

## RESEARCH ARTICLE

# A proteomic study of the major allergens from yellow jacket venoms

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The venoms of stinging insects belong to the most dangerous allergen sources and can cause fatal anaphylactic reactions. Reliable prediction of a patient's risk to anaphylactic reactions is vital, and diagnosis requires the knowledge of the relevant allergens. Recently, a new hyaluronidase-like glycoprotein from *Vespula vulgaris* (Ves v 2b) was identified. This led us to investigate hyaluronidases and also other major allergens from *V. germanica* and four additional *Vespula* species. By MALDI-Q-TOF-MS, the new hyaluronidase-like protein was shown to be the major component of the 43-kDa band in all *Vespula* species studied. LC-ESI-Q-TOF-MS/MS sequencing of Ves g 2a and Ves g 2b facilitated the cloning of their cDNA. Ves v 2b and Ves g 2b turned out to be essentially identical on protein level. Whereas the less abundant "a" form displayed enzymatic activity, the new "b" homologue did not. This is probably caused by amino acid exchanges in the active site, and it raises questions about the physiological role of this protein. Sequence comparisons by MS/MS of antigen 5 and phospholipases from *V. vulgaris*, *germanica*, *maculifrons*, *pen-sylvanica*, *flavopilosa* and *squamosa* revealed the latter as a taxonomic outlier and led to the discovery of several not previously reported amino acid differences.

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## 1 Introduction

Yellow jackets (*Vespula*) represent a latent risk for people allergic against the venom of these insects [1]. In contrast to

pollen-allergic patients, who suffer seasonally from diverse allergic symptoms, insect-allergic individuals are affected by insect stings only and may therefore not be aware of their disposition until stung for a second time. This makes insect allergy less predictable than pollen allergy and more dangerous, because without immediate treatment a single insect sting can lead to the death of the patient due to anaphylactic shock.

Venoms of the yellow jacket species considered so far contain three major allergens: antigen 5 (Ag5 or Ves x 5), phospholipase (PLA or Ves x 1) and hyaluronidase (Hyase or Ves x 2), the latter being a glycoprotein [2–6]. Other proteins such as Vmac1 and Vmac3 have additionally been described

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**Abbreviations:** Ag5, antigen 5; CCD, cross-reactive carbohydrate determinants; cDNA, complementary DNA; GlcNAc, N-acetyl glucosamine; GlcUA, glucuronic acid; Hyase, hyaluronidase; PLA, phospholipase

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as minor allergens from *V. maculifrons* [7]. The physiological role of Ag5 has not yet been discovered, however, the enzymatic activity of the two other major proteins has been revealed by Hoffman and King about 25 years ago [2, 8–10]. Their pioneering work led to the sequencing of several allergens from selected yellow jackets, but so far, Ag5 remained the only allergen where protein sequences have been obtained for all the usually considered yellow jacket species [2]. In the case of PLA, the protein sequence is known for *V. vulgaris* and *V. maculifrons* (and during the preparation of this paper the *V. germanica* PLA nucleotide sequence became available online), while for Hyase, only the *V. vulgaris* enzyme has hitherto been cloned and sequenced [11].

The structures of the Asn-linked oligosaccharides of Hyases from *V. vulgaris* have recently been determined and shown to be cross-reactive carbohydrate determinants (CCDs) due to the core  $\alpha$ 1,3-linked fucose [12, 13]. CCDs are a frequent cause of cross-reactions of patients' sera with allergens from insects and other sources [3, 14, 15] but appear to be of low clinical significance [13, 16, 17]. The relative contributions of the protein and the carbohydrate moiety of insect venom Hyases to *in vitro* IgE binding and to eliciting allergic symptoms *in vivo* has not yet been defined.

The most surprising finding of this glycoproteomic analysis was that the major component of the Hyase band was a protein dissimilar from but 76% homologous to the known allergen Ves v 2 [5]. Noteworthy, the two catalytically important residues in Ves v 2a, Asp 107 and Glu 109 [6, 18]), are both exchanged to His in Ves v 2b [5], raising doubts about the enzymatic activity of this homolog.

In the framework of this study, we have characterized the major allergens from six *Vespula* species by MS. The complementary DNA (cDNA) encoding the major allergens of *V. germanica* have been cloned and the enzymatic activity of the two Hyase homologues from *V. vulgaris* was examined.

## 2 Materials and methods

### 2.1 Materials

*Vespula* venoms in the form of venom sac extracts were purchased from Sweden Diagnostics (Uppsala, Sweden). Sequencing-grade trypsin was obtained from Roche (Basel, Switzerland). Specimens of *V. vulgaris* and *V. germanica* were collected in and around Vienna and stored at  $-80^{\circ}\text{C}$  until use.

### 2.2 Sample preparation, Western blotting and MS and MS/MS analysis

Sample preparation of wasp venom, SDS-PAGE, tryptic digest, MALDI and ESI-Q-TOF-MS and MS/MS for the identification and sequencing of tryptic peptides of the major allergen proteins was performed as described in detail elsewhere [5, 19]. Western blots with anti-HRP serum (Sigma) were performed as described previously [14].

### 2.3 cDNA cloning of *V. germanica* allergens

The cDNA cloning has been performed from venom sacs of wasps as previously published [5]. The primers used to amplify cDNA for *V. germanica* venom proteins were TCCGAGAGACCGAAAAGAGTC and TTAGTTGACGGCTTCTGTCACG for Ves 2a, GTGATTACAATCTGGCC-TAAG and CTAAAAGTTTAAACGGTGTG for Ves 2b, GAA-GAGTTAAGAAGAAACCTTTCG and AAAGGTCATTGTG-TAATCTTT for Ag5 and CATGGTGATCCGTTATCTTACG and TTAAATTATCTTCCCCTTGTTATTG for PLA.

### 2.4 Expression of Ves v 2 variants in insect cells

Ves v 2a and Ves v 2b constructs encoding amino acids 1–331 and 1–340, respectively, were generated by PCR from the corresponding cDNA [5], using the following primer combinations: 5'-AAAACTGCAGCCATCCGAGAGACCGAAAAGAGTCTT-3' and 5'-GGAATTCCTTAGTTGACGGCTTCTGTCACGTT-3' (Ves v 2a), and 5'-AAAACTGCAGCCAGACAGAACAATTTGGCCTAAGAAG-3' and 5'-GGAATTCCTTAAAGTTTAAACGGTGTGTTTCTTTG-3' (Ves v 2b). The PCR products were cleaved with *Pst*I and *Eco*RI restriction enzymes at the underlined sites and ligated into pVTBacHis-1 baculovirus transfer vector [20]. In this construct, the Ves v 2 proteins are placed downstream of the melittin signal peptide, a 6  $\times$  His-tag and an enterokinase cleavage site.

A pVTBacHis-1 construct encoding untagged Ves v 2b was produced as above, using a different forward primer (5'-CGGGATCCCGACAGACAATTTGGCCTAAGAAG-3'), the same reverse primer (5'-GGAATTCCTTAAAGTTTAAACGGTGTGTTTCTTTG-3'), and the restriction enzymes *Bam*HI and *Eco*RI. This procedure places the Ves v 2b sequence directly behind the melittin signal peptide, thereby removing the 6  $\times$  His-tag and the enterokinase cleavage site.

Expression in *Spodoptera frugiperda* Sf21 cells was performed exactly as described previously [21]. Briefly, the recombinant transfer vector (1  $\mu\text{g}$ ) was co-transfected with 200 ng of BaculoGold viral DNA (BD Biosciences, Erembodegem, Belgium) into Sf9 cells using Lipofectin (Invitrogen) as recommended by the manufacturer. After 5 days at  $27^{\circ}\text{C}$ , supernatants containing recombinant virus were used for infection of Sf21 cells. Cells and culture media were harvested after 4 days at  $27^{\circ}\text{C}$  and subjected to SDS-PAGE and immunoblotting analysis.

For immunoblotting analysis, baculovirus-infected and non-infected Sf21 cells were lysed in SDS-PAGE sample buffer. Cell lysates and culture supernatants of infected Sf21 cells were subjected to 12.5% SDS-PAGE under reducing conditions. Fractionated proteins were electrophoretically transferred onto NC membranes (ProtranR, Schleicher and Schuell Bioscience) and subsequently incubated with a mouse mAb recognizing the His-Tag (Sigma). Detection of bound antibodies was achieved with goat anti-mouse IgG antibodies conjugated to alkaline phosphatase (Jackson

ImmunoResearch, Soham, UK) using the NBT/BCIP development kit (Sigma).

## 2.5 Purification and activity assays of recombinant *Vespula* Hyases

Recombinant His-tagged Ves v 2a and 2b were purified using Ni-NTA agarose (Qiagen, Vienna) as described previously [22]. Besides the purified enzymes, diluted supernatants from insect cell cultures were also tested for Hyase activity. Total bee venom, purified bee venom Hyase [23] as well as bovine testes Hyase (Sigma) were used as positive controls. Hyaluronic acid (Sigma) degradation was measured using the BSA-test and the Morgan-Elson assay as described previously [24–26]. The latter assay was also performed with the substrates chondroitin sulfate A and chondroitin sulfate C (Sigma).

Weak anion-exchange chromatography of 2-amino-benzamide-labeled glycosaminoglycans obtained after degradation with purified Ves v 2a was performed as described previously [27].

## 3 Results

### 3.1 General remarks

SDS-PAGE separation of the six yellow jacket venoms yielded strong bands for two major allergens, Ag5 and PLA, and more or less closely migrating double bands of about 45 kDa for Hyase (Fig. 1). Whether the observed difference in concentration of Ag5 and PLA present especially in the venom of *V. flavopilosa* and *V. germanica* are just a

feature of the particular venom batch or are specific for the species has not been further investigated. The protein bands were excised as indicated in Fig. 1 and subjected to MS characterization.

### 3.2 *Vespula* venom Ag5

The majority of the MS/MS results obtained agreed with the published sequences. However, a number of single-amino acid differences were found. In *V. maculifrons* Ag5 (P35760) the predicted tryptic peptide 107–128 was detected, however, MALDI-Q-TOF MS showed an additional signal with a mass increment of 2. Using MS/MS this signal could be shown to be the same peptide, however, with Thr instead of Val in position 122. As deduced from signal intensities of the two peptides, approx. 80% of the Ag5 in the analyzed venom batch contained Thr instead of Val in the particular position. For *V. flavopilosa* Ag5 (P35783) an amino acid substitution in position 38 was identified, where a Glu replaced the Lys, resulting in a different tryptic cleavage pattern.

An interesting accumulation of changes in a particular region was detected in Ag5 from *V. squamosa*. While the majority of the sequence corresponded to the *in silico* predicted peptides (P35786), the tryptic peptide 108–132 could at first not be detected by either MALDI-Q-TOF-MS or ESI-Q-TOF-LC-MS and MS/MS. However, peptides of  $[MH]^+ = 1019.59$  and  $1676.90$  Da, respectively, as detected by MALDI-Q-TOF could not be explained by the *in silico* prediction. Sequencing by ESI-MS/MS identified them as the missing sequence but with a remarkable accumulation of six amino acid substitutions with one even introducing a tryptic cleavage site (Fig. 2).

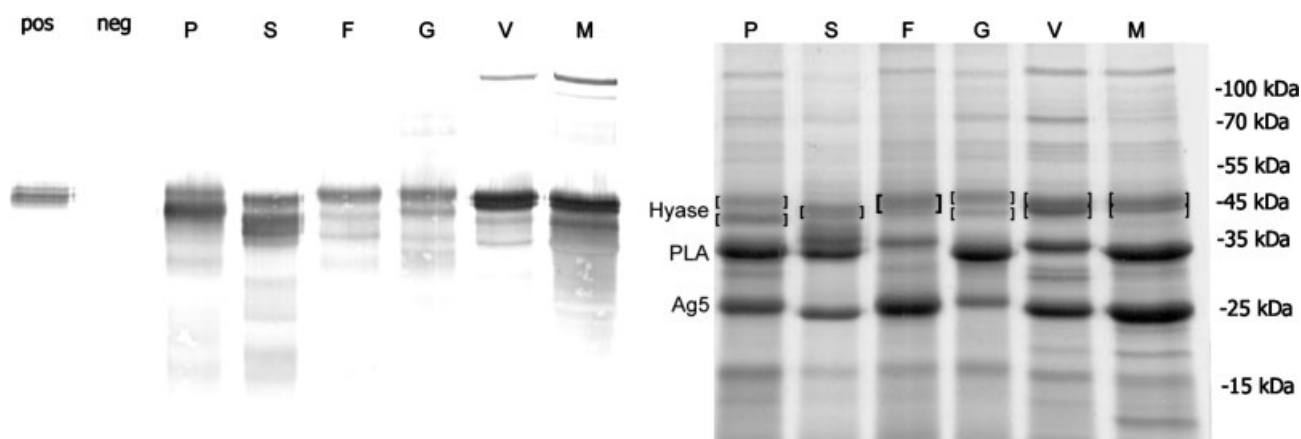


**Figure 1.** SDS-PAGE (right) and anti-HRP antibody Western blot (left) of six yellow jacket venoms. The three major allergen proteins were detected in all venoms: Ag5 (~25 kDa), PLA (~33 kDa) and Hyase (~44 kDa). The hyaluronidase bands analyzed are marked with brackets. MS of the Hyase double bands did not show any detectable differences between the upper and the lower bands. Blot staining using an antibody specific for core  $\alpha$ 1,3-linked fucose highlights the Hyase bands, however, especially in the *V. squamosa* venom also PLA-containing bands show a staining, that is not seen in the other five venoms. Abbreviations: P: *V. pensylvanica*; S: *V. squamosa*; F: *V. flavopilosa*; G: *V. germanica*; V: *V. vulgaris* and M: *V. maculifrons*. pos: positive control using HRP; neg: negative control using human transferrin.

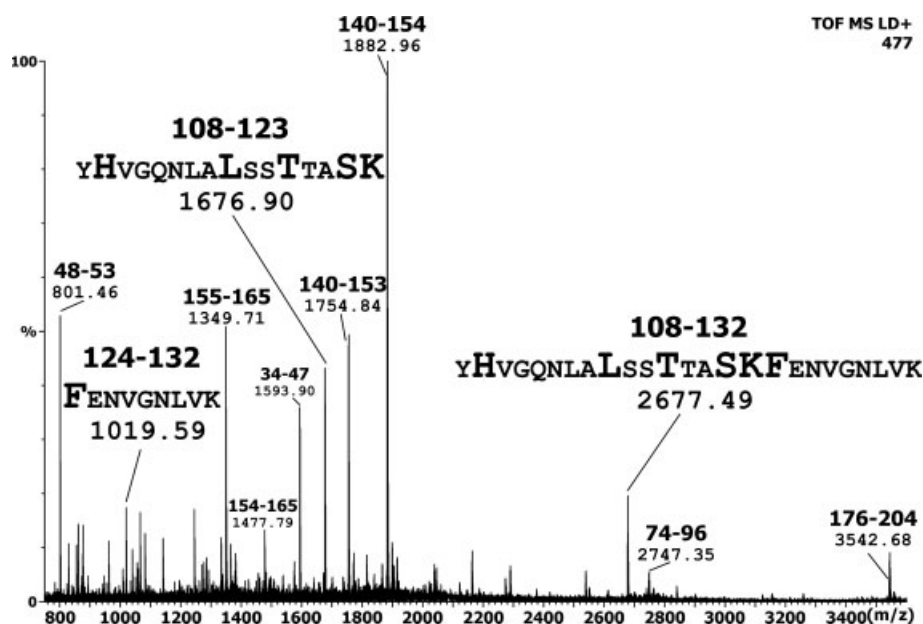


**Figure 2.** MALDI-Q-TOF MS spectrum of *V. squamosa* Ag5. The sequence of peptide 108–132 is given as revealed by LC-ESI-Q-TOF-MS/MS with the six amino acids different from database entry P35786 written in larger font. Because of the additional Lys, the peptide gave three signals. Other peptides were in accordance with the Swiss-Prot entry as confirmed by MS/MS. Leucine could equally be isoleucine, as they are not discriminated by their mass.

### 3.3 *Vespula* venom PLA

PLA sequences are just reported for *V. vulgaris* (P49369) and *V. maculifrons* (P51528) in the Swiss-Prot database and just recently (during the course of this work) the nucleotide sequence for *V. germanica* PLA became available (NCBI Entry AM083318). In a first survey, the six PLAs were subjected to a standard tryptic peptide mapping with MALDI-Q-TOF-MS. These preliminary results confirmed the database information for *V. vulgaris* and *V. germanica* PLA, whereas several peaks did not correspond to the *in silico* digest of *V. maculifrons* PLA. ESI-Q-TOF-LC-MS/MS indicated four yet unknown differences in the *V. maculifrons* sequence (96: K→T, 133: A→L, 238: I→M and 296: R→S; amino acid numbering see Fig. 3). Since the predicted peptides corresponding to these stretches of the previously reported sequence were not found, we conclude that the four exchanges occurred on one polypeptide and not on two or more isoforms as described above for *V. maculifrons* Ag5.

The close taxonomic relation of the different yellow jackets set the basis for the MS investigation of the hitherto unknown PLA from *V. pensylvanica*, *V. flavopilosa* and the partially described PLA from *V. squamosa*, respectively [28]. The peptide sequences identified for *V. pensylvanica* and *V. flavopilosa* displayed very high homologies at the protein level to the already known PLA sequences from *V. vulgaris*, *V. maculifrons* and *V. germanica* (Fig. 3). However, the peptide sequences for *V. squamosa* PLA differed significantly from those of the other five species. By MALDI-Q-TOF-MS and LC-ESI-MS/MS the *V. squamosa* Ag5 sequence described by Hoffman *et al.* [28] previously could be confirmed (Fig. 3) and one single-amino acid difference in position 136 (V→S) could be detected (see also Supporting Information). In contrast to the PLA sequences from the other yellow jacket spe-

cies analyzed in this study, *V. squamosa* PLA contains two N-glycosylation consensus sequences (N125 and N161, amino acid nomenclature adjusted to the *V. vulgaris* PLA, see Fig. 3). Although no glycopeptides could be detected by MS and peptide 156–168 (LVTDYNVSMADIR) was identified in its unglycosylated form by MS/MS, results from Western blot analyses using an antibody known to be specific for  $\alpha$ 1,3 bound fucose (and  $\beta$ 1,2 xylose) indicate that *V. squamosa* PLA might at least be partially glycosylated and contains CCD-reactive N-glycans (Fig. 1).

### 3.4 *Vespula* venom Hyases

The identification of *V. vulgaris* Hyase (Ves v 2a) and the detection of the major component of the Hyase band as Hyase-like protein (Ves v 2b) has already been described in a recent publication, which also dealt with the glycosylation of Hyase from various *Vespula* species [5]. The MALDI-Q-TOF-MS analysis of tryptic peptides from the Hyase bands of yellow jacket venoms revealed a striking similarity between all *Vespula* species (Fig. 4). Only *V. squamosa* gave a rather different picture (data not shown). Instead of continuing the MS sequencing of the highly conserved Hyases, we decided to determine the nucleotide sequences of the Hyases from *V. germanica*, the yellow jacket species endemic in Central Europe and to investigate the biological function of the two homologues.

### 3.5 cDNA cloning of *V. germanica* allergens

cDNA corresponding to *V. germanica* Ves v 2b and PLA could be amplified by PCR using primers designed on the basis of the high sequence homologies to the *V. vulgaris* homologues as revealed by the proteomic study. At first, an mRNA



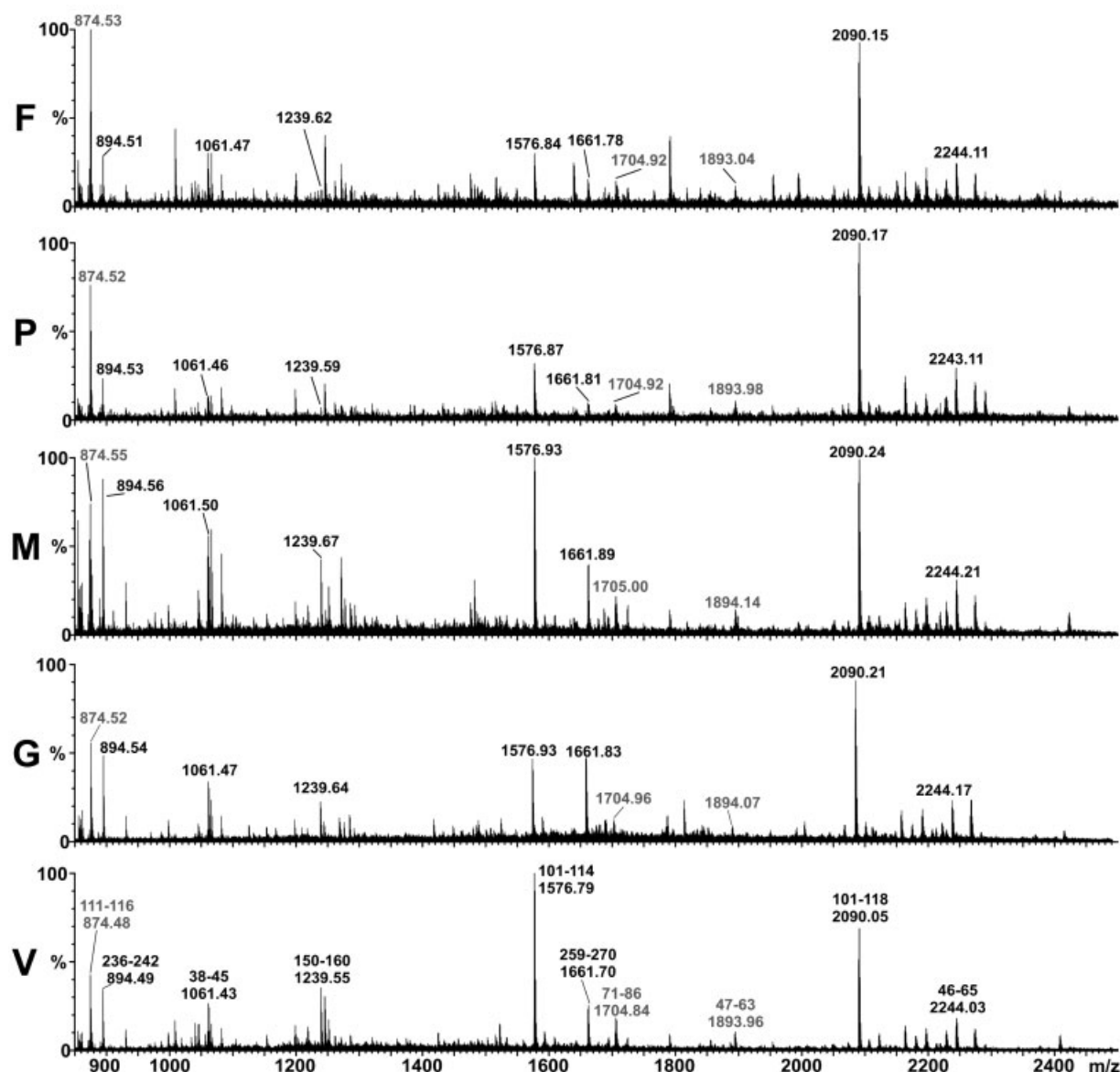


**Figure 4.** MALDI-Q-TOF-MS spectra of tryptic Hyase peptides. The Hyase bands from five different yellow jacket species were trypsin digested and analyzed by MS. The black labeled peaks correspond to protein homologues to Ves v 2b (amino acid numbering according to Swiss-Prot entry Q5D7H4), peaks labeled grey correspond to the Ves v 2a homologue (P49370).

clearly active, whereas neither tagged nor untagged recombinant Ves v 2b showed any activity against hyaluronic acid or against alternative substrates like chondroitin sulfate A and chondroitin sulfate C.

Notably, Ves v 2a, bee venom Hyase as well as bovine testis Hyase also degraded the chondroitin sulfates A and C, but the reactions reached an endpoint with only about 40% of maximally liberated hexosamine residues compared to hyaluronic acid. In contrast, the hyaluronic acid was apparently not quantitatively degraded by Ves v 2a (as well as bee or bovine Hyase) to tetramers (two repeating units). Ion exchange chromatography of 2-aminobenzamide-labeled

digestion products revealed that the major limit digestion product was glucuronic acid-*N*-acetyl glucosamine (GlcUA-GlcNAc)<sub>3</sub> accompanied by roughly equal amounts of (GlcUA-GlcNAc)<sub>2</sub> and (GlcUA-GlcNAc)<sub>4</sub> (data not shown).

## 4 Discussion

The detailed MS analysis of the major allergens from different yellow jackets led to the discovery of several conflicts between the data bank protein sequences and actually present allergens Ag5, PLA, and Hyase in venoms of most of the six

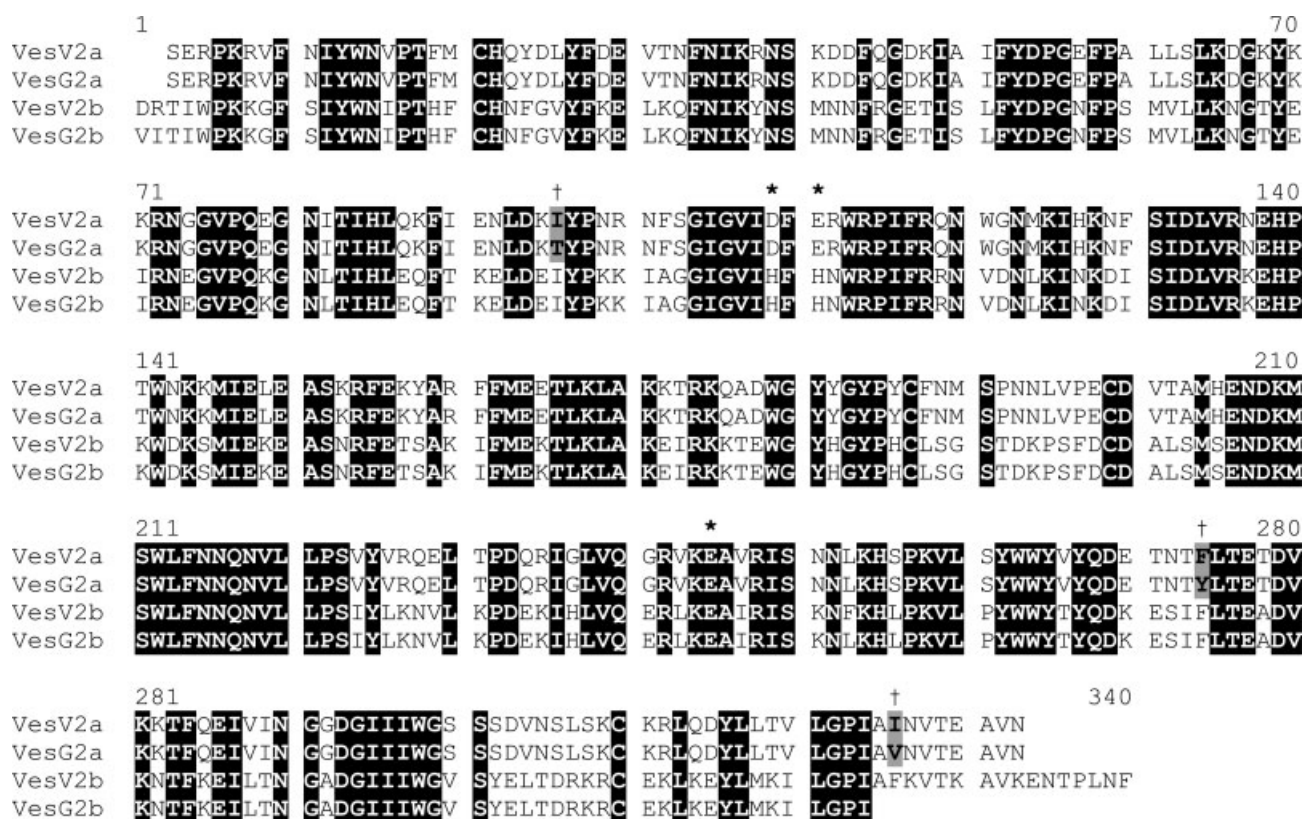


**Figure 5.** Alignment of the protein sequence from Ves 2a and 2b from *V. vulgaris* and *V. germanica*. Although both proteins share the major Hyase sites, two acidic amino acids in the active site are exchanged to a His (marked with an asterisk). The third Glu putatively involved in the active center, however, is present in all four forms. The a-forms differ in three residues (grey background and marked with a cross), whereas the b-forms from the two species are identical apart from the N terminus and possibly the not determined C terminus.

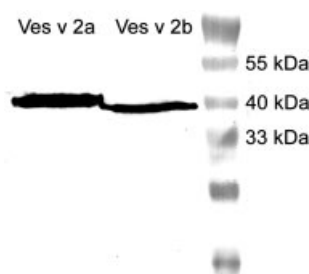


**Figure 6.** Western blot with anti His-Tag antibodies. Ves v 2a and 2b recombinantly expressed in insect cells was purified using metal chelate chromatography. The minor size difference detected on the Western blot is most probably due to Ves v 2a containing four and Ves v 2b only two potential N-glycosylation sites.

species analyzed. The majority of these differences are most likely due to individual single-amino acid differences resulting from the use of different venom batches. Nevertheless, problems or errors in the Edman sequencing [8] appear to be a better explanation for the observed accumulation of differences in peptide 108–132 of *V. squamosa* Ag5 and are likely to be due to the particular sequence in this region.

The precise MS characterization also facilitated the cDNA sequencing of PLA and Hyase from *V. vulgaris* and *V. germanica*, which belong to the most prominent yellow jacket species and are basically found all over the world.

The most remarkable finding was that all of the six species investigated contained mainly a probably inactive isoform of Hyase as the major protein in the 45-kDa bands hitherto supposed to consist of Hyase, whereas the active form, which appears to be as conserved among the species analyzed as the inactive “b” form, just constituted minor amounts.

Insect venom Hyases belong to the endo- $\beta$ -N-acetyl-hexosaminidase type Hyases that cleave after a GlcNAc residue yielding mainly tetrasaccharides [18, 29] or rather tetra- to octasaccharides as this study suggests. They are assumed to serve as a “spreading factor” facilitating the diffusion of other venom compounds thus they are playing a crucial biological role for insect defense. Surprisingly, the just recently identified Hyase-like protein Ves v 2b, which is detected in far higher concentrations than its active counterpart Ves v 2a is, did not show any activity towards various substrates. Recombinant expression of proteins in a particular host system does not always yield active enzymes. However, when

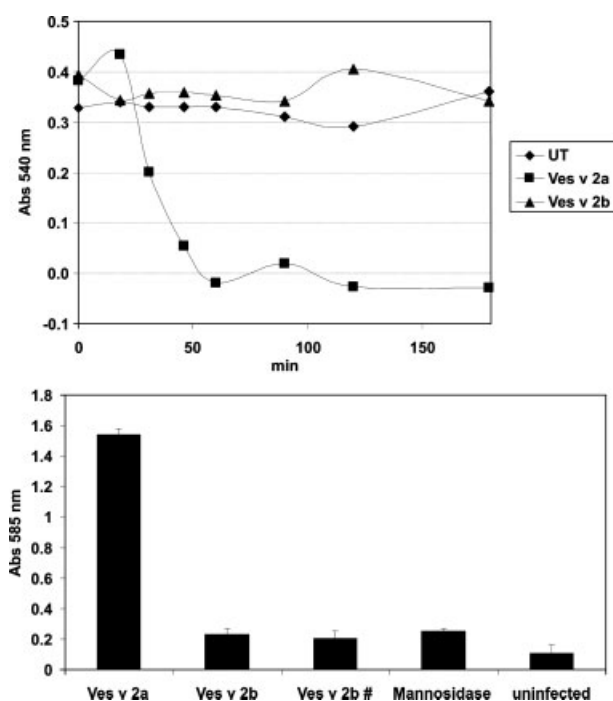


**Figure 7.** Determination of Hyase activity. Top: BSA-test. Enzymatic activity could be clearly measured in the supernatant from insect cells expressing Ves v 2a, whereas neither the supernatant from untransfected cells (UT) nor the one from cells transfected with Ves v 2b showed a detectable activity after 180 min. Bottom: Morgan-Elson assay for reducing HexNAc. Supernatants of insect cells expressing Ves v 2a with His-Tag, Ves v 2b with and without His-tag (#), an  $\alpha$ -mannosidase and the one of uninfected cells were tested for enzymatic activity. While Ves v 2a was clearly degrading hyaluronic acid, no activity could be detected for the different constructs of Ves v 2b.

the sequence of Ves v 2b is analyzed in the light of the 3-D structure of bee venom Hyase and its catalytic mechanism, this technical reason does not appear as the cause for the failure to obtain an enzymatically active protein. A key element of Hyases and many other glycohydrolases are two acidic residues in close proximity to each other [18, 30]. However, in the active site of Ves v 2b (and Ves g 2b) these amino acids are substituted with His (Fig. 5). Although the less acidic His could act as a hydrogen donor such as the acidic amino acids Asp and Glu, it is hardly imaginable how the contrarily charged His could substitute the role of Asp and Glu in this well-described and highly conserved catalytic mechanism.

This, however, raises the question on the biological role of these proteins in the venoms, since these proteins are conserved in the different yellow jacket species. It can be envisaged that the inactive Hyase isoform Ves v 2b operates as a lectin with the capacity to bind hyaluronic acid and/or related substances. Such a case has been reported for a family of mammalian chitinase-like lectins. There, substitution of two conserved acidic active-site residues by uncharged

amino acids abolishes enzymatic activity while preserving the ability of the proteins to bind to chitin and chito-oligosaccharides [31, 32]. However, the non-Hyases are not a component of all hymenoptera venoms. Blasting the Ves v 2b sequence against the recently published honey bee genome database did not reveal the presence of an analogous protein apart from the active and well-defined bee venom Hyase [33]. In agreement to this *in silico* data, we found that the bee venom Hyase band did not contain any noteworthy signals other than those assignable to the already known bee venom Hyase [23, 34].

We currently do not have any other suggestion for the biological role of the non-Hyases than the one mentioned above. This topic and the allergologic role of Hyase and its homologue and of their carbohydrate moieties pose interesting questions that have to await future studies.

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