previous microarray data with short peptides, but not with previous and our current Western blot results. Overall, dividing shrimp tropomyosin into five fragments did not reduce the allergenicity of the protein.

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