Differentiation between Natural and Commercial bee venoms using Fluorescence Spectroscopy

Hadeel Ahmed Ramahan[a], Alla M. Hashkel[b], Inass A Al sadawi[b], Asia Mohamed[b], Fathi M Sherif[c], Salah M. Bensaber[b], Anton Hermann[d] and Abdul M. Gbaj[b]

(a) Zoology Department, Faculty of Science, Tripoli University

(b) Department of Medicinal Chemistry, Faculty of Pharmacy, University of Tripoli, M16.

(c) Department of Pharmacology, Faculty of Pharmacy, University of Tripoli, M16, Libya.

(d) Department of Biosciences, University of Salzburg, Salzburg, A-5020, Austria.

abdulgbaj1@hotmail.com

Abstract

Honey bee venom (HBV, *A. m. scutellata*) contains polypeptides [melittin, mast cell degranulating peptide (MCD), adolapin and apamin] and enzymes.The aim of the present study was to differentiate between commercial and natural bee venoms using aqueous olive leaf extract (AOLE) employing fluorescence techniques. Tryptophan mainly responsible for the fluorescence and hence widely used as a tool to monitor conformational changes in proteins. Changes in the fluorescence intensities with blue shifts were obtained with bee venoms. Binding of AOLE constituents near the active site of the enzyme could be evidenced and possible modes of interaction are discussed. The fluorescence method used was rapid and sensitive and was able to differentiate between commercial and natural bee venoms utilizing AOLE.

Abbreviations: PLA_2: phospholipase A_2, AOLE: Aqueous olive leaf extract.

Introduction

Honey bee venom (HBV, *Apis mellifera*) is a colourless liquid with bitter taste, and it contains a combination of proteins act as anticoagulants and cause local inflammation. The honey bee venom also contains a mixture of polypeptides [melittin, mast cell degranulating peptide (MCD), adolapin and apamin] and enzymes such as phospholipase A_2 (PLA_2), phosphomonoesterase acid esterase, hyaluronidase, α-D-glucosidase, α-galactosidase lysopphospholipase, arylamidase and α-acetylamino-deosiglu-cosidase[1;2]. Many studies have indicated that the biological activity of HBV compounds are related to the peptides including melittin, apamin, adolapin and MCD) [1;2]. In addition to that HBV contains minor amounts of low molecular compounds that are dissimilar in nature: catecholamines, amino acids, minerals and sugars. The availability of sugars in the HBV depends on the way of HBV collection [3;4]. In the literature it has been reported that the bee venom is useful in the treatment of many pathologies conditions including skin diseases, rheumatism, malignant tumors and arthritis [5]. Also it is reported that bee venom stimulates the secretion of cortisol from the adrenal glands [6]. Recently it is reported that HBV is effective in the treatment of inflammatory and arthritis conditions [5;6].

Tryptophan (Trp) which is present in phospholipase A_2 and melittin is a naturally occurring amino acid, which exhibits fluorescence emission properties that are dependent on the polarity of the local environment around the Trp side chain [7]. The main objective of the this paper was to differentiate between commercial and natural bee venoms using aqueous olive leaf extract (AOLE) utilizing fluorescence spectroscopy.

Materials and methods

All experiments were conducted in Tris buffer (0.01M Tris, 0.1M NaCl, at pH 7.4). Glass-distilled deionized water and analytical grade reagents were used throughout experiments. pH values of solutions were measured with a

CC BY

calibrated Jenway pH-meter model 3510 (Staffordshire, UK). All buffer solutions were filtered through Millipore filters (Millipore, UK) of 0.45 mm pore diameter.

Absorbance spectra: Absorbance spectra were measured on a Jenway UV-visible spectrophotometer, model 6505 (London, UK) using quartz cells of 1.00 cm path length. The UV-Vis absorbance spectra were recorded in the 200-500 nm range, and spectral bandwidth of 3.0 nm. For the final spectrum of each solution analyzed baseline subtraction of the buffer solution was performed. The protein content of venoms samples was determined by the spectrophotometric method of Markwell *et al*. [8]. Bovine serum albumin (BSA, Sigma) was used for standard assays.

Fluorescence spectra: Fluorescence emission and excitation spectra were measured using a Jasco FP-6200 spectrofluorometer (Tokyo, Japan) using fluorescence 4-sided quartz *cuvettes* of 1.00 cm path length. The automatic shutter-on function was used to minimize photo bleaching of the sample. The selected wavelength chosen provided aggregate excitation of tryptophan and tyrosine residues. The emission spectrum was corrected for background fluorescence of the buffer.

Preparation of aqueous *Olea europaea* leave extracts

Leaves of olive trees (*Olea europaea*) were collected from the Novellien zone, Tripoli Centre, Tripoli, Libya during June 2018. The leaves (5 g) were cleaned and washed with distilled water and dried at a room temperature of 25°C for about 20 minutes. Dried leaves were grinded in a homogenizer (HO4A Edmund Buhler GmbH, UK) along with 15 ml of distilled water. The resulting aqueous solution was filtered using a Millipore filter (0.45 μm, GHD Acrodisc GF, UK).

Venoms

Bee (*A. m. scutellata*) venoms were extracted by manual stimulation and were obtained in liquid and semisolid forms from the Zoology Department, Faculty of Science, Tripoli University (Libya) and stored at -20°C until use. Venoms were added to 2 ml of 0.01 M Tris, 0.1 M NaCl at pH 7.4.

Results and discussion

The fluorescence spectrum shows a decrease of fluorescence intensity (Fig. 1) of the bee venoms (both the commercial and the natural bee venoms) due to addition of 100 μl of AOLE which could be related to various processes. It is well known that a decrease in fluorescence intensity can be caused by a range of molecular interactions such as molecular rearrangements, excited-state reactions, ground state complex formation, collisional quenching or energy transfer. The decrease in fluorescence emission intensity as shown in Fig. 1 was accompanied by a blue shift of 41 nm of the maximum emission (353 nm to 312 nm) in case of commercial bee venom and a blue shift of 27 nm of the maximum emission (353 nm to 326 nm) in case of natural bee venom which may indicate that Trp residues buried in a hydrophobic environment have moved into a relatively polar environment consistent with earlier reports [9] but the structural differences in the blue shift is also related to the different composition of both venoms. The packaged frozen dried bee venom (commercial bees venom) as a medicine usually contains fillers of high molecular weight compounds as polyvinylpyrrolidone, gum, polysaccharides (saccharose, lactose *etc*.), pectins, polyols (mannitol, sorbitol), topical anaesthetic (lidocaine hydrochloride), anticoagulant (heparin) and isotonicity regulator (sodium chloride), while the natural do not [10]. The filler might influence the Trp residues fluorescence for example the presence of heparin as a polymer which consists of sulfated repeating disaccharide unit might result in perturbations of geometry of Trp residues providing evidence that changes in the Trp residues environment effects the fluorescence spectra structure [11].

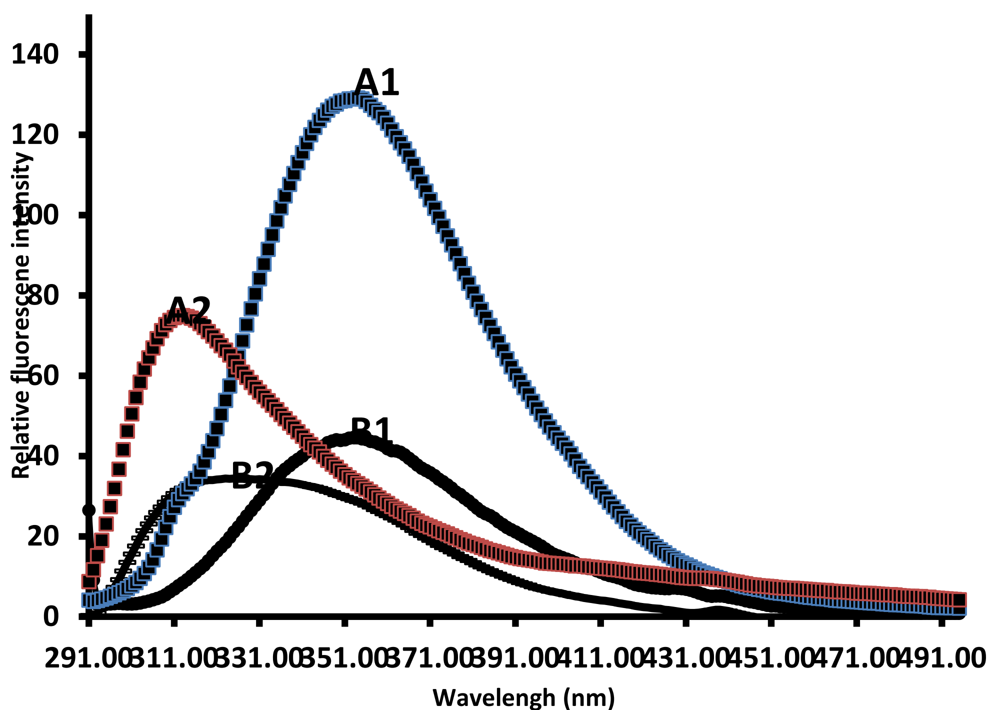

Figure 1. (A) Fluorescence perturbation of bee venom by addition of AOLE. Plot of fluorescence emission of commercial bee venom [**A1]** (50 ng/ml) and commercial bee venom with 100 µl AOLE (5g/15 ml) [**A2]** *vs* wavelength from 291 - 492 nm using excitation of λ_{280} nm in 0.01 M Tris, 0.1 M NaCl at pH 7.4. (**B)** Plot of fluorescence emission of natural bee venom [**B1**] (50 ng/ml) and natural bee venom with 100 µl AOLE (5g/15 ml) [**B2**] *vs* wavelength from 291 - 492 nm using excitation of λ_{280} nm in 0.01 M Tris, 0.1 M NaCl at pH 7.4. Spectra were corrected for small background fluorescence contributions from buffer solution and were scaled to visualize the shift.

It is well known that melittin has a tetrameric character at high concentration in solution but at low concentrations and low ionic strength the molecule has monomeric character, with the tryptophan at 19 totally exposed to the solvent water [12;13] and this is consistent with our results in which the intrinsic fluorescence intensity changed on the addition of AOLE. The AOLE might take some water from the environment and this lead to concentrating the melittin in the aqueous solution and hence aggregates are formed up to a tetramer, leading to a change of environment of the tryptophan residues from aqueous to hydrophobic and marked changes in secondary structure as proved by different studies [14;15]. In the literature the structural of the monomeric form has been studied by circular dichroism and NMR and both studies indicated that the polypeptide chain is of an extended, flexible form resembling a random coil and more ordered but on addition the AOLE an intrachain hydrogen bonding could form and this effect the florescence spectra and this is consist with the previous studies performed using ^{1}H NMR, circular dichroism[16]. In conclusion, The technique used in this paper employs inexpensive and easily available chemicals and instrument. It is simple, sensitive and rapid method to differentiate between commercial and natural bee venoms.

References

1. (1) Zolfagharian H, Mohajeri M, Babaie M. Honey Bee Venom (Apis mellifera) Contains Anticoagulation Factors and Increases the Blood-clotting Time. J Pharmacopuncture 2015; 18(4):7-11.

2. (2) Frick M, Fischer J, Helbling A, Rueff F, Wieczorek D, Ollert M, Pfutzner W, Muller S, Huss-Marp J, Dorn B, Biedermann T, Lidholm J, Ruecker G, Bantleon F, Miehe M, Spillner E, Jakob T. Predominant Api m 10 sensitization as risk factor for treatment failure in honey bee venom immunotherapy. J Allergy Clin Immunol 2016; 138(6):1663-1671.

3. (3) FISCHER FG, DORFEL H. [Honeybee venom. II. Amino acid composition of bee venom fractions]. Biochem Z 1953; 324(6):465-475.

4. (4) Danneels EL, Van Vaerenbergh M, Debyser G, Devreese B, de Graaf DC. Honeybee venom proteome profile of queens and winter bees as determined by a mass spectrometric approach. Toxins (Basel) 2015; 7(11):4468-4483.

5. (5) Son DJ, Lee JW, Lee YH, Song HS, Lee CK, Hong JT. Therapeutic application of anti-arthritis, pain-releasing, and anti-cancer effects of bee venom and its constituent compounds. Pharmacol Ther 2007; 115(2):246-270.

6. (6) Park HJ, Lee SH, Son DJ, Oh KW, Kim KH, Song HS, Kim GJ, Oh GT, Yoon DY, Hong JT. Antiarthritic effect of bee venom: inhibition of inflammation mediator generation by suppression of NF-kappaB through interaction with the p50 subunit. Arthritis Rheum 2004; 50(11):3504-3515.

7. (7) Kamceva T, Flemmig J, Damnjanovic B, Arnhold J, Mijatovic A, Petkovic M. Inhibitory effect of platinum and ruthenium bipyridyl complexes on porcine pancreatic phospholipase A2. Metallomics 2011; 3(10):1056-1063.

8. (8) Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 1978; 87(1):206-210.

9. (9) Kenoth R, Simanshu DK, Kamlekar RK, Pike HM, Molotkovsky JG, Benson LM, Bergen HR, III, Prendergast FG, Malinina L, Venyaminov SY, Patel DJ, Brown RE. Structural determination and tryptophan fluorescence of heterokaryon incompatibility C2 protein (HET-C2), a fungal glycolipid transfer protein (GLTP), provide novel insights into glycolipid specificity and membrane interaction by the GLTP fold. J Biol Chem 2010; 285(17):13066-13078.

10. (10) Poller L, Keown M, Shepherd SA, Shiach CR, Tabeart S. The effects of freeze drying and freeze drying additives on the prothrombin time and the international sensitivity index. J Clin Pathol 1999; 52(10):744-748.

11. (11) Hogg PJ, Jackson CM, Labanowski JK, Bock PE. Binding of fibrin monomer and heparin to thrombin in a ternary complex alters the environment of the thrombin catalytic site, reduces affinity for hirudin, and inhibits cleavage of fibrinogen. J Biol Chem 1996; 271(42):26088-26095.

12. (12) Shelley MY, Selvan ME, Zhao J, Babin V, Liao C, Li J, Shelley JC. A New Mixed All-Atom/Coarse-Grained Model: Application to Melittin Aggregation in Aqueous Solution. J Chem Theory Comput 2017; 13(8):3881-3897.

13. (13) Hagihara Y, Oobatake M, Goto Y. Thermal unfolding of tetrameric melittin: comparison with the molten globule state of cytochrome c. Protein Sci 1994; 3(9):1418-1429.

14. (14) Wilcox W, Eisenberg D. Thermodynamics of melittin tetramerization determined by circular dichroism and implications for protein folding. Protein Sci 1992; 1(5):641-653.

15. (15) John E, JÌ¤hnig F. Dynamics of melittin in water and membranes as determined by fluorescence anisotropy decay. Biophys J 1988; 54(5):817-827.

16. (16) Saitĺ´ H, Ando I, Ramamoorthy A. Chemical Shift Tensor â€" the Heart of NMR: Insights into Biological Aspects of Proteins. Prog Nucl Magn Reson Spectrosc 2010; 57(2):181-228.