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A NEW PEPTIDE FROM VENOM OF THE EAST-EUROPEAN HORNET *Vespa orientalis*. MASS SPECTROMETRIC *de novo* SEQUENCE

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The de novo *sequence of a new peptide from venom of the East-European hornet* Vespa orientalis *L. was determined by mass spectrometry. It was used as an example to show that time-of-flight mass spectrometry with an electrospray ion source can be used effectively to determine the amino-acid sequence of microquantities of peptides.*

Key words: *Vespa orientalis* L., new peptide, toxins, time-of-flight mass spectrometer, electrospray ion source.

Components of hymenoptera venoms are rich sources of biologically active substances and contain amines, short peptides, and high-molecular-weight proteins such as various enzymes, allergens, and toxins [1, 2]. The basic components of venoms from invertebrates have been well studied whereas the chemical composition of the minor components (especially peptides) is little studied. The de novo sequence of large proteins isolated from living organisms can be found using modern mass spectrometric methods with electrospray ionization and MALDI together with bhochemical digestion methods [3, 4]. Sequencing was previously carried out by the traditional method of Edman dehydration followed by mass spectrometric analysis [5]. This required rather large sample quantities and additional procedures.

Herein we establish the structures of the minor peptide components of invertebrate venoms by a combination of HPLC methods and time-of-flight mass spectrometry (TOFMS) with an electrospray ion source.

The basic components of venom from the East-European hornet *V. orientalis* are high-molecular-weight proteins orientotoxin 1 and orientotoxin 2 and low-molecular-weight histamine-releasing peptides HR-1, HR-2, and HR-3 [6, 7]. These peptides were isolated using preparative fractionation over Sephadex G-75 followed by HPLC, amino-acid analysis, and sequencing. We separated components of venom from East-European hornet using two-step reversed-phase HPLC without the use of preparative gel chromatography. This enabled all venom components to be isolated. The fractions from chromatography were analyzed using TOFMS to determine the molecular weights of their components. Known components and one unknown minor peptide were observed (Table 1).

The electrospray mass spectrum of the peptide was obtained using a custom TOF mass spectrometer with an orthogonal ion injector and electrospray ion (ESI) source [8, 9]. The interface between the ion source and the TOF mass spectrometer was a segmented radiofrequency quadrupole with a buffer gas at a pressure of 0.017 mbar. The segmented quadrupole was constructed in order to create a constant longitudinal electric field and to excite resonant ion rotation in a narrow *m*/*z* range, thereby causing them to fragment. Mass spectra of the fragments were obtained using just this selective excitation of singly and doubly charged protonated ions of the studied peptide. The TOF mass spectrometer had high resolution (10,000-15,000 at peak half-height) and rather high accuracy for *m*/*z* determination (~0.01 Da). Mass spectra of fragments that were obtained by resonant excitation in the radiofrequency quadrupole had, as a rule, a distinct "staircase" structure that corresponded to cleavage of peptide bonds in protonated molecular ions $[M + H]^+$ or $[M + 2H]^+$.

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TABLE 1. Low-Molecular-Weight Peptides from Venom of the East-European Hornet *V. orientalis*

Peptide	Amino-acid sequence	Molecular weight	
		Ref. [3]	Mass spectrometric determination
HR-1	INLKAIAALVKKVL-NH_2	1493	1492.7±0.3
HR-2	FLPLILGKLVKGLL-NH_2	1523	1523.3±0.3
HR-3	ASVHEFLVK(VKPGI)*	1523	1523.3±0.3
Minor peptide	AALAKLNL-NH_2		812.5±0.2

* - Amino-acid sequence partially established.

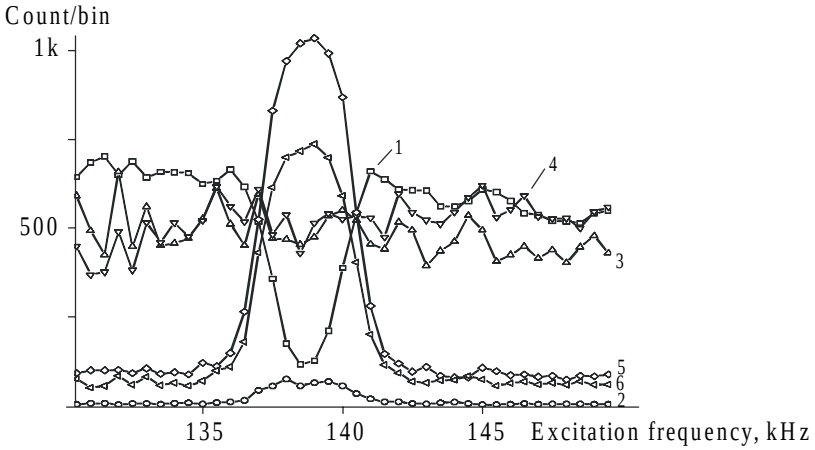
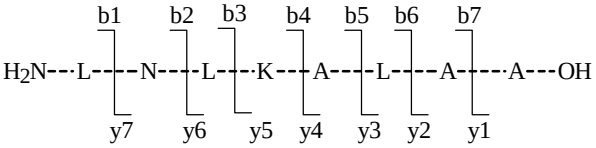


Fig. 1. Peak intensity of certain ions in the mass spectrum as a function of rotational excitation frequency in a radio-frequency quadrupole. The parent peak $[M + 2H]^{2+}$ (m/z = 407) decreases sharply and those of decomposition products increase simultaneously upon attaining resonance. Peaks with m/z = 279 and 431 are not fragments of the doubly charged studied peptide. m/z = 407, (×0.05) $[M + 2H]^{2+}$ (1); $274y_3^+$ (2), 279 (3), 431 (4), $473y_5^+$ (5), 724, (×0.2) b_7^+ (6).

Therefore, the masses of the amino-acid units could be found and the primary peptide structure deciphered from mass spectra of the fragments by determining the difference between the corresponding peaks. Even for amino acids with identical nominal molecular weights the accuracy of determining m/z using TOFMS could reliably identify the units because they had different defect masses (decimal units). Peaks in the high-resolution mass spectrum had resolved isotopic structure. Therefore, it was easy to figure out the ionic charge from the distance between isotopic peaks and to identify the "monoisotopic" peak. The mass spectrum of $[M + 2H]^{2+}$ fragments were more informative than those for $[M + H]^+$. Table 2 gives the initial electrospray mass spectrum of the peptide solution. The initial mass spectrum contained two main peaks corresponding to $[M + H]^+$ and $[M + 2H]^{2+}$. Table 2 also identifies peaks designated in the coded structure of the studied peptide.



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A series of mass spectra were recorded in small steps around the excitation frequency corresponding to m/z of the ion decomposing into fragments for the resonance excitation. The program used to record mass spectra could construct a curve of the studied peak intensity as a fucntion of excitation frequency. Comparison of such a curve with that of resonant decomposition of the parent ion could determine whether a certain peak was a fragment of the parent ion (Fig. 1). The results were analyzed to establish the structure of the minor peptide from venom of *V. orientalis* as AALAKLNL-NH_2. A literature search found that this peptide has not previously been reported. The data on the amino-acid composition of the new peptide were confirmed by amino-acid analysis using standard methods.

TABLE 2. Electrospray Mass Spectrum of Peptide with Assignment of Separate Fragments According to Formula 1

Diagnostic ion, *m*/*z*, experimental	Peak area	Diagnostic ion, *m*/*z*, calculated	Assignment
86.098	1602	86.096	Leu(Ile) immonium ion
90.057	1437	90.055	y1
97.067	23	97.065	b1-NH_3
114.092	184	114.091	b1
161.090	3677	161.092	y2
183.109	354	183.113	a2-NH_3
200.136	323	200.139	a2
211.104	477	211.108	b2-NH_3
228.129	3405	228.134	b2
229.115	3347	229.113	b2 (D instead of N)*
274.168	175	274.176	y3
296.181	224	296.197	a3-NH_3
313.212	159	313.223	a3
341.211	934	341.218	b3
345.204	308	345.213	y4
407.250	6041	407.263	$(M+2H)^{2+}$
469.311	138	469.313	b4
452.280	126	452.287	b4-NH_3
473.303	2164	473.308	y5
523.321	141	523.324	b5-NH_3
540.351	242	540.350	b5
586.396	8510	586.392	y6
636.417	610	636.408	b6-NH_3
653.442	2775	653.435	b6
700.453	2082	700.435	y7
707.462	687	707.445	b7-NH_3
724.487	3346	724.472	b7
813.534	10250	813.519	$(M+H)^+$

* - Asparagine partially converted to aspartic acid.

The isolation and establishment of the amino-acid sequence of the new peptide from *V. orientalis* venom were used to show that the combination of HPLC and TOFMS with an ESI source can be used to determine the amino-acid sequence of microquantities of peptides at a sensitivity up to 5×10^{-6} M.

Amino acid	Found	Mass spectrometry
A	3.04	3
L	3.07	3
K	0.98	1
N	1.00	1
Total units	8	8

EXPERIMENTAL

We used venom from the East-European hornet *V. orientalis* L. that was obtained by electrostimulation to isolate the peptides. Venom was dissolved in distilled water and used without further processing.

Two steps of preparative chromatography were performed on a Gilson instrument using 100 SS preparative pump heads, columns (24 × 250 mm) packed with Diasorb-130-C16/T [10 μm, 13,400 theoret. plates (tp); 5 μm, 25,500 tp]; analytical chromatography, on the Gilson instrument using 10 SS analytical pump heads and a column (4 × 250 mm) packed with

Diasorb-130-C10CN (5 μm). All chromatographic columns were from SP BioKhimMak (Moscow). The eluent was a gradient of CH_3CN in H_2O containing trifluoroacetic acid (0.1%). A Gilson 118 UV—Vis detector operating at 254 nm was used.

A preparative column of medium (13,400 tp) efficiency with a high capacity was used in the first step. The maximum loading without degrading the separation and increasing the time was up to 5 g of lyophilized venom. The fraction containing the minor peptide was injected without concentrating onto the HPLC column (25,500 tp) and eluted with a gradient to produce a fraction containing pure peptide. This fraction was lyophilized. The peptide yield was 90 ± 5% of its initial content in venom. Separation of whole venom (700 mg) afforded peptide (6.2 mg) of 99% purity. The minor peptide was then analyzed in the TOFMS. The peptide solution for TOFMS analysis was prepared in CH_3CN:H_2O:HCOOH (80:19:1) at 5×10^{-6} M peptide. The solution feed rate was <0.1 μL/min. The CH_3CN solution in the custom ESI source was fed into the capillary independently at the rate needed to give a stable electrospray.

The amino-acid analysis was performed on an Applied Biosystems 420A instrument. Hydrolysis was carried out in HCl (6 N) for 24 h [10].

Mass spectra were obtained and the peptide sequence was analyzed using a custom TOFMS with orthogonal ion injection and an ESI source. The instrument [8] was developed using earlier patented principles [9] and was built by staff members of the Laboratory of Prof. A. F. Dodonov (FIEPCP, RAS, Chernogolovka) together with the group of Prof. H. Wollnik (II Physical Institute of Justus-Liebig University, Gessen, Germany). The mass spectrometer had a resolution of 10,000-15,000 at peak half-height and *m*/*z* range 20-10,000 for detected ions.

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