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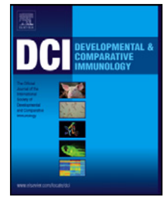

Cockroach allergens Per a 3 are oligomers

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ABSTRACT

Allergens from cockroaches cause major asthma-related health problems worldwide. Among them Per a 3 belongs to the most potent allergens. Although the sequences of some members of the Per a 3-family are known, their biochemical and biophysical properties have not been investigated. Here we present for the first time a thorough structural characterization of these allergens, which have recently been tested to induce an increase of allergy specific indicators in blood of Europeans. We isolated two Per a 3 isoforms, which occur freely dissolved in the hemolymph as hexamers with molecular masses of 465 ± 25 kDa (P II) and 512 ± 25 kDa (P I). Their sedimentation coefficients ($S_{20,W}$) were determined to be 17.4 ± 0.7 S (P II) and 19.0 ± 0.9 S (P I), respectively. Sequence analysis revealed that P II consists of two subunit types known as allergens Per a 3.01 and Per a 3.0201, while PI consists of a new allergenic subunit type designated as Per a 3.03. A 3D model of the hexameric allergen Per a 3 was obtained by homology modelling. Almost all of the recently predicted 11 putative antigenic peptides and reported IgE-epitopes could be located on the surface of the hexamer, thus being freely accessible in the hexameric structure of the native molecules. We propose this might contribute to their allergic potential as well as their extreme stability with respect to temperature.

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1. Introduction

Allergy against cockroaches is a worldwide problem. Since the first study of Bernton and Brown in 1964, numerous studies confirmed skin test sensitivities to cockroach allergens and an association between cockroach infestations and allergic sensitization was identified as a major risk factor for morbidity caused by asthma in children [1–6]. These studies have clearly demonstrated that asthma caused by cockroaches is antigen specific and similar to other types of atopic asthma [5,7]. Depending on the geographical location the incidence of asthmatic patients sensitized against cockroach allergens ranges from 40 to 70% [3,8].

Although more than 3500 different cockroach species are known, only a few of them are frequently found in homes the two most common species being the American cockroach (*Periplaneta americana*) and the German cockroach (*Blattella germanica*) [3,8–11]. Their aerosolized allergens are derived from several sources, such as saliva, fecal material, secretions, exuviae, egg cases, debris and dead bodies [7,9,11,12]. A high stability of their proteins probably enhances significant accumulation of their allergens in house dust. Several allergens from these cockroach species have recently been purified, sequenced, cloned and produced as recombinant proteins [3,13–21]. Immunological cross-reactivity between several allergens of these two species have been reported [20,22–27].

Per a 3, which does not exhibit cross-reactivity with other allergens, was identified as a major allergen in the American cockroach. Two isoforms of Per a 3 were identified as major allergens with molecular masses of 72 and 78 kDa. They bind to IgE in 100% of the sera of cockroach-allergic patients tested and cause T-cell proliferation of peripheral blood cells [3,8,28,29]. Four cDNA clones of these two slightly different Per a 3-isoformes (Per a 3.01, Per a 3.0201, Per a 3.0202, Per a 3.0203, formerly designated as C12, C20, C13, C28) were sequenced and analyzed [20,21]. The sequence comparison revealed a close relationship to storage proteins (hexamerins and arylphorins) from the hemolymph of insects and arthropod hemocyanins [20,21,30,31]. Further biophysical and structural information of Per a 3 allergens are not available.

The crystal structures of only three cockroach allergens have been described so far: Bla g 2 occurs as either monomer or dimer, Bla g 4 exists as a monomer and Per a 4 is found to be a dimer in

Abbreviations: ESI-QTOF, electrospray ionization-quadrupole time-of-flight mass spectrometry; HC, hemocyanin; CD, circular dichroism; ab, antibody; IEX, ion exchange chromatography; SEC, size exclusion chromatography.

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solution [32–35]. In recent years it has been proposed that the oligomeric structure of an allergen might play an important role with respect to its allergenicity (e.g. grass pollen allergens: Phl p 1, Phl p 5b, Phl p 7 and birch pollen Bet v 1) [36,37]. For the latter one, Bet v 1, it was recently demonstrated that oligomerization could enhance its cross-link capacity to receptor-bound IgE on basophils and mast cells [36].

Here we present the first physico-chemical characterization of the potent cockroach allergen Per a 3 and its 3D structure based on homology modelling. We were able to identify and isolate two Per a 3 isoforms: a mixture of Per a 3.01 and Per a 3.0201, designated as P II, and a new allergenic isoform, so far not known by sequence, named Per a 3.03. All of them occur as homohexamers with a close structural similarity to hemocyanins. This justifies to construct a hexameric 3D model of Per a 3, which was used to localize putative antigenic peptides suggested by Wu et al. and allergenic IgE-epitopes [8,21]. Both isoforms are thermal stable and might be able to remain for a longer period in the environment and accumulate in house dust as native allergens.

2. Experimental procedures

All chemicals were purchased from Roth (Karlsruhe, Germany) unless otherwise indicated and were of analytical grade. The pH of all buffers was adjusted at 20 °C.

2.1. Preparation of hemolymph

Unsexed nymphs and adults of the American cockroach (*P. americana*) were obtained from Bayer CropScience (Monheim, Germany). Prior to hemolymph withdrawal cockroaches were anesthetized with carbon dioxide and decapitated. Then ~100 μl hemolymph were collected with a pipette from each animal and immediately diluted in a ratio of 1:10 with ice-cold extraction buffer (100 mM Tris/HCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.01% (w/v) phenylthiourea, pH 7.8). After centrifugation at 4 °C for 10 min at 18.740 × *g* the yellowish-turbid supernatant was immediately stored at −32 °C.

2.2. Protein purification

Allergens were purified by a two-step protocol using ion exchange chromatography (IEX) and size exclusion chromatography (SEC). Briefly, hemolymph was prepared for IEX by dilution with loading buffer (20 mM Tris/HCl, pH 7.0) in a ratio of 1:3, centrifugation for 10 min at 18.740 × *g* and filtration with Acrodisc syringe filters (pore diameter 0.2 μm, PALL Gelman, Dreieich, Germany). Typically, 1 ml of diluted hemolymph was applied onto a Bio-Scale Q2 (2 ml, BioRad, Munich, Germany) at 4 °C. For ion exchange chromatography purification a "loading buffer" containing 20 mM Tris/HCl (pH 7.0) and a "high salt elution buffer" containing 20 mM Tris/HCl and 1.0 M NaCl (pH 7.0) were used. Bound proteins were eluted at a flow rate of 1 ml/min in a NaCl gradient with "high salt elution buffer". That way two isoforms of Per a 3 allergen were separated. Fractions containing the two isoforms of Per a 3 allergen were rechromatographed under the same conditions. Finally fractions containing the respective isoforms were chromatographed by size exclusion chromatography in stabilization buffer (100 mM Tris/HCl, 5 mM $CaCl_2$ and 5 mM $MgCl_2$, pH 7.3) using a S-300 16/60 column (Pharmacia, Erlangen, Germany) at a flow rate of 0.5 ml/min.

2.3. Electrophoresis

SDS-PAGE was performed using the system of Laemmli as modified by See and Jackowski [38,39]. Samples were denatured in sample buffer containing SDS and 2-mercaptoethanol for at least 10 min at 100 °C. "Plus Protein TM unstained Standard" (BioRad, Munich, Germany) or "Kaleidoscope prestained Standards" (BioRad, Munich, Germany) were used as the molecular-mass standards. For native PAGE the same system was used but SDS was omitted from all solutions and samples were not denatured prior to electrophoresis. Gels were stained either with Coomassie Brilliant Blue or with silver stain [40].

2.4. Crossed immuno-electrophoresis

Polyclonal antibodies against purified P II and Per a 3.03 in their native oligomeric state were raised in rabbits by Charles River GmbH (Sulzfeld, Germany). Crossed immuno-electrophoresis was performed as described elsewhere [41].

2.5. Western blot

Proteins were transferred after SDS-PAGE onto a nitrocellulose membrane (Protan BA83, 0.2 μm pore Size, Schleicher and Schuell, Germany) in a horizontal blotting chamber (Trans-Blot SD Semi-Dry Transfer Cell, BioRad, Germany) using the protocol of Kyhse-Andersen as supplied by the manufacturer [42]. Non-specific protein binding sites were blocked at 4 °C for 1–2 h with 5% (w/v) non-fat dry milk (Saliter, Germany) in TBST buffer (0.3% (v/v) Tween 20, 100 mM Tris/HCl, 1.4 M NaCl, pH 7.4). For immunodetection, the membrane was incubated overnight with rabbit antisera raised against P II or Per a 3.03 in a solution containing 5% (w/v) non-fat dry milk. Typically rabbit antisera were diluted in a ratio of 1:100,000 and human antisera were diluted 1:10 to prevent unspecific binding. Unbound antibodies were removed by washing four times for 10 min with TBST buffer.

Bound antibodies were detected with anti-rabbit-IgG-antibodies or anti-human-IgE-antibodies coupled with alkaline phosphatase (Sigma, Steinheim, Germany). Briefly, a solution of 0.01% (v/v) anti-rabbit-IgG-antibodies or 0.20% (v/v) anti-human-IgE-antibodies in TBST was incubated with the membrane for 90 min and the unbound antibodies were removed by washing. Bound antibodies were detected using a solution containing 10 mM Tris/HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.033% (w/v) p-nitrobluetetrazolium and 0.016% (w/v) 5-bromo-4-chloro-3-indolyl-phosphat and 0.8% (v/v) dimethyl formamide.

2.6. Analytical ultracentrifugation

Sedimentation equilibrium and sedimentation velocity experiments were performed in a Beckman Optima XL-I analytical ultracentrifuge (Palo Alto, CA, USA) using an An-50Ti rotor. For all sedimentation velocity experiments sample cells with 12 mm double-sector charcoal-filled epon centrepieces and quartz windows were used. All runs were performed at 25 °C and 28,000 rpm. During the run the absorbance of the cells was scanned at 280 nm every 3 min. Velocity data were analyzed using the method of van Holde and Weischet and the finite-element analysis of Demeler and Saber as implemented in the program ULTRASCAN 6.0 [43–45]. For sedimentation equilibrium runs six-channel charcoal-filled epon centerpieces with quartz windows were used. Samples were run at speeds of 6000, 8000 and 10000 rpm at 25 °C. Each sample was run for at least 24 h at each speed. To confirm that equilibrium was reached the absorbance of the cells was scanned at 280 nm after 23 h. When no significant change between the two scans could be detected it was assumed that equilibrium had been reached. Equilibrium data were analyzed with the program ULTRASCAN 6.0 employing the global-fit routine to integrate data taken at different speeds. In all calculations, a partial specific volume of 0.72 cm^3/g was assumed as commonly used [46].

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