Journal of Pharmaceutical and Biomedical Analysis 54 (2011) 273–278

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Characterization of honeybee venom by MALDI-TOF and nanoESI-QqTOF mass spectrometry

Jan Matysiak[a], Christian E.H. Schmelzer[b], Reinhard H.H. Neubert[b], Zenon J. Kokot[a,*]

[a] Department of Inorganic & Analytical Chemistry, Poznań University of Medical Sciences, Grunwaldzka 6 Street, 60-780 Poznań, Poland
[b] Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck-Straβe 4, 06120 Halle (Saale), Germany

ARTICLE INFO

Article history:
Received 28 April 2010
Received in revised form 30 July 2010
Accepted 15 August 2010
Available online 21 August 2010

Keywords:
Honeybee venom
MALDI
ESI
Mass spectrometry
Standardization

ABSTRACT

The aim of the study was to comprehensively characterize different honeybee venom samples applying two complementary mass spectrometry methods. 41 honeybee venom samples of different bee strains, country of origin (Poland, Georgia, and Estonia), year and season of the venom collection were analyzed using MALDI-TOF and nanoESI-QqTOF-MS.

It was possible to obtain semi-quantitative data for 12 different components in selected honeybee venom samples using MALDI-TOF method without further sophisticated and time consuming sample pretreatment. Statistical analysis (ANOVA) has shown that there are qualitative and quantitative differences in the composition between honeybee venom samples collected over different years. It has also been demonstrated that MALDI-TOF spectra can be used as a "protein fingerprint" of honeybee venom in order to confirm the identity of the product.

NanoESI-QqTOF-MS was applied especially for identification purposes. Using this technique 16 peptide sequences were identified, including melittin (12 different breakdown products and precursors), apamine, mast cell degranulating peptide and secapin. Moreover, the significant achievement of this study is the fact that the new peptide (HTGAVLAGV + Amidated (C-term), M_r = 822.53 Da) has been discovered in bee venom for the first time.

1. Introduction

Honeybee venom possesses diverse biological and pharmacological activities [1]. Its effectiveness has been demonstrated in treating pathological conditions such as arthritis [2], rheumatism, pain [3,4], cancerous tumors [5,6], and skin diseases. However, because bee venom is a toxin, a comprehensive information about its toxicology, side effects and chemical composition is needed in order to develop pharmaceutical formulations for its safe administration. On the European and global markets there are some registered pharmaceutical formulations with crude honeybee venom (Forapin, Germany; Virapin, Slovakia; Apiven, France; Melivenon, Bulgaria; Apifor, Russia) [7]. On the other hand, honeybee venom is used in venom immunotherapy. In both cases the standardization of the product is required. There are two approaches for standardization of complex natural products. If the pharmacological activity of the standardized product can be attributed to known constituents, they have to be quantified using appropriate method. If not all the active compounds are known or pharmacological activity of the product is caused by synergistic effect of all its constituents, some marker compounds must be used for standardization purposes [8]. Since honeybee venom is a very complex mixture of chemical compounds which are still not sufficiently characterized, analyzing marker compounds seems to be necessary. Unfortunately, there are still no uniform guidelines for honeybee venom standardization. There are only general methods to standardize allergen composition for preparations used in desensitization. These methods include: high performance liquid chromatography (HPLC) and high performance capillary electrophoresis (HPCE) to quantify the content of main proteins [9–11]; enzyme-linked immunosorbent assay (ELISA) inhibition to determine total allergenic potency [12]; sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and SDS-PAGE immunoblot to qualitatively analyze the total protein and IgE-binding components [13,14].

Honeybee venom has been intensively studied in the past and currently it is still subjected to modern venom profiling studies. Various analytical techniques for characterizing the properties of bee venom have been described in the literature: UV and IR spectroscopy [15,16], spectrofluorimetry [17], ^{1}H NMR spectroscopy [18,19], differential scanning calorimetry (DSC) [20], inductively coupled plasma mass spectrometry (ICP-MS) [21], high performance liquid chromatography (HPLC) [10,22–25], and high performance capillary electrophoresis (HPCE) [10,11,26]. Separa-

* Corresponding author. Tel.: +48 61 8546611; fax: +48 61 8546609.
E-mail address: zjk@ump.edu.pl (Z.J. Kokot).

0731-7085/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2010.08.020

tion techniques such as HPLC and HPCE with typical detectors like diode array detector (DAD) or fluorimetric detector (FLD) allow the analysis of no more than several honeybee venom constituents during a single experiment. However, bee venom is a very complex mixture and, hence, these methods can be insufficient for comprehensive characterization of this substance. This contrasts sharply with versatile and powerful mass spectrometric techniques, which can be used for determining many peptides or other compounds during a single experiment.

Mass spectrometry has become an indispensable tool for analyzing complex biological samples such as protein and peptide mixtures as a result of the development of the two soft ionization methods, MALDI (matrix-assisted laser desorption/ionization) and ESI (electrospray ionization) [27–30]. Most classical mass spectrometric protocols described for the analyses of honeybee venom go along with the employment of chromatographic techniques such as HPLC or two-dimensional gel electrophoresis (2-DE) [31,32]. These hyphenated techniques offer comprehensive results, especially when used for identification purposes. However, these methods are sample and time consuming when compared to direct analysis using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) or static nanoelectrospray quadrupole time-of-flight mass spectrometry (nanoESI-QqTOF-MS). The advantages of MALDI include high sensitivity, high speed of analysis, robustness, and the prevalence of singly charged ions [33]. For technical reasons MALDI is unsuitable for absolute quantitation purposes, but it has the potential of providing semi-quantitative information [34]. Furthermore, the measurement of individual samples can be easily automated and adapted for higher throughput with the use of MALDI as ionization method. The results from MALDI measurements compared to those from ESI are often complementary and thus applying both techniques in parallel allows a more comprehensive analysis of the samples. Therefore, we decided to use these two techniques to analyze different honeybee venom samples in order to characterize them conducting both qualitative and semi-quantitative analysis. This approach was to provide valuable information about the differences in the composition of honeybee venom samples from different strains of the bees, origin, year and season of the venom collection. Statistical evaluation of the obtained data was to determine the factors which influence the composition of the analyzed samples. Moreover, the results from direct honeybee venom analysis by MALDI-TOF-MS could be used as "fingerprints" of the product and therefore useful for bee venom standardization.

2. Experimental

2.1. Reagents and materials

Acetonitrile (ACN) of HPLC grade was supplied by Merck (Darmstadt, Germany), trifluoroacetic acid (TFA) of analytical grade was purchased from Fluka (Buchs, Switzerland) and the water used was doubly distilled. 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and α-cyano-4-hydroxycinnamic acid (CHCA) were obtained from Sigma–Aldrich (Steinheim, Germany). Glucagon-like peptide-1-(7-36) (GLP) was synthesized and purified in-house. Dithiothreitol (DTT) was obtained from Sigma Chemicals Co. (St. Louis, MO, USA). The membrane filters (0.45 μm pore size) were purchased from Agilent (Palo Alto, CA, USA).

Samples of honeybee venom were collected from an apiary of the Department of Inorganic and Analytical Chemistry, Poznan University of Medical Sciences by stimulating the bees with electric current pulses. Venom-collecting frames were placed in the upper body of the hive in the middle space of the hive super. The duration of the venom-collecting event was 2 h during full activity of bees. Such schedule allowed obtaining the highest efficiency of bee venom production. 28 samples were obtained from 2002 to 2007 during the whole beekeeping seasons from May until September. In 2006 and 2007, bee venom was collected from two different strains of *Apis mellifera* carnica (*niemka* – I strain, *singer* – II strain). Moreover, three archival samples were from 1991 (source: Georgia) and eight archival samples from 1995 (source: Poland) (the archival samples were supplied by the Department of Pharmacology and Biotechnology, Research Institute of Medicinal Plants, Poznan, Poland). Additionally, one bee venom sample (source: Estonia) was purchased from Sigma Chemicals Co. All samples were stored until analysis at 5 °C in darkness.

Due to the fact that there are insufficient data about honeybee venom stability in aqueous solutions in different storage temperatures, all the analyzed bee venom samples used in this study were freshly prepared.

2.2. MALDI-TOF-MS

MALDI experiments were carried out with delayed extraction time-of-flight mass spectrometer Voyager-DE PRO (AB Sciex, Weiterstadt, Germany) equipped with a pulsed nitrogen laser (λ = 337 nm, 3 ns pulse width, 20 Hz repetition rate). Two standard matrix systems were used in parallel: a solution of 10 mg mL^{-1} of α-cyano-4-hydroxycinnamic acid (CHCA) in ACN/0.1% TFA (1:1, v/v) and 20 mg mL^{-1} of sinapinic acid in the same solvent system. The honeybee venom solutions were prepared by dissolving 5 mg of the product in 1 mL of doubly distilled water. The samples were vortexed for 1 min followed by sonication for 10 min and then filtered. Then the internal standard (GLP) was added to a final concentration of 0.2 μg μL^{-1}. Bee venom solutions were mixed with the matrix solutions 1:10 (v/v). 1 μL of the resulting mixture was loaded on each spot of the sample plate. The samples were dried in a gentle stream of air (~35 °C).

Semi-quantitative analyses were performed in the positive ion linear mode by accumulating data from 500 laser shots for each spectrum. Mass spectra were acquired between *m/z* 750 and *m/z* 15000. Each measurement was carried out in triplicate. The instrument was calibrated using Calibration Mixture 2 of the Sequazyme Peptide Standards Kit (AB Sciex). The typical operating conditions for the mass spectrometer were as follows: acceleration voltage, 20 kV; grid voltage, 94%; guide wire voltage, 0.05% and extraction delay time, 300 ns. A low mass gate was set to *m/z* 750 to prevent detector saturation from matrix peaks. For semi-quantitative analysis 12 peptides were chosen and their intensities related to the internal standard intensity were calculated.

Furthermore, in order to achieve higher mass accuracy and better resolution the positive ion reflector mode was applied at 20 kV acceleration voltage, grid voltage set to 80%, 0.002% guide wire voltage and an extraction delay time of 400 ns. The low mass gate was set to 600 Da and analyses were performed in the mass range between 600 Da and 8000 Da. Survey spectra of the samples were obtained by accumulating data from 500 to 1000 laser shots.

The control of the instrument, data acquisition, and data evaluation were performed using Voyager Control Panel and Data Explorer software (AB Sciex).

2.3. NanoESI-QqTOF-MS

Static nanoESI experiments were conducted on a QqTOF mass spectrometer Q-TOF-2 (Waters/Micromass, Manchester, UK) equipped with a nanoESI Z-Spray source. The nanoESI glass capillaries were obtained precoated from DNU-MS (Berlin, Germany). The TOF analyzer was calibrated externally using a mixture of sodium iodide and cesium iodide.

Table 1
Semi-quantitative results of MALDI-TOF-MS of selected peptides (intensities in arbitrary units) in different honeybee venom samples. Min and max values refer to different months of bee venom collection.

Ordinal number	Monoisotopic peptide mass (Da)	1991 (Georgia)	1995	2002	2005	2006 bee strain I	2006 bee strain II	2007 bee strain I	2007 bee strain II	Sigma (Estonia)
1	2844.81	3.10–3.27	2.53–4.41	2.65–3.56	2.63–7.71	2.30–3.48	3.00–5.18	2.97–5.58	3.69–5.77	2.39
2	2859.84	0.07–0.12	0.05–0.16	0.00–0.05	0.00–0.04	0.03–0.09	0.00–0.08	0.00–0.08	0.00–0.04	0.05
3	2872.83	0.11–0.16	0.04–0.52	0.00–0.04	0.00	0.00–0.08	0.00–0.05	0.00–0.03	0.00	0.10
4	2886.80	0.03–0.06	0.00–0.20	0.00–0.14	0.00	0.00	0.00	0.00	0.00	0.10
5	2901.81	0.04–0.13	0.05–0.22	0.04–0.14	0.00–0.27	0.05–0.14	0.00–0.09	0.05–0.16	0.06–0.15	0.08
6	2941.14	0.07–0.12	0.04–0.13	0.08–0.15	0.12–0.20	0.09–0.13	0.11–0.21	0.14–0.18	0.14–0.16	0.12
7	2952.84	0.04–0.11	0.03–0.27	0.00–0.27	0.00–0.03	0.00–0.05	0.00	0.00	0.00	0.05
8	3006.84	0.33–0.67	0.00–0.65	0.20–0.88	0.20–0.59	0.29–0.55	0.00–0.44	0.17–0.49	0.29–0.37	0.35
9	3018.81	0.34–0.47	0.30–1.40	0.09–0.25	0.09–0.11	0.12–0.27	0.15–0.23	0.13–0.37	0.11–0.24	0.22
10	3050.87	0.12–0.21	0.05–0.30	0.03–0.16	0.11–0.22	0.08–0.19	0.15–0.29	0.08–0.20	0.13–0.25	0.12
11	3068.87	0.09–0.11	0.05–0.12	0.08–0.11	0.08–0.22	0.08–0.10	0.09–0.13	0.08–0.16	0.11–0.16	0.07
12	3643.12	0.04–0.15	0.02–0.18	0.03–0.09	0.05–0.12	0.07–0.09	0.05–0.09	0.04–0.09	0.06–0.11	0.07

For nanoESI analyses 1 mg of honeybee venom was dissolved in 1 mL of water with addition of 2.5% dithiothreitol (DTT) to reduce disulphide bonds in the peptides. Two microliters of the sample solution was loaded into the nanoESI glass capillaries using Microloader pipette tips (Eppendorf, Hamburg, Germany).

The typical operating conditions for the QqTOF mass spectrometer were as follows: capillary voltage, 900 V; sample cone voltage, 35 V; source temperature, 80 °C. The instrument was operated in the positive ion mode. Full scans were performed over the m/z range of 200–2500. Peptides of interest were selected manually for tandem MS experiments using collision-induced dissociation (CID) with argon as collision gas. The quadrupole mass filter was adjusted with low mass (LM) and high mass (HM) resolution settings of 10 (arbitrary units) and the CID collision energy was varied depending on charge state and mass-to-charge ratio between 20 eV and 60 eV.

2.4. Peptide sequencing

The fragment ion spectra obtained from the MALDI-PSD measurements were processed using Mascot Distiller (Matrix Science, London, UK). Mass spectra and tandem mass spectra of the nanoESI-QqTOF were analyzed by MassLynx (version 4.0, Waters/Micromass). The tandem MS spectra were subsequently processed by the MassLynx add-on Maximum Entropy 3 (MaxEnt3), and converted into PKL files, which were suitable for further analyses. Both the MALDI-PSD and QqTOF-MS/MS fragment ion peak lists, generated as described, were analyzed by searching the sequence database MSDB with Mascot (Matrix Science, London, UK) [35]. The searches were taxonomically restricted to "other metazoa". The mass error tolerances for precursor and fragment ions were set to 200 ppm for PSD data and to 0.1 Da for QqTOF-MS/MS data, respectively. The following variable modifications were considered: amidation of the C-terminus and formation of pyro-glutamate (pyro-Glu) from glutamate (Glu) at the N-terminus of peptides.

Additionally, automated *de novo* sequencing combined with database searching was performed on the QqTOF-MS/MS data using the software PEAKS Studio (version 4.5, Bioinformatics Solutions, Waterloo, Canada) [36] with precursor and fragment mass error tolerances of 0.10 Da. The same post-translational modifications as used for the Mascot search were considered.

3. Results and discussion

41 honeybee venom samples of different strains of the bees, country of origin (Poland, Georgia, and Estonia), year and season of the venom collection were analyzed using MALDI-TOF and nanoESI-QqTOF-MS. MALDI-TOF-MS was mainly used for semi-quantitative analyses because of its robustness when analyzing complex samples and the prevalence of singly charged peaks,

Table 2
Peptides identified by nanoESI-QqTOF-MS analysis conducted on representative honeybee venom samples.

Protein name	Experimental mass (Da)	Calculated mass (Da)	Mascot ion score	Peptide sequence
Melittin (honeybee), 44–52	868.51	868.57	56	GIGAVLKVL
Melittin (honeybee), 61–69	1227.68	1227.73	33	SWIKRKRQQ + Amidated (C-term)
Melittin (honeybee), 59–69	1453.76	1453.90	58	LISWIKRKRQQ + Amidated (C-term)
Melittin (honeybee), 57–69	1621.82	1621.99	76	PALISWIKRKRQQ + Amidated (C-term)
Melittin (honeybee), 48–69	2546.63	2546.59	117	VLKVLTTGLPALISWIKRKRQQ + Amidated (C-term)
Melittin (honeybee), 46–69	2674.71	2674.65	135	GAVLKVLTTGLPALISWIKRKRQQ + Amidated (C-term)
Melittin (honeybee), 44–69	2844.55	2844.75	125	GIGAVLKVLTTGLPALISWIKRKRQQ + Amidated (C-term)
Melittin precursor (honeybee), 36–69	3643.19	3643.06	136	DAEADPEAGIGAVLKVLTTGLPALISWIKRKRQQ + Amidated (C-term)
Melittin precursor (honeybee), 32–69	4025.33	4025.21	96	EAEADAEADPEAGIGAVLKVLTTGLPALISWIKRKRQQ + Amidated (C-term); Glu->pyro-Glu (N-term E)
Melittin precursor (honeybee), 24–69	4871.34	4871.58	64	EPEPAPEPEAEADAEADPEAGIGAVLKVLTTGLPALISWIKRKRQQ + Amidated (C-term); Glu->pyro-Glu (N-term E)
Apamine precursor (honeybee), 28–45	2029.91	2029.92	56	CNCKAPETALCARRCQQH + Amidated (C-term)
Mast cell degranulating peptide precursor (honeybee), 28–49	2589.56	2589.42	39	IKCNCKRHVIKPHICRKICGKN + Amidated (C-term)
Secapin precursor – honeybee	2866.49	2866.58	38	YIIDVPPRCPPGSKFIKNRCRVIVP
ORF for hypothetical protein – honeybee	822.53	822.47	32	HTGAVLAGV + Amidated (C-term)

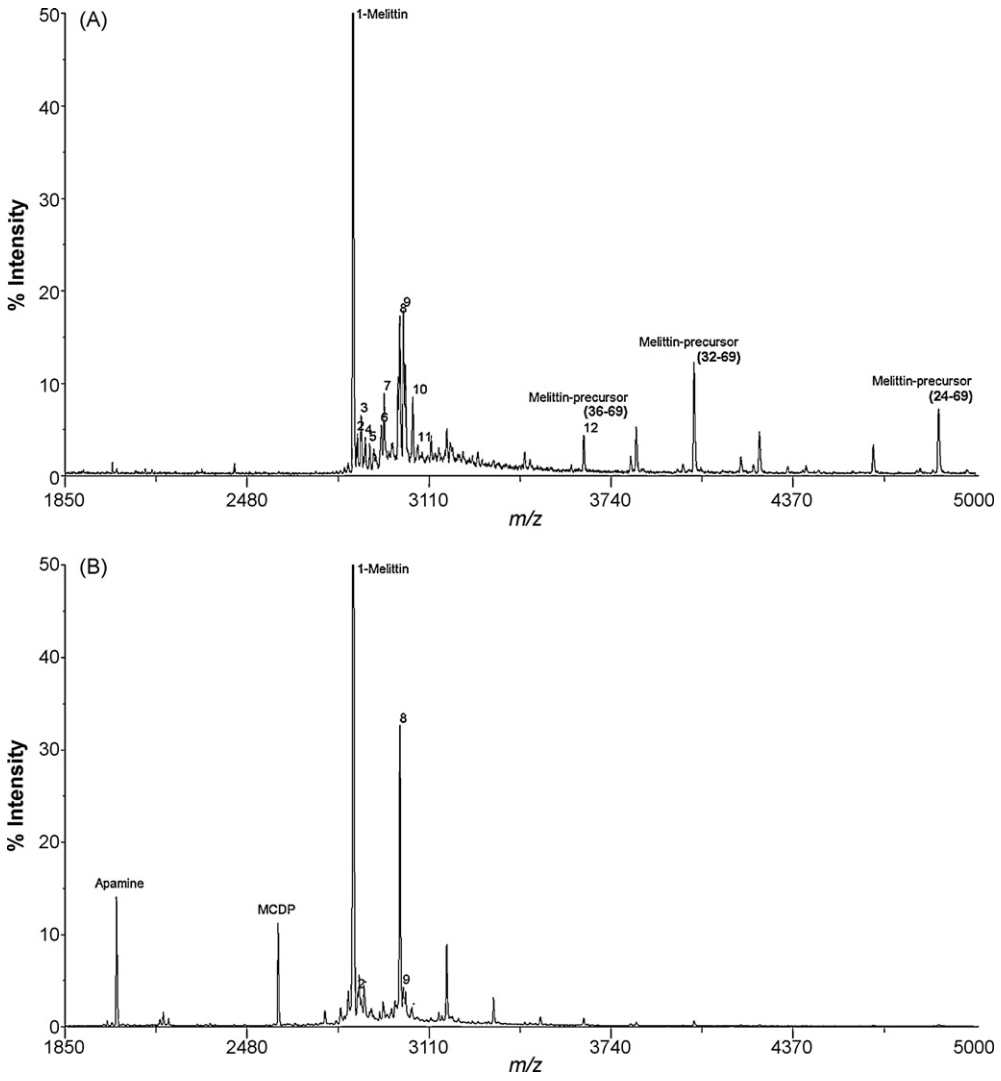

Fig. 1. Positive ion linear MALDI-TOF spectra of honeybee venom samples prepared using the dried droplet method, by mixing the sample with (A) sinapinic acid and (B) α-cyano-4-hydroxycinnamic acid, respectively. The peak numbering refers to the peptide numbers given in Table 1.

which simplifies the interpretation of the spectra [30]. NanoESI-QqTOF-MS and MS/MS were applied for qualitative analysis of representative honeybee venom samples.

The application of MALDI-TOF-MS to a wide variety of analytes is possible through the use of different matrices [37,38]. In this study, the application of the two matrix systems sinapinic acid and CHCA yields complementary results. Applying this methodology we have focused on a mass range lower than 15,000 Da however, generally higher masses could be also measured. The use of sinapinic acid resulted in the detection of many more peptides as compared to the analyses with CHCA (Fig. 1a and b). However, while applying CHCA two honeybee venom toxins (apamine, M_r = 2029.92 Da and mast cell degranulating peptide (MCDP), M_r = 2589.42 Da) were detected, which were not found when using sinapinic acid. This is in agreement with literature reporting that apamine and MCDP are present in different honeybee venom samples [9]. Therefore, to evaluate less characterized peptides sinapinic acid was used for further experiments.

About 20 peptide candidates were detected using MALDI-TOF-MS. The experiments with sinapinic acid allowed obtaining semi-quantitative data for the 12 most abundant peptides (in the range between 2845 Da and 3644 Da) in the 41 different honeybee venom samples. However, several peptides in the range from 3800 Da to 7000 Da were detected with very low abundance in most of the analyzed samples and, therefore, semi-quantitative analysis of these peptides was impossible.

Semi-quantitative analysis using MALDI is challenging due to the poor point-to-point repeatability, sample-to-sample reproducibility, and shot-to-shot signal degradation [39]. To minimize problems arising from these difficulties and to improve precision and accuracy of the results obtained, an internal standard was used (GLP). To obtain semi-quantitative information, the intensities of selected peaks were related to the intensity of the internal standard. Each sample was analyzed in triplicate (n = 3) and mean values were calculated. By applying this methodology, the average precision (expressed as relative standard deviation) of the results obtained was 15.5%. According to the obtained standard deviations a semi-quantitative evaluation was feasible.

The semi-quantitative results are shown in Table 1. The content of the analyzed peptides is presented in arbitrary units. The peptides of the monoisotopic masses 2859.84 Da and 2872.83 Da were observed mainly in the older bee venom samples collected in 1991 and 1995 while in the other samples from 2002 to 2007 the same peptides were detected with significant lower abundances. Similarly, the peptides of masses 2886.80 Da and 2952.84 Da were only observed in the older samples. Hence, it might be possible that these peptides are degradation products of others.

In order to verify significant differences between independent variables (the content of analyzed peptides), the data from analyzed samples were subjected to statistical analysis using the Analysis of Variance (ANOVA). Three factors were considered for the calculations: the strain of bees, month and year of venom collection. Multi-way statistical analysis did not show any significant differences. One-way ANOVA showed that there was significant influence of one factor: the year on the determination of analyzed peptides. The strongest influence was found for the peptides of following masses: 2859.84 Da, 2872.83 Da, 2886.80 Da, 2941.14 Da, 2952.84 Da, 3018.81 Da, and 3068.87 Da.

NanoESI-QqTOF-MS was applied as a complementary method especially for identification purposes. Due to the presence of disulphide bridges in the honeybee venom peptides [40,41], DTT was used as a reducing agent prior to nanoESI-MS analysis. Thus, 17 peptide sequences were identified including melittin (10 different breakdown products and precursors of melittin), apamine, mast cell degranulating peptide, and secapin. Additionally, one small protein (HTGAVLAGV) that has not been described in bee venom before [open reading frame (ORF) for hypothetical protein in *A. mellifera* – honeybee] was observed (shown in Table 2). The peptide identified could be assigned to a protein (LOC408363, accession number gi|118150480; obtained from genomic data of *A. mellifera*) that has been predicted, but until now there was no experimental evidence that this protein is expressed in vivo. Thus, the detection of a peptide of this precursor is a clear indication for the existence of this protein.

Four of the identified peptides which were degradation products and precursors of melittin were also detected by MALDI-TOF-MS with sinapinic acid as matrix (2844.53 Da, 3643.19 Da, 4025.33 Da, 4871.34 Da) and two peptides with CHCA (apamine and MCDP).

Moreover, some post-translational and chemical modifications could be confirmed: amidation of the C-termini of melittin, apamine, and mast cell degranulating peptide and transformation of glutamate (Glu) into pyro-glutamate (pyro-Glu) at the *N*-terminus of melittin precursors. It is likely that many more peptides could be identified using nanoESI-MS if further post-translational and chemical modifications were known.

Although not all peptides detected by MALDI-TOF were identified, it is obvious that monitoring of the content of them in the samples of bee venom is certainly necessary for medicinal purposes. It was demonstrated that some peptides occurring in extreme low quantities in honeybee venom samples had probably no biological function against the victim (they had only a local function in the venom duct or reservoir or they were other cell components released by leakage of the gland tissue) [42]. Therefore, mass spectrometric techniques applied in this study, which allowed obtaining broad data about different honeybee venom samples during one simple direct analysis, are powerful tools for standardization purposes.

4. Conclusions

By applying MALDI-TOF analysis, without further sophisticated and time consuming sample pretreatment it was possible to obtain semi-quantitative data for different components in selected honeybee venom samples. Statistical analysis (ANOVA) has shown that there are qualitative and quantitative differences in the composition between honeybee venom samples collected over different years. Moreover, the significant achievement of this study is the fact that the new peptide (HTGAVLAGV + Amidated (C-term), M_r = 822.53 Da) has been discovered in bee venom for the first time (open reading frame (ORF) for hypothetical protein in *A. mellifera* – honeybee).

On the basis of these considerations it can be stated that direct MALDI-TOF-MS is a very sensitive and rapid method for carrying out screening tests of honeybee venom samples. It has been demonstrated that spectra obtained from such a simple and fast analysis can be used as a "protein fingerprint" of honeybee venom in order to confirm the identity of the product. Applied methodology is also useful to screening degradation products of honeybee venom constituents. The detected peptides can be unambiguously identified using nanoESI-QqTOF or MALDI-TOF/TOF mass spectrometry.

References

[1] D.J. Son, J.W. Lee, Y.H. Lee, H.S. Song, C.K. Lee, J.T. Hong, Therapeutic application of anti-arthritis, pain-releasing, and anti-cancer effects of bee venom and its constituent compounds, Pharmacol. Ther. 115 (2007) 246–270.

[2] H.J. Park, S.H. Lee, D.J. Son, K.W. Oh, K.H. Kim, H.S. Song, G.J. Kim, G.T. Oh, D.Y. Yoon, J.T. Hong, Antiarthritic effect of bee venom: inhibition of inflammation mediator generation by suppression of NF-kappaB through interaction with the p50 subunit, Arthritis Rheum. 50 (2004) 3504–3515.

[3] Y.B. Kwon, T.W. Ham, H.W. Kim, D.H. Roh, S.Y. Yoon, H.J. Han, I.S. Yang, K.W. Kim, A.J. Beitz, J.H. Lee, Water soluble fraction (<10 kDa) from bee venom reduces visceral pain behavior through spinal alpha 2-adrenergic activity in mice, Pharmacol. Biochem. Behav. 80 (2005) 181–187.

[4] H.W. Kim, Y.B. Kwon, T.W. Ham, D.H. Roh, S.Y. Yoon, H.J. Lee, H.J. Han, I.S. Yang, A.J. Beitz, J.H. Lee, Acupoint stimulation using bee venom attenuates formalin-induced pain behavior and spinal cord fos expression in rats, J. Vet. Med. Sci. 65 (2003) 349–355.

[5] P.J. Russell, D. Hewish, T. Carter, K. Sterling-Levis, K. Ow, M. Hattarki, L. Doughty, R. Guthrie, D. Shapira, P.L. Molloy, J.A. Werkmeister, A.A. Kortt, Cytotoxic properties of immunoconjugates containing melittin-like peptide 101 against prostate cancer: in vitro and in vivo studies, Cancer Immunol. Immunother. 53 (2004) 411–421.

[6] T. Putz, R. Ramoner, H. Gander, A. Rahm, G. Bartsch, M. Thurnher, Antitumor action and immune activation through cooperation of bee venom secretory phospholipase A2 and phosphatidylinositol-(3,4)-bisphosphate, Cancer Immunol. Immunother. 55 (2006) 1374–1383.

[7] Z.J. Kokot, J. Matysiak, J. Kłos, B. Kędzia, E. Hołderna-Kędzia, Application of principal component analysis for evaluation of chemical and antimicrobial properties of honeybee (*Apis mellifera*) venom, J. Apicult. Res. 48 (2009) 168–175.

[8] V. Bankova, Chemical diversity of propolis and the problem of standardization, J. Ethnopharmacol. 100 (2005) 114–117.

[9] Z.J. Kokot, J. Matysiak, Simultaneous determination of major constituents of honeybee venom by LC-DAD, Chromatographia 69 (2009) 1401–1405.

[10] V. Pacakova, K. Stulik, P. Hau, J. Jelinek, I. Vins, D. Sykora, Comparison of high performance liquid chromatography and capillary electrophoresis for determination of some bee venom components, J. Chromatogr. A 700 (1995) 187–193.

[11] V. Pacakova, K. Stulik, Validation of a method for determination of phospholipase A2 and melittin in bee venom preparations by capillary electrophoresis, J. AOAC Int. 83 (2000) 549–554.

[12] L. Xing, X. Liping, Z. Rongqing, C. Dawei, S. Lan, A sensitive enzyme-linked immunosorbent assay for evaluating the concentration of bee venom in rat plasma, J. Pharm. Pharmacol. 55 (2003) 1359–1363.

[13] M.C. Pereira Santos, E. Pedro, A. Spinola Santos, M. Branco Ferreira, M.L. Palma Carlos, A.G. Palma Carlos, Immunoblot studies in allergic patients to hymenoptera venom before and during immunotherapy, Eur. Ann. Allergy Clin. Immunol. 37 (2005) 273–278.

[14] M.D. Wiese, R.W. Milne, N.W. Davies, T.K. Chataway, S.G. Brown, R.J. Heddle, Myrmecia pilosula (Jack Jumper) ant venom: validation of a procedure to standardize an allergy vaccine, J. Pharm. Biomed. Anal. 46 (2008) 58–65.

[15] A. Follenius-Wund, Y. Mely, D. Gerard, Spectroscopic evidence of two melittin molecules bound to Ca^{2+}-calmodulin, Biochem. Int. 15 (1987) 823–833.

[16] C.R. Flach, F.G. Prendergast, R. Mendelsohn, Infrared reflection-absorption of melittin interaction with phospholipid monolayers at the air/water interface, Biophys. J. 70 (1996) 539–546.

[17] P. Kaszycki, Z. Wasylewski, Fluorescence-quenching-resolved spectra of melittin in lipid bilayers, Biochim. Biophys. Acta 1040 (1990) 337–345.

[18] W.E. Steinmetz, T.I. Bianco, M. Zollinger, D. Pesiri, Characterization of the multiple forms of mast cell degranulating peptide by NMR spectroscopy, Pept. Res. 7 (1994) 77–82.

[19] X. Xu, J.W. Nelson, Solution structure of tertiapin determined using nuclear magnetic resonance and distance geometry, Proteins 17 (1993) 124–137.

[20] T.D. Bradrick, E. Freire, S. Georghiou, A high-sensitivity differential scanning calorimetric study of the interaction of melittin with dipalmitoylphosphatidylcholine fused unilamellar vesicles, Biochim. Biophys. Acta 982 (1989) 94–102.

[21] Z.J. Kokot, J. Matysiak, Inductively coupled plasma mass spectrometry determination of metals in honeybee venom, J. Pharm. Biomed. Anal. 48 (2008) 955–959.

[22] G. Szokan, J. Harvath, M. Almas, G. Saftics, A. Palocz, Liquid chromatographic analysis and separation of polypeptide components from honeybee venoms, J. Liq. Chromatogr. 17 (1994) 3333–3349.

[23] K. Rader, A. Wildfeuer, F. Wintersberger, P. Bossinger, H.W. Mucke, Characterization of bee venom and its main components by high performance liquid chromatography, J. Chromatogr. 408 (1987) 341–348.
[24] J. Gauldie, J.M. Hanson, F.D. Rumjanek, R.A. Shipolini, C.A. Vernon, The peptide components of bee venom, Eur. J. Biochem. 61 (1976) 369–376.
[25] B.E. Banks, C.E. Dempsey, F.L. Pearce, C.A. Vernon, T.E. Wholley, New methods of isolating been venom peptides, Anal. Biochem. 116 (1981) 48–52.
[26] S. Pattanaargson, J. Roboz, Determination of hyaluronidase activity in venoms using capillary electrophoresis, Toxicon 34 (1996) 1107–1117.
[27] H.F. Alomirah, I. Alli, Y. Konishi, Applications of mass spectrometry to food proteins and peptides, J. Chromatogr. A 893 (2000) 1–21.
[28] G. Baggerman, P. Verleyen, E. Clynen, J. Huybrechts, A. De Loof, L. Schoofs, Peptidomics, J. Chromatogr. B 803 (2004) 3–16.
[29] H. John, M. Walden, S. Schäfer, S. Genz, W.G. Forssmann, Analytical procedures for quantification of peptides in pharmaceutical research by liquid chromatography–mass spectrometry, Anal. Bioanal. Chem. 378 (2004) 883–897.
[30] M. Mann, R.C. Hendrickson, A. Pandey, Analysis of proteins and proteomes by mass spectrometry, Annu. Rev. Biochem. 70 (2001) 437–473.
[31] N. Peiren, D.C. de Graaf, F. Vanrobaeys, E.L. Danneels, B. Devreese, J. Van Beeumen, F.J. Jacobs, Proteomic analysis of the honeybee worker venom gland focusing on the mechanisms of protection against tissue damage, Toxicon 52 (2008) 72–83.
[32] N. Peiren, F. Vanrobaeys, D.C. de Graaf, B. Devreese, J. Van Beeumen, F.J. Jacobs, The protein composition of honeybee venom reconsidered by a proteomic approach, Biochim. Biophys. Acta 1752 (2005) 1–5.
[33] C.E. Schmelzer, R. Schöps, R. Ulbrich-Hofmann, R.H. Neubert, K. Raith, Mass spectrometric characterization of peptides derived by peptic cleavage of bovine beta-casein, J. Chromatogr. A 1055 (2004) 87–92.
[34] K. Tang, S.L. Allman, R.B. Jones, C.H. Chen, Quantitative analysis of biopolymers by matrix-assisted laser desorption, Anal. Chem. 65 (1993) 2164–2166.
[35] D.N. Perkins, D.J. Pappin, D.M. Creasy, J.S. Cottrell, Probability-based protein identification by searching sequence databases using mass spectrometry data, Electrophoresis 20 (1999) 3551–3567.
[36] B. Ma, K. Zhang, C. Hendrie, C. Liang, M. Li, A. Doherty-Kirby, G. Lajoie, PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry, Rapid Commun. Mass Spectrom. 17 (2003) 2337–2342.
[37] F. Gonnet, G. Lemaitre, G. Waksman, J. Tortajada, MALDI/MS peptide mass fingerprinting for proteome analysis: identification of hydrophobic proteins attached to eucaryote keratinocyte cytoplasmic membrane using different matrices in concert, Proteome Sci. 1 (2003) 1–7.
[38] M. Karas, R. Krüger, Ion formation in MALDI: the cluster ionization mechanism, Chem. Rev. 103 (2003) 427–440.
[39] Y.C. Ling, L. Lin, Y.T. Chen, Quantitative analysis of antibiotics by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 12 (1998) 317–327.
[40] E.M. Dotimas, K.R. Hamid, R.C. Hider, U. Ragnarsson, Isolation and structure analysis of bee venom mast cell degranulating peptide, Biochim. Biophys. Acta 911 (1987) 285–293.
[41] R.A. Shipolini, A.F. Bradbury, G.L. Callewaert, V.C.A., The structure of apamin, Chem. Commun. (1967) 679–680.
[42] D.C. de Graaf, M. Aerts, E. Danneels, B. Devreese, Bee, wasp and ant venomics pave the way for a component-resolved diagnosis of sting allergy, J. Proteomics 72 (2009) 145–154.