

The N-glycans of yellow jacket venom hyaluronidases and the protein sequence of its major isoform in *Vespula vulgaris*

Daniel Kolarich¹, Renaud Léonard¹, Wolfgang Hemmer² and Friedrich Altmann¹

¹ Department of Chemistry, University of Natural Resources and Applied Life Sciences (BOKU), Vienna, Austria

² Floridsdorf Allergy Center (FAZ), Vienna, Austria

Keywords

cross-reactive carbohydrate determinant; hyaluronidase; *Hymenoptera*; insect sting allergy; *Vespula*

Correspondence

F. Altmann, Department of Chemistry, BOKU, Muthgasse 18, A-1190 Vienna, Austria

Fax: + 43 136006 6059

Tel: + 43 136006 6062

E-mail: friedrich.altmann@boku.ac.at

Note

The nucleotide sequence of Ves v 2b can be found at: EMBL AJ920395 The protein sequence for Ves v 2b (fragment) can be found at ExPASy Q5D7H4.

(Received 11 May 2005, revised 22 June 2005, accepted 1 July 2005)

doi:10.1111/j.1742-4658.2005.04841.x

Almost 50% of patients with suspected hymenoptera allergy turn out to be sensitized to both honeybee and yellow jacket venom [1]. Contrasting with these *in vitro* results, only very few patients show adverse reactions to both venoms. In two recent studies, protein-bound carbohydrate was shown to be a major, but not the only, cause for the simultaneous reactivity of patients' IgE with honeybee and yellow jacket venoms [2,3]. Apart from cases where true double sensitization to both venoms or antibodies against cross-reactive protein appears to be involved, a frequently found situation is that patients sensitized to one of the venoms cross-react with the other venom on the sole basis of protein-linked carbohydrate, which forms the so-called cross-reactive carbohydrate determinants (CCDs) [2].

Hyaluronidase (E.C. 3.2.1.35), one of the three major allergens of yellow jacket venom, is a glycoprotein of 45 kDa that is largely responsible for the cross-reactivity of wasp and bee venoms with sera of allergic patients. The asparagine-linked carbohydrate often appears to constitute the common IgE-binding determinant. Using a combination of MALDI MS and HPLC of 2-aminopyridine-labelled glycans, we found core-difucosylated paucimannosidic glycans to be the major species in the 43–45 kDa band of *Vespula vulgaris* and also in the corresponding bands of venoms from five other wasp species (*V. germanica*, *V. maculifrons*, *V. pensylvanica*, *V. flavopilosa* and *V. squamosa*). Concomitant peptide mapping of the *V. vulgaris* 43 kDa band identified the known hyaluronidase, Ves v 2 (SwissProt P49370), but only as a minor component. *De novo* sequencing by tandem MS revealed the predominating peptides to resemble a different, yet homologous, sequence. cDNA cloning retrieved a sequence with 58 and 59% homology to the previously known isoform and to the *Dolichovespula maculata* and *Polistes annularis* hyaluronidases. Close homologues of this new, putative hyaluronidase b (Ves v 2b) were also the major isoform in the other wasp venoms.

Probably, CCDs also cause some of the cross-reactivity observed for hyaluronidases from hornet (*Dolichovespula* sp.), paper wasp (*Polistes* sp.) [4] and even bumble bee and fire ant venom [5,6].

The protein responsible for most of the carbohydrate based reactivity on immunoblots of yellow jacket venom migrates at 43 kDa under denaturing conditions [2]. A rabbit serum raised against plant glycoproteins (oilseed rape extract) likewise primarily bound to the 43 kDa protein [2]. This band is believed to represent hyaluronidase for which, in the case of *Vespula vulgaris*, the primary structure is known from cDNA sequencing (Swissprot P49370) [4] and which is known to be glycosylated [7]. However, in a recent investigation, by MALDI-TOF MS, of the 43 kDa

Abbreviations

CCD, cross-reactive carbohydrate determinant; MUF³F⁶ and MMF³F⁶, N-glycan structures.

band, this hyaluronidase P49370 constituted only a minor component and the strong signals could not be assigned to a known protein sequence [2]. In this study, a mixture of venoms from *V. vulgaris* and *V. germanica* was employed and thus the unknown peptide peaks were interpreted as possibly arising from *V. germanica* hyaluronidase. This explanation was wrong, as shown below.

Irrespective of the nature of the protein, protein-linked glycans can bind IgE, which turns many proteins, especially those of higher molecular mass, into apparent allergens. Even though some studies have shown *in vitro* histamine release by plant glycoprotein glycans [8–10], the contribution of such carbohydrate determinants to clinical symptoms is unclear and is believed, by many researchers, to be negligible [2,3,5,11,12]. However, patients' sera containing anti-glycan immunoglobulin can bind to a variety of plant and insect glycoproteins and even to human proteins unrelated to any allergen in the peptide part when the glycan has been modified with core α 1,3-fucose, as shown for patients 9–14 in a previous study [11]. The glycan-based cross-reaction of honeybee-allergic patients' sera with wasp venom, and vice versa for that of wasp venom-allergic patients' sera with bee venom, could be inhibited by oilseed rape pollen extract [2]. Furthermore, the inhibition could be performed with BSA, to which small glycopeptides containing core α 1,3-fucose (and xylose) had been attached [2]. Thus, it can be speculated that yellow jacket hyaluronidase (or, more precisely, the 43 kDa protein) carries core α 1,3-fucosylated N-glycans, such as the honeybee venom hyaluronidase [13]. However, *Vespula* venom and the proteins therein have never been subjected to any structural analysis of protein-linked carbohydrate. Moreover, not even the polypeptide of hyaluronidase has been analyzed, to date, and the data on its sequence have been obtained exclusively from cDNA cloning [4].

Here we report on the identification of the major polypeptide in the 43 kDa band of *V. vulgaris* as a new isoform of hyaluronidase. Furthermore, we analyzed the hyaluronidase band in five other common yellow jacket species and we investigated the N-glycan structures of hyaluronidase from all six species.

Results

Immunological detection of venom glycoproteins

Electrophoretic separation of *V. vulgaris* venom under reducing and denaturing conditions yielded three major bands (Fig. 1). Antigen 5 migrated at 28 kDa,

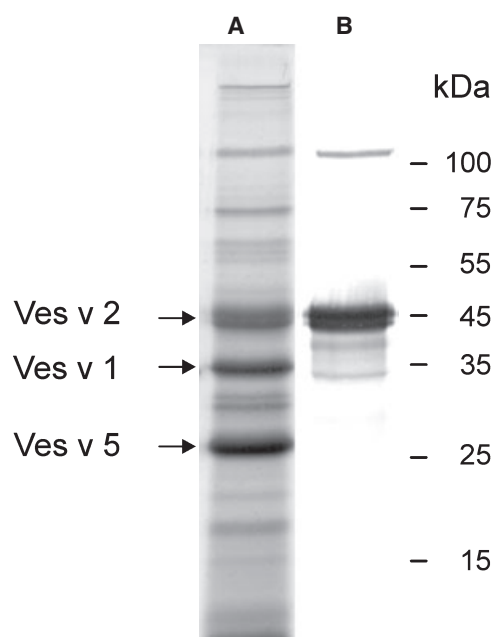


Fig. 1. Reducing SDS/PAGE (lane A) and immunoblot (lane B) of the venom from *Vespula vulgaris*. The double band at 43–45 kDa, usually considered to represent hyaluronidase, is the object of the present study. In the immunoblot, the serum specific for cross-reactive plant and insect N-glycans essentially only bound to the presumed hyaluronidase. The 100 kDa glycoprotein could not be identified. The diffuse bands of $\approx$ 40 kDa are regarded as degradation products of hyaluronidase.

phospholipase at 34 kDa and the putative hyaluronidase formed a double band at 43–45 kDa. On immunoblots with a rabbit serum that binds complex plant N-glycans (CCDs), one major band (at 43 kDa), believed to be hyaluronidase, became visible. Faint bands of lower molecular mass (34–40 kDa) were believed to represent degradation products of hyaluronidase. A sharp band at *c.* 105 kDa was shown by MS to be a glycoprotein (data not shown) but could not be identified. Only the 43 kDa band was subjected to N-glycan analysis.

Analysis of N-glycans of the 43 kDa band and of whole venom

The major glycan species found by MALDI-TOF MS had masses indicating them to consist of two GlcNAc, either two or three mannose residues and two fucose residues (Fig. 2). Hence, they resembled the difucosylated paucimannosidic N-glycans (Fig. 3), known from honeybee venom phospholipase A₂ and hyaluronidase [13,14]. Apart from quantitative differences, essentially the same results were obtained when this analysis was conducted with the hyaluronidase bands from

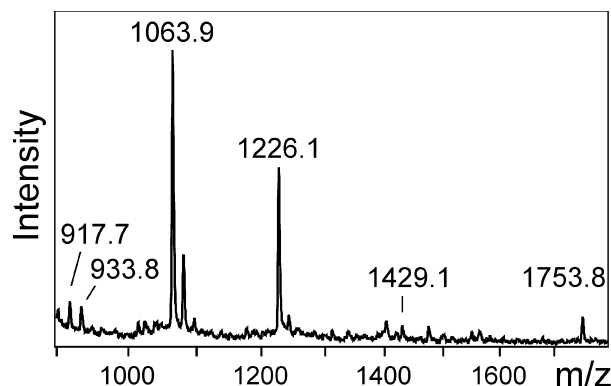


Fig. 2. Analysis of N-glycans from the *Vespula vulgaris* 43 kDa protein. Enzymatically released N-glycans from the 43 kDa gel band, which was later shown to consist of two hyaluronidase isoforms, were analyzed by linear MALDI-TOF MS. The major species represent difucosylated oligosaccharides (see Fig. 3 and Table 1).

V. maculifrons (Table 1). In this case, the structure of the presumed N-glycan structure MMF^3F^6 (Fig. 3) was verified by 2D HPLC and fucosidase digestion. However, for this purpose, whole venom from *V. maculifrons* was employed in order to obtain the necessary amount of glycans, as this venom is the least expensive.

As hyaluronidase is the dominating glycoprotein in the venom, we believe that the glycans from whole venom essentially stem from, and therefore credibly represent, those from hyaluronidase. First, pyridylaminated glycans were fractionated according to size (Fig. 4). A large peak eluting at 21.7 min was collected and further analyzed. Its elution time on reverse-phase HPLC matched that of MMF^3F^6 from honeybee phospholipase. A moderate dose of bovine kidney α -fucosidase caused a shift to a lower retention time, which is consistent with the removal of an α 1,6-linked fucose residue from the reducing end GlcNAc [14,15]. The glycan mass and the removal of one fucose residue were confirmed by MS (Fig. 4).

In summary, we conclude that wasp venom hyaluronidases contain the difucosylated paucimannosidic N-glycans MUF^3F^6 and MMF^3F^6 (Fig. 3) as the major species. In addition, glycans containing one or two GlcNAc residues at the nonreducing end and trace amounts of other, probably hybrid-type, N-glycans were present, but have not been further analyzed.

Proof that MMF^3F^6 is, in fact, bound to hyaluronidase came from tandem MS of a glycopeptide with a mass of 2037.0 Da, which at first could not be assigned to any known sequence. Only in retrospect was it recognized as having the peptide sequence NGTYEIR (which does not occur in P49370, see below), with a glycan of the composition $\text{Man}_3\text{GlcNAc}_2\text{Fuc}_2$. Among

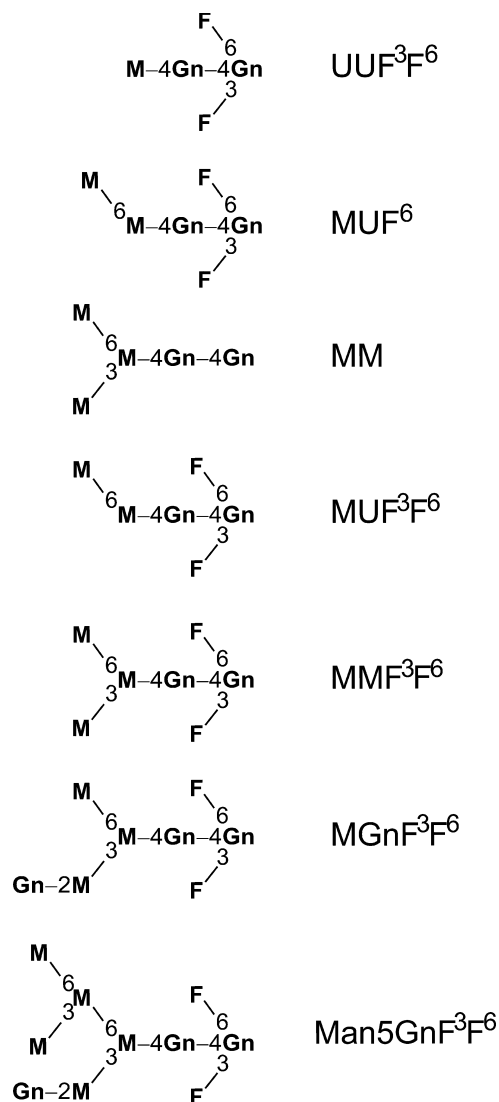


Fig. 3. Structures of the major N-glycan species found on yellow jacket hyaluronidases. Oligomannosidic structures are not depicted. Abbreviations are made according to the 'proglycan' system (<http://www.proglycan.com>). M, mannose; F, fucose; Gn, N-acetylglucosamine. The distribution of the glycans in the different venoms is listed in Table 1. The two complex-type structures at the bottom were only tentatively assigned; other isomers might also be present.

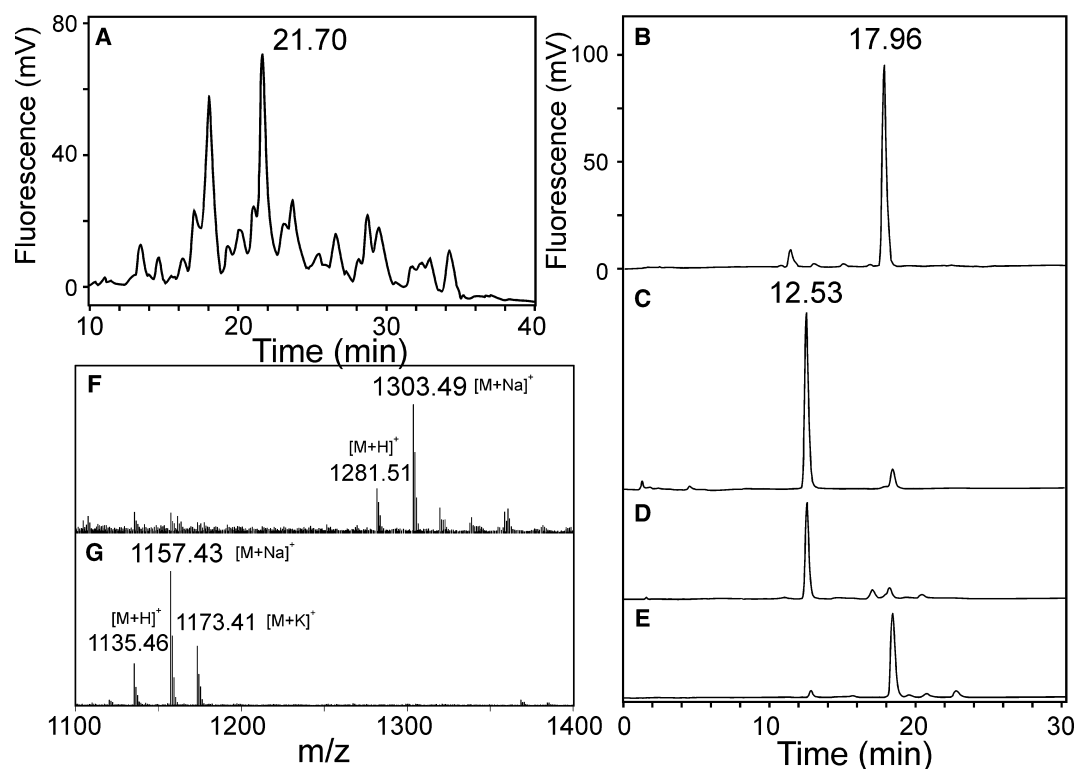
the various glycopeptide and oligosaccharide fragments, the fragment at $m/z = 1347.6$, which still contained two fucoses but only one GlcNAc residue, exemplified best the difucosylation of the asparagine-linked GlcNAc residue (Fig. 5).

Proteomic characterization of the 43 kDa band

As mentioned in a previous publication [2], peptide mapping of the 43 kDa band revealed considerable

Table 1. N-glycan analysis of the hyaluronidases from venoms of different wasp species. The structures are depicted in Fig. 3. The glycan structures shown in parenthesis were tentatively assigned and other isomers may be present.

Mass (Da)	Glycan structure	<i>V. vulgaris</i> %	<i>V. maculifrons</i> %	<i>V. germanica</i> %	<i>V. pensylvanica</i> %	<i>V. flavopilosa</i> %	<i>V. squamosa</i> %
901.8	UU ³ F ⁶				14.7	7.1	
917.8	MUF ⁶	3.7	3.8	9.9	8.8		24.7
933.8	MM	2.6	3.0		4.5	8.6	1.5
1064.0	MUF ³ F ⁶	47.7	55.3	48.9	33.7	45.1	32.4
1080.0	MMF ⁶	11.5	10.2	9.0	7.0	13.8	8.4
1096.0	Man4	0.8			1.1		
1226.1	MMF ³ F ⁶	28.1	22.3	32.1	23.5	23.9	26.9
1258.1	Man5				1.3		
1420.3	Man6				0.6		
1429.3	(MGnF ³ F ⁶)	1.2	3.3		4.8	1.5	6.0
1753.5	(Man5GnF ³ F ⁶)	4.3	2.0				

**Fig. 4.** Structural analysis of the N-glycans from whole *Vespa vulgaris* venom. (A) Size fractionation of pyridylaminated glycans by normal phase HPLC. (B) Analysis of the normal phase peak at 21.7 min by reversed phase HPLC. (C) Reanalysis of the same peak after treatment with bovine kidney α -fucosidase, which preferentially cleaves the α 1,6-linked fucose residue. (D) and (E) Chromatograms of reference glycans MMF³ and MMF³F⁶, respectively, from honeybee venom phospholipase [14]. (F) and (G), MALDI spectra of the normal phase peak at 21.7 min, before and after fucosidase digestion, respectively.

inconsistency between the data bank entry for hyaluronidase and the tryptic peptides obtained from the 43 kDa band of *V. vulgaris* venom (Fig. 6). Only a few, smaller, signals could be assigned to SwissProt entry P49370 (Fig. 6). Hence, the tryptic peptides were

subjected to nano-HPLC separation followed by tandem mass spectrometric peptide sequencing. BLAST analysis of the sequences deduced from the fragment spectra soon revealed moderate, but obvious, sequence homologies with hyaluronidase P49370 (Fig. 7). We

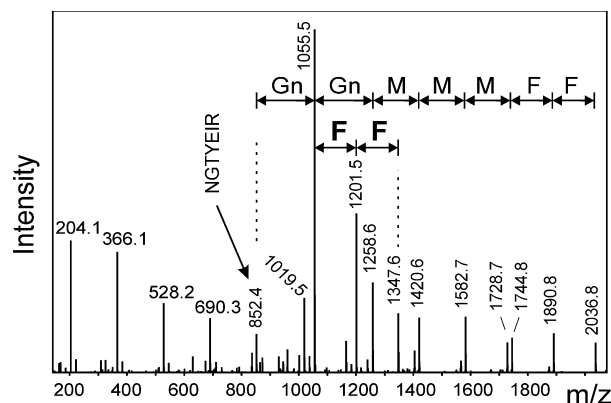


Fig. 5. Fragment ion spectrum of a glycopeptide from the 43 kDa band. The masses of (glyco-)peptides are written vertically, those of free glycans horizontally, and that of the mother ion tilted. From the loss of two fucose, of three mannose and, finally, of two GlcNAc residues, the composition of the glycan moiety of this peptide could be deduced. The fragment at 1347.6 Da implied two fucose residues to be linked to the reducing terminal GlcNAc. After acquisition of the sequence of the new isoform, Ves v 2b, the peptide moiety could be identified as comprising residues 66–72. Corresponding peptides in the other hyaluronidases (see Fig. 7) lack the glycosylation sequon.

concluded that the 43 kDa band contained a second isoform of hyaluronidase which – from the intensity of MALDI and ESI peaks – actually constituted the major form. To assess the true relative amount of the isoforms, the band was subjected to Edman sequen-

cing, which revealed one sequence that deviated from the sequence of P49370 (Fig. 7), but no clear indication for the presence of the known hyaluronidase (data not shown). It may be added here that both potential glycosylation sites were found as glycopeptides by either MALDI-TOF MS (site 81, data not shown) or ESI MS (site 66, see also previous chapter). Furthermore, the two glycopeptides were detected in both the upper and the lower part of the double band, at 43–45 kDa, thus ruling out underglycosylation as a reason for the different migration behaviour.

Molecular cloning of the new hyaluronidase isoform, Ves v 2b

Aiming at the molecular cloning of the cDNA of the newly discovered hyaluronidase isoform (referred to as Ves v 2b in the present article, in contrast to the known form, Ves v 2), the N-terminal sequence and internal fragments near the C terminus were translated into degenerate primers for PCR. Extension by 3'-RACE led to identification of the entire sequence. This new sequence was found to contain two glycosylation sites. Site 66 yields a tryptic peptide of mass 851.44 Da, which is identical to the peptide mass observed for the (glyco-)peptide dealt with in Fig. 5. It is notable that from the same cDNA we could also isolate a clone of the 'old' isoform, Ves v 2 (which probably should be referred to as Ves v 2a from now on).

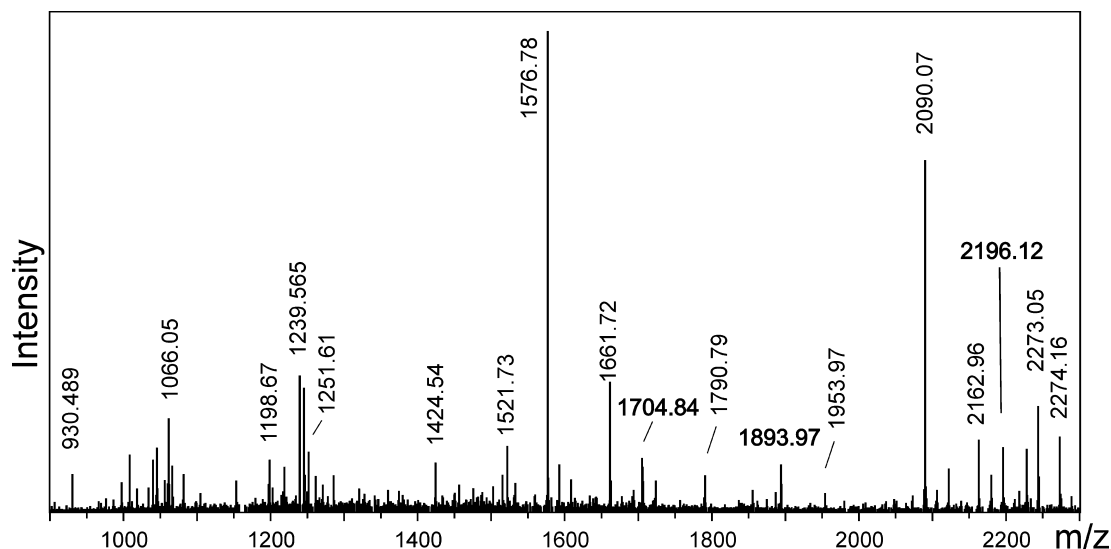


Fig. 6. MALDI spectrum of tryptic peptides from the 43 kDa band. The upper and lower parts of the double band, at 43–45 kDa (Fig. 1), were investigated separately but the spectra were identical. Only the three signals with mass numbers written horizontally match the data bank entry, P49370.

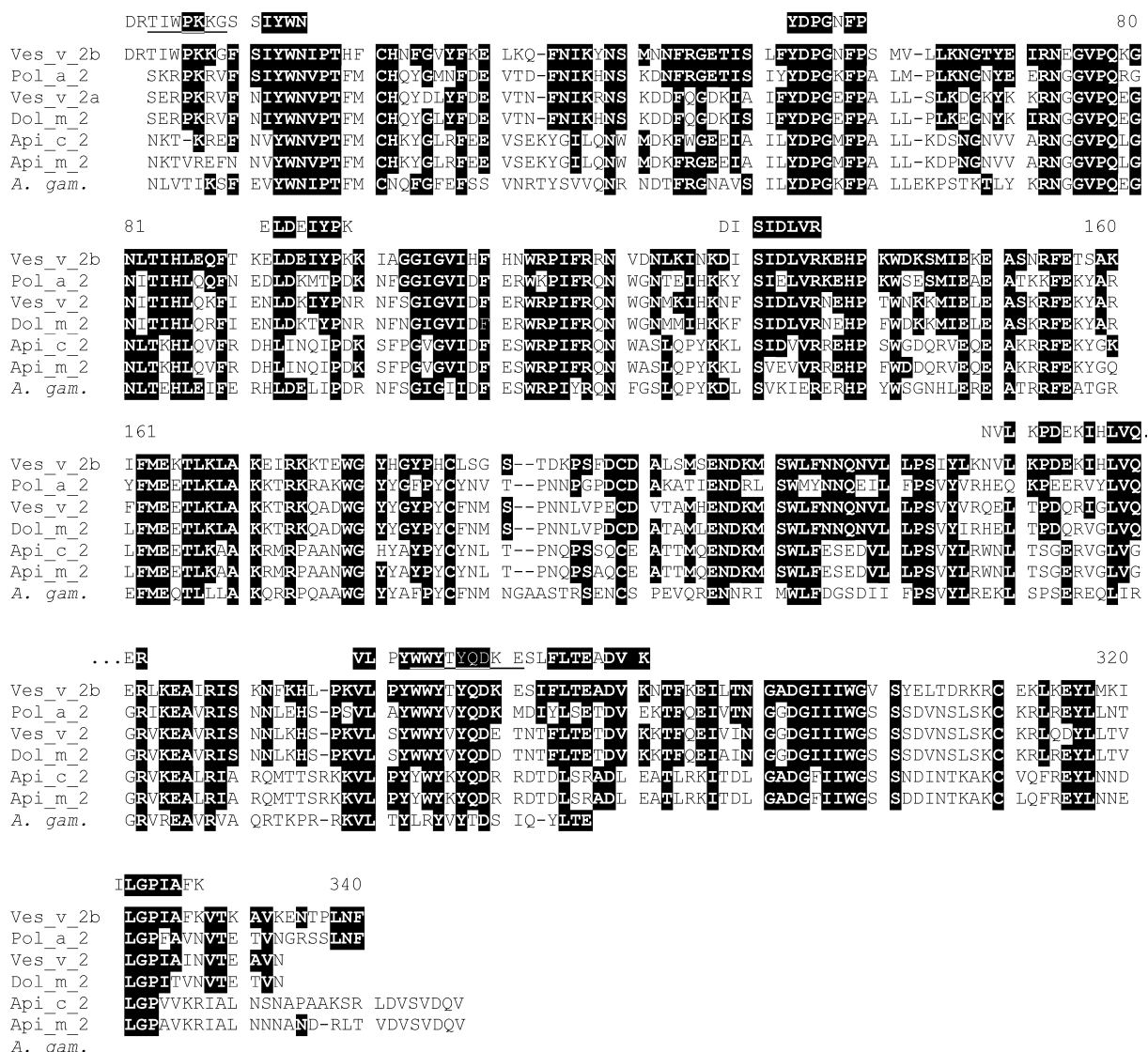


Fig. 7. Alignment of insect venom hyaluronidases and of MS sequence tags: The partial sequences in upper lines represent those deduced from fragment spectra of the unassignable peptides in the tryptic digest of the 43 kDa band of *Vespa vulgaris* venom. These pieces were aligned with the known hyaluronidase Ves v 2a (or Ves v 2; P49370) and matching residues are printed white on black. While leucine (Leu) is suggested by default in MS sequencing, isoleucine (Ile) is written when this residue occurs in the known protein sequence. The N terminus was obtained by Edman sequencing. The peptide stretches chosen for primer design are underlined. In the actual protein alignment, the new isoform, Ves v 2b, is shown at the top with the other sequences given in the order of decreasing similarity: Pol a 2 (*Polistes annularis*), Ves v 2a (= Ves v 2; *V. vulgaris*), Dol m 2 (*Dolichovespula maculata*), Api c 2 (*Apis cerana cerana*), Api m 2 (*A. mellifera*), and even from *Anopheles gambiae*. The highlighted positions correspond to amino acids that occur in Ves v 2b and in at least one of its homologues.

Simple BLAST search with the new hyaluronidase protein yielded 58% homology with P49370 and 59% similarity with the hyaluronidases from *P. annularis* and *D. maculata*. Sequence alignment with the currently known insect venom hyaluronidases is shown in Fig. 7. Like its homologues, Ves v 2b contained several hyaluronan-binding motifs (i.e. two basic amino acids spaced by seven other residues) [16].

Ves v 2b-homologues in other *Vespa* species

The MALDI spectra of the hyaluronidase bands from other wasp venoms (*V. germanica*, *V. flavopilosa*, *V. maculifrons* and *V. pensylvanica*) were almost identical to that from *V. vulgaris*. This corroborates the close relatedness of these wasp species and, at the same time, identifies hyaluronidase b (possibly

with a few species-specific differences) as the major isoform in these species. In contrast, the hyaluronidase band from *V. squamosa* yielded a totally different MALDI peptide map, which suggests a low sequence homology with the hyaluronidases from the other species.

Discussion

The similarity of the glycan structures on wasp and bee venom hyaluronidases explains – at least to a large extent – their allergological cross-reactivity. The role of venom hyaluronidases as major cross-reactive allergens in honeybee and *Vespula* venom has been recognized previously [17] and was thought to be a result of the significant sequence identity ($\approx 50\%$) between these allergens [4]. Although there is some evidence for cross-reactivity between honeybee and *Vespula* hyaluronidases at the protein level [2,7], its significance in relation to the cross-reactivity mediated by glycans should be redefined in future studies.

The cross-species survey performed in this study showed that the venoms from all six of the species usually considered (*V. vulgaris*, *V. germanica*, *V. flavopilosa*, *V. maculifrons*, *V. pensylvanica* and *V. squamosa*) contained MUF³F⁶ and MMF³F⁶ as the major glycan structures (Fig. 3). Sera from patients with antibodies directed to the core $\alpha 1,3$ -fucose determinant will therefore inevitably react with the hyaluronidase in venoms of all types of hymenoptera, irrespective of the underlying protein. These sera may be expected to bind also with other venom components bearing similar glycans, such as phosphatases [18], serine proteases [19], and honeybee and fire ant phospholipases [6,14,20].

Moreover, in such cases it is not even clear whether the original sensitizing agent was an insect venom or a plant allergen, which also contain this carbohydrate determinant [21]. Especially in the case of such ‘plant-allergic’ patients, a positive *in vitro* test against insect venoms can be expected to be unassociated with clinical symptoms from insect stings. Likewise, IgE against carbohydrates induced by *Hymenoptera* stings leads to a positive *in vitro* test with pollen allergens but is not associated with symptoms of pollinosis [5]. This shows, once again, the importance of a discrimination between carbohydrate- and protein-based IgE binding.

Incidentally, analysis of glycans from the wasp venom hyaluronidase led to the identification and molecular cloning of Ves v 2b, the true major protein in the 43 kDa band of *V. vulgaris* venom. It is currently unknown whether Ves v 2b represents a ‘true’ allergen,

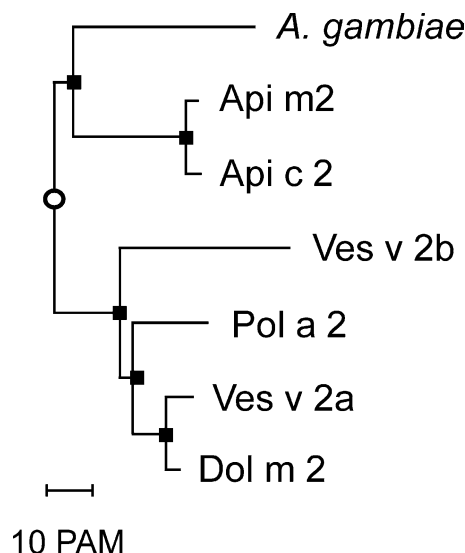


Fig. 8. Phylogenetic tree of hyaluronidases from several insect species: *Anopheles gambiae* (*A. gambiae*), *Apis cerana cerana* (*Api c 2*), *Apis mellifera* (*Api m 2*), *Dolichovespula maculata* (*Dol m 2*), *Polistes annularis* (*Pol a 2*) and *Vespula vulgaris* (*Ves v 2* and *Ves v 2b*). The tree was obtained by using the program MULTALIN (prodes.toulouse.inra.fr/multalin).

or binds with IgE only through its carbohydrate determinants. The other isoform, Ves v 2 (or Ves v 2a, as we would like to call it), was originally isolated by using primers that had been designed for the hyaluronidase of *D. maculata* [4]. In fact, the higher similarity of the original Ves v 2 to the *D. maculata* homolog than to Ves v 2b is depicted in a phylogenetic tree of insect hyaluronidases (Fig. 8). As confirmed in the present study, the cDNA amplified by these primers does code for a hyaluronidase which occurs in *V. vulgaris* venom, but as a minor isoform only. This example nicely demonstrates the merits of proteomic analysis in parallel with genomic work. As *V. vulgaris* does not stand high on the list of organisms whose genomes will be fully sequenced in the near future, the composition of the 43 kDa band would not have been revealed otherwise. Cloning of the two isoforms may allow their expression without immunogenic sugars, so that IgE binding to the polypeptides alone can be measured, which would clarify the question regarding the significance of hyaluronidase-based cross-reactions between wasp and honeybee venom. Furthermore, the individual role of the two isoforms for binding of IgE of insect venom-allergic patients can be studied.

Besides, the comparative analysis (e.g. by MALDI peptide mapping of several wasp venoms) revealed high homology between the hyaluronidases of all species, except that of *V. squamosa*.

Experimental procedures

Materials

Vespula venoms in the form of venom sac extracts were purchased from Sweden Diagnostics (Uppsala, Sweden). Sequencing grade trypsin and peptide N-glycopeptidase A were obtained from Roche (Basel, Switzerland). Rabbit anti-horseradish serum and bovine kidney α -fucosidase were from Sigma-Aldrich (Vienna, Austria). Specimens of *V. vulgaris* and *V. germanica* were collected in Vienna with assistance of the beekeeper of the local fire brigade.

Glyco-proteomic work

Vespula venom samples (approximately 10 μ g of protein) were separated by reducing SDS/PAGE on 12.5% (w/v) gels. Carboxamidomethylation, tryptic digestion, extraction of peptides and preparation of N-glycans were performed as described previously [11,22]. Oligosaccharides were analyzed either by MALDI-TOF MS by using an instrument operating in the linear mode or – after labeling with 2-aminopyridine – by reverse-phase HPLC, as described for honeybee hyaluronidase [13,22]. Peptides and glycopeptides were analyzed by MALDI or ESI MS on a Q-TOF ULTIMA GLOBAL (Waters-Micromass, Manchester, UK). Nano-LC was performed by using an Opti-Pak trap column (Waters, Vienna, Austria) and a Xterra[®] MS C18 reverse-phase analytical column (75 μ m $\times$ 10 cm; Waters, Vienna). The analytical column was eluted with a linear gradient from 5 to 40% (v/v) of acetonitrile in 0.1% (v/v) formic acid at a flow rate of 200 nL min⁻¹ postsplit. The column was directly coupled to PicoTips[™] (10 μ m I.D.; New Objectives, Woburn, MA, USA) to generate the nanospray. The mass spectrometer had been previously tuned with human [Glu1]-fibrinopeptide B (Sigma-Aldrich) to give the highest possible sensitivity and a resolution of approximately 10 000. Mass tuning of the TOF analyzer was carried out in the MSMS mode by using the y-ion series of [Glu1]fibrinopeptide B. Fragment spectra were finally processed manually by using BioLynx Peptide sequencing software (Waters-Micromass). Sequence alignments were performed by using the MULTALIN program.

N-terminal sequencing was performed by Laurent Coquet (UMR 6522 CNRS – Université de Rouen, France) on a Procise protein sequencing system (Applied Biosystems, Foster City, CA, USA).

Immunological methods

Western blots with anti-(horseradish peroxidase) serum (Sigma) were performed as described previously [11].

cDNA cloning

RNA from 10 *V. vulgaris* venom bags was purified by Trizol extraction, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNA was performed by using Superscript III reverse transcriptase (Roche) and a T¹⁸ oligonucleotide as an anchor. The degenerated primers used to obtain a partial hyaluronidase II cDNA were designed according to the peptide sequences obtained either by N-terminal sequencing or by tandem MS. Forward primers, corresponding to the N-terminal end of hyaluronidase II (peptide TIWPKKG), had the sequences 5'-ACNATHTGCCNAARAAGG-3' and 5'-ACNATHTGCCNAARAAGGG-3'. The degenerate reverse primers, corresponding to an internal peptide (WWY-TYQDKE), had the sequences 5'-TCYTTRTCYTGR TANGTGTACCACC-3' and 5'-CYTTRTCYTGR TANGTATACCACC-3'.

The PCR fragments obtained were purified by using the DNA and Gel purification kit from Amersham Pharmacia Biotech (Uppsala, Sweden) and cloned in pGEM-T (Invitrogen) before being sequenced by using Big Dye (Perkin Elmer, Boston, MA, USA). According to the sequence information obtained this way, primers were designed for a 3'-RACE PCR. The anchor used to synthesize the cDNA for the RACE PCR had the sequence 5'-AAGCAGTGGTATCAACGCAGAGTACT30VN-3'. The RACE PCR was then conducted by using the forward primer 5'-ACATTCCTACTCACTTTTGCCACAACCTTCGGCGTCTAT-3' and a mixture of the reverse universal primers 5'-CTAATACGACTACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' and 5'-CTAATACGAC-TCACTATAGGGC-3', at a ratio of 1 : 40 w/w. The obtained PCR fragments were purified, cloned and sequenced as described above.

Acknowledgements

This work was supported by the Joint Research Project S88-MED (S8802, S8803, S8808) of the Austrian Science Fund. We gratefully acknowledge the indispensable technical help of Karin Polacsek and Thomas Dalik and the proofreading by Jayakumar Singh Bondili.

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