HPLC STUDY OF CHEMICAL COMPOSITION OF HONEYBEE (*Apis mellifer*a L.) VENOM*

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S u m m a r y

The aim of the study was to the development of the method for the separation, identification and determination quantitatively of the major constituents of honeybee (*Apis mellifera* L.) venom by high performance liquid chromatography (HLPC) and determination of the chemical composition of the domestic product by running routine tests.

In the study to develop an HPLC method to assay the major venom compounds the following elements were tested: chromatographic columns with C18 packing materials of different pore size (100, 180 and 300Å), separation temperature (25, 30 35°C), flow rate (1.0; 1.5; 2.0 ml/min.), conditions of gradient elution (0%B - 45%B, 60 min and 0%B - 60%B, 60 min, 5%B - 80%B, 40 min). Chromatographic separation was performed using the following mobile phases: A - 0.1% trifluoroacetic acid (TFA) in water, B - 0.1% TFA in acetonitrile : water (80:20). The assays of the separated venom compounds were made using UV detector at 220 nm wavelength.

The best separation of the bee venom protein fraction was obtained on 180 and 300Å pore size columns at a temperature of 25°*C*. The routine assays of bee venom chemical composition involved 29 samples collected from the apiary of the Apiculture Division, Institute of Pomology and Floriculture in Puławy over three consecutive beekeeping season (2002-2004). Bee venom samples were analyzed for the following major protein fraction compounds: melittin, phospholipase A_2 and apamine. Melittin content varied from 61.15 to 70.15 and averaged 64.40%. Phospholipase A_2 content came within a range of 11.24 to 15.05, and averaged 13.00%, and apamine content was between 2.09 to 4.18, averaging 3.10%. Statistically significant year-to-year differences were found for melittin.

Keywords: bee venom, *Apis mellifera* L., chemical composition, melittin, apamine, phspholipase A_2, HPLC.

INTRODUCTION

Investigations of the chemical composition of bee venom are relatively recent origin. In the 50's of the last century mainly due to the use of chromatographic methods of compound separation and identification (liquid chromatography, capillary electrophoresis) several venom fractions were isolated and within those fractions main components were identified. The enzymes phospholipase A_2 and hyaluronidase, and the polipeptyde melittin were identified in the protein fraction. Peptides were another fraction in which apamine and peptide MCD were found. In yet another fraction amines were found including histamine, dopamine and noradrenaline. In addition, some small amounts of sugars, phospholipides, free amino acids and pheromones were identified (Benton et al. 1963, Habermann and Reitz 1965, Tolksdorf et al. 1949, Zalewski 1959).

Further researches on the chemical composition of bee venom proceeded along several directions: detailed separation and

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identification of compounds in pre-isolated fractions, investigations of compounds structure and mode of action e.g. those of phospholipase A_2 (King et al. 1976, Shipolini et al. 1971), hyaluronidase (Kemeny and Vernon 1983), melittin (Bazzo et al. 1988). Many studies were also concerned with the biological activity of venom and its components (Owen and Sloley 1988, Rybak-Chmielewska et al. 1994, Sikora et al. 1987, Yoshimoto 1985).

The latest researches concerning the chemical composition of various venoms was aimed at elaboration of fast, economical and efficient methods for sample preparation to be used in HPLC separation or in capillary electrophoresis and at obtaining a high-degree of purity of the separated components. In those studies the investigators tested different columns for chromatographic separation conditions (Ameratunga et al. 1995, Dotimas and Hider 1987, Fenton et al. 1995, Packova et al. 1995, Szokan et al. 1994). None-the-less, in the literature there are no reliable and unified methods for the purification, separation, identification and assay of the major bee venom compoounds. Up to now in Poland, the product has not been tested for its chemical composition.

The aim of the study was the development of an HPLC method for separation, identification and quantitative assays of individual bee venom components and the determination the chemical composition of domestic bee venom by routine assays of venom samples.

MATERIAL AND METHODS

MATERIAL

Analytical standards of venom compounds - apamine, phospholipase A_2 and melittin isolated from bee venom, freeze-dried honeybee (*Apis mellifera* L.) venom, hyaluronidase from bovine testes were supplied by Sigma Chemicals Co. (St Luis, MO, USA). Acetonitrile also came from Sigma, trifluoroacetic acid was obtained from Aldrich Chemicals Co. (Milwaukee WI USA) and membrane filters 0.45 μm were manufactured by Pall Gelman Laboratory (USA).

Standard solutions were prepared by dilution: 5 mg of freeze-dried venom, 4.00 mg of melittin, 0.25 mg apamine, 1.00 mg phospholipase A_2 and 1.00 mg hyaluronidase in 1 ml of deionised water.

Bee venom for analysis were sampled over three consecutive beekeeping seasons (2002-2004) in the apiary of the Apiculture Division, Institute of Pomology and Floriculture in Puławy, a total of 29 samples. The samples were collected by stimulating the bees with electric current pulses (Rybak et al. 1995) and stored until analsis at 5°C in the darkness. The bee venom solution for HPLC studies was prepared by dilution 5 g of that product in 1ml of deionised water. Prior to loading onto the column, the solution was filtered through a 4.5 μm membrane filter.

METHODS

HPLC studies were carried out using the KNAUER (Dr. Ing. Herbert Knauer GmbH, Berlin, Germany) instrument made up of the following elements: HPLC PUMP K-1001, detector (UV K-2501), column oven, manual injector with 20 μm loop and EUROCHROM 2000 V 2.05 computer software.

In the investigations for the development of the method for separation and identification of bee venom components the following parameters were checked using literature data (Szokan et al. 1994, Packova et al. 1995):

— chromatographic columns with C18 packing, 25 cm x 4 mm (4.6 mm), 5 μm, of different pore size: 100Å (Knauer Eurosfer-100 C18, Dr. Ing. Herbert Knauer GmbH, Berlin, Germany), 180Å (DISCOVERY®C18, Supelco Park, Ballefonte PA, USA)

and 300Å (SUPELCOSIL™ LC-318, Supelco Park, Ballefonte PA, USA);
— conditions for linear gradient elution: 0%B - 45%B for 60 min, 0%B - 60%B, for 60 min, 5%B - 80%B for 40 min;
— flow rate of mobile phase 1.5 ml/min, 2.0 ml/min and 2.5 ml/min;
— separation temperature: 25°C, 30°C and 35°C;

Chromatographic separation was performed using the following eluents: eluent A - 0.1% TFA in water, B - 0.1% TFA in the solution: acetonitrile:water (80:20). Venom compounds were identified using UV detector at 220 nm wavelength.

The external standard method (one--point calibration) was used for quantitation of apamine, phospholipase A_2, and melittin. The whole procedure was validated by determining detection limit, precision, accuracy, repeatability and linearity of the method. Validation was performed for the assay on the column DISCOVERY®C18.

The assay data of 29 venom samples were subjected to statistical analysis including one-way ANOVA. The significance of year-to-year differences for individual venom components were examined using Duncan's test at $\alpha = 0.05$.

RESULTS AND DISCUSSION

The HPLC study for development of the method for separation, identification and assay individual components of honeybee (*Apis mellifera* L.) venom showed that chromatographic separation obtained using any of the columns packed with C18 material (SUPELCOSIL™ LC-318, DISCOVERY®C18, Knauer Eurosfer-100 C18) was similar. The best separation conditions were those of 5-80%B linear gradient elution at 2.0 ml/min flow rate of mobile phase, and a temperature of 25°C. The analytical column with pore size, 300Å (SUPELCOSIL™ LC-318), gave the best separation of analytes, good separation was also obtained on a 180Å (DISCOVERY®C18) column (Fig. 1). The column with the smallest pore size, 100Å (Knauer Eurosfer-100 C18, gave a much inferior separation. Using the DISCOVERY®C18, 180Å column average retention time for apamine was 8.617 min, for phospholipase A_2 20.467 min and for melittin 28.075 min (Table 1). The average retention time for the mentioned analytes using the SUPELCOSIL™ LC-318, 300Å column was shorter by a factor of 2.3 min.

The accuracy and repeatability of the method as applied to the bee venom components, apamine, phospholipase A_2, and melittin, were adequate. The variation coefficients were 1.5; 1.8 and 1.2%, respectively for retention time and 5.9; 5.4 and 1.0% (Table 1 and 2) for quantitative assays. The detection limit was 35 μg/ml for apamine, 60 μg/ml for phospholipase A_2 and 40 μg/ml for melittin. The results indicate a linear relationship between detector reactions (peak area) and concentration of the bee venom components. The linear correlation coefficient was over 0.994 for all studied compounds. The HPLC separation profile of the protein fraction of honeybee (*Apis mellifera* L.) venom may be used to assess the identity of that bee product (Fig. 1). The results from this study of the HPLC separation of the protein bee venom fraction are confirmed by an earlier study by Hungarian investigators (Szokan et al. 1994).

By using the mentioned conditions and hyaluronidase from bovine testes as a standard the enzyme was failed to be detected in bee venom. At the site of the hyluronidase peak from the standard solution no peak was detected in the venom samples. In the study by Szokan et al. (1994) hyaluronidase was detected before (Econospher 300Å C4, DeltaPak 300Å C18) or after (Hypersil 300Å C18)

Journal of Apicultural Science

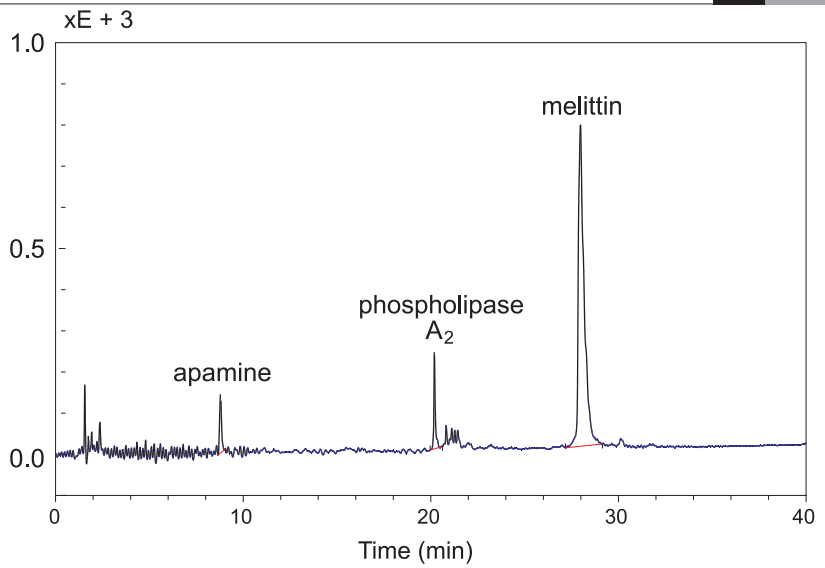

Fig. 1 Bee venom protein fraction chromatogram - column DISCOVERY®C18

melittin in the bee venom chromatogram and its average content was reported as 2.8%.

In our study the content of melittin in bee venom samples collected during beekeeping season 2002-2004 ranged from 61.15 to 70.15% with the mean value of 64.61% (Table 3). The average content of phospholipase A_2 was at 12.98% (11.24--15.05%). Apamine content averaged 3.13% and came within a range of 2.09--4.18%. No significant year-to-year (2002-2004) differences in phospholipase A_2 and apamine contents were found. Significant differences were recorded for melittin.

The results on the principal bee venom constituent, melittin, obtained in this study, differ from those measured by Szokan et al. (1994) who found 50-60% of that compound in bee venom. In this study melittin content was ca. 65%. Apamine and phospholipase A_2 contents found in this study were similar to those reported by mentioned investigators. The reason behind the differences in melittin contents between this and other study (Szokan et al. 1994) was probably the different origin of

Table 1

Retention time for individual bee venom components separated on two chromatographic columns (min)

Component	Column type			
	DISCOVERY®C18 180Å		SUPELCOSIL™ LC-318 300Å	
	Average ± SD	Variation coefficient (%)	Average ± SD	Variation coefficient (%)
Apamine	8.617 ± 0.132	1.53	6.425 ± 0.190	2.97
Phospholipase A_2	20.467 ± 0.366	1.79	18.100 ± 0.170	0.94
Melittin	28.075 ± 0.341	1.21	25.727 ± 0.200	0.77

Table 2

Repeatability and reproducibility of apamine, phospholipase A_2 and melittin assays in bee venom (n=8)

Apamine		Phospholipase A_2		Melittin	
Average ± SD	Variation coefficient (%)	Average ± SD	Variation coefficient (%)	Average ± SD	Variation coefficient (%)
1.02 ± 0.08	7.46	9.95 ± 0.34	3.44	63.30 ± 0.32	0.51
3.72 ± 0.16	4.30	12.91 ± 0.94	7.29	75.26 ± 1.09	1.44

Table 3

Protein fraction composition of honeybee (*Apis mellifera* L.) venom sampled in the years 2002-2004 (%)

Sampling year	Apamine		Phospholipase A_2		Melittin	
	from-to	average	from-to	average	from-to	average
2002	2.34 - 4.09	3.37 a	11.24 - 15.05	12.71 a	62.15 - 70.15	66.23 a
2003	2.09 - 4.18	3.14 a	11.91 - 13.41	12.66 a	61.15 - 65.32	63.24 ab
2004	2.90 - 3.45	2.88 a	11.67 - 14.02	12.83 a	62.30 - 67.80	64.37 b

samples and the use of different sampling methods. Alongside with a venom collection method similar to ours that consisted in stimulating the bee to sting with electric current the Hungarian investigators assayed bee venom that was extracted with water and with other solvents from powdered venom glands. In this study raw untreated venom stored until analyzed at 5°C in the dark was assayed.

The qualitative and quantitative composition of the protein fraction of honeybee venom supplements the description of the product that is obtained by a method developed earlier at the Apiculture Division Institute of Pomology and Floriculture in Puławy and used to gather venom from bees on a larger scale. The HPLC separation profile of the bee venom protein fraction can be used to develop a bee venom standard as an identifying method (to establish identity). The results from quantitative assays of individual compounds, melittin, phospholipase A_2 and apamine, are meant, first of all, to be used by the pharmaceu- tical industry.

SUMMARY AND CONCLUSIONS

1. The chromatographic columns with C18 packing material of pore size of at least 180Å gave the best separation of bee venom compounds (apamine, phospholipase A_2, melittin) during HPLC analysis. Under linear gradient elution 5-80%B (eluent A - 0.1% TFA in water, eluent B - 0.1% TFA in acetonitrile : water (80:20)) for 40 min, at flow rate of 2.0 ml/min and at temperature of 25°C these columns showed the highest stability and repeatability of results.
2. The average percent composition of major honeybee (*Apis mellifera* L.) venom compounds is as follows: 65% - mellitin, 13% - phospholipase A_2 and 3% - apamine.

3. Bee venom sampled in different beekeeping seasons differs significantly for the content of the main component - melittin.
4. HPLC separation profile analysis of the protein fraction of honeybee (*Apis mellifera* L.) venom can be used to qualify the identity of the product.
5. The quantitative composition of the protein fraction (melittin, phospholipase A_2 and apamine) may provide a basis for the development of the domestic standard for honeybee venom.

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BADANIA SKŁADU CHEMICZNEGO JADU PSZCZOŁY MIODNEJ *Apis mellifera* L. ZA POMOCĄ HPLC*

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S t r e s z c z e n i e

Celem badań było opracowanie metody rozdziału, identyfikacji i ilościowego oznaczania głównych składników jadu pszczoły miodnej *Apis mellifera* L. za pomocą chromatografii cieczowej (HPLC) oraz określenie składu chemicznego krajowego produktu poprzez przeprowadzenie seryjnych badań.

W badaniach nad opracowaniem metody HPLC oznaczania głównych składników jadu sprawdzono: kolumny chromatograficzne z wypełnieniem C18 o różnym rozmiarze porów (100, 180 i 300Å), temperaturę rozdziału (25, 30 35°*C*), przepływ fazy ruchomej (1,0; 1,5; 2,0 ml/min.), warunki elucji gradientowej (0%B - 45%B, 60 min oraz 0%B - 60%B, 60 min., 5%B - 80%B, 40 min). Rozdział chromatograficzny wykonywano stosując następujące eluenty: eluent A - 0,1% kwas trójfluorooctowy w wodzie, eluent B - 0,1% kwas trójfluorooctowy w roztworze: acetonitryl : woda (80:20). Identyfikację rozdzielanych składników jadu prowadzono za pomocą detektora UV przy długości fali 220 nm.

Najlepszy rozdział frakcji białkowej jadu pszczelego uzyskano na kolumnach o rozmiarze porów 180 i 300Å w temperaturze 25°C. Do seryjnych badań składu chemicznego jadu wykorzystano 29 próbek pozyskanych w pasiece Oddziału Pszczelnictwa ISK w Puławach w trzech kolejnych sezonach pszczelarskich (2002-2004). W próbkach jadu określono zawartości głównych składników frakcji białkowej produktu: melittyny, fosfolipazy A_2 i apaminy. Zawartość melittyny wahała się w granicach od 61,15 do 70,15 i średnio wynosiła 64,40%. Zawartość fosfolipazy A_2 mieściła się granicach od 11,24 do 15,05, średnio - 13,00%, a zawartość apaminy w przedziale od 2,09 do 4,18, średnio 3,10%. Porównując wyniki z poszczególnych lat zbioru uzyskano różnice statystycznie istotne dla zawartości melittyny.

Słowa kluczowe: jad pszczeli, *Apis mellifera* L., skład chemiczny, melittyna, apamina, fosfolipaza A_2, HPLC.

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