

Characterization of the major allergens purified from the venom of the paper wasp *Polistes gallicus*

Barbara Pantera^a, Donald R. Hoffman^b, Lara Carresi^a, Gianni Cappugi^a, Stefano Turillazzi^c, Giampaolo Manao^a, Maurizio Severino^d, Igino Spadolini^e, Giuseppe Orsomando^f, Gloriano Moneti^g, Luigia Pazzagli^{a,*}

^aDepartment of Biochemical Sciences, University of Florence, Viale Morgagni 50, 50134 Florence, Italy

^bBrody School of Medicine at East Carolina, University of Greenville, Greenville, NC, USA

^cDepartment of Animal Biology and Genetics, University of Florence, Italy

^dAllergology and Clinical Immunology Unit, S. Giovanni di Dio Hospital, Florence, Italy

^eAnallergo s.r.l, Florence, Italy

^fInstitute of Biochemical Biotechnology, University of Ancona, Italy

^gInterdepartmental Mass Spectrometry Center, University of Florence, Italy

Received 2 May 2003; received in revised form 25 July 2003; accepted 30 July 2003

Abstract

Allergic reactions to vespid stings are one of the major causes of IgE-mediated anaphylaxis. *Vespa* and *Vespula* venoms are closely related; *Polistes* venom is more distantly related and its allergens are less well studied. There is limited cross-reactivity between *Polistes* and the other vespid venoms because of differences in the epitopes on the allergen molecules.

In this study, the major allergens of *Polistes gallicus* are isolated and characterized. *P. gallicus* venom contains four major allergens: phospholipase, antigen 5 (Ag5), hyaluronidase and protease that were characterized by mass spectrometry and specific binding to IgE. The complete amino acid sequence of Ag5 and the sequence of the N-terminal region of phospholipase were also determined. The alignment of Ag5 from *P. gallicus* (European species) and *Polistes annularis* (American species) shows an 85% identity that increases to 98% within the same subgenus. This could suggest the presence of specific epitopes on Ag5 molecule being the variations on the superficial loops. The features of the *P. gallicus* allergens could explain the partial cross-reactivity found between the American and European *Polistes* venoms, and suggest that the use of European *Polistes* venoms would improve the diagnostic specificity and the therapy of European patients and of North American patients sensitized by European *Polistes*.

© 2003 Elsevier B.V. All rights reserved.

Keywords: Allergen; Venom; *Polistes*; Purification; Amino acid sequence; Antigen 5

1. Introduction

Allergic reactions to Hymenoptera stings are one of the major causes of IgE-mediated anaphylaxis. Apidae, Formicidae and Vespidae cause sting allergies in sensitized individuals by the interaction of cell-bound specific IgE antibodies with allergens [1,2]. The Vespidae family includes hornets (genera *Vespa* and *Dolichovespula*), yellow jackets (genus *Vespula*) and paper wasps (genus *Polistes*). The species *Polistes dominulus* and *Polistes gallicus* are

European paper wasps belonging to the subgenus *Polistes*, while the species *Polistes exclamans*, *Polistes annularis* (subgenus *Aphanilopterus*) and *Polistes fuscatus* (subgenus *Fuscopolistes*) are typical of North America [3].

Vespid venoms have similar venom compositions and their major venom allergens are phospholipase A1 (PL), hyaluronidase (HY) and antigen 5 (Ag5) a protein of unknown biological function [4,5]. Significant amounts of a fourth allergen, a serine-protease has been detected in *P. exclamans* and *P. dominulus* venoms [6,7]. In order to study the molecular basis of the allergic response, the primary structure of several hornet and yellow jacket allergens were determined, but there are only a few known sequences of *Polistes* allergens [5,8–11].

* Corresponding author. Tel.: +39-55-413-765; fax: +39-55-422-2725.
E-mail address: luigia.pazzagli@unifi.it (L. Pazzagli).

Most vespid-allergic patients exhibit reactions to more than one vespid venom that may be caused by cross-reactivity to one or more allergens [12–15]. Cross-reactivity can be due to homologous allergens from different species that lead to the production of IgE-recognizing common epitopes [16,17]. The pattern of cross-reactivity among different allergens is related to their sequence similarity, and cross-reactive antibodies are produced against the highly conserved regions [18,19]. On the other hand, venom allergens with <70% sequence identity do not usually have a high degree of cross-reactivity and species-specific antibodies result from responses to the variable regions of the molecules. *Vespa* and *Vespula* venoms are closely related, but *Polistes* are more distantly related and their allergens show a lower degree of cross-reactivity [5,13]. The cross-reactivity of *Polistes* venoms and allergens were studied by RAST-inhibition assays [9,14,20]: there are some cross-reacting antigens between *Polistes* and other vespids, although to a much lesser degree than among the *Vespa*, *Dolichovespula* and *Vespula* species; furthermore, the amino acid sequence similarities between *Polistes* and other vespid allergens are lower [5,20].

The primary diagnostic tests (intradermal skin tests, prick tests, and venom-specific serum IgE) and the conventional therapy for allergic patients (specific immunotherapy) require the use of venom extracts [21–23]. To avoid false-negative and false-positive responses to diagnostic tests and in order to completely protect allergic patients from the effects of subsequent stings, the commercial extracts of venom should be prepared with appropriately selected species [24]. Choosing the proper venom is particularly important for diagnosis and therapy of *Polistes* allergic patients, since this genus is the most distantly related in the Vespidae family and since there is only partial cross-reactivity between European and American *Polistes* venoms [25–27]. Previously reported immunological studies suggest that there may be species-specific epitopes in *Polistes* allergens, the structures of which are yet to be defined.

The European species, *P. dominulus*, has spread to much of the northeastern United States; this adds even more interest to the characterization of venoms from European *Polistes* [28,29].

In this report, we present the biochemical characterization of the proteins contained in the venom of *P. gallicus*, one of the most common European species, which could be useful in improving the diagnosis and treatment of European patients with paper wasp venom allergy.

2. Materials and methods

2.1. Venom

P. gallicus workers were collected in the countryside around Florence (Tuscany, Italy). The insects were killed by

freezing and stored at -80°C until used. The venom from 3000 wasps was obtained by squeezing dissected sacs and it was diluted twice in cold water.

2.2. Purification procedure

The venom was concentrated and exchanged with 0.1 M borate buffer pH 8.0 containing 0.15 M NaCl using a Centricon YM-10 ultrafilter (Millipore, Bedford, MA) and then applied to an affinity chromatography column (5×50 mm) containing benzamidine agarose adsorbent (Sigma Chemical, St. Louis, MO) [7]. The column was pre-equilibrated with borate saline buffer and eluted with 0.1 M benzamidine. The eluted fraction was dialyzed against water to remove benzamidine; the flow-through fraction was concentrated and then applied to a reversed phase C-18 HPLC column ($5 \mu\text{m}$, 4.6×250 mm) (Vydac, Grace, Columbia, MD). All fractions collected were assayed for total protein, phospholipase, hyaluronidase and protease activities. Protein concentration was determined by the Bradford method (BioRad, Hercules, CA, USA).

Phospholipase activity was measured by a potentiometric method using phosphatidylcholine as substrate and phospholipase A2 from *Apis mellifera* as standard [30]. Protease was assayed by a colorimetric method using resorufin-labeled casein as substrate [31] (Roche Diagnostics, Basel, Switzerland). *N*-acetylglucosamine produced from hyaluronic acid was determined by a colorimetric method to assay hyaluronidase activity [32]. The enzymatic activities were expressed in international units.

The purity of proteins was assessed by SDS-PAGE on 15% polyacrylamide gels according to Laemmli [33]; proteins were stained with BioRad silver stain. The amino acid composition of purified native and carboxymethylated proteins was determined after acid hydrolysis by the procedure of Spackman et al. [34] using an amino acid analyzer and a post column derivatization with *o*-phthaldialdehyde and 2-mercaptoethanol [35].

2.3. Mass spectrometry

Both electrospray (ESI) and matrix-assisted laser desorption (MALDI) ionization techniques were used to obtain the molecular masses of purified allergens. Ag5 (200 pmol) was diluted 1:2 in 50% acetonitrile containing 0.1% TFA and 1% formic acid; the spectrum was obtained by electrospray ionization in a triple quadrupole mass analyzer (API 365 Applied Biosystems). PL (1 nmol) was dissolved in 100 μl of 50% acetonitrile and 1% formic acid and injected by HPLC (Reverse Phase, C5 column, $5 \mu\text{m}$, 2×30 mm, Jupiter) on an ESI/TOF (time of flight) mass spectrometer (Mariner, Applied Biosystems). PR and HY (8–10 pmol), dissolved in 70% acetonitrile containing 0.1% TFA, were diluted 1:1 with sinapinic acid matrix and deposited on the target to be analyzed on a MALDI/TOF mass spectrometer (Reflex III, Bruker-Franzen Analytik, Bremen, Germany).

2.4. Sera and patients

Most of the sera were collected from patients in the San Giovanni di Dio Hospital (Florence, Italy). Specific sensitization to *Polistes* venoms was determined by intradermal skin tests using from 1/100 to 1 µg of venom from *Polistes*, *A. mellifera*, *Vespula* (mix) and *Vespa crabro*. Only patients mono-positive to the *Polistes* venom were selected. Sera were collected before any therapy and stored at -20°C until used.

The sera from Spanish patients were generously contributed by Dr. Miguel Blanca.

2.5. Enzymatic Allergo Sorbent Test (EAST) and Radio Allergo Sorbent Test (RAST)

EAST (Allercoat™ 6, BioRad) is an in vitro method of detecting specific IgE antibodies. Whole venom or purified allergens (0.2 and 0.1 mg/ml) were bound to nitrocellulose CNBr-activated paper disks and incubated with human sera from five allergic patients. Specific IgE were detected with alkaline phosphatase labeled anti-human IgE. Absorbance at 405 nm is compared to absorbance obtained with IgE Standard (0.35–100 UI/ml) and the results are referred as Allercoat™ 6 classes as follows: Class 0, <0.35 UI/ml; Class 1, 0.35–0.70 UI/ml; Class 2, 0.7–3.5 UI/ml; Class 3, 3.5–17.5 UI/ml; Class 4, 17.5–50 UI/ml; Class 5, 50–100 UI/ml; Class 6, >100 UI/ml. Negative and positive control sera were also assayed.

RAST assays were performed with protease from *P. gallicus* and *P. dominulus* venom. The allergens were coupled to CNBr-activated paper disks and the specific IgEs in the sera of seven Italian and three Spanish patients were detected with ^{125}I -labeled anti-IgE according to Hoffman et al. [36].

2.6. Enzymatic digestions and peptide separation

Purified allergens (2–10 nmol) to be digested were first carboxymethylated (CM) as previously described [37]. Before protease addition, the CM-protein solution was incubated for 3 min in a boiling water bath and then chilled in ice. All sequencing grade proteases were purchased from Sigma.

Lys-C endoproteinase digestion was carried out on 4 nmol of CM-Ag5 dissolved in 50 µl of 25 mM Tris–HCl buffer pH 8.5. The protease/protein molar ratio was 1:50; the mixture was incubated at 37°C for 18 h. Glu-C endoproteinase was added to 4 nmol of CM-Ag5 dissolved in 30 µl of 0.05 M ammonium bicarbonate buffer. The protease (10 µg) was added to this solution which was kept at 37°C for 24 h. Chymotrypsin digestion was performed by dissolving 5 nmol of CM-Ag5 in 60 µl of 0.2 M ammonium bicarbonate buffer adding the protease (2.5% w/w) and incubating the mixture at 37°C for 8 h.

Peptides produced after each enzymatic digestion were purified by reversed phase HPLC on a C18 Vydac column (5 µm, 4.6×250 mm) using a trifluoroacetic acid/acetonitrile gradient [37].

2.7. Sequence determination and models of three-dimensional structure

Intact CM-proteins and digested peptides, immobilized on different polyvinylidene difluoride membranes (Immobilon P, Sequelon DITC, Sequelon AA, Millipore) were subjected to automated Edman degradation on a Milligen model 6600 protein sequencer (Millipore), or on a Procise model 491 gas-phase protein sequencer (Applied Biosystems, Foster City, CA) with on-line HPLC for the identification of phenylthiohydantoin derivatives of released amino acids [38].

The alignments of allergen sequences from different species and genera of vespids were made using the CLUSTAL W program at <http://www.ebi.ac.uk/clustal> (the European Bioinformatics Institute); and they were used to construct a Phylip phylogenetic tree [39].

Three-dimensional structure of Ag5 was modeled using Swiss-Model system [40]. The crystal structure of *Vespula vulgaris* Ag5 (Protein Databank 1QNX) was used as a template [41]. Models were visualized and compared with Swiss-Pdb Viewer [42,43].

3. Results

3.1. Allergen purification

P. gallicus venom was first purified by a benzamidine-agarose chromatography [7]. The venom protease (PR) was obtained in native form by elution with 0.1 M benzamidine (Fig. 2). The flow-through fraction containing the other venom components was then applied to a Vydac C18 column (Fig. 1). This method enabled the purification of phospholipase (peak E, PL), antigen 5 (peak B, Ag5) and hyaluronidase (peak D, HY). The structure integrity was checked assaying the enzymatic activity of purified proteins.

The peaks labeled as A and C, named RP1 and RP3 fraction according to their retention times on the Reverse Phase column, contain two unidentified protein components (Fig. 2); they have only weak allergenic activity (data not shown) and they were consequently not investigated further.

As reported in literature [10,44], other purification methods were used, but no increase of purification yield or specific activity of allergens was observed.

3.2. Characterization of the major allergens

Identification of the four major allergens was performed by SDS-PAGE (Fig. 2), amino acid composition (Table 1)

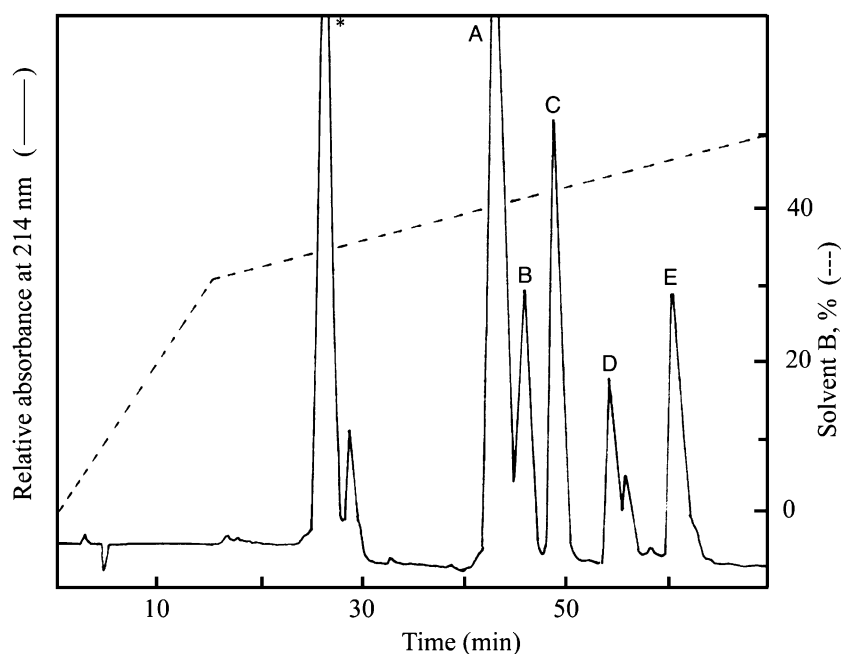


Fig. 1. RP-HPLC of the flow-through fraction from affinity chromatography. Peak A, RP1; peak B, Ag5; peak C, RP3; peak D, hyaluronidase; peak E, phospholipase. The peak labeled as * was not characterized. Column: Vydac C-18 (5 μ m, 4.6 $\times$ 250 mm). Solvent A, 10 mM TFA in water; Solvent B, 10 mM TFA in acetonitrile. Flow rate was 0.8 ml/min. The elution was performed by a linear acetonitrile gradient as indicate in the figure (— — —).

and enzymatic activity. *P. annularis* is the only *Polistes* species whose major allergen sequences are known: sequence data of Ag5, PL and HY are available at the Swiss-Prot databank with the accession numbers Q05109, Q9U6W0 and Q9U6V9, respectively. The amino acid composition of Ag5, PL and HY from *P. gallicus* shows a good degree of similarity to that of the homologous allergens from *P. annularis*. No amino acid composition of PR from other *Polistes* species has been reported.

The specific activity of PR, PL and HY was 179.4, 283.2 and 0.53 U/mg, respectively.

The molecular weights of Ag5 and PL, determined by ESI mass spectrometer are 23 135 and 33 475 Da, respectively (Fig. 3). The molecular weights of PR (33 669 Da) and HY (42 173 Da) were determined by MALDI/TOF mass spectrometry because these two allergens were not ionized with the electrospray methods (Fig. 4). The determination of the exact molecular weights may be a suitable method to obtain a rapid preliminary structural characterization, but until now, the molecular weights of the majority

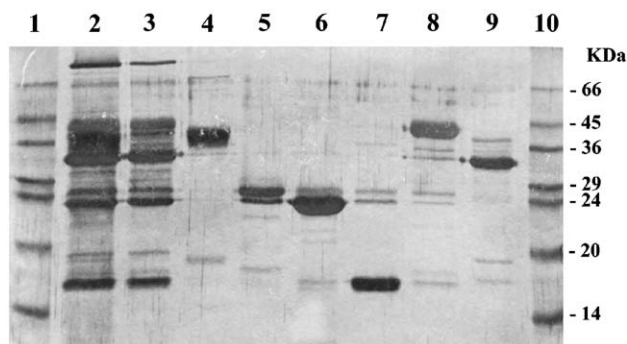


Fig. 2. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of the main purification steps: lanes 1 and 10, Sigma VII L low molecular weight standards; lane 2, whole venom; lane 3, flow-through fraction from affinity chromatography; lane 4, elution fraction (protease) from affinity chromatography. lanes 5–9 are from RP-HPLC purification step: lane 5, RP1 (peak A); lane 6, Ag5 (peak B); lane 7, RP3 (peak C); lane 8, hyaluronidase (peak D); lane 9, phospholipase (peak E).

Table 1

Amino acid composition of the major allergens from *P. gallicus* venom

	Ag5	PL	HY	PR
Cysteine	3.4	4.5	1.4	2.2
Aspartic acid	11.6	11.5	12.2	12.9
Threonine	5.3	4.6	4.2	9.4
Serine	5.3	5.7	6.5	5.6
Glutamic acid	12.9	9.4	11.2	10.5
Glycine	9.6	8.7	7.7	10.4
Alanine	5.5	7.9	5.5	7.6
Valine	8.3	8.3	7.1	7.3
Methionine	1.4	0.8	1.9	1.8
Isoleucine	6.5	7.5	8.1	6.9
Leucine	6.0	8.9	7.9	6.0
Tyrosine	5.7	6.1	5.5	5.3
Phenylalanine	2.2	4.9	4.5	3.2
Lysine	11.4	9.5	9.5	4.5
Histidine	2.5	2.9	2.4	3.2
Arginine	2.0	3.1	5.2	5.3

The results are expressed as percentage. Values for serine and threonine were corrected for loss (15% and 5%, respectively) during hydrolysis. Cysteine was determined as carboxymethyl cysteine.

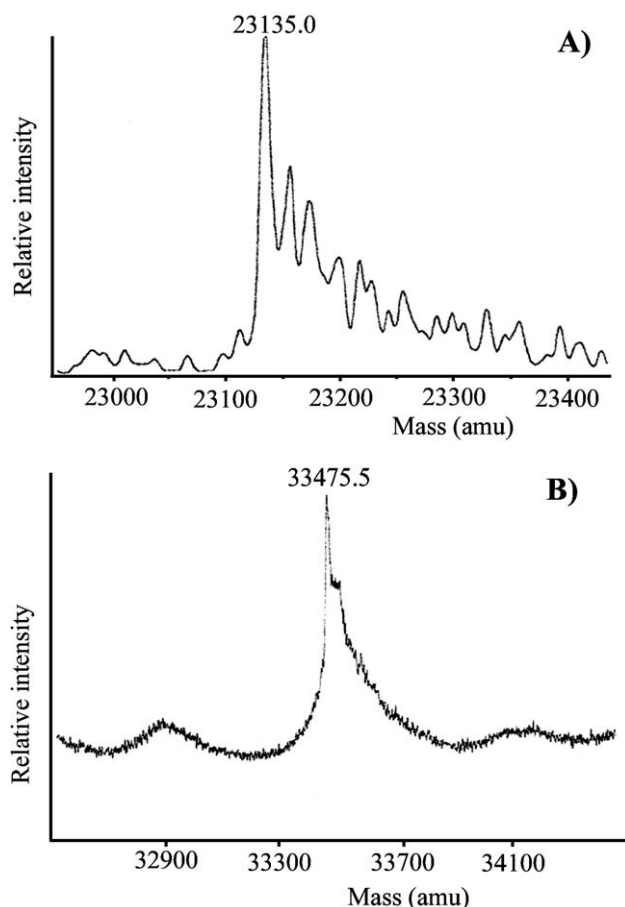


Fig. 3. Deconvolution of electrospray mass spectra of purified Ag5 and phospholipase. (A) 200 pmol of Ag5 were diluted 1:5 in 50% acetonitrile containing 0.1% TFA and 1% formic acid and injected by infusion on an ion source electrospray mass spectrometer equipped with a triple quadrupole mass analyzer (IS 5000 V, OR 100 V, RNG 300 V, scan range: 1200–2700 m/z). (B) 1 nmol of PL was dissolved in 100 μ l of 50% acetonitrile and 1% formic acid and injected by HPLC on an ESI/TOF spectrometer (IS 5000 V nozzle potential, 140 V scan range: 500–3500 m/z, Mariner, Applied Biosystems).

of allergens have been calculated from the sequence. However, it can be observed that the molecular weights of Ag5, PL and HY are similar to that of their homologues from *P. annularis* (23 293, 33 483 and 43 019 Da, respectively); no sequence or mass spectrometry data are available for *Polistes* protease.

The *P. gallicus* venom and the purified allergens were bound to CNBr-activated nitrocellulose disks and the amount of allergen-specific IgE in the sera of five allergic patients was determined by the EAST technique (Fig. 5). Ag5 and phospholipase bind specific IgE (EAST classes 5 and 6, respectively) and are strong allergens, similar to those observed in other vespids [9,14,16]. A smaller amount of IgE against hyaluronidase was found, confirming the lower allergenic activity of this protein [11]. Protease, a fourth allergen also identified in *P. dominulus* and *P. exclamans* venoms [6,7], is an EAST class 2–3

allergen in the *P. gallicus* venom and RAST assays show that protease from *P. gallicus* and *P. dominulus* binds about the same amount of IgE in the sera of European patients (Fig. 6).

3.3. Amino acid sequencing

High yield of the Ag5 from *P. gallicus*, named Pol g 5 according to the international allergen nomenclature [45], was obtained from the purification procedure (0.72 mg) and its complete amino acid sequence was determined (Fig. 7A). Automated Edman degradation was first performed on 1 nmol of the whole CM-protein to obtain the N-terminal sequence (residues 1–39). The peptides from Lys-C and Glu-C proteinase digestions were sequenced to obtain the most of the sequence. Among the chymotryptic peptides, only the overlapping ones were sequenced. The sequence obtained (accession number P83377 in the Swiss-Prot databank) was compared to the homologous

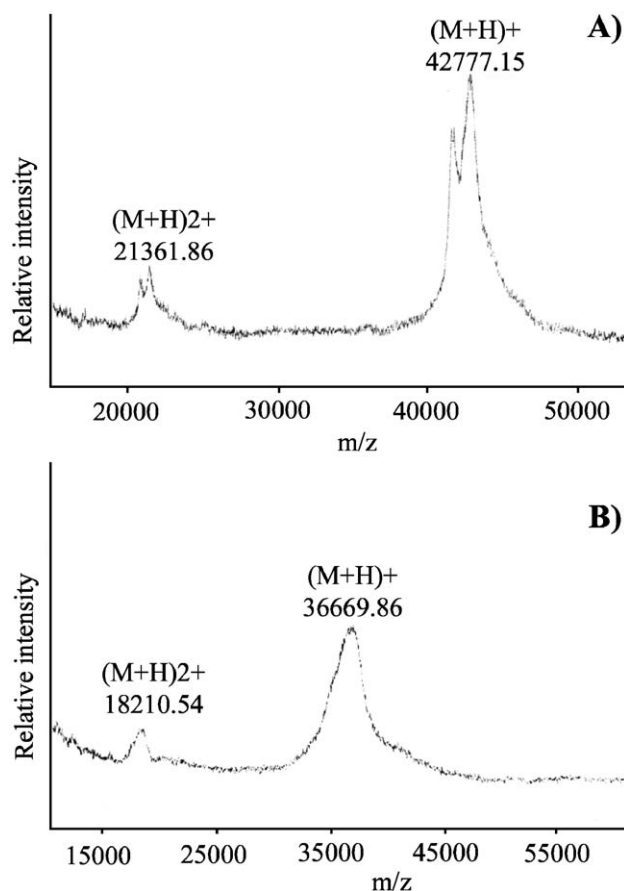


Fig. 4. MALDI/TOF mass spectra of purified hyaluronidase and protease. Mass spectrometer was a Reflex III, Bruker. Analysis were performed using a LINEAR method. (A) 8.0 pmol of HL were diluted 1:5 with 70% acetonitrile containing 0.1% TFA, then diluted 1:1 with sinapinic acid matrix and carried on the target to be analysed. (B) 7.5 pmol of PR were diluted 1:4 with 70% acetonitrile containing 0.1% TFA then diluted 1:1 with sinapinic acid matrix.

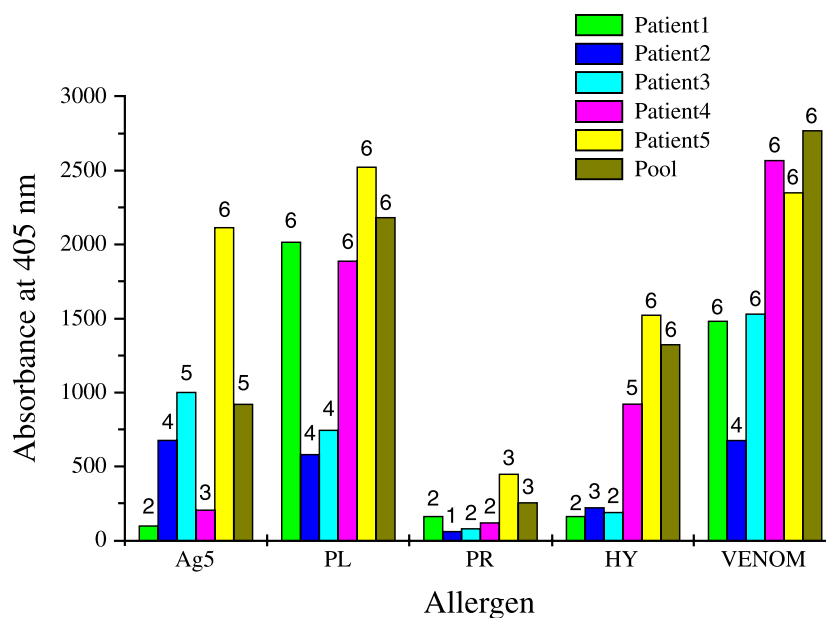


Fig. 5. IgE antibody binding activity of five sera from sensitized patients obtained by EAST assay. The sera were from Italian patients and were incubated with the whole venom and with the purified allergens (Ag5, phospholipase, protease, hyaluronidase). Anti-IgE antibodies are labeled with alkaline phosphatase and results are reported as absorbance at 405 nm. The value of the EAST Class, according to the BioRad Allercoat™ 6 method, is reported above each column. *pool: obtained by mixing the sera from five patients.

ones from *Vespula* and *Polistes* species: there is a 98% identity between Ag5s from *P. dominulus* (accession number P81656 Swiss-Prot databank) and *P. gallicus* (European paper wasps); the identity decreases to 85% between

Pol g 5 and the Ag5s from American paper wasps. A phylogenetic tree was constructed using the known amino acid sequences of Ag5s in order to highlight the phylogenetic relations among *Polistes* and other vespidae species (Fig. 8).

The low purification yield (0.42 mg) obtained for phospholipase (Pol g 1) was only adequate for N-terminal amino acid sequencing of the first 43 residues (Fig. 7B). The alignment of this partial sequence (accession number P83542) with the 1–43 stretch of the homologous allergens shows an identity of 75.6% with the PL from *P. annularis* and a 29% identity with the PLs from *V. vulgaris* and *Vespula maculifrons*. These results show that the N-terminal region of PLs have a lower degree of homology than Ag5s.

P. gallicus protease (300 pmol) was used to obtain the 1–16 N-terminal sequence: IVNGVXTEIN EFPXMV, where X indicates the uncertain residues. Information about venom protease is still limited and no sequence data are available for other Vespidae venom proteases. Until now, only the complete sequence of two Apidae venom proteases from *A. mellifera* [46] and *B. pennsylvanicus* (accession number A56338) and of the N-terminal 20 residues from *B. terrestris* [36] are known.

3.4. Three-dimensional models of Pol g 5

The crystallographic structure of Ag5 from *V. vulgaris*, Ves v 5, [41] was used as a template to obtain three-dimensional models of Pol g 5 and Pol a 5 (the Ag5 from *P. annularis*). These last two sequences have a 58.7% and a

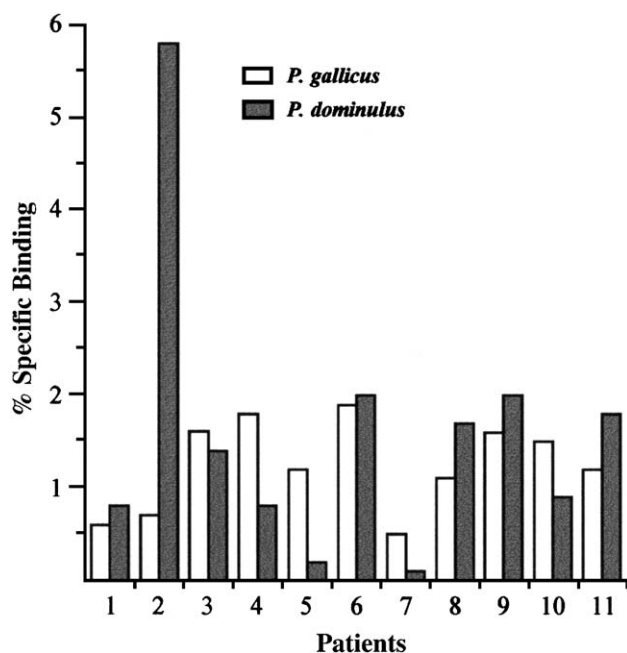


Fig. 6. RAST assay of binding of IgE from sera with the pure protease from *P. gallicus* (white) and *P. dominulus* (gray). Sera 1–7 were from Italian patients; sera 8–11 were from Spanish patients. Anti-IgE antibody was labeled with ^{125}I . Results are expressed as percentage of the counts added minus non-specific binding obtained from negative control sera.



Fig. 7. (A) The complete amino acid sequence of Ag5. (B) The partial N-terminal amino acid sequence of phospholipase. (—) Sequence results obtained from the whole proteins; (---) sequence results obtained from peptides; (LC) endoproteinase Lys-C peptides; (GC) endoproteinase Glu-C peptides; (CH) chymotryptic peptides.

56.8% identity with Ves v 5, respectively; the putative functional and structural residues (disulfide bridges, His 98, His156, Trp133) and the topologies of the secondary structure are conserved. Models of Pol g 5 and Pol a 5 were then superimposed in order to highlight the structural

differences (Fig. 9). The loops 6–10, 98–102 and 121–126 are not completely overlapped: the Pro9, Phe98 and Leu125 of Pol a 5 are replaced by Ser9, Ile98 and Phe126, respectively, in Pol g 5; moreover, there is an additional His122 in Pol g 5 molecule. The amino acid substitutions in

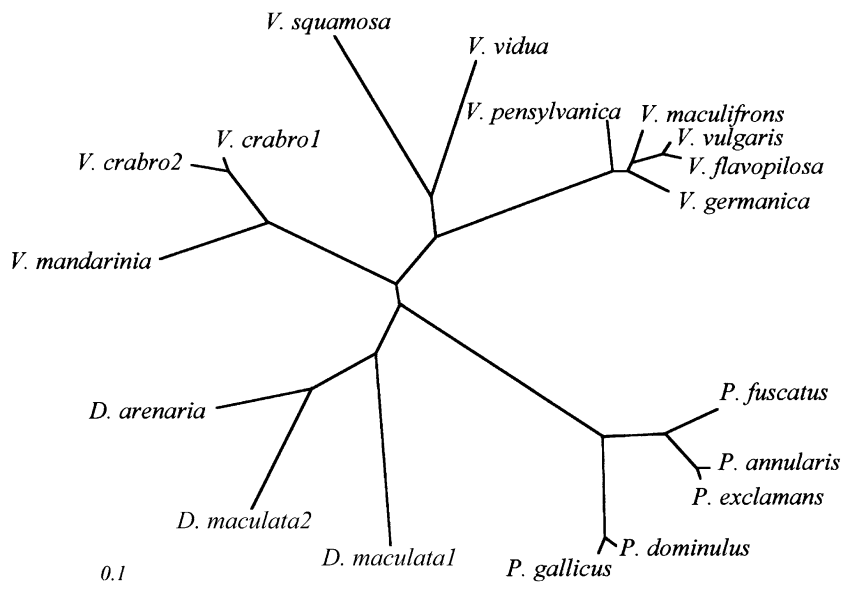


Fig. 8. Phylogenetic tree of Vespidae. The tree was obtained by the alignment of Ag5 sequences from various Vespidae. It was obtained with the TreeView plot program using the data from the Clustal W alignment employing the neighbour joining (NJ) method. The sequences were taken from the Swiss-Prot Database. The scale bar (its length corresponds to 0.1 substitutions per site) below the tree indicates the evolutionary distance.

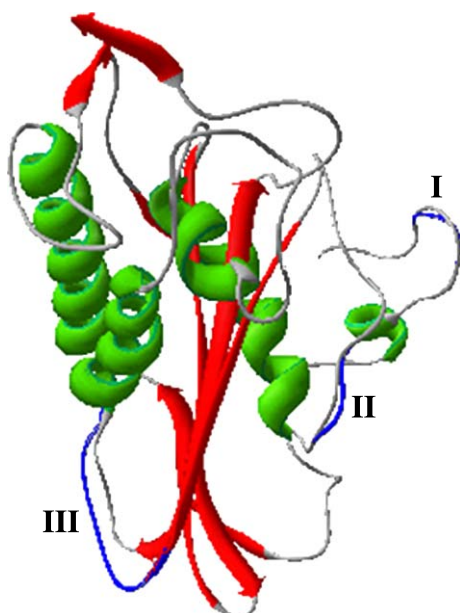


Fig. 9. Comparison of the three-dimensional structures of *P. annularis* and *P. gallicus* Ag5s. Models were obtained with the Swiss-Pdb Viewer using the Ag5 from *V. vulgaris* as a template. The loops labeled in blue are the sequence regions of *P. gallicus* that are not superimposed with the respective sequences from *P. annularis*. (I) 6–10 loop; (II) 98–102 loop; (III) 121–126 loop.

the 121–126 loop produce a noticeable twist at the end of the fourth β -sheet.

4. Discussion

The limited cross-reactivity of European allergic patients to venoms of American species could be due to structural differences in the allergens of the *Polistes* species that lead to the production of specific antibodies directed against the more variable regions of the allergen molecules. Among North American species, the amino acid sequences of Ag5 from *P. annularis* [9], *P. exclamans* [9] and *P. fuscatus* [8], and sequences of PL and HY from *P. annularis* (accession number Q9U6W0 and Q9U6V9 in Swiss-Prot databank) are known. Ag5 from *P. dominulus* (accession number P81656) is the only sequence available among European *Polistes*. Moreover, the partial cross-reactivity observed among the subgenera of *Polistes* [26,27] means that allergic patients sensitized by European paper wasps (*P. dominulus* and *P. gallicus*) respond to a different epitope spectrum than patients sensitized by the North American *Polistes* and, suggests that there may be some important differences in *Polistes* subgenera allergens.

Therefore, the characterization and the amino acid sequence determination of other European *Polistes* allergens is of value in shedding light on this matter.

The venom of the European paper wasp *P. gallicus* contains four major allergens: phospholipase, antigen 5, hyaluronidase and a serine protease. The molecular weights

of PR and PL, determined by mass spectrometry, are 36 669 and 33 475 Da, respectively; in their native forms, the two proteins can be separated only by benzamidine agarose affinity chromatography.

Hyaluronidase was only analyzed for amino acid composition, enzymatic activity and molecular mass (42 173 Da).

Protease has been detected in significant amount in *P. exclamans* and *P. dominulus* venoms [6,7] and it is distantly related to honeybee venom proteases. The quantity of PR obtained enabled us to determine the amino acid composition (Table 1), the exact molecular weight, the N-terminal sequence and the specific binding to IgE. In EAST assay, the protease is a class 2–3 allergen (Fig. 5) and in most of the sera from European patients, the amounts of IgE bound by *P. gallicus* and *P. dominulus* protease is quite similar (Fig. 6). Both sera from European and American patients were compared with protease indicating that it is a weak sensitizer in North American *Polistes* venom [7] while it is a significant allergen in both of the European *Polistes* species (as showed by RAST assay) and can partially explain the differences in reactivity observed among European and American *Polistes* venoms.

Further studies of *P. gallicus* venom allergens have focused on PL (Pol g 1) and Ag5 (Pol g 5), which appear to bind the largest amounts of specific IgE in the sera of five allergic patients (Fig. 5). The molecular weight of Ag5 calculated from the sequence is 23 153 Da, a value that agrees with that determined by mass spectrometry (23 135 Da), suggesting that, like other Ag5s, no post-translational modifications occur. The sequence data of Pol g 5 were used to construct a phylogenetic tree and to obtain three-dimensional models. The tree shows that *Polistes* wasps are distantly related to yellow jackets and that European and North American paper wasps are on different branches of it, pointing out a divergence that could lead to a different epitope spectrum and also confirming the positions they have in the cladograms of *Polistes* subgenera, based on morphological differences, proposed by Carpenter [3].

When the tertiary structure models of Pol g 5 and Pol a 5 are compared, a number of differences appear on superficial loops which are putative species-specific epitopes. The twist at the end of the fourth β -sheet and the consequent shift of the next loop cause a variation in the surface of the molecule. However, these structural differences are not sufficient to explain the low RAST-inhibition value observed [27], and to suggest that Ag5 is the major cause of the different immunological responses between American and European venoms.

There must be additional differences in the structure of *Polistes* European allergens. These differences could also be due to phospholipase. Vespidae phospholipases are relatively conserved in the C-terminal region (residues 130–300) but are quite variable in the N-terminal portion; this variability may account for the insect-specific reactivity observed in many patients [24,34]. The PL from *P. annularis* (a North

American species) is the only completely known amino acid sequence [9]. Some partial sequence data are known for PL from *P. dominulus* (Hoffman D.R., data not published). The 62% sequence identity, found in the 1–43 stretch between *P. gallicus* (or *P. dominulus*) and *P. annularis* PLs, indicate a higher degree of evolutionary divergence and strongly suggests the presence of species-specific epitopes.

Consequently, (i) the degree (85%) of sequence homology between European and North American Ag5s, which is lower than that observed within the same subgenus (98%); (ii) the differences found in the PL molecules and (iii) the finding that protease is an important allergen in *P. gallicus* and *P. dominulus* venoms, can help us to elucidate the molecular basis of the limited cross-reactivity.

Diagnosis and immunotherapy are performed with a standard *Polistes* mix including venoms from the North American species *P. annularis*, *P. exclamans*, *P. fuscatus* and *P. metricus*. The findings in this study suggest that the addition of venom from *P. gallicus* and *P. dominulus* to the *Polistes* mix currently available could lead to an improvement in the diagnosis and the therapy of *Polistes* allergic European patients. Moreover, in other studies, venom from European *Polistes* has been used in diagnosis and therapy of 130 Italian patients with a reduction of false-negative responses and an improved efficacy of therapy (98.3%) (Severino M. et al., unpublished data).

These clinical studies confirm the in vitro RAST inhibition assays [27] and corroborate our observations that some species-specific epitopes are present on European *Polistes* allergens.

A mix including the venoms from European *Polistes* species could also be useful for specific immunotherapy of patients in the northeastern United States where *P. dominulus* has become well established.

Acknowledgements

We are grateful to Dr. Miguel Blanca, Allergy Unit, La Paz Hospital, Madrid, Spain, for providing the sera from Spanish patients. Our thanks also to Dr. Francesco Ranaldi, Department of Biochemical Sciences, University of Florence for his assistance in the preparation of this manuscript.

References

- [1] D.B.K. Golden, D.G. Marsh, A. Kagey-Sobotka, Epidemiology of insect venom sensitivity, *JAMA* 262 (1989) 240–244.
- [2] C. Incorvaia, S. Pucci, E.A. Pastorello, Clinical aspects of Hymenoptera venom allergy, *Allergy* 54 (1999) 50–52.
- [3] J.M. Carpenter, Phylogeny and biogeography of *Polistes*, in: S. Turillazzi, M.J. West-Eberhard (Eds.), *Natural History and Evolution of Paper Wasps*, Oxford Univ. Press, UK, 1996, pp. 18–57.
- [4] T.P. King, A.K. Sobotka, A. Alagon, L. Kochoumian, L.M. Lichtenstein, Protein allergens of white-face hornet, yellow hornet and yellow jacket venoms, *Biochemistry* 17 (1978) 5165–5174.
- [5] T.P. King, M.D. Spangfort, Structure and biology of stinging insect venom allergens, *Int. Arch. Allergy Immunol.* 123 (2000) 99–106.
- [6] D.R. Hoffman, M.G. Severino, P. Campi, S. Turillazzi, R. Zerboni, Protease is an important allergen in European *Polistes* wasp venom allergy, *J. Allergy Clin. Immunol.* 101 (1998) S33.
- [7] M.M. McNairy, J. Gastmeyer, B. Pantera, D.R. Hoffman, Isolation of paper wasp venom protease by affinity chromatography, *J. Allergy Clin. Immunol.* 105 (2000) S57.
- [8] D.R. Hoffman, Allergens in Hymenoptera venom: XXV. The amino acid sequences of antigen 5 molecules and the structural basis of antigenic cross-reactivity, *J. Allergy Clin. Immunol.* 92 (1993) 707–716.
- [9] G. Lu, M. Villalba, M.R. Coscia, D.R. Hoffman, T.P. King, Sequence analysis and antigenic cross-reactivity of a venom allergen, antigen 5, from hornets, wasps, and yellow jackets, *J. Immunol.* 150 (1993) 2823–2830.
- [10] D.R. Hoffman, Allergens in Hymenoptera venom: XXVI. The complete amino acid sequences of two vespid venom phospholipases, *Int. Arch. Allergy Immunol.* 104 (1994) 184–190.
- [11] G. Lu, L. Kochoumian, T.P. King, Sequence identity and antigenic cross-reactivity of white face hornet venom allergen, also a hyaluronidase, with other proteins, *J. Biol. Chem.* 270 (1995) 4457–4465.
- [12] T.P. King, A.C. Alagon, J. Kuan, A.K. Sobotka, L.M. Lichtenstein, Immunochemical studies of yellow jacket venom proteins, *Mol. Immunol.* 20 (1983) 297–308.
- [13] D.R. Hoffman, Allergens in Hymenoptera venom: XV. The immunologic basis of vespid venom cross-reactivity, *J. Allergy Clin. Immunol.* 75 (1985) 611–613.
- [14] T.P. King, G. Lu, M. Gonzalez, N. Qian, L. Soldatova, Yellow jacket venom allergens, hyaluronidase and phospholipase: sequence similarity and antigenic cross-reactivity with their hornet and wasp homologous and possible implication for clinical allergy, *J. Allergy Clin. Immunol.* 98 (1996) 588–600.
- [15] S.O. Stapel, J. Waanders-Lijster de Raadt, A.W. van Toorenbergen, H. de Groot, Allergy to bumblebees venom: II. IgE cross-reactivity between bumblebee and honeybee venom, *Allergy* 53 (1998) 769–777.
- [16] D.R. Hoffman, Allergens in Hymenoptera venom: XIV. IgE binding activities of venom proteins from three species of vespids, *J. Allergy Clin. Immunol.* 75 (1985) 606–609.
- [17] D.R. Hoffman, Allergens in Hymenoptera venom: XVI. Studies of the structure and cross-reactivities of vespids venom phospholipases, *J. Allergy Clin. Immunol.* 78 (1986) 337–443.
- [18] D.J. Barlow, M.S. Edwards, J.M. Thorton, Continuous and discontinuous protein antigenic determinants, *Nature* 322 (1986) 747–748.
- [19] C. Chothia, A.M. Lesk, The relation between the divergence of sequence and structure in proteins, *EMBO J.* 5 (1986) 823–826.
- [20] R.E. Reisman, J.I. Wypych, U.R. Muller, J.A. Grant, Comparison of the allergenicity and antigenicity of *Polistes* venom and other vespid venoms, *J. Allergy Clin. Immunol.* 70 (1982) 281–287.
- [21] U.R. Muller, New development in the diagnosis and treatment of Hymenoptera venom allergy, *Int. Arch. Allergy Immunol.* 124 (2001) 447–453.
- [22] D.B.K. Golden, K.A. Kwitrovich, A. Kagey-Sobotka, L.M. Lichtenstein, Discontinuing venom immunotherapy: extended observations, *J. Allergy Clin. Immunol.* 101 (1998) 298–305.
- [23] F. Ruëff, A. Wenderoth, B. Przybilla, Patients still reacting to a sting challenge while receiving conventional Hymenoptera venom immunotherapy are protected by increased venom doses, *J. Allergy Clin. Immunol.* 108 (2001) 1027–1032.
- [24] U.R. Muller, Recombinant Hymenoptera venom allergens, *Allergy* 57 (2002) 570–576.
- [25] F. Sanchez, M. Blanca, J. Fernandez, A. Miranda, A. Terrados, M.J. Torres, et al., Comparative study between European and American species of *Polistes* using sera from European sensitized subjects, *Clin. Exp. Allergy* 25 (1995) 281–287.
- [26] D.R. Hoffman, Antigen 5 and phospholipase from *Polistes dominulus*

- differ significantly in amino acid sequence from those of North American *Polistes* venoms, *J. Allergy Clin. Immunol.* 99 (1997) S377.
- [27] M. Severino, European *Polistes* venom allergy, *Ital. J. Allergol. Immunol. Clin.* 8 (1998) 527–534.
- [28] D.R. Hoffman, R. Jacobson, M. Blanca, Allergy to venom of *Polistes dominulus*, a paper wasp introduced from Europe, *J. Allergy Clin. Immunol.* 85 (1990) S211.
- [29] R. Cervo, F. Zacchi, S. Turillazzi, *Polistes dominulus* (Hymenoptera, Vespidae) invading North America: some hypothesis for its rapid spread, *Insectes Soc.* 47 (2000) 155–157.
- [30] T.P. King, L. Kochoomian, A. Joslyn, Wasp venom proteins: phospholipase A and B, *Arch. Biochem. Biophys.* 230 (1984) 1–12.
- [31] S.S. Twining, Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes, *Anal. Biochem.* 143 (1984) 30–34.
- [32] A. Linker, Hyaluronidase, in: H.U. Bergmeyer (Ed.), *Methods of Enzymatic Analysis*, vol. 4, Verlag Chemie, Germany, 1984, pp. 256–262.
- [33] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680–685.
- [34] D.H. Spackman, W.H. Stein, S. Moore, Automatic recording apparatus for use in the chromatography of amino acids, *Anal. Biochem.* 30 (1958) 1190–1206.
- [35] J.R. Benson, P.E. Hare, *O*-phthalaldehyde: fluorogenic detection of primary amines in the picomole range. Comparison with fluorescamine and ninhydrin, *Proc. Acad. Sci. U. S. A.* 72 (1975) 619–622.
- [36] D.R. Hoffman, S.E. El-Choufani, M.M. Smith, H. de Groot, Occupational allergy to bumblebees: allergens of *Bombus terrestris*, *J. Allergy Clin. Immunol.* 108 (2001) 855–860.
- [37] L. Pazzagli, G. Cappugi, G. Manao, G. Camici, A. Santini, A. Scala, Purification, characterization and amino acid sequence of cerato-platanin, a new phytotoxic protein from *Ceratocystis fimbriata* f. sp. *platani*, *J. Biol. Chem.* 274 (1999) 24959–24964.
- [38] J.B.C. Findlay, D.J. Pappin, S.N. Keen, in: J.B.C. Findlay, M.D. Geisow (Eds.), *Automated Solid-Phase Microsequencing in Protein Sequencing: A Practical Approach*, IRL Press at Oxford University Press, Oxford, 1989, pp. 69–84.
- [39] R.D.M. Page, TREEVIEW: an application to display phylogenetic trees on personal computers, *Comput. Appl. Biosci.* 12 (1996) 357–358.
- [40] N. Guex, M.C. Peitsch, SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modelling, *Electrophoresis* 18 (1997) 2714–2723.
- [41] A. Henriksen, T.P. King, O. Mirza, R.I. Monsalve, K. Meno, H. Ipsen, et al., Major venom allergen of Yellow jackets, *Ves v 5*: structural characterization of a pathogenesis-related protein superfamily, *Proteins* 45 (2001) 438–448.
- [42] M.C. Peitsch, ProMod and Swiss-Model: internet-based tools for automated comparative protein modelling, *Biochem. Soc. Trans.* 24 (1996) 274–279.
- [43] M.C. Peitsch, Protein modeling by e-mail, *Bio/Technology* 13 (1995) 658–660.
- [44] D.R. Hoffman, Allergens in Hymenoptera venom: XIII. Isolation and purification of protein components from three species of vespid venoms, *J. Allergy Clin. Immunol.* 75 (1985) 599–605.
- [45] J.N. Larsen, H. Lowenstein, Allergen nomenclature, *J. Allergy Clin. Immunol.* 97 (1996) 577–578.
- [46] M. Schmidt, K.M. Winningham, D.R. Hoffman, The bee venom protease allergen contains a CUB domain, *J. Allergy Clin. Immunol.* 109 (2002) S79.