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Characterization of the major allergens of *Pachycondyla chinensis* in ant sting anaphylaxis patients

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Clinical and Experimental Allergy

Summary

Background The ant species *Pachycondyla chinensis*, which has spread from Far Eastern Asia to New Zealand and North America, induces anaphylactic reactions in human with its sting. However, the major allergens of *P. chinensis* have not yet been characterized.

Methods We selected seven patients with histories of anaphylaxis induced by *P. chinensis*. Two-dimensional electrophoresis (2-DE) was used to identify the major allergens. We subsequently performed Western blots for *P. chinensis*-specific IgEs, N-terminal amino acid sequencing, ESI-MS/MS, and RT-PCR using primers based on the N-terminal sequence.

Results Six of the anaphylactic subjects had an IgE specific to a 23 kDa allergen of *P. chinensis*. Two candidates for major allergens, 23 kDa (pI 8.7) and 25 kDa (pI 6.2), were revealed by 2-DE using *P. chinensis*-specific IgE immunoblotting. In N-terminal sequencing and ESI-MS/MS analysis, 23 kDa (pI 8.7) and 25 kDa (pI 6.2) allergens, belonging to the protein families of antigen 5, were identified and share marked amino acid sequence similarity. The 23 kDa allergen is 206 amino acids in length and homology searches showed 54.0% and 50.0% homology with Sol i 3 and Ves v 5, respectively.

Conclusion The major allergens of *P. chinensis* are 23 kDa (pI 8.7) and 25 kDa (pI 6.2) proteins that belong to the antigen 5 family of proteins.

Keywords ant sting anaphylaxis, major allergen, *Pachycondyla chinensis*

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Introduction

Allergies related to ant sting anaphylaxis are a major problem; species from a total of 10 genera in six subfamilies of ants have been reported to induce anaphylaxis [1]. Imported fire ants (*Solenopsis invicta* and *Solenopsis richteri*), which originated from South America and belong to the Myrmicinae subfamily, are frequently identified as a major cause of anaphylaxis in the south-eastern United States. *Pogonomyrmex*, known as harvester ants, also in the Myrmicinae subfamily, elicit anaphylaxis in the USA. In Australia, Jack jumper ants and bull ants are the major species responsible for ant sting anaphylaxis; both of these species are also members of the Myrmecinae subfamily. Stings from several species in the Ponerinae subfamily including species in the genera *Rhyridoponera*,

Hypoconera, *Odontomachus*, and *Pachycondyla* can also cause anaphylaxis [1, 2] (Fig. 1).

Pachycondyla chinensis is native to Far Eastern Asian countries such as Korea, Japan, China, Taiwan, and now occurs in New Zealand, and the USA. This species invaded the United States 70 years ago and has since successfully colonized the south-eastern states [3]. The sting of *Pachycondyla* species has occasionally induced anaphylactic reactions. All reported cases of ant sting anaphylaxis in Korea were induced by this species [4–6], and sting anaphylaxis patients stung by *Pachycondyla* species have been reported in Japan [7], the United Arab Emirates [8], and the USA [3, 9]. *Pachycondyla sennaarensis* was responsible for four fatal cases in the United Arab Emirates [8]. The sensitization rate to *P. chinensis* was found to be about 23% in residents from the ant's endemic area, and 2.1% of the residents were reported to have a history of systemic allergic reactions to the ant [5].

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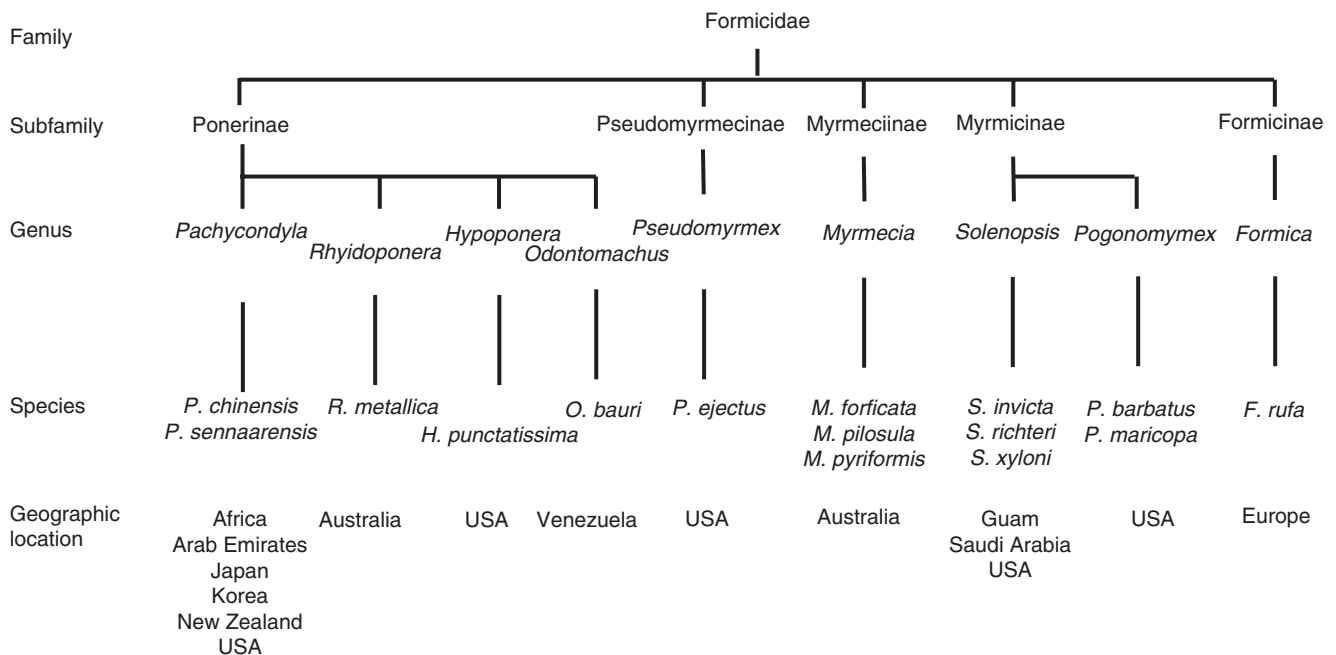


Fig. 1. Taxonomy of ants that can induce sting anaphylaxis.

The prevalence of sensitization and allergies to *P. chinensis* are similar to those seen in the endemic area of *Solenopsis*.

However, the major allergens of the *P. chinensis* venom have not yet been identified. In this study, we identified the major venom allergens of *P. chinensis* using two-dimensional PAGE, N-terminal amino acid sequencing, and ESI-MS/MS analysis, and cloned the cDNA of the suspected major allergen.

Material and methods

Preparation of *P. chinensis* ant extract

P. chinensis were defatted by ether. Subsequently, the abdomen of *P. chinensis* containing the venom sac was separated from the thorax and head and mixed with phosphate-buffered saline (PBS; 1:50 w/v). After ultrasonication, the extract was centrifuged at 9000 g for 30 min. The supernatant was dialyzed (pore size 3 kDa; Spectrum, Houston, TX, USA) against distilled water for 72 h and then used as crude extract. The protein concentration of the extract was measured using the Bradford method (Bio-rad, Hercules, CA, USA; 1980 µg/mL), and was then lyophilized for use.

Patients and measurement of specific immunoglobulin E

The sera of seven ant sting anaphylaxis patients were used for this study. All patients had visited the emergency room due to anaphylaxis after a *P. chinensis* sting. Two patients had histories of anaphylaxis related to bee stings. Serum

Table 1. Clinical features of the enrolled subjects

Patient no.	Sex	Age (year)	Causes of anaphylaxis	SPT (mm)	slgE (OD)
1	F	51	Ant Sting	ND	1.12
2	F	47	Bee and ant sting	5	0.178
3	M	38	Ant sting	3	0.048
4	F	33	Ant sting	3	0.032
5	M	22	Bee and ant sting	5.5	0.059
6	F	34	Ant sting	4	0.145
7	F	40	Ant sting	ND	1.189

ND, not done; SPT, skin prick test – the values indicate the mean wheal size; OD, optic density. Mean and SD of negative control OD ($n = 10$), 0.004 ± 0.002 .

was collected at the initial diagnoses. For a negative control, sera from 10 non-atopic asthma patients were used. A skin prick test was performed in five patients with the *P. chinensis* extract described above (1 mg/mL in 50% glycerol phenol saline), using histamine (1.7 mg/mL) as a positive control and physiologic saline as a negative control. Mean weal size larger than 3 mm compared with the negative control was considered a positive response. Two other patients who visited other hospitals were not tested using the skin prick test and only clinical data and sera were available. ELISA tests for the detection of the IgE specific to *P. chinensis* were performed as described previously [4]. All enrolled subjects had specific IgE to *P. chinensis* and all five patients who received the skin prick test also showed positive skin responses; conversely, all of the 10 negative control subjects showed negative responses (Table 1).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoglobulin E immunoblotting

Antigens were dissolved in a reducing sample buffer (12.5 mM Tris–HCl, pH 6.8, 5% glycerol, 0.4% SDS, 1% mercaptoethanol, and 0.02% bromophenol blue) and boiled at 100 °C for 5 min. Fifty micrograms of the allergen samples were separated by electrophoresis on 4–12% polyacrylamide gels (Hoeffer, San Francisco, CA, USA) at 50 V for 30 min and at 180 V for 2 h. Proteins were transferred onto nitrocellulose membranes (Amersham, Buckinghamshire, UK) using a transfer unit (Hoeffer) in buffer (25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3) at 350 mA for 2 h. The membranes were blocked using 5% non-fat dried milk in TBS-T (50 mM Tris, 0.1% Tween 20, pH 7.5), then incubated with 1 : 10 diluted sera at room temperature overnight and washed with TBS-T. The membranes were incubated with 1 : 1000 diluted alkaline phosphatase-conjugated goat anti-human IgE (Sigma, St. Louis, MO, USA) for 60 min. After washing, the colorimetric reaction was developed by the BCIP/NBT system (Promega, Madison, WI, USA). For inhibition immunoblotting, the patient's sera was incubated with 20 µg/mL of *P. chinensis* antigen for overnight at 4 °C, and immunoblotting was done with the incubated sera as described above.

Two-dimensional polyacrylamide gel electrophoresis, ESI-MS/MS, and N-terminal sequencing

One hundred micrograms of *P. chinensis* antigen was loaded onto a two-dimensional cleanup strip (Bio-Rad) with a pH range from 3.0 to 10.0. The strip was electrophoresed, equilibrated, and then loaded onto the 12% SDS–polyacrylamide gel. The electrophoresed SDS gel was transferred to a polyvinylidene difluoride membrane (PVDF; Micro Separations Inc., Westborough, MA, USA) as described above. The gel and/or membrane were stained with Coomassie brilliant blue, or immunoblotting was performed. Proteins of interest were excised, and in-gel digestion was performed. The tryptic digests were separated by high-performance liquid chromatography, followed by Q-TOF (Micro-mass, Manchester, UK) analysis of selected peaks and their N-terminal sequences were determined by Edman degradation (Procise 492 CLC Protein Sequencer, Applied Biosystems, Foster City, CA, USA). The spots in the transferred PVDF membrane were also extracted and then analysed using the N-terminal amino acid sequence

Molecular cloning of cDNA encoding a 23 kDa allergen

To obtain the full sequence information for the 23 kDa (pI 8.7) allergen, a degenerate primer (Primer 23-2, ACNGARGGGNGCNGTNCAYAC, where N stands for

A, C, G, or T; R for A or G; Y for C or T) was designed on the basis of the N-terminal sequence information. PCR was performed using a 3'-Full RACE Core set kit (TAKARA) according to the manufacturer's instruction. The PCR-amplified DNA fragment was ligated into the pCR[®]II-TOPO[®] vector Invitrogen (Carlsbad, CA, USA) before sequence determination. A homology search for other allergens was done through the National Center for Biological Information (NCBI) program. Sequence alignments were carried out using the CLUSTAL X program (<ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX>) [10].

Results

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoglobulin E immunoblotting

SDS–PAGE analysis of the ant abdomen extracts, including the venom sac and the other portions, were performed. The SDS–PAGE features differed between the two extracts (Fig. 2a). The extract from the abdomen and venom sac of *P. chinensis* was used for the characterization of the IgE binding. Nine allergenic components were found in the IgE immunoblotting with molecular sizes of 12, 23, 25, 28, 40, 45, 50, 70, and 85 kDa. Six out of the seven allergic patients had specific IgEs to the 23 kDa protein, two patients to the 25 kDa protein (Fig. 2b, patients 5 and 7), and one patient had a specific IgE to the 12 kDa protein of *P. chinensis* (Fig. 2b). Inhibition immunoblotting with 20 µg/mL of *P. chinensis* extract attenuated the specific IgE binding to the 23 kDa protein (Fig. 3c).

Two-dimensional polyacrylamide gel electrophoresis and immunoglobulin E immunoblotting

IgE immunoblotting against the two-dimensional PAGE membrane was done with the pooled atopic sera ($n = 5$; patients 1, 3, 5, 6, 7 in Table 1) and four IgE binding spots were identified at 25 kDa (spot a in Fig. 3b; pH 6.2), 23 kDa (spot b; pI 8.7), 25 kDa (spot c; pH 9.0), and 45 kDa (spot d; pH 7.0) (Fig. 3b).

N-terminal amino acid sequencing and ESI-MS/MS analysis

A partial amino acid sequences analysis was done using N-terminal amino acid sequencing and ESI-MS/MS analysis. The analyses showed that spots a (25 kDa, pI 6.2) and b (23 kDa, pI 8.7) in Fig. 3b share a similar peak of peptides, and these two allergens have 27 of 31 amino acids in common as determined by N-terminal and ESI-MS/MS, and the NCBI database analysis suggested homology with the group 3 major allergen of *S. invicta* (Sol i 3) and the group 5 major allergen of *Vespula vulgaris* (Ves v 5) (Fig. 4).

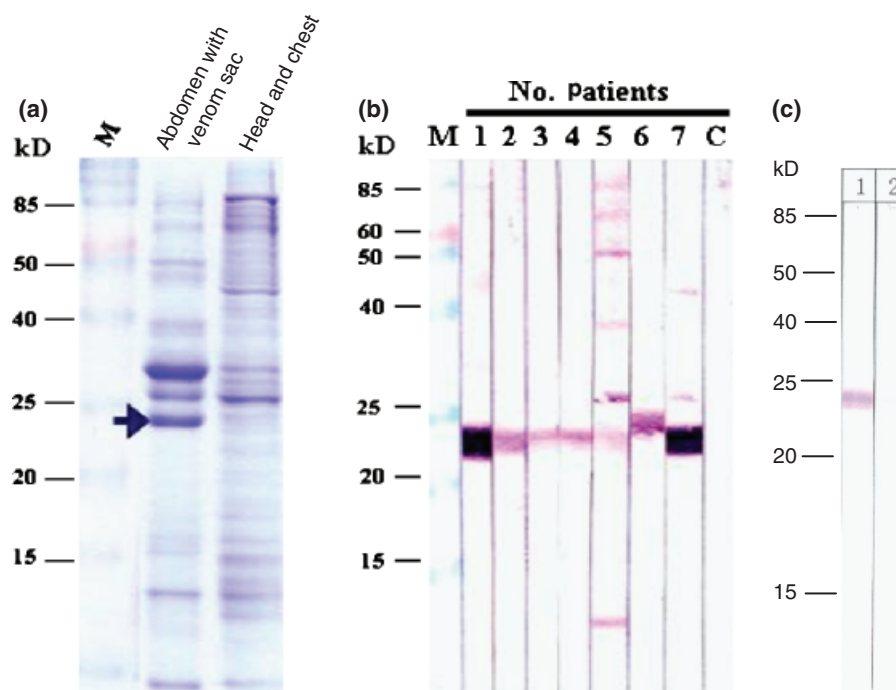


Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (a), immunoblotting for individual patients (b) and inhibition immunoblotting (c). SDS–PAGE was done with the extracts from abdomen including the venom sac and the other parts of the ant. Lane 1 in part (c) is immunoblotting features and lane 2 is inhibition of the patient's sera with 20 µg of the *Pachycondyla chinensis* extract. M: marker.

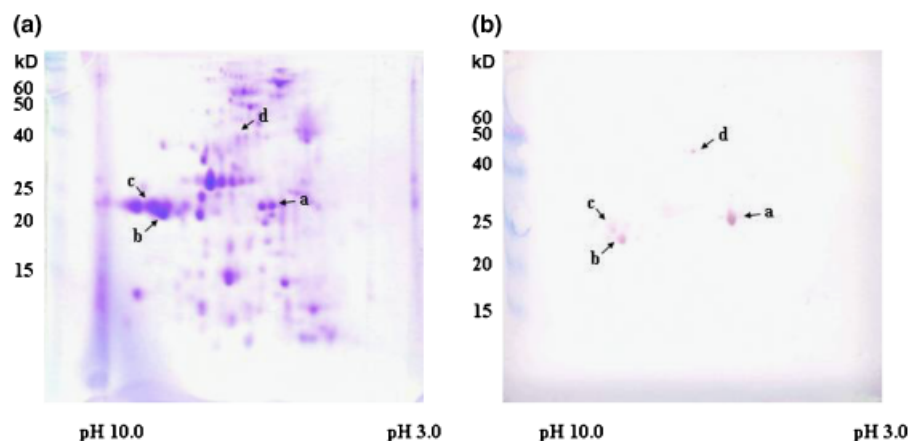


Fig. 3. Two-dimensional polyacrylamide gel electrophoresis (PAGE) (a) and IgE immunoblot (b) for *Pachycondyla chinensis*. Four allergen spots were identified: spot a – 25 kDa (pI 6.2), spot b – 23 kDa (pI 8.7), spot c – 25 kDa (pI 9.0), and spot d – 45 kDa (pI 7.0).

Identification of a 23 kDa allergen

A cDNA sequence of 761 bp encoding the 23 kDa allergen, which corresponds to 199 amino acids, was cloned by RT-PCR using a degenerate primer designed on the basis of the N-terminal amino acid sequence. The mature form of the 23 kDa allergen is estimated to have 206 amino acids, including the seven amino acids that were identified by the N-terminal sequencing. The calculated molecular weight is 22.59 kDa, the isoelectric point is estimated to be 6.56, and no potential glycosylation sites were found (deposited in GenBank under accession number

EU516327). Homology searches suggested that the 23 kDa allergen belongs to the antigen 5 family. It has 54% homology to Sol i 3, 48.5–51.2% homology to antigen 5 of *Vespula* species, 47.3–47.5% homology to antigen 5 of *Dolichovespula* species, and 45.5–50.5% homology to antigen 5 of *Polistes* species (Fig. 5).

Discussion

Pachycondyla species have been recognized as an important cause of ant sting anaphylaxis in Asia, the Middle

Four major allergens have been identified in *S. invicta*; Sol i 1 (phospholipase A1), Sol i 2, Sol i 3 (antigen 5), and Sol i 4. Proteins with sequence homology to Sol i 2 and Sol i 4 are not present in the honeybee or vespid genome databases [12]. The 23 kDa allergen has moderate homology to Sol i 3. High cross-reactivities of allergenicity have been found within genera and various cross-reactivities are found between different genera of Hymenoptera [13]. Antigen 5 from *Polistes* is less cross-reactive with that of *Vespula* and antigen 5 of *Dolichovespula* and *Vespa* is not always cross-reactive with that of *Vespula* species. The antigen 5 allergen of the imported fire ant (Sol i 3) is known not to be cross-reactive with antigen 5 of *Vespid*s [13, 14]. We did not evaluate the cross-reactivity between the 23 kDa allergen of *P. chinensis* and Sol i 3 in this study. We previously reported little cross-reactivity between whole extracts of *P. chinensis* and *S. invicta* [4] and others have reported similar results [6]. We are currently attempting to express the 23 kDa allergen in a *Pichia* yeast strain because *Escherichia coli* only expressed low levels of the 23 kDa allergen. Amino acid sequence analysis showed only 54% sequence identity between the 23 kDa allergen of *P. chinensis* and Sol i 3, suggesting a lower possibility of cross-reactivity between these two allergens. However, the 10 sera from the patients who had anaphylaxis to *P. sennaarensis* could bind to the 24 kDa allergen of *S. invicta* venom extract, which may be Sol i 3, suggesting the possibility of cross-reactivity between Sol i 3 and body extracts of *P. sennaarensis* [11]. So further studies are required to evaluate the cross-reactivity between Sol i 3 and the Pac c 23 kDa allergen with the recombinant or native form of these allergens.

In conclusion, we showed that the 23 kDa allergen is the major allergen of *P. chinensis*; this allergen belongs to the antigen 5 family of proteins. Further studies are required to characterize this allergen.

Acknowledgements

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