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Research Article

High level expression, purification and physico- and immunochemical characterisation of recombinant Pen a 1: A major allergen of shrimp

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Well-characterised and immunologically active recombinant allergens are of eminent importance for improvement of diagnostic tools and immunotherapy of allergic diseases. The use of recombinant allergens has several advantages such as the more precise quantification of the active substance compared to allergen extracts and the reduced risk of contamination with other allergenic proteins compared to purified natural allergens. Optimised standard protocols for expression and purification and a detailed physico-chemical characterisation of such recombinant allergens are necessary to ensure consistent quality and comparability of results obtained with recombinant material. In this study the major allergen Pen a 1 of brown shrimp (*Penaeus aztecus*) was expressed in *E. coli* and purified in two steps by immobilised metal chelate-affinity chromatography (IMAC) and size-exclusion chromatography. Identity and purity were verified with N-terminal sequencing and peptide mass fingerprinting. Circular dichroism and NMR-spectroscopy indicated an alpha-helical flexible structure of rPen a 1 which is in accordance with the known structure of tropomyosins. Finally, the recombinant allergen proved to be immunologically reactive in IgE Western blot analysis and ELISA. This study provides a protocol for the preparation of recombinant shrimp tropomyosin in standardised quality.

Keywords: Allergen / High yield expression / Immunological characterisation purification / Pen a 1 / Tropomyosin

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1 Introduction

The majority of relevant allergens from foods have been identified, sequenced and expressed in recombinant form. Recombinant allergens are the basis to improve *in vitro* diagnosis and therapeutic approaches, since large quantities with high purity and standardised quality can be produced. But when recombinant allergens are to be used in diagnosis or therapy it has to be proven that the immunological reactivity of the chosen isoform is representative when compared to the natural protein.

Consumption of seafood such as fish, lobster, crab and molluscs can cause life-threatening symptoms in allergic individuals [1]. Most shellfish species that elicit allergic food reactions belong to the taxonomic class Crustacea and include shrimp, crab, crawfish and lobster; the shrimp genera *Penaeus* and *Metapenaeus* have been two of the most frequently studied.

Only two classes of immunological relevant allergens from shrimp have been identified and characterised so far: the arginine kinase Pen m 2 identified by proteomic analysis of *Penaeus monodon* extract [2] and the muscle protein tropomyosin, a major allergen, identified – among other species – in *Peneaus aztecus* (Pen a 1) and *Penaeus indicus* (Pen i 1) [3, 4]. Tropomyosins from other invertebrata such as arachnids (house dust mites), insects (cockroaches) and molluscs (squid, snail, oyster) were also shown to be allergenic and crossreactive against each other, suggesting invertebrate tropomyosins as important allergens [5 – 12]. Despite a very high degree of sequence identity and functional similarity with the latter, vertebrate tropomyosins are nonallergenic [13, 14]. Tropomyosins and the troponin C, I and T subunits regulate the Ca^{2+} dependent muscle contrac-

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Abbreviations: CD, circular dichroism; **IMAC,** immobilised metal affinity chromatography; **SEC,** size exclusion chromatography

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tion [15]. Tropomyosin is a homodimer of two parallel alpha-helical molecules with a molecular weight of approximately 33 kDa each forming a coiled-coil structure of about 40 nm length.

Even though numerous publications describe cloning, sequencing, expression and purification of recombinant allergens including invertebrate tropomyosin [16, 17] the practical experience often shows that the production of larger amounts that are required for experiments under well controlled conditions (*e.g.* T cell stimulation or epitope studies) is not always simple. Different proteins may need different approaches to expression and purification to obtain the best results regarding purity, structural integrity and immunological reactivity. The aim of this study is to describe in detail "large scale, preindustrial" production of a single batch of Pen a 1 that is well-characterised to be used in a multicentre Europe-wide study to characterise the allergen-specific immune response of allergic subject, investigate the effects of food processing and digestion on the allergenic potential and improve component-resolved diagnosis of food allergy. Identical allergen preparations of constant quality will also be used in other *in vitro* studies, such as different basophil activation tests allowing side-by-side comparison of results obtained by different techniques and laboratories but excluding the possibility that differences may be due to batch variation or diverging quality of the allergen preparations used. For this reason the purified recombinant Pen a 1 was characterised in detail in regard to its structural and immunological properties.

2 Materials and methods

2.1 cDNA synthesis, amplification and cloning

Total RNA was isolated from pulverised, snap-frozen *P. aztecus* (RNeasy-Total RNA-Isolation kit; Qiagen, Hilden, Germany), and 5′ RACE was performed using a gene-specific internal primer (5′-CTGCTCTTAACCGCCGCATC-CAGC-3′) deduced from published partial Pen a 1 cDNA sequence. Full-length cDNA was obtained by PCR, purified, cloned into pCR4-TOPO vector, expanded in TOP10 cells and sequenced [16]. It is our experience that RNA isolated snap-frozen live shrimp is an important step since prolonged storage (1–2 h) even at 4°C results in rapid degradation of mRNA. Competent *E. coli* BL21(DE3) (Novagen, Darmstadt, Germany) were transformed by heat shock with a pET-101/D-TOPO vector (Novagen) coding for Pen a 1 and a C-terminal hexahistidine tag.

2.2 Expression and purification of rPen a 1

Single colonies were grown in LB medium containing 50 μg/mL ampicillin (Carl Roth, Karlsruhe, Germany) at 37°C in a 2 L-benchtop fermenter (MoBiTec, Göttingen, Germany) for 2–4 h. Protein expression was induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG; Carl Roth) to a final concentration of 1 mM and incubation was continued for 2–4 h. Cells were harvested by centrifugation (20 min, 5000 × *g*, 4°C), resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 8 M urea) and stored at −80°C overnight. The lysate was thawed at 37°C in a water bath and centrifuged at 20 000 × *g* at RT for 1 h. The supernatant was filtrated (cellulose acetate filters, pore sizes 5 and 1.2 μm, Sartorius, Göttingen, Germany) and diluted 1:2 with lysis buffer without urea to reduce the urea concentration to 4 M. The recombinant Pen a 1 was purified by immobilised metal affinity chromatography (IMAC) using metal chelate spin columns (Vivascience/Sartorius) loaded with Co^{2+} ions and equilibrated two times with equilibration buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole). After binding of the protein to the column Pen a 1 refolded by several washing steps (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole) with decreasing amounts of urea. Recombinant Pen a 1 was eluted stepwise with buffer containing 250 mM, 500 mM and 1 M imidazole, respectively. Final purification and thereby buffer exchange to MOPS (20 mM, 0.5 M NaCl) was performed by size exclusion chromatography (SEC, Superdex 75 prep grade (26/60)). Protein content was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

2.3 SDS-PAGE and immunoblotting

Proteins were separated by SDS-PAGE on 13%T, 2.67%C gels under nonreducing conditions using the Laemmli buffer system and were detected by CBB staining (Pierce) or by silver staining according to Heukeshoven and Dernick [18]. For immunoblot analysis proteins were transferred to nitrocellulose membrane (pore size 0.2 μm, Schleicher & Schuell, Dassel, Germany) by semidry blotting [19]. Protein on the membrane was visualised by reversible Ponceau S staining (Fluka, Seelze, Germany). After blocking of free binding sites on the membrane (TBS containing 0.3% Tween-20), IgE-binding of rPen a 1 was shown with sera from shrimp-allergic patients (1:10 in TBS plus 0.05% Tween-20, 0.1% BSA) and a serum pool of mice sensitised to shrimp (1:100). Additionally, IgG-binding was shown with a polyclonal rabbit-anti-Pen a 1 serum (1:50 000) and the mouse serum pool (1:1000). The bound antibodies were detected with alkaline phosphatase labelled antibodies and visualised by adding the substrate containing 5-bromo,4-chloro,3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT) [20].

2.4 ELISA for the detection of Pen a 1 specific IgG antibodies

Recombinant Pen a 1 or shrimp extract was coated on microtitre plates (0.5 and 10 μg/well, respectively; 50 mM

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$Na_2CO_3/NaHCO_3$, pH 9.6; Nunc Maxisorp, Wiesbaden, Germany) overnight at 4°C. After blocking with TBS containing 0.05% v/v Tween and 1% w/v BSA for 1 h at room temperature, the coated proteins were incubated with sera from shrimp-sensitised mice (dilution series starting at 1:100) for 1 h at room temperature. Bound IgG was detected with alkaline phosphatase labelled anti-mouse IgG (1:5000) and developed with *p*-nitrophenyl phosphate substrate. Colour development was measured with an ELISA reader (Spectra MAX 340_{PC}, Molecular Devices, Sunnyvale, CA, USA) at 405 and 650 nm as reference wavelength.

2.5 Antibodies and sera

The human serum for Western blot analysis of an individual with history of shrimp allergy was bought from PlasmaLab (Everett, WA, USA). Human IgE was detected with alkaline phosphatase labelled anti-human IgE (1:1 000; Southern Biotechnologies Associates, Birmingham, USA). Murine serum pools were obtained from mice sensitised i.p. (adjuvant: aluminium hydroxide) with 100 µg (Western blot, ELISA) and 10 µg (ELISA) shrimp extract (4 mice *per* group). As secondary antibodies served alkaline phosphatase labelled rat-anti-mouse IgE (1:1000; Southern Biotechnologies Associates) and alkaline phosphatase labelled rabbit-anti-mouse IgG antibodies (1:5000; Sigma–Aldrich, Munich, Germany). The polyclonal rabbit-anti-Pen a 1 serum provided by Jonas Lidholm (1:50 000; Phadia, Uppsala, Sweden) was detected with an alkaline phosphatase labelled sheep-anti-rabbit IgG antibody (1:20 000; Sigma).

2.6 Circular dichroism (CD) spectroscopy

Recombinant Pen a 1 was dialysed against 10 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.4), and protein concentrations were adjusted to 5.2 µM. CD spectroscopy was performed on a J-180 S spectropolarimeter (Jasco, Gross-Umstadt, Germany) with constant nitrogen flushing at 20°C. For wavelength analysis, Pen a 1 was scanned with a step width of 0.2 nm and a band width of 1 nm. The spectral range was 185–255 nm at 50 nm/min. Ten scans were accumulated, and the mean residue molar ellipticity (Θ_{MRV}) was calculated.

2.7 N-terminal sequencing

A protein solution volume corresponding to 350 ng of protein was applied to Prosorb Inserts (Applied Biosystems) and washed three times with 30 µL of 0.1% TFA. The adsorbed protein was analysed on a Procise 492 sequencer (Applied Biosystems), using the standard PL PVDF Protein method.

2.8 Mass spectrometry

Purified rPen a 1 was subjected to SDS-PAGE and stained with CBB for subsequent MS analysis. In gel digestion was performed according to Shevchenko with several modifications [21]. Briefly, gel pieces were excised and destained in 25 mM NH_4HCO_3 containing 40% of ethanol. The samples were reduced and alkylated at ambient temperature for 20 min each with 10 mg/mL DTT and 50 mg/mL iodoacetamide in 50 mM NH_4HCO_3, respectively. The gel pieces were washed one time for 15 min in 25 mM NH_4HCO_3 before two cycles of incubation in 100% ACN for 15 min. Residual bicarbonate and ACN was removed in a vacuum centrifuge at ambient temperature for 30 min. Trypsin digestion was carried out at 37°C for 4 h by adding 10 µL of 75 ng/µL of trypsin (proteomics grade, Sigma–Aldrich, St. Louis, USA) to reswell the gel pieces followed by addition of 10 µL of 25 mM NH_4HCO_3. The peptides were eluted with 10 µL of 25 mM NH_4HCO_3 containing 75% of ACN. One microlitre of undiluted sample was mixed with 1 µL of peptide standard I (Bruker Daltonic, Bremen, Germany) diluted 1:5 in TA solution (one part of ACN and two parts of ultrapure water containing 0.1% of TFA) and 2 µL of matrix solution (a saturated solution of alpha-cyano-4-hydroxycinamic acid HCCA in TA solution). One microlitre of sample plus standard in matrix solution was allowed to air-dry on a ground steel target plate. Peptide mass fingerprints were recorded on a FLEX III MALDI-TOF mass spectrometer (Bruker) using standard parameters. FlexAnalysis 2.4 software (Bruker) was used for data analysis. The identity of rPen a 1 was proven by comparing experimental peptide masses with masses that were obtained by *in silico* digestion of Pen a 1. A maximum difference of 30 ppm was allowed for a match between experimental and theoretical data.

2.9 NMR spectroscopy

Pen a 1 was analysed by NMR. Before and after the NMR experiments, the allergen was stored at 253 K (−20°C). A solution was prepared in 0.45 mL H_2O plus 0.05 mL of D_2O. Starting form the experimental conditions described above (final purification), it was necessary to increase the concentration of rPen a 1 five-fold, by centrifugation coupled with a proper centrifugal filter unit (Centricon®). The final concentration was 0.13 mM. The solution was transferred into a high-quality NMR tube with argon as head-space gas. Two high resolution NMR experiments were carried out, using a Bruker Avance 700 spectrometer operating at a proton resonance frequency of 700 MHz (11.7 Tesla), at 298 K (25°C). Both experiments differed in the method to manage the water signal. The zgesgp experiment included a 1-D sequence to suppress the water signal using excitation sculpting with gradients. The pulse program of the zgpr experiment included a 1-D sequence with f1 presaturation to minimise the water signal.

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3 Results

3.1 Recombinant expression of Pen a 1

Recombinant Pen a 1 was expressed by *E. coli* BL21(DE3) cells that were transformed with the pET-101/D-TOPO vector (Novagen) coding for Pen a 1 and a C-terminal hexahistidine tag [16], in a bench top fermenter (MoBiTec) at 37°C under constant stirring and air supply.

3.2 Establishment of an efficient first purification step

Purification of his-tagged protein by immobilised metal chelate-affinity chromatography (IMAC) with Ni-NTA columns is a very common and mostly efficient technique. Hence, this was the method of choice to purify the his-tagged recombinant tropomyosin Pen a 1 (rPen a 1). Unfortunately, the binding efficacy to the Ni-NTA column and thus the amount of rPen a 1 in the elution fractions of the affinity chromatography was very low. To evaluate whether the usage of another capture ion as Ni^{2+} would improve the binding and elution properties of this method a comparative purification of rPen a 1 in bacterial lysate with four different capture ions (Ni^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}) was performed. A small volume of clarified bacterial lysate (400 μL) was applied onto Mini Spin Columns (Vivascience), which were loaded with the above-mentioned ions. The flow-through after binding and washing steps, as well as the elution fractions (250 and 500 mM imidazole) were collected. The proteins were separated by SDS-PAGE and visualised by silver nitrate staining. The comparison revealed that the column loaded with Co^{2+} ions showed the highest binding capacity for his-tagged rPen a 1, because there was almost no tropomyosin (36 kDa) detectable in the flow-through after binding (Fig. 1). There was more his-tagged tropomyosin found in the flow-through of the column loaded with Ni ions (Fig. 1), indicating a lower binding capacity of nickel for the his-tagged rPen a 1 compared with cobalt. The amount of target protein of in the elution fractions was strongly increased with the cobalt loaded columns compared to nickel loaded columns. Efficacy and specificity of binding to zinc or copper loaded columns turned out to be not preferable (not shown).

Based on these results his-tagged rPen a 1 was purified in larger scale (2 L fermenter culture) with Metal Chelate Mega Spin Columns (Vivascience) loaded with cobalt as capture ion and eluted with three different imidazole concentrations (250 mM, 500 mM, 1 M). Analysis of the different fractions by SDS-PAGE and Coomassie stain (Pierce) showed a high amount of purified rPen a 1 (Fig. 2) in the elution fractions. Processing the cell pellet for a 2-L-fermenter run yielded approximately 10 mg Pen a 1. Nevertheless, there was some tropomyosin detectable in the flow-through, which could be due to an overloading of the IMAC columns. More Pen a 1 was obtained in a subsequent purification step of this flow-through fraction even though amounts were lower than before (Fig. 2).

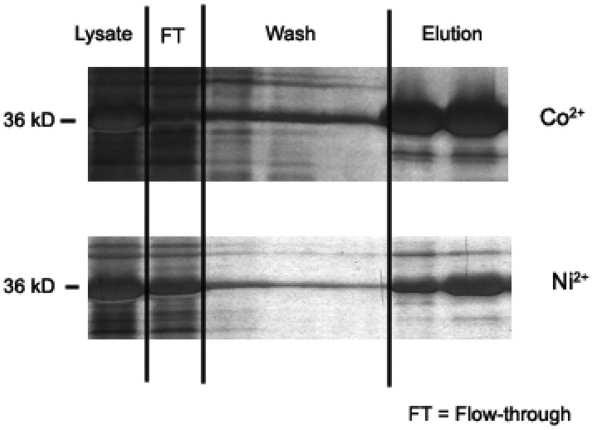

Figure 1. Comparison of purification efficacy using Co^{2+} or Ni^{2+} as capture ion. Bacterial lysates (Lysate) from rPen a 1-expressing *E. coli* cells were purified by affinity chromatography. Samples of flow-through (F), wash (Wash) and elution fractions (Elution) were analysed with SDS-PAGE and silver staining.

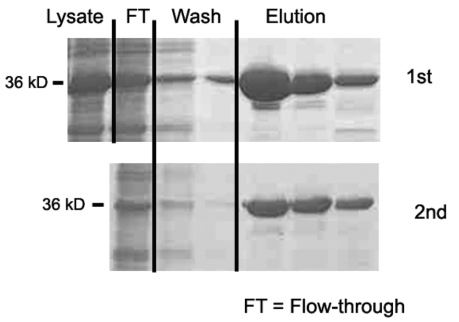

Figure 2. Increased yield of rPen a 1 with two-fold purification. Bacterial lysates (Lysate) from rPen a 1-expressing *E. coli* cells were purified by affinity chromatography. Samples of flow-through (FT), wash (Wash) and elution fractions (Elution) were analysed with SDS-PAGE and Coomassie stain. The flow-through from the first purification step (1st purification step) was reapplied to affinity chromatography (2nd purification step).

3.3 Second purification step by SEC

Approximately 30 mg of rPen a 1 were required for the planned studies [22]. To obtain a sufficient amount of rPen a 1, elution fractions of purifications based on a total of 6 L bacterial culture were pooled and concentrated with ultrafiltration spin columns (Vivaspin, MWCO 10 kDa, Vivascience). The concentrated samples were applied to an SEC column, to further purify the recombinant tropomyosin. During this process a buffer exchange to MOPS (20 mM, 0.5 M NaCl, pH 7.6) took place. The chromatogram showed a peak reaching about 70 mAU (Fig. 3A).

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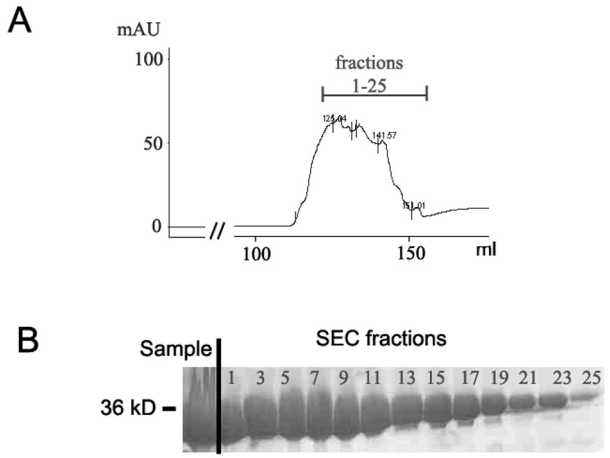

Figure 3. Purification by SEC. (A) Prepurified rPen a 1 was applied to SEC. Emergence of the separated protein was monitored by measurement of extinction. Fractionation started at beginning of the peak. (B) Relevant fractions were analysed by SDS-PAGE and Coomassie stain, compared to the sample applied to SEC.

Fractionation started at the onset of the peak as indicated in the histogram. Analysis of the relevant fractions by SDS-PAGE and Coomassie staining showed a high amount of purified rPen a 1 (Fig. 3B). Protein determination of the pooled tropomyosin containing fractions by the BCA (bicinchoninic acid) method revealed a concentration of 1 mg/mL in a volume of about 30 mL, resulting in a total amount of approximately 30 mg rPen a 1.

3.4 Authentication and identity

The apparent molecular mass of his-tagged rPen a 1 as determined by SDS-PAGE was approximately 37 kDa which corresponded well to the theoretical molecular mass of the fusion protein (36.4 kDa). The identity of the purified recombinant protein was confirmed by N-terminal microsequencing, revealing an amino acid sequence of MDAIKK that represent the first six N-terminal amino acids of Pen a 1 (Fig. 4). No contamination was detectable. In the mass fingerprint analysis 16 peptides representing 161 amino acid residues were identified, representing a sequence coverage of 49% of the tagged, 316 amino acid residue-long rPen a 1 (Fig. 4, Table 1).

3.5 Folding and stability of rPen a 1

Further analyses concerning the physicochemical properties of rPen a 1 were performed in order to demonstrate whether the structural properties were in accordance with known structural features of tropomyosins. In addition such data are required to obtain a constant batch-to-batch quality of recombinant material.

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1   MDAIKKKMQA MKLEKDNAMD RADTLEQQNK EANNRAEKSE  40
41  EEVHNLQKRM QQLENDLDQV QESLLKANIQ LVEKDKALSN  80
81  AEGEVAALNR RIQLLEEDLE RSEERLNTAT TKLAEASQAA  120
121 DESERMRKVL ENRSLSDEER MDALENQLKE ARFLAEEADR  160
161 KYDEVARKLA MVEADLERAE ERAETGESKI VELEEELRVV  200
201 GNNLKSLEVS EEKANQREEA YKEQIKTLTN KLKAAEARAE  240
241 FAERSVQKLQ KEVDRLEDEL VNEKEKYKSI TDELDQTFSE  280
281 LSGYKGELNS KLEGKPIPNP LLGLDSTRTG HHHHHH
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Figure 4. Amino acid sequence of rPen a 1 peptides detected by MALDI-TOF-MS. Peptides detected by MALDI-TOF-MS are underlined. Amino acids printed in bold letters are confirmed by N-terminal microsequencing. The amino acids residues 285–316 belong to the C-terminal His-tag.

Table 1. Summary of MS data of recombinant Pen a 1

Peptide position		Peptide position		Peptide position	
1–5	–	113–125	+	214–217	–
8–12	–	129–133	–	218–222	–
13–21	–	134–140	–	223–226	–
22–30	–	134–149	+	227–231	–
31–35	–	141–149	–	234–238	–
36–38	–	150–152	–	239–244	–
39–48	–	153–160	+	245–248	–
50–66	+	162–167	–	249–251	–
67–74	–	169–178	+	252–255	–
75–76	–	169–182	+	256–264	–
77–90	–	179–182	–	269–285	+
77–91	+	183–189	–	269–291	+
91–101	+	190–198	+	286–291	–
92–101	+	190–205	+	292–308	+
92–105	+	199–205	–	296–308	–
106–112	–	206–213	–	309–316	+

+ indicates peptides which were detected by MALDI-TOF-MS.

NMR-analysis of rPen a 1 showed that in the aromatic/amide region of the spectra (Fig. 5), there were no peaks above 9 ppm and below 6.7 ppm. The same was observed for the 5–6 ppm region, where alpha protons of tertiary structures can have their signals dispersed. In these spectra they were noticed only between 4 and 4.5 ppm. In the methyl region only few peaks were observed, especially near 1 ppm and no more peaks appeared downfield. The region of CH_2 and of aliphatic CH showed few peaks, together with peaks of the buffer and of small organic molecules. Therefore there was no evidence of tertiary structure of the recombinant protein but the spectra were consistent with the presence of secondary structure and flexibility.

To further assess the secondary structure of the recombinant protein, CD spectroscopy was performed. The CD spectrum of rPen a 1 showed minima at 222 and 208 nm (Fig. 6), typical for alpha-helical proteins such as tropomyosins. These data are in accordance with the folding data from the purified natural Pen a 1 showing the same CD spectrum [16].

Recombinant Pen a 1 was stored at different temperatures (–20, 4, RT and 37°C) for 1 and 2 wk, to determine its stability. The samples were analysed with SDS-PAGE and

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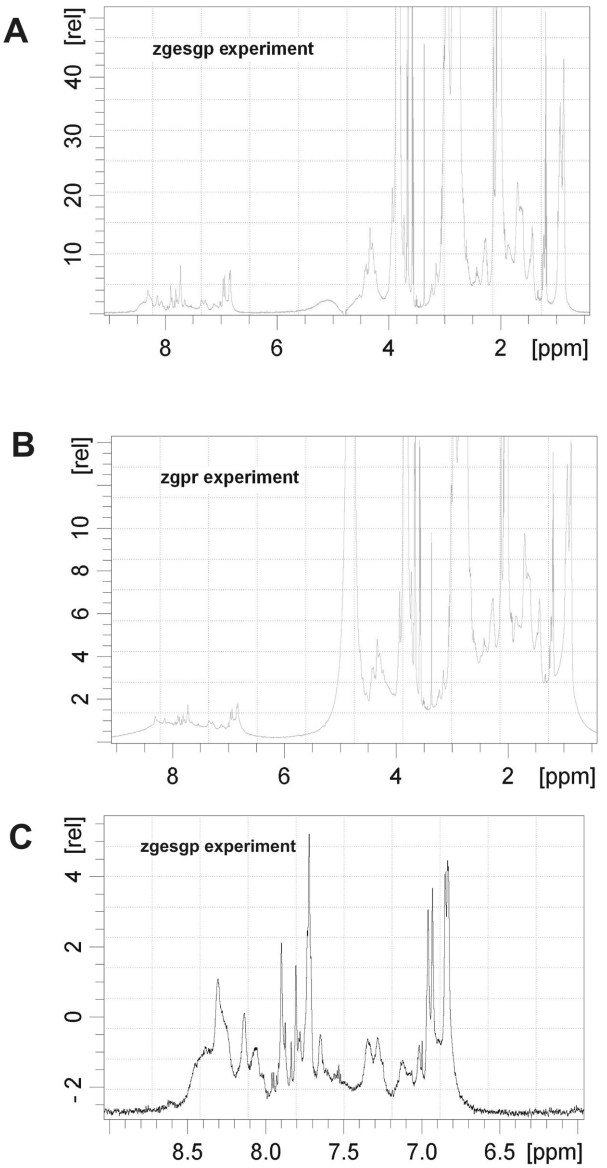

Figure 5. NMR-spectra of rPen a 1. ^{1}H 700 MHz spectra of Pen a 1, 256 scans, 298 K; zgesgp (A,C) and zgpr (B) experiments. The spectra provide no evidence of tertiary structure, but the spectra are typical for tropomyosin and are consistent with the presence of secondary structure. The pane (C) shows, enlarged, the 6–9 ppm region of the zgesgp spectrum.

silver staining or by immunoblot. As expected, no degradation of the tropomyosin was observed in the silver stained gel after storage at 4 and −20°C for 1 and 2 wk (Fig. 7A). Surprisingly this was also true for storage at room temperature and even at 37°C after 1 wk. The sample incubated for 2 wk at 37°C showed a slight smear in the silver stain, indicating some degradation. Immunoblot analysis of the samples with a polyclonal antibody directed against Pen a 1 showed that the recombinant protein was not degraded and also kept its immunological reactivity after the indicated storage conditions (Fig. 7B).

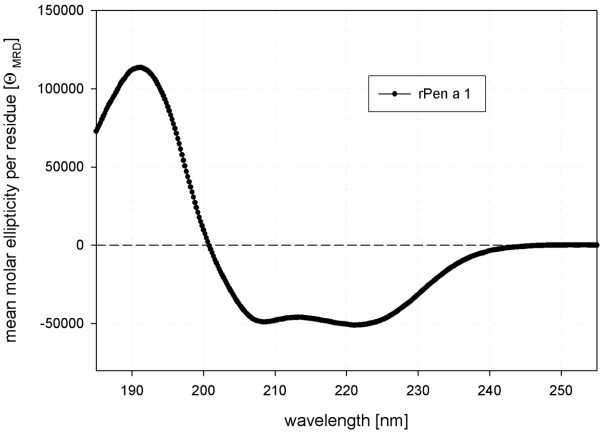

Figure 6. CD-spectrum of rPen a 1. The secondary structure of the purified rPen a 1 was confirmed by CD analysis showing minima at 222 and 208 nm, typical for alpha-helical proteins such as tropomyosins.

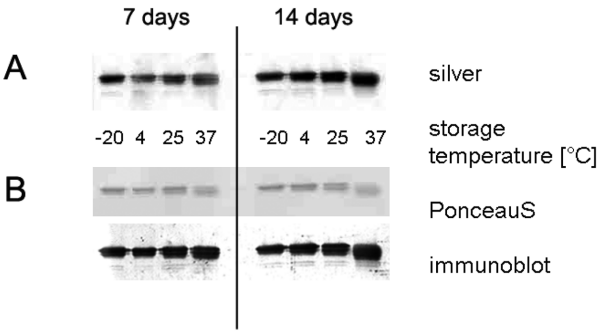

Figure 7. Stability of rPen a 1 at different storage conditions. The rPen a 1 was stored at the indicated temperatures for 1 and 2 wk, respectively. Stability was checked by SDS-PAGE and silver stain (A) and Western blotting with polyclonal rabbit-anti-Pen a 1 serum (B). A Ponceau S stain of the nitrocellulose membrane was performed to confirm correct protein transfer.

3.6 Recombinant Pen a 1 is immunologically reactive

When analysed by Western blotting rPen a 1 was detectable with a polyclonal IgG antibody directed against Pen a 1 (Fig. 9A). Specific IgG and IgE antibodies in sera from mice that were immunised with shrimp extract [23] reacted with the recombinant protein (Fig. 8A). Additionally the binding of human IgE antibodies from a shrimp-allergic patient to the rPen a 1 was observed (Fig. 8A). Binding of the polyclonal anti-Pen a 1 antibody and IgE from a shrimp-allergic subject (Pen a 1-specific IgE measured with ImmunoCAP, Phadia) to the natural tropomyosin in shrimp extract was inhibited in a dose-dependent manner by preincubation with the recombinant tropomyosin (Fig. 8B).

Furthermore, binding of rPen a 1 in comparison with shrimp extract in an ELISA-test was investigated. For this

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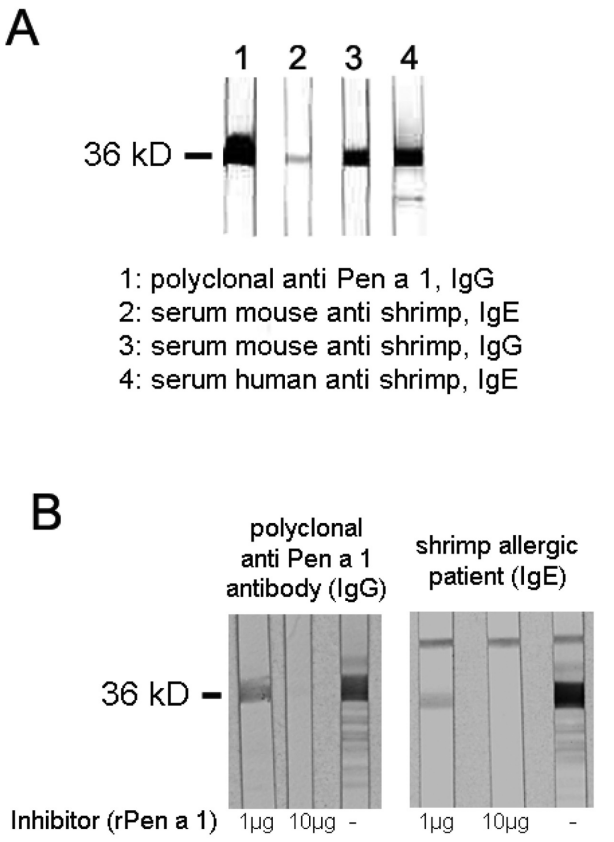

Figure 8. Immunological reactivity of rPen a 1 determined by Western blot analysis. (A) Immunological reactivity of rPen a 1 in Western blot analysis was demonstrated with different sera from rabbit, mouse and human. (B) Reactivity to Pen a 1 in shrimp extract of sera from a shrimp allergic patient (IgE) and a polyclonal rabbit-anti-Pen a 1 serum (IgG) was inhibited by preincubation with 1 and 10 µg of rPen a 1.

purpose the recombinant tropomyosin or an extract from *P. aztecus* [24] were coated on 96 well microtitre plates. As first antibody served the dilution series of two different pools from mice immunised with shrimp extract. Bound IgG antibodies were detected with an alkaline phosphatase-labelled antibody directed against mouse IgG. After incubation with the substrate, the OD of the developed colour was measured with an ELISA reader. The resulting curves showed that binding to the extract was approximately tenfold lower than to rPen a 1 (Fig. 9).

4 Discussion

Expression and purification of recombinant allergens that resemble their natural counterparts in structure, folding and immunological reactivity is of increasing importance in terms of component-resolved diagnosis and specific immunotherapy of allergic diseases [25, 26], and for studying the behaviour of food allergens under conditions of the gastro-

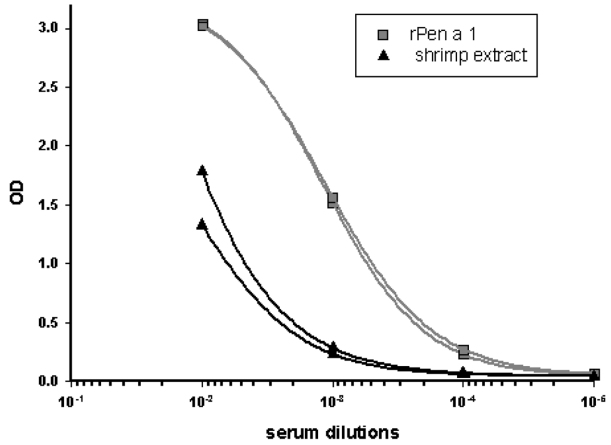

Figure 9. Immunological reactivity of rPen a 1 in ELISA. Two serum pools from mice sensitised with shrimp extract were titrated in IgG-ELISA with rPen a 1 (0.5 µg/well; triangle) or shrimp-extract (10 µg/well; rectangle) coated on the microtitre plate.

intestinal tract or during food processing. Thus, standardised protocols for reproducible production of recombinant allergens are of high interest.

Although other publications describing cloning, expression and purification of recombinant Pen a 1 and other crustacean tropomyosins are already available [3, 8, 27–29] this study sought to develop a detailed protocol resulting in a high amount of purified protein combined with evidence for correct folding and demonstration of IgE-binding properties, allowing the production of subsequent batches in constant quality. Given the high degree of similarity among different invertebrate tropomyosins in regard to amino acid identity and immunological reactivity [12, 30, 31], this protocol might be useful as a guide on how to obtain other recombinant allergenic tropomyosins for research applications.

The commonly used IPTG-inducible *E. coli* B21(DE3) expression system was successfully applied in this investigation. In contrast to many other studies, where bacteria were grown in shaker flasks, in this study, bacterial growth and induction of expression took place in a 2 L bench top fermenter system, since this system turned out to be more controllable regarding temperature, stirring and air supply and led to reproducible growth curves. Utilisation of this method yielded in approximately 10 mg rPen a 1 per 2-L-culture after purification, which corresponds with the amounts obtained by other groups expressing recombinant tropomyosins by *E. coli* [7, 32–34]. Thus, it seems that recombinant invertebrate tropomyosins could be easily manufactured in *E. coli* at "mg-scale". In some studies even higher yields have been reported [17].

Lysis of the bacterial pellet in presence of 8 M urea yielded in a higher amount of soluble recombinant his-tagged Pen a 1 than lysis under native conditions indicating

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a considerable amount of protein was present in inclusion bodies. The issue using urea in the lysis is often to obtain finally correctly folded proteins in the buffer of choice. In this case, the protein remained completely soluble and presented a fully alpha-helical secondary structure, indicating that a folding corresponding to the known structure of tropomyosins had been adopted. Correct 3-D (secondary structure) folding of rPen a 1 could be demonstrated by CD-spectroscopic analysis (Fig. 6).

Separation of the protein of interest from the other bacterial components present in the lysate is an important step of the protocol, since this is the basis for purity of the recombinant protein. A contaminated preparation of a recombinant allergen might lead to false outcomes of research experiments and might therefore cause wrong conclusions. Thus, the process of separation should lead to a high purity of the protein. On the other hand it is desirable to obtain high amounts of the protein for some applications (*e.g.* mouse models), because in terms of comparability one should use the same batch of material within one study. Often these two needs contradict each other. To address this problem a comparison of four different capture ions for purification of his-tagged proteins was performed at small scale. As Ni^{2+} is the ion of choice for purification of his-tagged proteins in most studies, it was surprising that Co^{2+} ions maximised purity and protein yield. Taken into account that in the majority of cases a different capture ion than Ni^{2+} is probably not considered, purification with Co^{2+} ions might lead to better results with other recombinant proteins as well. This investigation shows that the choice of metal ion that works best for the protein of interest should be investigated. Moreover this test could be done with the minute volume of 400 μL of bacterial lysate for each ion, so the benefit seems to overcome the minimal loss of expressed protein needed in the test. Another difference of our study to several purification protocols of his-tagged proteins is the use of metal chelate spin columns (Vivascience) instead of agarose gravitation flow columns. Any desirable capture ion can be loaded onto the membrane of the spin columns. The procedure comprises several centrifugation steps, at which the ion-loaded membrane is equilibrated, the protein is loaded onto the membrane and eluted after some washing steps. It is possible to process lysate of 2 L bacterial culture on one column which is very useful for larger scale purifications performed at research laboratories. This method is quick, convenient and resulted in comparable amounts of purified protein in different experiments.

Subsequently, the rPen a 1 was applied to a second purification step by SEC. This purification relies on another separation principle, which ensures that contaminating proteins that might have been eluted together with rPen a 1 in the affinity chromatography, due to similar binding properties to Co^{2+}, could be separated from rPen a 1 in SEC by different size. N-terminal sequencing of the rPen a 1 preparation confirmed the identity of the desired protein without any detectable contamination, as did MALDI-TOF MS, indicating that the purification protocol was efficient.

The two methods used to characterise the conformation of rPen a 1 provided evidence that the recombinant protein adopted the overall alpha-helical structure expected for tropomyosins: The CD spectrum of rPen a 1 showed minima at 222 and 208 nm in the experimental conditions adopted for CD analysis (Fig. 6) and, according to literature, the [θ]222 to [θ]208 ratio of more than 1 (1.067) indicates a coiled-coil structure [35–37]. Even though the NMR analysis showed no evidence of tertiary structure, the spectra are consistent with the presence of secondary structure (Fig. 5). However, the absence of a signal in the HN backbone region above 8.7 ppm (Fig. 5C) argues against a coiled-coil structure, indicating that rPen a 1 exists as a alpha-helical monomer – at least in the experimental conditions that were adopted for NMR analysis [38–41].

A feature often associated with classical food allergens is the high stability [42]. Although tropomyosins are known to be heat-stable [28, 43], which might also be related to its coiled-coil structure [44], it has to be proven, if preparations of recombinant allergens remain stable and retain their immunological reactivity during storage in the buffer used. As expected, investigations with rPen a 1 revealed that it is stable and immunological reactive in MOPS-buffer at different storage conditions for at least 2 wk even at increased temperatures. Further monitoring over longer periods will be required, but the results on hand suggest that Pen a 1 remains stable even as a recombinant protein.

The immunological reactivity, especially the IgE-binding properties, of a recombinant produced allergen is of great relevance for its use in research applications, since it should resemble those of the natural allergen. Immunological properties of rPen a 1 were assessed in this study by Western blot analysis, inhibition experiments and ELISA. The recombinant protein could be detected with sera from mice and human that were sensitised to shrimp containing the natural form of Pen a 1 and the binding of IgG and IgE antibodies to natural tropomyosin in shrimp extract could be inhibited by preincubation with rPen a 1. Reactivity of mouse sera from shrimp-sensitised mice measuring specific IgG antibodies by ELISA titration with both rPen a 1 and shrimp extract suggest that the immunological properties of rPen a 1 may be similar to those of nPen a 1 in shrimp extract. These data are consistent with previous studies on rPen a 1 prepared and purified by a different approach [16].

A complicacy not addressed in this study is the endotoxin level in the preparations, which are probably high due to expression in a bacterial system. Contaminations with endotoxin could play a role when the recombinant allergen is used in cell culture (*e.g.* T cell studies involving analysis of cytokine expression pattern) or in *in vivo* models of food allergy, since endotoxin is known to modulate the immune response and to influence allergic sensitisation [45–47]. Some preliminary experiments showed that a washing step

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with isopropanol-containing buffer during the affinity chromatography somewhat reduced the endotoxin level, but this was accompanied with a high loss of the desired protein as well (data not shown). Here, the major portion of endotoxin in the rPen a 1 preparation might have been eliminated by the second purification step (SEC), but if a preparation of recombinant protein expressed in bacterial systems is to be applied to the aforementioned methods, measurement of endotoxin levels should be considered.

In conclusion, this study provides the protocol for an expression system and a two-step purification method for production of high amounts recombinant Pen a 1 reflecting its natural counterpart in structure, folding an immunological reactivity. Furthermore, this protocol might serve as a guide for expression of other recombinant tropomyosins.

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