REVIEW ARTICLE

Analytical methods for honeybee venom characterization

Iouraouine El Mehdi[1,2,3], Soraia I. Falcão[3], Saïd Boujraf[1,2], Harandou Mustapha[1], Maria G. Campos[4,5], Miguel Vilas-Boas[3]

[1]Prof. Said, Clinical Neurosciences Laboratory, Faculty of Medicine and Pharmacy, [2]Department of Biophysics and Clinical MRI Methods, Faculty of Medicine and Pharmacy, University Sidi Mohamed ben Abdellah, Fez, Morocco, [3]Centro de Investigação de Montanha, Instituto Politécnico de Bragança, Campus de Santa Apolónia, Bragança, [4]Observatory of Drug-Herb Interactions, Faculty of Pharmacy, University of Coimbra, Health Sciences Campus, Azinhaga de Santa Comba, Coimbra, [5]Coimbra Chemistry Centre (CQC, FCT Unit 313) (FCTUC), Univ Coimbra, Rua Larga, Coimbra, Portugal

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ABSTRACT

The discovery of new drugs has benefited significantly from the development of research in venomics, increasing our understanding of the envenomation processes. It has been previously reported that honeybee venom (HBV) exhibits several pharmacological activities such as anti-inflammatory, antibacterial, antimutagenic, radioprotective, and anticancer activity and may inclusively act as a complementary treatment for SARS-CoV-2. It composition consists mainly on melittin, phospholipase A2, and apamin but other constituents such as hyaluronidase, mast cell degranulating peptide and secapin are also relevant for its bioactivity. However, and because HBV is not officially recognized as a drug, until now, the international community did not establish quality standards for it. To uncover its exact composition, and boost the discovery of HBV-derived drugs, a significant number of techniques were developed. In this review, a relevant overview of the so far published analytical methods for HBV characterization is organized with the aim to accelerate its future standardization. The literature search was performed within PubMed, Google Scholar, and Science Direct by selecting specific documents and exploring HBV evaluation.

Key words: Analytical methods, apamin, enzymes, mast cell degranulating peptide, peptides, venomics

INTRODUCTION

Honeybee venom (HBV) or apitoxin is a biotoxin produced in the venom gland of honeybees (*Apidae* family), under its abdominal cavity.[1] It has been suggested that HBV represents much more than a classical stereotype of defense against predators,[2] acting also as a medium of social antisepsis contributing to the colony collective immunity. Even without a complete scientific proof of its efficacy/safety, the use in traditional medicine is recurrent for the prevention and treatment of many diseases such as arthritis, rheumatism, pain cancer, skin problems, among others. Moreover, it acts as anti-inflammatory, leishmanicidal agent, antimicrobial, antiviral, antiapoptotic, wound healer, antifibrinolytic and antielastolytic. More recently HBV has been appointed in complementary treatments against SARS-CoV-2.[3-5]

In this review, a brief information about the HBV compounds and its extraction will be provided before the presentation of the various analytical methods described in the literature. The information discussed within this manuscript was searched using PubMed, Google Scholar, and Science Direct filtering with common terms such as "*apitoxin*," "*apis*," "*bee venom*," "*bee products*," and "honey *bee*." The documents were analyzed to allow a deeper description on the most

Address for correspondence:
Prof. Dr. Saïd Boujraf,
Department of Biophysics and Clinical MRI Methods, Faculty of Medicine and Pharmacy, University Sidi Mohamed ben Abdellah, BP. 1893; Km 2.200, Sidi Hrazem Road, Fez 30000, Morocco.
E-mail: sboujraf@gmail.com

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relevant compounds present on HBV, considering the number of studies on the literature and the use the different techniques applied in the evaluation.

HBV COMPONENTS

HBV compounds have a wide range of pharmacological targets and have been extensively studied as sources for new drugs. An overview has been published on the therapeutic potential and some preclinical trials using HBV.[6,7] This toxin, with more than 80% of water, is a liquid mixture of active substances covering proteins, peptides, enzymes, and other small molecules[4,8,9] [Figure 1]. To guarantee the reproducibility of the results during bioactivity assays or clinical trials with HBV, its crucial to perform the identification of these compounds and standardize the HBV extract.

Melittin, the main component of HBV, is the most studied active membrane protein, consisting of 26 amino acid residues. Its amphiphilic property makes it water soluble and naturally associable with any type of membranes, including artificial.[10]

The major neurotoxin in HBV is apamin. This compound is an octadecapeptide that contains four cysteine residues with two disulfide bonds,[10] resulting in stabilization of its tertiary structure for the expression of biological activity.

The mast cell degranulating peptide (MCDP), a 22 residue polypeptide stable over the pH 2-8, presents two disulfide bridges and a terminal amide group. MCDP is not a true anti-inflammatory agent, but employs its activity via the mediation of the anti-inflammatory effect.[11]

Another important compound of HBV is secapin, a polycationic peptide with 25 amino acid residues. It is stabilized by an intramolecular disulfide bridge formed between cysteine residues. Secapin has been shown in two isoforms, secapin-1 and secapin-2 with a similar secondary structure.[12-14]

Other peptides found in lower quantities in HVB are adolapin and tertiapin. The first is a basic polypeptide with 103 amino acids residues, exhibiting an antinociceptive and anti-inflammatory activity through inhibition of cyclo-oxygenase function.[15] While tertiapin is a presynaptic neurotoxin composed by 21 amino acid residues. The single methionine residue in tertiapin can be readily oxidized, and consequently, its affinity for inhibiting certain types of inward-rectifier K + channels will be 4–5-fold lower.[16]

The major allergen compound of HBV is phospholipase A2 (PLA2), a glycoprotein. A revised study based on the analysis of the cDNA for PLA2 from HBV gland showed that the sequence of the mature PLA2 comprises 134 amino acids with a single glycosylation site which is cross-linked by four disulfide bridges responsible for their stability and their folding mechanism. PLA2 belongs to the secreted group III sPLA2 enzymes and shows its inflammatory activity by inducing the biosynthesis of prostaglandin, arachidonic, and lysophosphatidic acids.[17]

The last compound highlighted in this review is not involved in the toxic effect but can contribute for the characterization of the product. Hyaluronoglucosaminidase (Hya) plays a role as a spreading factor since the depolymerization and the hydrolysis of the substrate enable the diffusion of the venom constituents.[18] Hya is a glycoprotein with 349 amino acids and four cysteine bridged by two disulfides, with significant similarity with human hyaluronidase, which is tangled in fertilization and the hyaluronan turnover.[19]

The volatile composition of HBV is also a key point in its characterization, since it is directly linked with the social behavior of the insect. Qualitative and quantitative differences were reported depending on the bee species,[20] but also depending on the cast or age within the same species. For instance, isoamyl acetate, absent in queens and newly hatched bee workers, is one of the main components of adult bee sting volatiles.[21] Over than 20 volatile components have been unveiled, which the major of them are (z)-11-eicosen-1-ol, iso-pentyl acetate, n-octyl acetate, benzyl acetate, iso-pentanol, 2-nonanol, n-hexyl acetate, benzyl alcohol, n-butyl acetate, and n-decyl acetate.[22,23]

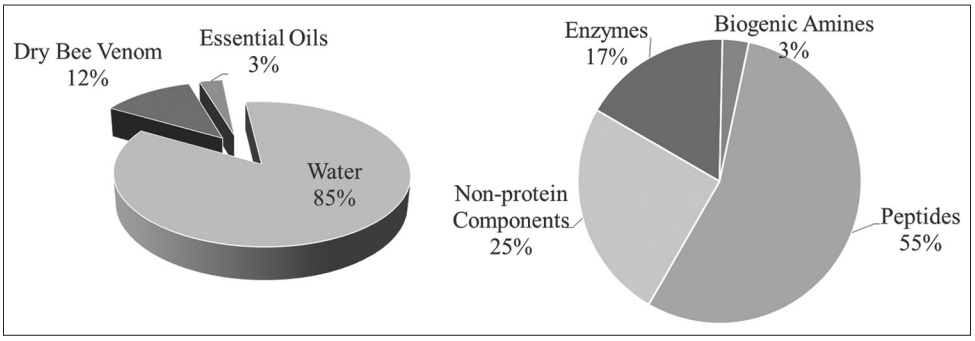

Figure 1: Chemical composition of honeybee venom (left) and for the dry extract (right)

Several metal elements were also reported in HBV including K, Na, Ca, a, Mg, Cu, Zn, B, Al, V, Mn, Co, Ni, Sr, and Mo, but also toxic metals such as As, Ba, Cd, Sb, Cr, and Pb.[24] Its content was suggested to be dependent on floral species and season, but its quantities should not surpass the levels established on pharmacopeias. The identification of metal contamination, mainly the most toxic, is highly recommended to guarantee the safety, conformity, and quality of HBV as a pharmaceutical raw material. Unfortunately, there is a noteworthy insufficiency of data in literature concerning metal contamination in this product.

HONEYBEE VENOM COLLECTION

Collecting fresh HBV requires careful work with the highest degree of cleanliness. From a single bee, the collection of venom can be performed using a glass capillary and gently squeezing the venom sac or, alternatively, the sac and gland can be dissected and opened by repeatedly puncturing with a sharp needle.[25-27] For collecting HBV on a commercial scale, several collector devices, based on the stimulating of the bees with electrical current pulses, have been built. The first, was built on a wood frame crossed by metallic wires, with an inner glass plate covered tightly with a nylon sheet, and subject to a specific voltage as a stimulating tension.[28] However, further developments allowed to optimize the position of venom-collecting frames in beehive, and the coupling of stimulating factors: electrical and sound stimulus.[29]

HONEYBEE VENOM CHARACTERIZATION: ANALYTICAL TECHNIQUES

Several methods have been applied in bee venom characterization, which include biological tests, chemical approaches based on typical protein reaction, and separation techniques. The choice may depend on several factors, particularly the non-proportionality of HBV components: the high level of melittin and PLA2 may mask the detection of minor compounds.[4] Unfortunately, there are still no methods that allow product standardization,[27,30,31] and the purification stage of HBV components remains a problem, due to the combinations of factors such as the highly alkaline profile, the similar molecular weight of the most relevant compounds, and the mixture complexity. Table 1 resumes the analytical methods used to access the main components.

PEPTIDES AND PROTEINS

Gel filtration and thin-layer chromatography were the most important techniques used for separation, purification, and characterization of HBV peptides, which implies selected conditions for eluents, pH, temperature conditions, and gel filtration resin types, particularly during purification. A strategy without the need of preliminary purification steps relies on the fractionation with columns of Heparin Sepharose CL-6P at pH 6.8 and an extremely high concentration of salt gradient, able to elute melittin once it bounds to the column.[42] The heparin's ability to precipitate melittin and MCDP made this technique the choice to purify apamin. To do this, it is possible to associate a propionic acid/urea polyacrylamide gel electrophoresis with gel filtration, which improves the quality of the electrophoretic separation, reproducibility, and robustness of the analysis, but limits the evaluation of the molecular weight of peptides.[35] Thin-layer isoelectric focusing polyacrylamide gel was also used to study the variability of melittin and other protein components in HBV.[26]

Table 1: Analytical techniques to access honeybee venom components

Type	Technique	HBV components	References
Biochemical			
Enzymatic	Phospholipase assay	PLA2	[26,32,33]
	Melittin assay	Melittin	[26]
	Allergosorbent assay	PLA2	[25]
Electrophoresis	SDS-PAGE	PLA2; melittin	[34]
	Propionate acid/urea PAGE	Melittin; apamin; MCDP	[35]
	Isoelectric focusing	PLA2; melittin	[26]
Chromatography	HPLC	PLA2; hyaluronidase; melittin; apamin; MCDP	[36,37]
	HPLC-MS	Melittin; apamin; MCDP; secapin; histamine; free amino acid; sugars, PLA2	[8,31,38]
	GC-MS	Volatiles	[9,20]
Spectroscopy	Liquid scintillation counting	PLA2	[32]
	Fluorescence	Melittin; apamin; MCDP	[38]
	Infrared	Biological active components	[37,39]
	ICP-MS	Metals	[24]
Electrochemistry	Stripping voltammetry	Melittin Metals	[40,41]

HBV: Honeybee venom, PAGE: Polyacrylamide gel electrophoresis, SDS-PAGE: Sodium dodecyl sulfate-PAGE, HPLC: High-performance liquid chromatography, MS: Mass spectrometry, GC-MS: Gas chromatography-MS, ICP-MS: Inductively coupled plasma-MS, PLA2: Phospholipase A2, MCDP: Mast cell degranulating peptide

Table 2: Chromatography conditions used for honeybee venom peptides/enzymes identification

Chromatography technique	Column type and elution conditions	Detection	Components	References
SEC-HPLC RP-HPLC	I-125/isocratic LiChrospher Si100, RP-18/isocratic	DAD	Apamin, hya, MCDP, melittin, PLA2	[33]
RP-HPLC	Hypersil WP-300 RP-C18/linear gradient	UVD	Apamin, hya, MCDP, melittin, PLA2, procamine, tertiapin, secapin	[36]
RP-HPLC	C18 (100/180/300Å)/linear gradient	UVD	Apamin, melittin, PLA2	[46]
RP-HPLC	Synchropack C8/linear gradient	DAD	Apamin, MCDP, melittin, PLA2	[45]
RP-HPLC	Sepax Bio-C18	IFD	Melittin	[38]
RP-HPLC	XSelect CSH130 C18/linear gradient	DAD	Apamin, melittin, PLA2	[47]

PLA2: Phospholipase A2, MCDP: Mast cell degranulating peptide, DAD: Diode array detection, IFD: Intrinsic fluorescence detection, UVD: Ultraviolet detector, RP-18: Reverse phase with C18 columns, HPLC: High-performance liquid chromatography, SEC-HPLC: Size exclusion chromatography-HPLC, RP-HPLC: Reversed phase-HPLC

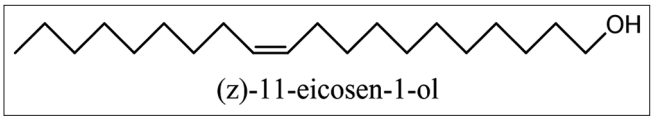

Figure 2: Major compound on HBV volatile fraction

Capillary electrophoresis is another option for the determination of melittin. The separation occurs in a double capillary system with UV-detection at two different wavelengths.[43] The output revealed a good performance for systematic analysis of HBV.

Despite this, HPLC is the main chromatographic technique used for peptides and proteins, either for quality control purposes, fingerprinting, or standardization of the product [Table 2]. Exclusion HPLC (SEC-HPLC) under isocratic conditions can identify melittin, apamin, and MCDP; nevertheless, due to melittin/apamin ratio (30:1) and the chemical behavior, it additionally requires reverse phase with C18 columns (RP-18) to determine apamin alone, implying quantification of melittin by subtraction.[33] Different modifications to improve these methods can be found in the literature, such as the use of RP-18 preparative columns, addition of cytochrome c as internal standard, or coupling with intrinsic fluorescence detection (IFD).[36,38,44,45]

Spectrometry has become the key tool for HBV analysis. Multiple platforms with distinct capabilities were explored, such as matrix-assisted laser desorption/ionization with time-of-flight detector (MALDI-TOF), liquid chromatography with electrospray ionization detector coupled to mass spectrometry (LC-ESI/MS), ion trap instruments, and mass spectrometry with Fourier transform systems mass spectrometry (FT-MS).[8,46,47] Melittin and apamin were simultaneously quantified through their precursor ions $[M + 5H]^{5+}$ (*m*/z 570.2) and $[M + 4H]^{4+}$ (*m*/z 507.7), respectively, using a triple quadrupole tandem mass spectrometer combined with an ESI interface with multiple reaction monitoring.[10] The combination of two complementary mass spectrometry platforms, MALDI-TOF-MS and nano-electrospray ionization quadrupole TOF MS (nanoESI-QqTOF-MS), enabled the development of a precise and accurate HBV characterization process, using an internal standard and two different matrices, sinapinic acid and α-cyano-4-hydroxycinnamic acid (CHCA): apamin and MCDP were detected with CHCA but not with sinapinic acid, which instead enabled the detection of four degradation products of melittin.[48]

The spectroscopic techniques have been used mainly for melittin, apamin, and tertiapin structural analysis, which involve peptides in solution at different temperature conditions. The three-dimensional structure of melittin was detected by X-ray crystallography, indicating a tetramer holding at least 2-fold axis of rotation. Proton nuclear magnetic resonance spectroscopy (^{1}HNMR) was also employed to understand melittin behavior at different temperature, pH, and ionic strength confirming an amphiphilic spatial structure, which is stabilized by forming mixed micelles in ionic solution or self-aggregation.[49] Two-dimensional nuclear magnetic resonance spectroscopy was also useful for evaluation of the apamin structure, enabling the observation of an α-helical core with fraying residues, and a β-turn,[50] however, some differences where reported when comparing with the outputs of distance geometry derived from NMR.[51]

Electrochemical methods were also used for HBV characterization and its introduction was achieved exploring proportionality between the decrease on oxidation current of ferrocene at 110 mV and the increase of melittin concentration. This indirect activity, measured by square wave striping voltammetry on gold electrodes, using nonaqueous solvents as dimethyl sulfoxide and acetonitrile, proved to be a fast and low cost approach, avoiding preconcentration procedures.[40]

Enzymes

The allergenic effect of HBV is due to the presence of enzymes, with fifthy five already described, including PLA2, phospholipase B, hyaluronidase, phosphatase acid, and α-glucosidase.[17,52] In this review, we focus on the major, PLA2 and hyaluronidase.

The first attempt on fractioning PLA2 and hyaluronidase from HBV was made with dialysis membranes using cellophane tubing and gel filtration on sephadex columns. This procedure allowed the majority of the HBV components to pass through the column, but retaining hyaluronidase and partially PLA2.[52] The use of a stepped-gradient open column with ethanol/water elution, proved also to be adequate for the purification and further quantification of PLA2.[30]

To analyze PLA2 and its isoforms, electrophoresis is a common methodology either using propionic acid/urea gels, thin-layer isoelectric focusing polyacrylamide gels, or sodium dodecyl sulfate polyacrylamide gels.[26,35] The combination of capillary electrophoresis with UV-DAD proved to be a suitable tool for monitoring PLA2 and assessing traceability and authenticity of HBV.[43]

The characterization of HBV enzymes using chromatography techniques is also recurrent, and most of the protocols applied for peptide analysis are frequently optimized for PLA2 [Table 2].[33,44,45] For hyaluronidase, exclusion chromatography on I-125 protein column, under isocratic conditions, enabled its identification at 200–230 nm,[33] however, a better performance was achieved when using DeltaPak, which reduces, specifically, the elution time of hyaluronidase due to its interaction with the hydrophobic stationary phase.[36]

ESI-MS^n, nanoESI-MS, and ESI-QTOF-MS are among the many different spectrometric methods explore for the evaluation of HBV enzymes.[47] NanoHPLC-nanoESI-MS was used to evaluate PLA2 production at particular climatic conditions, while MALDI mass spectrometry imaging was applied to map the diffusion and distribution of PLA2 over time.[53,54] Electrospray ionization linear quadrupole FT ion cyclotron resonance (ESI-LTQ-FT-ICR), MALDI-TOF/TOF, ESI–QTOF, can also be used for characterization of hyaluronidase.[8,44]

The secondary and tertiary structural information on PLA2 has been revealed using X-ray diffraction, molecular dynamics and NMR spectroscopic technics,[55] whereas its primary structure was identified by tandem MS and bioinformatics, exploring the protein sequence derived from cDNA and mRNA.[56] X-ray crystallography was also employed to evaluate the crystal structure of hyaluronidase oligomer reveling more than 50% sequence identity with hyaluronidase from other hymenoptera.[19]

Volatiles

Three techniques have been explored to collect the HBV volatile fraction, either recovering the volatile compounds from the whole sting apparatus,[57] using a solid phase microextraction fiber to collect the volatiles from the airspace around caged bees[20] or the collection of the volatile fraction while bees are stinging.[9] The last one requires a dry air pulsating device which creates an airflow carrying away the HBV volatile components while bees are sting and pierce a porous membrane. Subsequently, by condensation at −90°C, the volatiles are brought into solution and analyzed by GC-MS.[9] Results produced showed quantitative and qualitative differences of volatile substances either in stings or in fresh HBV. This variation is related to the denaturation process which immediately triggers after the extrusion of HBV next stinging or extraction. Over than 20 volatile components have been unveiled from HBV, which the most significant beeing the (z)-11-eicosen-1-ol, Figure 2, and the esters fraction, higly represented by the isoamyl acetate.[9,20-22,57]

Metals and minerals

Metal content monitoring in HBV is an extremely important issue; nonetheless, the literature information on this topic is limited.[24,41] Inductively Coupled Plasma MS (ICP-MS) was applied in the evaluation of twenty contaminant elements, revealing the year to year variation. In this work, the toxic metals levels in HBV were below the permissible for drug substances.[24] A more recent approach for the evaluation of heavy metals in HBV explored the anodic stripping voltammetry using either mercury drop electrode or PLA/carbon filament after a previous wet pressure digestion to enable sample dissolution. The results between the two electrodes were equivalent but with the mercury drop exhibiting higher sensitivity and lower detection limits.[41]

Sugars

Structural resemblances, hydrophilic characteristics, low proton affinity, and ionizability of the oligosaccharides make their characterization tricky. In HBV, they are frequently analyzed in the context of protein-linked sugars. *N*-linked oligosaccharides of PLA2 and hyaluronidase were the most investigated using GC after hydrolysis of the glycoprotein/glycopeptide in methanolic hydrogen chloride.[58] Glucose, fructose, and vanilloloside were identified and quantified in HBV using LC-Q-Orbitrap-MS untargeted metabolomics analysis,[59] while mannose, galactose, fructose, and *N*-acetylglucosamine were the main PLA2-linked monosaccharides detected after hydrolysis by an endoglycosidase-H.[60]

Free amino acids

Amino acids constitute the major precursors of HBV catecholamines, peptides, and proteins biosynthesis.[61] Gel filtration, paper, and thin-layer chromatography are used to estimate HBV-free amino acids (less than 1%), and among them, arginine, cystine, glutamic acid, and histidine are the most reported.[62] Others, such as aspartate, glutamate, serine, alanine, glycine, phenyl-ethanolamine, gamma-aminobutyric acid, and tyrosine, can also be detected using RP-HPLC and electrochemical detection.[62]

CONCLUSION AND FUTURE PERSPECTIVES

The interest on bee venom research is increasing due to its potential therapeutic use as anti-inflammatory, antimicrobial, or cytotoxicity. The complexity of its composition demands a constant development on methodologies and techniques that may fraction and detect the different constituents enabling to identify its biopotential at low risk. This review highlights the different techniques and analysis which have been used to characterize this matrix. Various enzymatic, electroanalytical, spectroscopic, and spectrometric techniques can be applied for the standardization of the product and inference on its content. Those techniques allow the separation, purification, quantification, chemical, and structural determination of the biocompounds, adding important quality value to the product, proving its feasibility in quality control and specific fingerprinting. Moreover, coupled methods and big data engineering undone a new task to identify the whole product proteome and therefore set up a standardization approach, to design good manufacturing practices of pharmaceutical products.

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Conflicts of interest

There are no conflicts of interest.

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