

ORIGINAL PAPER

Structural, immunological and functional properties of natural recombinant Pen a 1, the major allergen of Brown Shrimp, *Penaeus aztecus*

G. Reese*, S. Schick Tanz*, I. Lauer*, S. Randow*, D. Lüttkopf*, L. Vogel*, S. B. Lehrer† and S. Vieths*

*Paul-Ehrlich-Institut, Department of Allergology, Langen, Germany and †Tulane University School of Medicine, Department of Clinical Immunology, Allergy and Rheumatology, New Orleans, LA, USA

Clinical and Experimental Allergy

Summary

Background Recombinant allergens are considered the basis for new diagnostic approaches and development of novel strategies of allergen-specific immunotherapy. As Pen a 1 from brown shrimp *Penaeus aztecus* is the only major allergen of shrimp and binds up to 75% of all shrimp-specific IgE antibodies this molecule may be an excellent model for the usage of allergens with reduced IgE antibody-binding capacity for specific immunotherapy.

Aim The aim was to clone, express and characterize a full-length recombinant Pen a 1 molecule and compare it with natural Pen a 1 in regard to structural and immunological parameters such as IgE antibody capacity and ability to induce IgE-mediated mediator release.

Methods Total RNA was isolated from *P. aztecus* and a rapid amplification of cDNA ends (5' RACE) was performed to obtain full-length cDNA coding for Pen a 1. Using a gene-specific primer, PCR was performed and full-length cDNA was cloned and sequenced. Recombinant His-tagged Pen a 1 was isolated from *Escherichia coli* under native conditions by immobilized metal affinity chromatography. Secondary structure of natural and recombinant Pen a 1 was compared by circular dichroism (CD) spectroscopy, and the IgE antibody-binding capacity evaluated by RAST. The allergenic potency was tested by the capability of natural and recombinant Pen a 1 to induce mediator release in a murine and human *in vitro* model of IgE-mediated type I allergy.

Results The deduced amino-acid sequence was 284 residues long and amino-acid sequence identities with allergenic and non-allergenic tropomyosins ranged from 80% to 99% and 51% to 58%, respectively. The analysis of the secondary structure of natural and recombinant Pen a 1 by CD spectroscopic analysis showed that both nPen a 1 and rPen a 1 had α -helical conformation that is typical for tropomyosin. The IgE antibody binding capacities of nPen a 1 and rPen a 1 were found to be essentially identical by RAST. The mediator release experiments using both wild-type and humanized rat basophilic leukaemia 30/25 cells showed that rPen a 1 and nPen a 1 induced a similar level of mast cell activation.

Conclusions Recombinant Pen a 1 and natural Pen a 1 are structurally and immunologically identical and rPen a 1 may be used as the basis for component-resolved diagnosis and the generation of modified shrimp tropomyosin for allergen-specific immunotherapy. The results of the animal studies indicate that C3H/HeJ mice that were sensitized with shrimp extract in combination with cholera toxin as adjuvant may be a suitable model to study shrimp allergy.

Keywords C3H/HeJ, circular dichroism, mediator release, mouse model, rat basophilic leukaemia cells, RBL assay, shrimp allergen Pen a 1, tropomyosin

Submitted 12 October 2004; revised 19 October 2005; accepted 13 December 2005

Correspondence:

Gerald Reese, Paul-Ehrlich-Institut,
Department of Allergology, Paul-
Ehrlich-Str. 51–59, D-63325 Langen,
Germany.
E-mail: reese@pei.de

Introduction

Recombinant proteins are considered the basis for new approaches to the diagnostic use of allergens and the

development of novel strategies of allergen-specific immunotherapy. As recombinant allergens are easily produced in large quantities they may serve as reference

material for allergen standardization. It has been proposed that only a few recombinant allergens may be sufficient to detect the vast majority of allergen-specific sensitizations owing to the predominance of some allergens and the high degree of allergen cross-reactivity [1–3]. For therapeutic use recombinant allergens and mutated variants may be a way to deliver standardized amounts of allergen during specific immunotherapy (SIT) and/or allow for the first time SIT of allergies that have not been treated because natural extracts contain allergens with such a high IgE antibody-binding capacity that their administration may cause a severe adverse reaction such as in the treatment of allergies to foods [4, 5].

Shellfish is a frequent cause of allergic reactions to foods [6, 7]. Most shellfish species that elicit allergic food reactions belong to the class Crustacea and include shrimp, crab, crawfish and lobster; the shrimp genera *Penaeus* and *Metapenaeus* have been two of the most frequently studied. The only major allergen identified in shrimp is the muscle protein tropomyosin. At least 80% shrimp-allergic subjects react to tropomyosin and it binds approximately 75% of the shrimp-specific IgE from shrimp-allergic subjects [8–10]. Previous studies have demonstrated that tropomyosin is an important allergen in other crustaceans such as lobster *Panulirus stimpsoni* and *Homarus americanus* (Pan s 1, Hom a 1) [11, 12], crab *Charyabdis feriatus* (Cha f 1) [13], mollusk such as squid *Todareus pacificus* (Tod p 1) [14], snail *Turbo cornutus* (Tur c 1) [15] and oyster *Crassostrea gigas* (Cra g 1) [16, 17], and in other invertebrates such as house dust mite *Dermatophagoides farinae* (Der f 10) and *Dermatophagoides pteronyssinus* (Der p 10) [18, 19], and cockroach *Periplaneta americana* (Per a 7) [20, 21]. Furthermore, concomitant clinical and *in vitro* hypersensitivity to crustaceans, insects, arachnids, mollusks and even nematodes have implied tropomyosins as the cause of clinical cross-sensitivity among invertebrates [22–25]. These studies taken together suggest that tropomyosin is an invertebrate panallergen [26].

Tropomyosins are present in all eukaryotic cells, where they are associated with the thin filament in muscle, and microfilaments in many non-muscle cells. Together with actin and myosin, tropomyosin plays a role in the contractile activities of these cells, as well as in the regulation of cell morphology and motility [27]. Tropomyosin is present in phylogenetically unrelated vertebrate and invertebrate species, with several tropomyosin isoforms being found in different tissues and cell varieties. Even though the degree of sequence identity and functional similarity is very high among tropomyosins, vertebrate tropomyosins are non-allergenic [28–30]. Nevertheless, the basis for these differences in allergenicity among tropomyosins has not yet been elucidated. Thus, tropomyosin molecules provide an excellent opportunity to study the relationship between protein structure and

allergenicity. The aim of the current study was to clone, sequence and express a full-length recombinant Pen a 1, and characterize its structural and allergenic properties.

Material and methods

Patients' sera

Sera collected from five atopic shrimp-allergic patients were used. All subjects fulfilled the following criteria [31]: (1) history of respiratory (wheezing or shortness of breath), dermatologic (urticaria or angio-oedema), or gastrointestinal (nausea, vomiting, and/or diarrhoea) symptoms occurring within 1 h following ingestion of shrimp; (2) positive skin prick test (SPT, weal > 3 mm) to extract of cooked brown shrimp (*Penaeus aztecus*); (3) elevated shrimp-specific IgE levels demonstrated by RAST (binding > 3%); and (4) strong IgE reactivity to purified shrimp tropomyosin (Pen a 1) by immunoblot analysis.

Preparation of shrimp extract and purification of natural and recombinant Pen a 1

Natural Pen a 1 was purified by preparative SDS-PAGE. Briefly, extract from locally (New Orleans, LA, USA) purchased brown shrimp, *P. aztecus*, was prepared as described previously [32]. Pen a 1 was purified from shrimp extract by preparative SDS-PAGE (Model 491 PrepCell, BioRad, Hercules, CA, USA). Briefly, shrimp extract was separated on the 28 mm ID column using Laemmli discontinuous SDS-PAGE buffer system [33]. A 15 mm high stacking gel (5%T, 1.5%C) poured on top of the 65 mm high separation gel (11.5%T, 1.5%C) was used to separate shrimp proteins, and the fractions containing Pen a 1 were identified by SDS-PAGE, pooled and dialysed against water. Approximately 50 mg of shrimp extract was applied onto the preparative SDS-PAGE. Between 1.0 and 2.5 mg of Pen a 1 with a purity of at least 95%, as judged by analytical SDS-PAGE and silver staining, was obtained. Estimated additional 3.0–4.0 mg of Pen a 1 with a purity of more 50% were obtained.

To obtain recombinant Pen a 1, total RNA was isolated from pulverized, snap-frozen *P. aztecus* using the RNeasy-Total RNA-Isolation kit (Qiagen, Hilden, Germany). A rapid amplification of cDNA ends (5' RACE) to obtain 5' cDNA sequence using the GeneRacer kit (Invitrogen, Karlsruhe, Germany) was performed. 5'-CTGCTCTTAACCGCCGATC-CAGC-3' was used as reverse gene-specific internal primer deduced from already published partial Pen a 1 cDNA sequence [26, 34]. PCR was performed under the following conditions: initial denaturation: 5 min at 94 °C; amplification: 1 min at 94 °C, 1 min at 50 °C, 1 min, 45 s at 72 °C, 31 cycles; final elongation: 10 min at 72 °C. Full-length cDNA was obtained by PCR using 5'-terminal primer (5'-CACCATGGACGCCATCAAGAAGAAG-3') deduced from the

results of the 5' RACE, and 3'-terminal primer (5'-GTAGC-CAGACAGTTCGCTGAAAGT-3') according to the manufacturer's instructions under the following conditions: initial denaturation: 5 min at 94 °C; amplification: 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, 31 cycles; final elongation: 10 min at 72 °C. Full-length PCR products were purified (QIAquick Gel Extraction kit, Qiagen), and the cDNA was cloned into the pCR4-TOPO vector, expanded in TOP10 cells and sequenced. For the production of His-tagged rPen a 1, PCR was performed, the amplified cDNA, ligated into the pET101/D-TOPO vector, and was used to transform BL21 Star (D3) (Invitrogen) cells. The bacteria were cultured for 4 h in Luria Broth at 37 °C. The recombinant Pen a 1 carried a C-terminal His tag and the V5 epitope. The C-terminal His tag was chosen, to ensure that only full-length Pen a 1 was purified by Ni-NTA affinity chromatography. The additional V5 epitope was added to verify Pen a 1 expression if an immunologically inactive Pen a 1 were expressed. Recombinant Pen a 1 was isolated from lysate under native conditions by Ni-NTA affinity chromatography (Qiagen). Purity was tested by SDS-PAGE and silver staining [35].

Radioallergosorbent test

For comparison of the IgE antibody-binding capacity of natural and recombinant Pen a 1, RAST was performed [36]. Briefly, nPen a 1 and rPen a 1 were coupled to CNBr-activated paper disks (Schleicher & Schuell, Dassel, Germany) at 10.0, 1.0, 0.1 and 0.01 µg protein per disk. After blocking the disks were incubated with 100 µL undiluted sera of five subjects of shrimp-allergic subjects overnight at RT. The disks were washed with 0.9% NaCl and 100 µL (15 000 c.p.m.) of ¹²⁵I-labelled equine anti-human IgE (Sanofi Diagnostics Pasteur, Chaska, MN, USA) was added to each tube, and disks incubated overnight and washed as described above. Bound ¹²⁵I-IgE was counted in a Beckman 5500 γ counter (Beckmann Coulter, Fullerton, CA, USA). Binding was expressed as a percent of total ¹²⁵I added.

Sensitization of C3H/HeJ mice

Four-week-old female C3H/HeJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and sensitized four times orally by gavage with 1.0, 0.1 and 0.01 mg of shrimp extract using cholera toxin as adjuvant at 1-week intervals [37]. The mice were bled 2 weeks after the last injection and pooled sera stored at -20 °C.

Mediator release from wild-type and transfected rat basophilic leukaemia cells

The mediator release assays followed a protocol established by Hoffmann and colleagues [38–41]. Briefly, RBL-2H3 (DSMZ, Braunschweig, Germany) or RBL 30/25 cells expressing the α-chain of the human Fcε-RI receptor [42]

were plated in 96-well cell culture plates (Nunc, Wiesbaden, Germany) at 1.5×10^5 cell/well. The cells were passively sensitized with IgE-containing mouse (1 : 50) and human (1 : 10–1 : 20) sera, respectively. After washing, the cells were stimulated with antigen (*P. aztecus* extract, rPen a 1, nPen a 1). The antigen-specific release was quantified by measuring β-hexosaminidase activity and expressed as percent of the total β-hexosaminidase content that was obtained by lysing the cells with Triton X100. For measurements of spontaneous release and possible non-specific effects, naïve RBL cells were incubated with Tyrode's buffer, cell culture medium and antigen without specific antibodies, respectively.

Circular dichroism spectroscopy

Natural and recombinant Pen a 1 were dialysed against 10 mM KH₂PO₄/K₂HPO₄ buffer, pH 7.4 and protein concentrations adjusted to 5.2 µM (0.17 mg/mL). Circular dichroism (CD) spectroscopy was performed on a J-810 S spectropolarimeter (Jasco, Groß Umstadt, Germany) with constant N₂ flushing. The temperature of 20 °C of the optic cell chamber was controlled by a circulating water bath; the rectangular cell had a optical path length of 1 mm. For wavelength analysis nPen a1 and rPen a 1 were scanned with a step width of 0.2 nm, and a bandwidth of 1 nm. The spectral range was 185–255 nm at 50 nm/min. Ten scans were accumulated. Mean residue molar ellipticity (θ_{MRV}) was calculated [43].

Results

Pen a 1 sequence and rPen a 1

The cDNA of Pen a 1 (GenBank accession no. DQ151457) is 855 bp in length, the deduced amino-acid sequence was 284 residues long (Fig. 1) with a calculated molecular mass of 32 848.5 Da and a theoretical isoelectric point of pI 4.72. Amino-acid sequence identities with allergenic and non-allergenic tropomyosins ranged from 80% to 99% and 51% to 58%, respectively (Table 1). No amino-acid sequence differences with previously published partial sequence [26, 34] were detectable. The N-terminal sequence of Pen a 1 was identical with allergenic crustacean tropomyosins from *H. americanus* (Hom a 1), *Metapenaeus ensis* (Met e 1), *Charybdis feriatus* (Cha f 1), and the cockroach tropomyosin (Per a 7) from *P. americana*. After induction with 1 mM IPTG for 4 h, approximately 2 mg His-tagged rPen a 1 were isolated from a 1 L culture by Ni-NTA affinity chromatography.

Comparison of secondary structure of natural and recombinant Pen a 1 by circular dichroism spectroscopy

The CD spectroscopic analysis (Fig. 2) showed that both natural and recombinant Pen a 1 had α-helical structures.

Both curves were almost identical and showed the characteristic minima at approximately 208 and 222 nm. The shapes of the spectra were consistent with α -helically folded proteins. The secondary structure estimation that resulted from fitting predicted 67% (nPen a 1) and 66% (rPen a 1) α -helical and 2.2% (nPen a 1), 2.3% (rPen a 1) β -sheet, 30.8% (nPen a 1) and 31.7% (rPen a 1) random-coiled structures.

```

ATG GAC GCC ATC AAG AAG AAG ATG CAG GCG ATG AAG CTG GAG AAG GAT AAC GCC ATG GAC
M D A I K K K M Q Q A M K F L E K D N A M D
AGG GCG GAT ACC CTC GAA CAG CAG AAC AAG GAG GCC AAC AAG GCT GAG AAG AGC GAG
R A D T L E Q Q N K E A N N R A E K S E
GAG GAG GTT CAC AAC CTT CAG AAG AGG ATG CAG CAA CTT GAG AAC GAC CTT GAC CAG GTG
E E V H N L K A N I Q L V E K D K A L S N
CAG GAA TCC TTG CTG AAG GCT AAC ATC CAG CTT GTG GAG AAG GAC AAG GCC CTC TCT AAC
Q E S L L K A N I Q L V E K D K A L S N
GCT GAG GGC GAG GTG GCC GCT CTG AAC CCG CCG ATC CAG CTG CTC GAG GAG GAC CTG GAG
A E G E V A A L N R R I Q L L E E D L E
CGC TCT GAG GAG CCG CTC AAC ACC GCC ACC ACC AAG CTG GCC GAG GCC TCC CAG GCC GCC
R S E E R L N T A T T K L A E A S Q A A
GAC GAG TCC GCG CAG ATG CCG AAG GTG CTC GAG AAC CCG TCC CTG TCC GAG GAG GAG CCG
D E S E R M R K V L E N R S L S D E E R
ATG GAC GCC CTG GAG AAC CAG CTC AAG GAG GCT CGA TTC CTG GCT GAG 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 GTT GCC CGT AAG CTG GCC ATG GTT GAG GCC GAC CTT GAG CGT GCT GAG
K Y D E V A R K L A M V E A D L E R A E
GAG CGT GCC GAG ACT GGT GAA TCA AAG ATC GTC GAG CTT GAG GAA GAG CTG CGT 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 TCC CTT GAG GTG TCT GAG GAG AAG GCC AAC CAG CCG GAG GAA GCC
G N N L K S L E V S E E K A N Q R E E A
TAC AAG GAG CAG ATT AAG ACA CTT ACC AAC AAG CTG AAG GCG GCT GAG GCC CGT GCT GAG
Y K E Q I K T L T N K L K A A E A R A E
TTC GCC GAG AGG TCT GTG CAG AAG CTC CAG AAG GAG GTC GAC AGG CTT 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 TCC ATT ACC CAG GAG CTG 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

```

Fig. 1. cDNA sequence and deduced amino-acid sequence of full-length Pen a 1 (GenBank accession no. DQ151457). Pen a 1 is 284 amino acids long, has a theoretical pI of 4.72 and a calculated molecular mass of 32 849.5 Da.

Table 1. Sequence identities of Pen a 1 with other allergenic and non-allergenic tropomyosins

	Pen a 1	Met e 1	Hom a 1	HomaTMs	Pan s 1	Per a 7	Der f 10	Der p 10	GalzTM	GalgβTM	SaltTM	OryczTM
Met e 1	99											
Hom a 1	98	98										
HomaTMs	93	93	94									
Pan s 1	98	98	98	94								
Per a 7	82	82	81	82	82							
Der f 10	81	81	82	83	81	80						
Der p 10	81	81	81	82	81	80	98					
GalzTM	58	58	58	58	58	55	58	58				
GalgβTM	54	54	54	55	54	51	55	55	85			
SaltTM	54	54	54	54	54	51	53	53	89	82		
OryczTM	56	56	56	57	56	54	58	58	96	84	88	
OryczβTM	55	55	56	56	56	53	57	57	95	84	88	99

Crustacea: *Metapenaeus ensis* (greasy-back shrimp, Met e 1, acc. no. Q25456), *Homarus americanus* (Atlantic lobster, Hom a 1, acc. no. AAC48288), *H. americanus* slow muscle tropomyosin (HomaTMs, accession no. AAS98885), *Panulirus stimpsoni* (spiny lobster, Pan s 1, accession no. O61379); Insecta: *Periplaneta americana* (American cockroach, Per a 7, accession no. AAD19606, Q9UB83); Arachnida: *Dermatophagoides pteronyssinus*, *D. farinae* (house dust mites, Der p 10, accession no. AAB69424, accession no. O18416; Der f 10, accession no. Q23939); Vertebrata: *Gallus gallus* (chicken, alpha-tropomyosin GalgβTM, CAA41056), *Oryctolagus cuniculus* (rabbit, OryczTM, P58772), *Salmo trutta* (Brown Trout, SaltTM, accession no. CAA91251).

Comparison of immunoglobulin E antibody reactivity of natural and recombinant Pen a 1 by radioallergosorbent test

An amount larger than 0.1 µg/disk of Pen a 1 did not increase the IgE antibody reactivity (data not shown). Thus, this concentration was used to compare the IgE antibody reactivity of natural and recombinant Pen a 1. Linear regression analysis was performed (Fig. 3) showing an excellent correlation between the reactivities of five sera of shrimp-allergic subjects to natural and recombinant Pen a 1 ($r^2=0.997$). The slope of 1.010 indicated essentially identical IgE antibody-binding properties of both rPen a 1 and nPen a 1.

Mediator release from wild-type and transfected rat basophilic leukaemia cells

Sensitization of wild-type RBL cells with sera of immunized C3H/HeJ mice and sensitization of humanized RBL

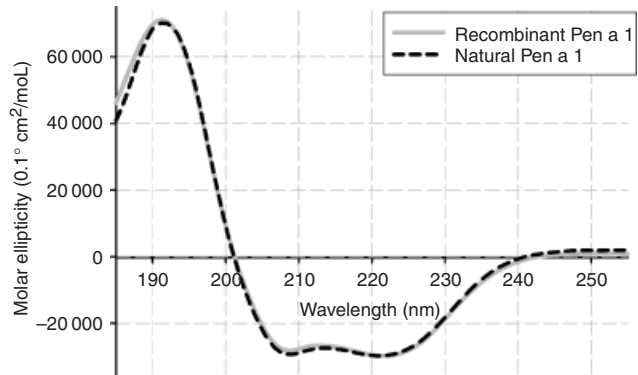


Fig. 2. Circular dichroism spectra of natural and recombinant Pen a 1. Both spectra are almost identical and show the characteristic minima of α helical proteins at approximately 208 and 222 nm.

30/25 cells with sera from shrimp-allergic patients followed by a challenge with shrimp extract, natural and recombinant Pen a 1 resulted in dose-dependent mediator release. The release data were fitted to four-parameter-logistic curves by non-linear regression using the Sigma-Plot software package (SPSS, Ekrath, Germany). For better comparison the mediator release induced by the murine serum pool and the human subject's IgE antibodies, the release curves of natural and recombinant Pen a 1 were normalized to the maximum of the release curves obtained with shrimp extract. The results are summarized in Fig. 4 and Table 2. The mediator releases induced by

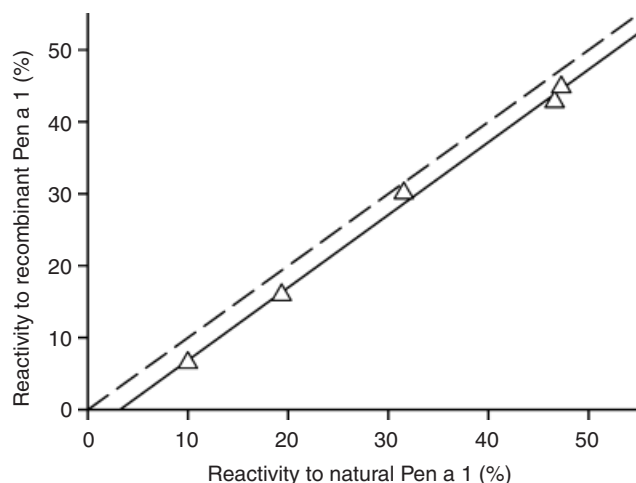


Fig. 3. Comparison of natural and recombinant Pen a 1 by RAST. Linear regression analysis shows an excellent correlation between the IgE reactivities of five sera of shrimp-allergic subjects to natural and recombinant Pen a 1 ($r^2 = 0.997$).

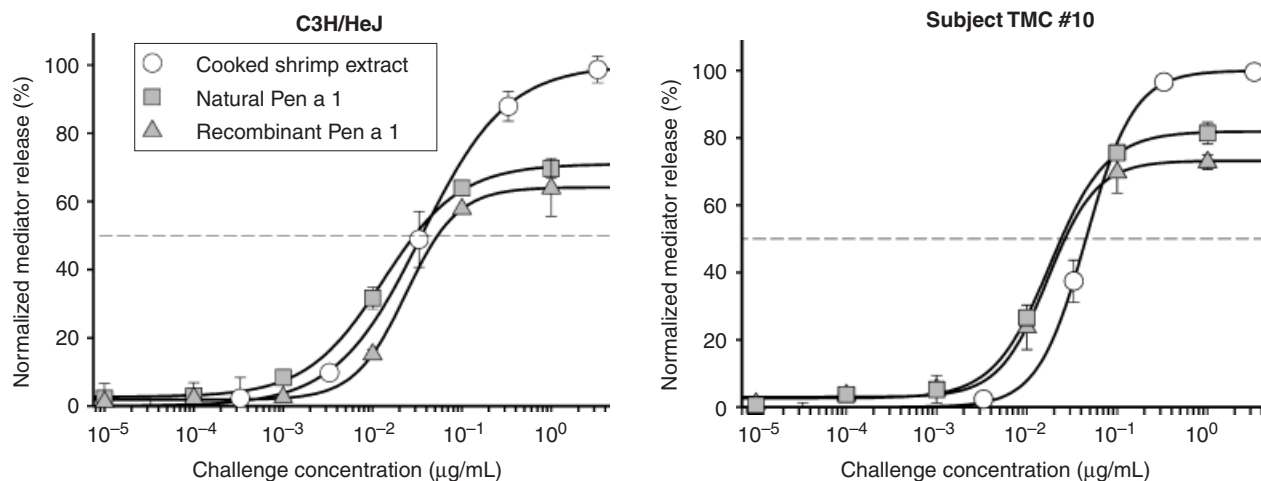


Fig. 4. β -hexosaminidase release from wild-type and humanized rat basophilic leukaemia (RBL) cells by shrimp extract, natural and recombinant Pen a 1. The mediator releases that are induced by natural and recombinant Pen a 1 are very similar in both systems (RBL 2H3 cells sensitized with a pool of C3H/HeJ mice immunized with shrimp extract; RBL 2H3 cells sensitized with the serum of the shrimp-allergic patient TMC #10). Furthermore, the murine and human IgE antibody reactivities directed towards Pen a 1 were similar; in the mouse model 64.1% (rPen a 1) and 71.0% (nPen a 1) of anti-shrimp activity is owing to Pen a 1. For the patient TMC #10 the amounts are slightly higher, but similar: 73.2% (rPen a 1) and 81.9% (nPen a 1) of anti-shrimp activity is owing to Pen a 1, respectively.

natural and recombinant Pen a 1 were very similar. The crucial parameter that defined the four-parameter-logistic curves showed that murine and human IgE antibody activity directed towards Pen a 1 was substantial; in the mouse model 64.1% (rPen a 1) and 71.0% (nPen a 1) of anti-shrimp activity was owing to Pen a 1. For the patient TMC #10 the amounts were slightly higher, but similar 73.2% (rPen a 1) and 81.9% (nPen a 1) of anti-shrimp activity was owing to Pen a 1, respectively. Additionally, the calculated amounts of natural and recombinant Pen a 1 that induced 50% of the maximal mediator release of shrimp extract were in the same range in both the murine and human model. In the murine model, 29.3 ng/mL of natural and 53.2 ng/mL of recombinant Pen a 1 was sufficient to induce 50% of the maximal mediator release of shrimp extract, respectively. In the human model the values were closer together; 23.9 ng/mL of natural and 26.4 ng/mL of recombinant Pen a 1 were sufficient to induce 50% of the maximal mediator release of shrimp extract, respectively.

Discussion

As the brown shrimp *P. aztecus* and probably other Crustacea contain only one major allergen that is recognized by the majority of shrimp-allergic subjects and Pen a 1 binds up to 75% of all shrimp-specific IgE antibodies this molecule may be more easily used as proof-of-principle that the usage of allergens with reduced IgE antibody-binding capacity (= hypoallergenic variants) for SIT is a feasible strategy to treat food-allergic individuals. To study the effect of mutations on the IgE antibody

Table 2. Parameters (minima, maxima, EC50 values, slopes) of logistic curve fits

Parameters	C3H/HeJ Mice			Subject TMC #10			Unit
	Extract	nPen a 1	rPen a 1	Extract	nPen a 1	rPen a 1	
Minimum	0.0202	2.7191	1.846	0.0000	2.6356	2.9818	%
Maximum	100.0	71.0	64.1	100.0	81.9	73.2	%
EC ₅₀	0.0352	0.0131	0.0236	0.0451	0.018	0.017	µg
Slope	-0.9074	-1.0096	-1.507	-1.6311	-1.3899	-1.6109	
Conc _{50%}		29.3	53.2		23.9	26.4	ng/mL

Conc_{50%} were the concentrations of nPen a 1 and rPen a 1 that were needed to induce 50% of the maximal release caused by shrimp extract.

binding capacity, it is of utmost importance that the recombinant natural allergen is essentially identical to the natural allergen that was purified from the allergenic source material.

The use of a purification procedure that comprised of denaturation by the detergent SDS and simple dialysis may raise the question whether this procedure could affect the structure of natural Pen a 1, especially as the dialysis may not remove the entire SDS. Our experience has shown that the SDS concentration was reduced below an amount that had a negative effect on the allergenic properties of Pen a 1 and other food allergens that were more sensitive in regard to changes of the native conformation. Inhibition experiments showed that SDS-PAGE-purified Pen a 1 is able to inhibit IgE antibody binding to shrimp extract in a similar manner (e.g. slope of the inhibition curve) as shrimp extract which is consistent with the notion that most of shrimp-specific IgE antibodies were directed towards Pen a 1 and that the SDS-PAGE-purified natural Pen a 1 is immunologically identical to the native Pen a 1 found in shrimp extract. Furthermore, Bet v 1-homologous food allergens purified by preparative SDS-PAGE under non-reducing conditions showed immunological activities and secondary structures that were consistent with native conformation, (manuscript in preparation) even though the allergenic potency of this group of allergens depended on native conformation. Thus, the purification procedure based on preparative SDS-PAGE and dialysis can provide pure allergens with full allergenic potency.

The amino-acid sequence of the full-length Pen a 1 was according to expectations; the amino acid of the N-terminal sequence of Pen a 1 was identical to those of other allergic crustacean tropomyosins from *H. americanus* (Hom a 1), *M. ensis* (Met e 1), *C. feriatius* (Cha f 1), and the cockroach tropomyosin (Per a 7) from *P. americana*. In the rest of the Pen a 1 molecule no amino acid sequence differences with previously published partial sequences [26, 34] were found. The analysis of the secondary structure of natural and recombinant Pen a 1 by CD spectroscopic analysis showed that both nPen a 1 and rPen a 1 have α -helical conformation that is typical for tropomyosin. The analysis of the immunological

properties of rPen a 1 was aimed at the IgE antibody-binding capacity and its ability to induce mediator release.

The IgE antibody-binding capacity was studied by RAST, nPen a 1 and rPen a 1 were covalently bound to paper disks, and the RAST activity sera of five shrimp-allergic subjects measured. The linear regression analysis indicated that both rPen a 1 and nPen a 1 have identical IgE antibody binding properties. It seemed that natural Pen a 1 might be a bit more active, however, the difference to the IgE antibody reactivity was always less than 5%, and, thus, in the range of variation for this method.

The mediator release experiment using the humanized RBL 30/25 cells that carry the α -chain of the human Fc ϵ -RI receptor showed rPen a 1 and nPen a 1 induced mediator release and that Pen a 1 carried the most of the mediator release capacity as compared with shrimp extract. This result is in accordance with previous findings obtained by RAST inhibition that at least 75% of shrimp-specific IgE antibodies are directed towards Pen a 1 [9]. Our initial experiments with a mouse model of shrimp allergy using C3H/HeJ mice and cholera toxin as adjuvant were performed according to Li et al. [37] showed promising results in that high amounts of Pen a 1-specific IgE antibodies were induced, however, this response may be mouse strain dependent [44] and other adjuvants such as alum may be considered. To further validate this model, the IgE-reactive murine epitopes must be determined so that Pen a 1 variants which may be considered for therapeutic use in humans can be tested in this model. It probably will be not necessary that all epitopes are identical as humans show also a variety of individual patterns, however the epitopes recognized by murine IgE antibodies should at least bind to the major IgE-binding regions [31].

In conclusion, the recombinant Pen a 1 and natural Pen a 1 are structurally and immunologically identical. The initial results of the animal model using C3H/HeJ mice and cholera toxin as adjuvant demonstrated characteristics that were similar to those of shrimp allergy in humans. However, more work has to be performed to validate the model before it can be used to study hypoallergenic variants of Pen a 1 as new reagents for SIT of shrimp-allergic individuals.

Acknowledgement

This study was performed in part by a grant from the German Office for Education and Research (BMBF-0312216).

References

- Kazemi-Shirazi L, Niederberger V, Linhart B, Lidholm J, Kraft D, Valenta R. Recombinant marker allergens: diagnostic gatekeepers for the treatment of allergy. *Int Arch Allergy Immunol* 2002; 127:259–68.
- Valenta R, Kraft D. Recombinant allergens for diagnosis and therapy of allergic diseases. *Curr Opin Immunol* 1995; 7:751–6.
- Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D, Gronlund H. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). *Clin Exp Allergy* 1999; 29:896–904.
- Nelson HS, Lahr J, Rule R, Bock A, Leung D. Treatment of anaphylactic sensitivity to peanuts by immunotherapy with injections of aqueous peanut extract. *J Allergy Clin Immunol* 1997; 99:744–51.
- Oppenheimer JJ, Nelson HS, Bock SA, Christensen F, Leung DY. Treatment of peanut allergy with rush immunotherapy. *J Allergy Clin Immunol* 1992; 90:256–62.
- FDA. *Report of the FDA ad hoc committee on hypersensitivity to food constituents*. Washington, DC: S. Food and Drug Administration, 1996.
- Bush RK, Hefle SL. Food allergens. *Crit Rev Food Sci Nutr* 1996; 36 (Suppl.):S119–63.
- Shanti KN, Martin BM, Nagpal S, Metcalfe DD, Rao PV. Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. *J Immunol* 1993; 151:5354–63.
- Daul CB, Slattery M, Reese G, Lehrer SB. Identification of the major brown shrimp (*Penaeus aztecus*) allergen as the muscle protein tropomyosin. *Int Arch Allergy Immunol* 1994; 105:49–55.
- Leung PS, Chu KH, Chow WK *et al*. Cloning, expression, and primary structure of *Metapenaeus ensis* tropomyosin, the major heat-stable shrimp allergen. *J Allergy Clin Immunol* 1994; 94:882–90.
- Mykles DL, Cotton JL, Taniguchi H, Sano K, Maeda Y. Cloning of tropomyosins from lobster (*Homarus americanus*) striated muscles: fast and slow isoforms may be generated from the same transcript. *J Muscle Res Cell Motil* 1998; 19:105–15.
- Leung PS, Chen YC, Mykles DL, Chow WK, Li CP, Chu KH. Molecular identification of the lobster muscle protein tropomyosin as a seafood allergen. *Mol Mar Biol Biotechnol* 1998; 7:12–20.
- Leung PS, Chen YC, Gershwin ME, Wong SH, Kwan HS, Chu KH. Identification and molecular characterization of *Charybdis feriatus* tropomyosin, the major crab allergen. *J Allergy Clin Immunol* 1998; 102:847–52.
- Miyazawa H, Fukamachi H, Inagaki Y *et al*. Identification of the first major allergen of a squid (*Todarodes pacificus*). *J Allergy Clin Immunol* 1996; 98 (Part 1):948–53.
- Ishikawa M, Ishida M, Shimakura K, Nagashima Y, Shiomi K. Purification and IgE-binding epitopes of a major allergen in the gastropod *Turbo cornutus*. *Biosci Biotechnol Biochem* 1998; 62:1337–43.
- Ishikawa M, Ishida M, Shimakura K, Nagashima Y, Shiomi K. Tropomyosin, the major oyster *Crassostrea gigas* allergens and its epitopes 1998; 63:44–7.
- Leung PS, Chu KH. cDNA cloning and molecular identification of the major oyster allergen from the Pacific oyster *Crassostrea gigas*. *Clin Exp Allergy* 2001; 31:1287–94.
- Aki T, Kodama T, Fujikawa A *et al*. Immunochemical characterization of recombinant and native tropomyosins as a new allergen from the house dust mite, *Dermatophagoides farinae*. *J Allergy Clin Immunol* 1995; 96:74–83.
- Asturias JA, Arilla MC, Gomez-Bayon N, Martinez A, Martinez J, Palacios R. Sequencing and high level expression in *Escherichia coli* of the tropomyosin allergen (Der p 10) from *Dermatophagoides pteronyssinus*. *Biochim Biophys Acta* 1998; 1397:27–30.
- Asturias JA, Gomez-Bayon N, Arilla MC *et al*. Molecular characterization of American cockroach tropomyosin (*Periplaneta americana* allergen 7), a cross-reactive allergen. *J Immunol* 1999; 162:4342–8.
- Santos ABR, obias KR, Ferriani VPL *et al*. Identification of tropomyosin from *Periplaneta americana* as a major cockroach allergen. *J Allergy Clin Immunol* 1999; 103:S122.
- Martinez A, Martinez J, Palacios R, Panzani R. Importance of tropomyosin in the allergy to household arthropods. Cross-reactivity with other invertebrate extracts. *Allergol Immunopathol (Madrid)* 1997; 25:118–26.
- Banzet ML, Adessi B, Vuitton DA. L'Amecoforcal. Manifestations allergiques après ingestion d'escargots chez 12 malades allergiques aux acariens. une nouvelle allergie croisée? *Rev Fr Allergol* 1992; 32:198–202.
- Carrillo T, de Rodriguez F, Blanco C, Castillo R, Quiralte J, Cuevas M. Anaphylaxis due to limpet ingestion. *Ann Allergy* 1994; 73:504–8.
- van Ree R, Antonicelli L, Akkerdaas JH, Garritani MS, Aalberse RC, Bonifazi F. Possible induction of food allergy during mite immunotherapy. *Allergy* 1996; 51:108–13.
- Reese G, Ayuso R, Lehrer SB. Tropomyosin: an invertebrate pan-allergen. *Int Arch Allergy Immunol* 1999; 119:247–58.
- Smillie LB. Structure and functions of tropomyosins from muscle and non-muscle sources. *Trends Biochem Sci* 1979; 4:151–5.
- Restani P, Beretta B, Fiocchi A, Ballabio C, Galli CL. Cross-reactivity between mammalian proteins. *Ann Allergy Asthma Immunol* 2002; 89 (Suppl. 1):11–5.
- Ayuso R, Lehrer SB, Tanaka L *et al*. IgE antibody response to vertebrate meat proteins including tropomyosin. *Ann Allergy Asthma Immunol* 1999; 83:399–405.
- Ayuso R, Lehrer SB, Lopez M *et al*. Identification of bovine IgG as a major cross-reactive vertebrate meat allergen. *Allergy* 2000; 55:348–54.
- Ayuso R, Lehrer SB, Reese G. Identification of continuous, allergenic regions of the major shrimp allergen Pen a 1 (tropomyosin). *Int Arch Allergy Immunol* 2002; 127:27–37.
- Lehrer SB, McCants ML. Reactivity of IgE antibodies with crustacea and oyster allergens: evidence for common antigenic structures. *J Allergy Clin Immunol* 1987; 80:133–9.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–5.
- Reese G, Jeoung BJ, Daul CB, Lehrer SB. Characterization of recombinant shrimp allergen Pen a 1 (tropomyosin). *Int Arch Allergy Immunol* 1997; 113 (1–3):240–2.

- 35 Heukeshoven J, Dernick R. Improved silver staining procedure for fast staining in PhastSystem Development Unit. I. Staining of sodium dodecyl sulfate gels. *Electrophoresis* 1988; **9**:28–32.
- 36 Ceska M, Lundkvist U. A new and simple radioimmunoassay method for the determination of IgE. *Immunochemistry* 1972; **9**:1021–30.
- 37 Li XM, Serebrisky D, Lee SY *et al*. A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J Allergy Clin Immunol* 2000; **106** (Part 1):150–8.
- 38 Hoffmann A, Jamin A, Foetisch K *et al*. Determination of the allergenic activity of birch pollen and apple prick test solutions by measurement of beta-hexosaminidase release from RBL-2H3 cells. Comparison with classical methods in allergen standardization. *Allergy* 1999; **54**:446–54.
- 39 Vieths S, Hoffmann A, Holzhauser T, Muller U, Reindl J, Hausteine D. Factors influencing the quality of food extracts for *in vitro* and *in vivo* diagnosis. *Allergy* 1998; **53**:65–71.
- 40 Hoffmann A, Vieths S, Hausteine D. Biologic allergen assay for *in vivo* test allergens with an *in vitro* model of the murine type I reaction. *J Allergy Clin Immunol* 1997; **99**:227–32.
- 41 Ballmer-Weber BK, Hoffmann A, Wuthrich B *et al*. Influence of food processing on the allergenicity of celery: DBPCFC with celery spice and cooked celery in patients with celery allergy. *Allergy* 2002; **57**:228–35.
- 42 Vogel L, Luttkopf D, Hatahet L, Hausteine D, Vieths S. Development of a functional *in vitro* assay as a novel tool for the standardization of allergen extracts in the human system. *Allergy* 2005; **60**:1021–8.
- 43 Kwok SC, Hodges RS. Clustering of large hydrophobes in the hydrophobic core of two-stranded alpha-helical coiled-coils control protein folding and stability. *J Biol Chem* 2003; **278**:35248–54.
- 44 Morafo V, Srivastava K, Huang CK *et al*. Genetic susceptibility to food allergy is linked to differential TH2–TH1 responses in C3H/HeJ and BALB/c mice. *J Allergy Clin Immunol* 2003; **111**:1122–8.