

Review article

Recombinant Hymenoptera venom allergens

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Hymenoptera venoms, instead of the previously used whole body extracts, were introduced in the late 1970s for the diagnosis and treatment of IgE-mediated allergic reactions to stings by insects of the order Hymenoptera, such as honey bees and vespids (1, 2). Insect venom allergy is often considered as a model for IgE-mediated allergy: Diagnostic tests (skin tests and radioallergo-sorbant assays, RAST) are thought to be reliable, and specific immunotherapy with venoms is claimed to be safe and highly effective. However on closer looks the specificity of the main diagnostic tests (prick tests or intracutaneous skin tests with insect venom extracts, and tests for venom-specific serum IgE antibodies) is far from perfect. Up to 20% of individuals with no history of systemic sting reactions have positive tests. On the other hand only 30–50% of those with positive tests will react to a subsequent sting by the respective insect (3). According to a sting provocation test during venom immunotherapy around 95% of patients allergic to vespid stings are completely protected and do not develop any generalized allergic symptoms, while the complete protection rate achieved for those allergic to honey bee venom is only 80–90% (3, 4). Systemic allergic side-effects to immunotherapy injections may occur in 20–40% of patients during immunotherapy with honey bee venom and 5–10% during immunotherapy with vespid venoms (4). There is therefore considerable potential for improvement of both diagnosis and immunotherapy of Hymenoptera venom allergy. Thanks to modern molecular biology technology, recombinant venom allergens are available today and offer several promising approaches to achieve such an improvement.

Expression of various recombinant allergens from Hymenoptera venoms

The cDNA of a number of major venom allergens from the honey bee (*Apis mellifera*), various vespids (*Vespula vulgaris*, *Dolichovespula maculata*, *Polistes annularis*) and from the fire ant (*Solenopsis invicta*) have been cloned, and recombinant allergens were expressed in various prokaryotic and eukaryotic systems. The data concerning the respective allergens are summarized in Table 1 (5–34).

Recombinant bee venom allergens

The amino acid sequence of four allergens from honey bee venom is known and three of them have been cloned and expressed in various systems: phospholipase A2 (Api m 1)(11, 12), hyaluronidase (Api m 2) (13, 14) and, at least partially, acid phosphatase (Api m 3) (15). Melittin (Api m 4) on the other hand is available as a synthetic peptide. The crystal structures of the bee venom allergens phospholipase A2 (PLA), hyaluronidase (HYA) and melittin have been elucidated and published (16–18).

The biologic properties of recombinant bee venom PLA, a 16–20 kDa enzyme expressed in *E. coli* were found to be comparable to that of natural purified PLA (12, 19): The enzymatic activity of the recombinant affinity purified and refolded PLA (rPLA) was similar to that of natural purified PLA (nPLA) from honey bee venom (12). Point mutation in the center of enzymatic activity of rPLA completely destroyed this activity, but had no influence on the allergen binding in skin tests (20), indicating that B-cell epitopes of PLA are not

Table 1. Recombinant Hymenoptera venom allergens

Species	Allergen	MW kDa	References
<i>Apis mellifera</i>	Api m 1 phospholipase A2	16–20	11, 12, 16, 19, 20
	Api m 2 hyaluronidase	43	13, 14, 17, 25, 26
	Api m 3 acid phosphatase	49	15
<i>Vespula vulgaris</i>	Ves v 1 phospholipase A1	35	8, 29
	Ves v 2 hyaluronidase	45	8, 29
	Ves v 5 antigen 5	25	9, 10, 30
<i>Dolichovespula maculata</i>	Dol m 1 phospholipase A1	35	7
	Dol m 2 hyaluronidase	45	27
	Dol m 5 antigen 5	25	6
<i>Polistes annularis</i>	Pol a 5 antigen 5	25	28
<i>Solenopsis invicta</i>	Sol i 2 antigen 5	30	31, 33
	Sol i 3 antigen 5	25	32, 33
<i>Myrmecia pilosula</i>	Myr p 1	7.5	34

related to its enzymatic activity. This does not, however, exclude the possibility that this activity could be of relevance during the sensitization process.

In intracutaneous skin test end-point titration, the allergenic activity of rPLA was about 10 times higher than that of whole-bee venom, reflecting the fact that the major bee venom allergen PLA makes up about 10% of the protein content of the whole venom (19). Unrefolded rPLA, however, was completely devoid of allergenic activity in this system. It could further be shown that the allergenic activity in skin testing was similar for rPLA, nPLA and natural purified deglycosylated PLA, indicating that glycosylation, a post-translational process which is not provided by expression in *E. coli*, is of little relevance also for the allergenic activity of PLA. The oligosaccharide side chain of PLA has, however, been shown to function occasionally as a B-cell epitope for IgE and IgG responses in patients and in animals (21) and also to be able to induce a specific T-cell response (22). Similar oligosaccharide side chains are observed in glycoproteins from plant origin and have been made responsible for *in vitro* crossreactions between PLA and certain plant allergens, which are of unknown clinical significance (23).

Förster et al. (24) compared nPLA and rPLA from honey bee venom in their ability to release histamine from blood basophils of bee venom allergic patients *in vitro*. They found no significant difference between the two preparations. By comparing the histamine-releasing capacity of rPLA with that of its enzymatically inactive mutant they confirmed that the catalytic activity of PLA is not a requirement for allergenicity in the effector phase.

Also, recombinant bee venom HYA, a 43 kDa enzyme, was first expressed in *E. coli* (14). The enzymatic activity of this preparation was, however, clearly inferior and amounted to only about 30% of that of the natural purified allergen (nHYA). Likewise the IgE-binding capacity of the recombinant HYA expressed in *E. coli* (EchYA) was strongly reduced when compared to the nHYA (14, 25): In RAST

inhibition experiments, an almost 10-fold higher concentration was needed for comparable inhibition of IgE binding to discs coupled with nHYA. Therefore another expression system was chosen, Baculovirus infected insect cells which, through transmembranous secretion, provide post-translational changes such as refolding and glycosylation (14). It resulted in a preparation (BvHYA) with an enzymatic activity and an IgE-binding capacity similar to that of the natural purified allergen (Fig. 1). This observation, which differs from similar studies with PLA, indicates that in individual allergens post-translational changes may be essential for the correct three-dimensional conformation of the molecule, its biologic activity and the correct conformation of its B-cell epitopes.

Fig. 2 shows the crystal structure of bee venom rHYA as described by Markovic-Housley (17), consisting of an (α/β)₇ barrel composed of seven β strands and 10 α helices. By substituting the active site residues Glu 113 or Asp 111 in mutant recombinant HYA preparations, their enzymatic activity was completely abolished, while in immunoblot studies, binding to IgE and IgG human serum antibodies seemed preserved. In RAST-inhibition assays it was, however, shown to be reduced significantly, when compared to nHYA or BvHYA (26).

Molecular cloning and sequencing of about 70% of the gene of acid phosphatase, a 49 kDa glycoprotein and the third major allergen of honey bee venom, has recently been described (14). This allergen seems to be related to the lysosomal, but not the prostatic, family of acid phosphatases. Melittin, a 2.9 kDa basic peptide made up of 26 amino acids, is quantitatively the main component of bee venom, but is only a minor allergen. It is available in synthetic form (1, 5, 18).

Recombinant vespid venom allergens

There are four clinically important genera of the family Vespidae

1 *Vespula* (*V. vulgaris*, *V. germanica*, *V. maculifrons*, etc.) is certainly the most important. Its popular name in Europe is wasp, and yellow jacket in the US

2 *Dolichovespula* (*D. maculata*, *D. arenaria*, *D. media*, etc.)

3 *Vespa* (*V. crabro*, *V. orientalis*), the hornet

4 *Polistes* (*P. annularis*, *P. gallicus*, etc.).

The genera 2–4 are of limited importance for human insect venom allergy because, in contrast to *Vespula*, these insects do not compete with men for food and stings are mostly observed only near their nests.

The venoms of the four genera all contain the three major allergens phospholipase A1 (for *Vespula vulgaris*: Ves v 1), hyaluronidase (Ves v 2) and antigen 5 (Ves v 5 5).

Antigen 5 (Ag5), a 23 kDa protein with unknown function so far, has been expressed in *E. coli* and in yeast

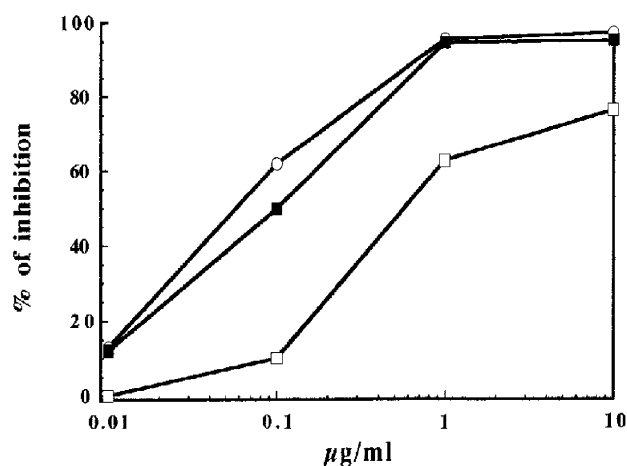


Figure 1. RAST-inhibition assay with serum from a bee venom allergic patient with high titers of hyaluronidase-specific IgE. Discs are coupled with natural purified hyaluronidase (nHYA). Complete inhibition is obtained at comparable concentrations with nHYA (○), recombinant HYA from Baculovirus infected insect cells (■), but not from recombinant HYA expressed in *E. coli* (□).

and is available in recombinant un-refolded and refolded forms from *Vespula vulgaris* (8, 9), *D. maculata* (28) and *P. annularis* (9, 28). In contrast to Ag5 expressed in the eukaryotic system (*Pichia pastoris*) Ag5 expressed in bacteria did not inhibit binding of natural Ag5 to human IgE from allergic patients, or to mouse IgG-antibodies in a significant way. This suggests, as with rHya from bee venom, incorrect folding of the bacterial preparation, with at least partially lacking conformational B-cell epitopes (9). Another group (10) however, was able to produce Ag5 in *E. coli* which after special refolding procedures exhibited IgE-reactivity similar to the natural purified allergen.

The Ag5s of various *Vespula* species have a sequence identity of about 95%, those of the genera *Vespula*, *Dolichovespula* and *Polistes* of 58–67% (29). This corresponds to almost complete crossreactivity within the *Vespula* genus but only partial crossreactivity between various genera of the *Vespidae* family. The structure of Ag5 from *V. vulgaris* has been determined by X-ray crystallography (30).

Phospholipase A1 (PLA1), a 35 kDa enzyme and hyaluronidase (HYA), a 45 kDa glycoprotein from *V. vulgaris* and *D. maculata* have so far only been expressed in prokaryotic systems (7, 27, 29). Sequence identity of PLA1 from the two species was found to be 67%, that of HYA 80%, and crossreactivity between the respective proteins of *V. vulgaris* and *D. maculata* has been demonstrated by antibody binding studies (27, 29). While vespid PLA1 has no sequence identity and hence no crossreactivity with PLA2 from bee venom, a 50% sequence identity between HYA from vespids and the honey bee has been found and is thought to be

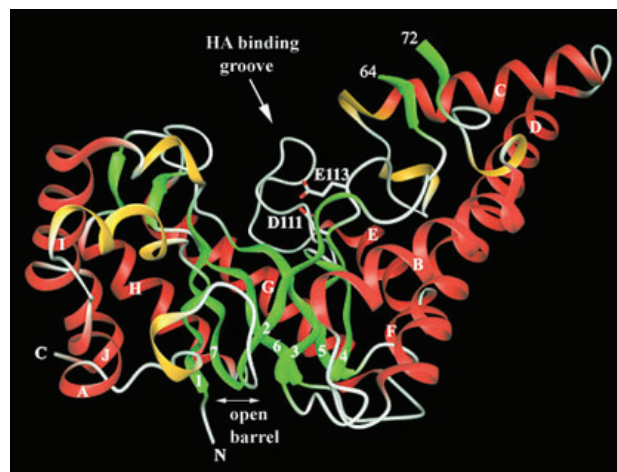


Figure 2. Ribbon representation of the three-dimensional structure of bee venom hyaluronidase. Side view of the (α/β)₇ barrel composed of βstrands 1–7 (green) and 10 αhelices A–J (red). The active site residues Glu 113 and Asp 111 are shown as stick models. The loops of the C-terminal end of the β barrel form the substrate binding groove which is large enough to accommodate a hexamer of hyaluronic acid. The barrel is open between strands β1 and β2 (We thank Dr Zora Markovic-Housley, Biocenter Basel, Switzerland for kindly making this picture available).

responsible for the limited crossreactivity which is observed between the venoms of these two different Hymenoptera families.

Recombinant formicidae venom allergens

The stings by several ant species, especially of the genera *Solenopsis* and *Pogonomyrmex* in America, and *Myrmecia* in Australia, may cause systemic IgE mediated allergic disease similar to that caused by stings of bees or vespids. The ant venoms resemble vespid venoms in their composition and contain a phospholipase A1 and a protein with about 50% sequence identity with antigen 5 from *Vespula vulgaris*. A 30 kDa protein (Sol i 2) and Antigen 5 (Sol i 3) have been cloned and expressed in Baculovirus infected insect cells (31–33). The IgE binding capacity of these recombinant proteins was similar to that of the respective natural purified proteins isolated from *Solenopsis* venom. Molecular cloning and characterization of a major allergen from the venom of the Australian jumper ant (*Myrmecia pilosula*) has also been described. IgE binding determinants of this allergen were localized in the C-terminal domain (34).

Recombinant venom allergens in diagnosis

The IgE-binding capacity of recombinant and natural purified PLA and HYA was studied *in vitro* in sera from a large number of bee venom allergic patients and controls (35, 36). As Fig. 3 shows, a very close correlation between specific IgE to nPLA and rPLA

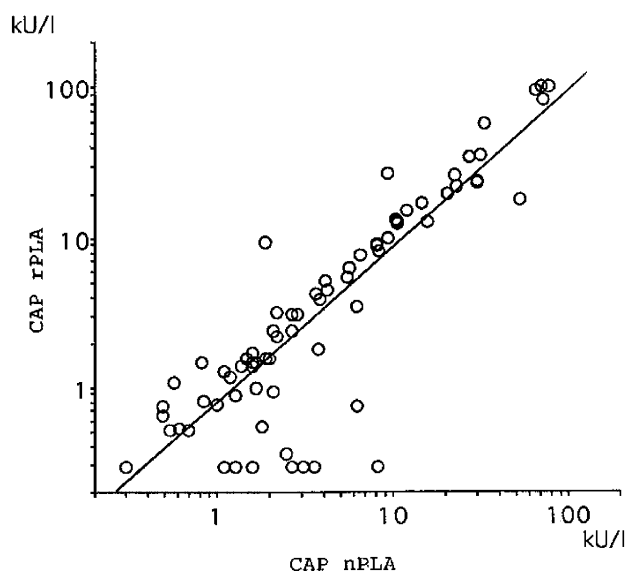


Figure 3. Correlation of specific IgE to recombinant PLA (rPLA) and natural purified PLA (nPLA) obtained by ImmunoCAP (Pharmacia, Uppsala, Sweden) in 85 bee venom allergic patients ($r=0.932$). A small group of seven patients had only specific IgE to nPLA.

was found. There was, however, a small group of patients who had only specific IgE antibodies to the natural purified preparation. Similar results were obtained for nHYA and BvHYA (14, 36). Further analysis of the sera from bee venom allergic patients by Western blot revealed, that six of the seven rPLA negative patients had no IgE to PLA, but exclusively bound to other bee venom allergens which contaminated

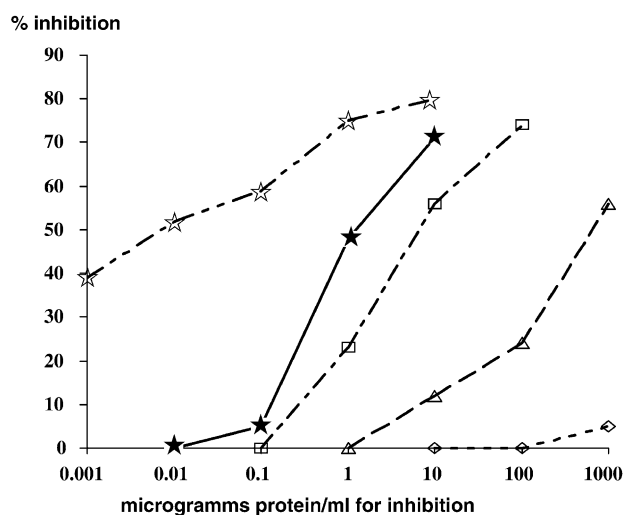


Figure 4. RAST-inhibition assay with serum from a bee venom allergic patient with a high titer of specific IgE to HYA. Recombinant HYA expressed in baculovirus infected insect cells (rHYA) is coupled to the discs. rHYA (★) but also three commercially available preparations of natural purified PLA, nPLA1 (□), nPLA2 (△), nPLA3 (☆) show significant inhibition of IgE-binding to the discs, indicating that these preparations contain significant amounts of HYA. No relevant inhibition is obtained with rPLA (◇).

the natural purified preparation (35). It can be speculated that the only patient who reacted to PLA did so on the basis of specific IgE to the glycosylated B-cell epitope of this allergen. This observation could be further confirmed by RAST-inhibition studies which are shown in Fig. 4: The IgE-binding to BvHYA coupled to RAST discs could be inhibited completely by all three commercial preparations of natural purified PLA, but not to a significant extent by the recombinant PLA. We also compared the IgE-binding capacity of natural purified and recombinant HYA, and of synthetic and natural purified melittin from honey bee venom (36). In a similar way RAST-inhibition studies showed that IgE-binding to PLA could be inhibited by natural purified HYA, and by natural purified melittin, but not by recombinant hyaluronidase and synthetic melittin. Recombinant allergens will therefore be superior to highly purified natural preparations when it comes to determination of the true clinical relevance of an individual allergen.

Recombinant technology has already been very helpful in clarifying crossreactivities between venom allergens from different species, genera or even families of Hymenoptera (29, 33). It has also revealed partial sequence homology between proteins from unrelated biologic sources, like hyaluronidase from Hymenoptera venoms and from mammalian testes (37) and it may in the future help to detect other sequence homologies between unrelated proteins which could also be of clinical relevance.

Another potential advantage of the use of recombinant allergens for diagnosis is increased specificity. As shown in Table 2 no false positive results were found when specific serum IgE antibodies were estimated by RAST with a diagnostic cocktail prepared from three recombinant or synthetic bee venom allergens. On the contrary, three of 20 control persons with no history of systemic reactions to honey bee stings and negative intracutaneous skin tests to whole-bee venom at 1 µg/ml reacted to the commercial whole-bee venom, and one to nPLA (36). The use of this cocktail resulted thus in a specificity of 100% which compared favorably with the 85% specificity of whole-bee venom, and the sensitivity of 87% was close to that of the whole venom. By the addition of recombinant acid phosphatase, the third major bee venom allergen, a sensitivity

Table 2 Specific IgE to whole bee venom (BV), natural purified (n) and recombinant (r)/synthetic (s) BV allergens phospholipase A2 (PLA), hyaluronidase (HYA) and melittin (Mel), as well as to a combination of the 3 recombinant/synthetic allergens (panel)

Population	% with specific IgE to							Recombinant panel rPLA + rHYA = sMel
	BV	nPLA	rPLA	nHYA	rHYA	nMel	sMel	
Patients $n=85$	96	86	78	88	71	66	28	87
Controls $n=20$	3	1	0	0	0	0	0	0

equaling that of whole honey bee venom could probably be reached, hopefully without loss of specificity. Such a diagnostic cocktail might thus replace natural whole-bee venom for *in vivo* and *in vitro* diagnosis of bee venom allergy in the future. Similar diagnostic cocktails could of course be prepared with recombinant major allergens from vespid venoms. Because most of the IgE-binding epitopes are conformational, recombinant allergens expressed in eukaryotic systems such as *Pichia pastoris* or Baculovirus infected insect cells should preferably be used for diagnostic cocktails.

Perspectives for recombinant venom allergens in treatment

Until now, recombinant venom allergens have not been used for immunotherapy. Theoretically, however, a number of promising approaches are conceivable:

1 Once all relevant allergens of a venom are available in recombinant form, the sensitization pattern of an individual patient can be determined exactly by estimating specific IgE antibodies to them all. A patient-tailored cocktail containing all the allergens to which the patient has IgE antibodies could then be prepared for immunotherapy (36, 38).

2 The mostly conformational B-cell epitopes have been shown to be strongly reduced in un-refolded recombinant allergens (10, 19). Such preparations, in which all relevant T-cell epitopes of the allergen are preserved since they are linear, will have a strongly reduced reactivity to IgE antibodies fixed on effector cells, and will therefore induce far less mediator release, and be better tolerated. On the other hand their capacity to interact with T cells and thus to induce protective immunologic effects will be preserved.

3 A similar concept consists of point mutations of conformational B-cell epitopes resulting in hypoallergenic mutants, such as the active site mutants of bee venom hyaluronidase mentioned above (26).

4 Finally T-cell epitope peptides could be expressed as recombinant fragments and used for immunotherapy (39): T-cell epitopes consist of short linear sequences of 10–15 amino acids of an allergen, which are recognized by specific T lymphocytes from allergic patients. By using overlapping peptides covering the whole 136 amino acid sequence of phospholipase A₂, the most important bee venom allergen, major T-cell epitopes have been identified by several groups (40–42). These linear short peptides were unable to bind to PLA specific IgE-antibodies in sera from bee venom allergic patients, but induced strong proliferation of the T lymphocytes of bee venom allergic patients *in vitro*. In a pilot study (43), five bee venom allergic patients with predominant sensitization to PLA were treated by weekly injections of increasing doses of an equimolar mixture of three

major T-cell epitope peptides of PLA thus identified. Over 2 months a total dose of 397 µg protein was given without causing any allergic side-effects. This observation contrasts with the reported late side-effects during peptide immunotherapy with T-cell peptides of the major cat allergen Fel d 1 (44) in a considerable proportion of patients. It may be explained by the fact that much longer peptides were used in the cat study. One week after the last treatment injection all five patients tolerated a first provocation test consisting of subcutaneous injection of 10 µg of natural purified PLA, corresponding approximately to the PLA content of one to two bee stings, without any systemic allergic symptoms. Another week later, three patients tolerated well a second provocation test by a live bee sting, while two developed minor cutaneous symptoms consisting of singular urticarial wheals or mild angioedema, which were much less severe than before the treatment. *In vitro* studies of lymphocyte cultures from the patients revealed a strong allergen-specific reduction of proliferation and secretion of both Th1 and Th2 cytokines following stimulation, which was less prominent in the two only partly protected individuals. This finding suggests the induction of PLA-specific tolerance by this form of peptide immunotherapy (45). During peptide immunotherapy PLA-specific serum IgE antibodies decreased slowly while the quotient of specific IgG4 to specific IgE increased sharply. Further serological studies indicated that the two patients with incomplete protection were sensitized to other bee venom allergens besides PLA as well. Immunotherapy with T-cell peptides from all allergens to which the patient is sensitized may thus be required in order to achieve complete protection.

Concluding remarks

Recombinant technology has opened a wide range of possibilities to improve the diagnosis and treatment of Hymenoptera venom allergy. A number of experimental studies have analyzed some of these possibilities. Nearest to clinical practice are probably recombinant cocktails for diagnosis which could lead to a very significant increase in the specificity of commonly used diagnostic tests, such as immediate type skin tests and the estimation of serum specific IgE antibodies. Somewhat farther away, but even more exciting, are the possible therapeutic implications, such as the use of T-cell epitope peptides or of hypoallergenic mutants of major venom allergens for immunotherapy of Hymenoptera venom allergy.

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