Journal of Chromatography B, 1002 (2015) 139–143

Contents lists available at ScienceDirect

Journal of Chromatography B

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

High-performance liquid chromatography combined with intrinsic fluorescence detection to analyse melittin in individual honeybee (*Apis mellifera*) venom sac

Jiangtao Dong [a,b,c], Bihua Ying [a,b,c], Shaokang Huang [a,b,c], Shuangqin Ma [a,b,c], Peng Long [a,b,c], Xijuan Tu [a,b,c], Wenchao Yang [a,b,c], Zhenhong Wu [a,b,c], Wenbin Chen [a,b,c,*], Xiaoqing Miao [a,b,c,*]

[a] *College of Bee Science, Fujian Agriculture and Forestry University, Fuzhou, China*
[b] *State and Local Joint Engineering Laboratory of Natural Biotoxin, Fujian Agriculture and Forestry University, Fuzhou, China*
[c] *Engineering Research Center of Bee Products Process and Application, Ministry of Education, China*

ARTICLE INFO

Article history:
Received 3 December 2014
Received in revised form 11 August 2015
Accepted 12 August 2015
Available online 20 August 2015

Keywords:
Melittin
HPLC
Intrinsic fluorescence
Bee venom
Individual honeybee

ABSTRACT

Melittin is the major toxin peptide in bee venom, which has diverse biological effects. In the present study, melittin was separated by reverse-phase high-performance liquid chromatography, and was then detected using intrinsic fluorescence signal of tryptophan residue. The accuracy, linearity, limit of quantitation (LOQ), intra-day and inter-day precision of the method were carefully validated in this study. Results indicate that the intrinsic fluorescence signal of melittin has linear range from 0.04 μg/mL to 20 μg/mL with LOQ of 0.04 μg/mL. The recovery range of spiked samples is between 81.93% and 105.25%. The precision results are expressed as relative standard deviation (RSD), which is in the range of 2.1–7.4% for intra-day precision and 6.2–10.8% for inter-day precision. Because of the large linear dynamic range and the high sensitivity, intrinsic fluorescence detection (IFD) can be used for analyzing melittin contents in individual venom sac of honeybee (*Apis mellifera*). The detected contents of melittin in individual bee venom sac are 0.18 ± 0.25 μg for one-day old honeybees ($n = 30$), and 114.98 ± 43.51 μg for 25-day old ($n = 30$) honeybees, respectively. Results indicate that there is large bee-to-bee difference in melittin contents. The developed method can be useful for discovering the melittin related honeybee biology information, which might be covered in the complex samples.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Bee venom (BV) is a complicated transparent liquid, which is secreted by bee venom glands. The main components of BV are peptides and enzymes, such as melittin, apamin, mast cell degranulating peptide, phospholipase A_2, and hyaluronidase [1]. Melittin is the major toxin of BV, which is weighted about 50% in dry venom. Melittin consists of 26 amino acids, within which 6 hydrophilic amino acids residue in the C-terminal region, and 20 hydrophobic amino acids residue in the N-terminal region [2]. The special property of amphilicity makes melittin a nonspecific cytolytic peptide with multiple effects, including anti-inflammatory effect [3], antibacterial effect [4], antiviral effect [5], and antitumor effect [6].

Recent studies showed that melittin also played an important part in the honeybee biology. Melittin was observed on the body surface of female honeybees and on the comb wax [7], and it may be related to social immunity of honeybee [8]. Additionally, contents of melittin in bee venom vary due to climatic, seasonal and feeding factors [9].

Determination of melittin have been done by combining high performance liquid chromatography (HPLC) [10–15] and capillary electrophoresis (CE) [16,17] with ultra-violet (UV) [10–14,16,17] or mass spectrometry (MS) detectors [15,18]. When UV detector is applied with RP-HPLC, as the UV absorption of peptides in bee venom is similar, rigor elution condition and long analysis time are generally required for the quantitative determination [10]. In addition, the low sensitivity obtained using UV detector limits the application in samples with low contents of melittin. HPLC–MS/MS were also used for the rapid and high sensitive determination of melittin in bee venom lyophilized powder [15]. Under the multiple-reaction monitoring (MRM) mode in triple-quadrupole

* Corresponding authors. Fax: +86 591 83789482.
E-mail addresses: wbchen@fafu.edu.cn (W. Chen), mxqsf88@126.com (X. Miao).

http://dx.doi.org/10.1016/j.jchromb.2015.08.014
1570-0232/© 2015 Elsevier B.V. All rights reserved.

mass spectrometer, the full chromatographic separation becomes uncritical. Thus, the analysis time could be drastically shortened. MALDI-TOF and nanoESI-QqTOF MS were also developed for the rapid semi-quantitative analysis of melittin [18]. However, there are several drawbacks of LC–MS quantification, such as suppression of ionization and the difficulty of reproducible quantification between MS instruments [19–21].

Recently, HPLC in combination with intrinsic fluorescence detection (HPLC–IFD) has been explored for the highly selective and highly sensitive quantification of peptides and proteins [22,23]. The intrinsic fluorescence of peptides and proteins mainly comes from three aromatic amino acid residues- tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe). HPLC–IFD has been demonstrated to be able to provide better sensitivity and linearity than the UV detection [24,25]. Compared with MS detection, intrinsic fluorescence detection provides similar sensitivity, better linearity and repeatability of quantification for natively fluorescent peptides and proteins [22,23]. Moreover, as only the fluorescent compounds under the selected excitation wavelength could be detected, fluorescence detection exhibited higher selectivity and simpler chromatograms than UV detection [26].

Melittin is composed of 26 amino acids, and single tryptophan residue is located at position 19 [27]; therefore, melittin shows the intrinsic fluorescence. This property of melittin provides powerful means for studying the aggregation behavior of melittin [28] and for analyzing the interaction between melittin molecules and membranes [29]. However, there was no report that applied this intrinsic fluorescence for the determination of melittin in bee venom. In this study, we take advantage of the properties of intrinsic fluorescence detection to establish a rapid, highly sensitive, and highly selective determination method for melittin. To the best of our knowledge, this is the first report of melittin analysis using HPLC–IFD. Benefit from the enhancement of sensitivity, melittin contents in individual venom sac from bees of different ages are successfully demonstrated.

2. Materials and methods

2.1. Chemicals

Standards of melittin and phospholipase A_2 were purchased from Sigma–Aldrich, acetonitrile was obtained from Merck, trifluoroacetic acid was supplied by Sinopharm Chemical Reagent Co., Ltd., and ultra-pure water (18.25 MΩ) was used in the experiments.

2.2. Chromatographic conditions

The HPLC analyse were performed using Shimadzu HPLC instrument, which is equipped with high pressure pump (LC-20AT), fluorescence detector (RF-10AXL), ultraviolet detector (SPD-20A), controllers (CBM-20 Alite), chromatography workstation (LC Solution), HT-220A column temperature box, and 20 μL sample loop. Samples were separated using Sepax Bio-C18 column (4.6 × 150 mm, 5 μm, 300 Å) at the flow rate of 0.8 mL/min. The mobile phase was 0.1% trifluoroacetic acid (TFA) in water (mobile phase A) and acetonitrile (mobile phase B) at 25 °C. Intrinsic fluorescence emission of 340 nm was detected with an excitation wavelength of 280 nm. And UV detector was set at 280 nm.

2.3. Sample preparation

Melittin stock solution was prepared by dissolving melittin standard in water to yield the concentration of 200 μg/mL. Then a certain amount of stock solution was diluted to solutions with concentrations of 0.04, 0.08, 0.4, 0.8, 4, 8, and 20 μg/mL with water.

Crude bee venom (obtained from a beekeeper at Mudanjiang city in Heilongjiang Province, China) was dissolved in water and was filtrated with 0.45 μm membrane filters. The obtained bee venom liquid was freeze-dried to gain bee venom lyophilized powder (BVLP). Then a certain amount of BVLP was diluted into 200 μg/mL with water as stock solution for the recovery study.

Determination of melittin in individual bee venom sac of honeybee (*Apis mellifera*): In March, the capped brood combs close to emergence was withdrawn, and then was cultured in constant temperature incubator (temperature: 35 °C; humidity: 55%). 54 newly emerged worker bees were collected randomly after 24 h incubation. Then several hundreds of newly emerged workers were painted on the dorsal thorax and then returned to the colonies. After anesthesia on the ice, newly emerged worker bees were anatomized carefully for venom sacs by drawing out the last segment including the sting with forceps under stereomicroscope. Intact venom sac was individually put into centrifuge tube, and contents in the sac were squeezed out and rinsed with certain amount of water, and then were centrifuged for 10 min at 12,000 rpm at 4 °C. The supernatant was analyzed with HPLC–IFD. Thirty 25-day old adult workers were re-collected from colonies, and then processed using above procedures, and analyzed by HPLC–IFD.

3. Results and discussion

3.1. Optimized the HPLC–IFD conditions

RP-HPLC with isocratic condition was used to perform rapid separation of melittin from bee venom. Both size-exclusion chromatography (SEC) [11] and RP chromatography [10,12–14] have been described for the separation of melittin from bee venom. SEC can be used for the rough characterization and final purification. However, separation efficiency of SEC is insufficient for quantitative purposes. C_{18} chromatographic column was examined here for the separation of melittin with high resolution and efficiency. Mobile phases in RP-HPLC consisted of 0.1% trifluoracetic acid (TFA) in water (mobile phase A) and acetonitrile (mobile phase B). Acetonitrile is a better eluent for RP-HPLC separation of bee venom than methanol, and the TFA is the required ion suppressor and ion pairing reagent for the basic amino acid residues in melittin to receive reproducible results [10]. TFA–water–acetonitrile mobile phase can be used for the fingerprint analysis and quantitative characterization of peptides and enzymes in bee venom under the gradient elution [13], and also for the quantitative determination of a single component in bee venom under isocratic conditions [10].

In this study, the isocratic elution combined with selective intrinsic fluorescence detection was carefully optimized to shorten the analysis time of melittin. Results indicated that the resolution and retention time of peptides in bee venom are increased with the reduction of acetonitrile/water ratio. As shown in Fig. 1a–d, when the mobile phase consists 48% of acetonitrile, the chromatograms of melittin and phospholipase A_2 (PLA_2) in BV sac appear high degree of overlap at RT 3 min. The proportion of mobile phase B was adjusted down to 44%, 41% and 38% in sequence. When the content of acetonitrile in mobile phase is 44%, chromatograms of melittin and PLA_2 in BV sac can be separated completely, but melittin cannot be isolated from other ingredients in BV sac adequately. When the ratio of acetonitrile/water is lower than 41%, the isolation of melittin is good for the quantitative analysis (resolution > 1.5). For 38% of acetonitrile in mobile phase, retention time of melittin is increased to 13.56 min. As the content of acetonitrile in mobile phase is 41%, retention time of melittin is about 6.82 min, and it is more suitable for the rapid determination of melittin in BV sac. Compared with published results, the retention time obtained in this study is much faster than those obtained by using HPLC–UV methods, which are

Download English Version:

https://daneshyari.com/en/article/1212065

Download Persian Version:

https://daneshyari.com/article/1212065

Daneshyari.com