Università degli Studi di Firenze

HONEYBEE VENOM DETECTION AND DIFFUSION IN ENVENOMED TISSUE BY DIRECT MALDI MSI

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INTRODUCTION

Honeybee venom (HBV) is the best characterised amongst Hymenoptera's. As commonly know, it can trigger either allergenic or toxic reactions. Allergens are generally proteins and may trigger life threatening and sometimes fatal IgE-mediated anaphylactic reactions. Toxins are generally peptides and cause local reactions. Melittin (Api m 4) is in this context an exception being a peptidic allergen. A deep understanding of HBV composition, action and interaction with the biological tissue are very important aspects, especially when this information can be used for the development of more efficient Venom Immunotherapy (VIT). Here it is described the use of MALDI Mass Spectrometry Imaging (MSI) for the on tissue direct detection and over time diffusion of two HBV toxins (Apamine and MCD) and three allergens (Api m 1, Api m 4, Api m 6) upon honeybee sting.

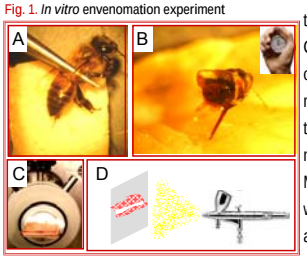
EXPERIMENTAL

In vitro model

Materials- living honeybees, pig ear pieces

Method- 4 stages procedure (Fig 1): A) the honeybee is forced to sting the pig ear; B) prior freezing,the sting is left in the skin for 1, 30, 60 and 120 min; C) tissue sections (12 µm) are obtained in a -25°C cryostat and mounted on an ITO glass slide; D) the sections are spray coated in matrix by means of an airbrush. For MALDI MSI peptide analysis matrix was 20 mg/ml αCHCA in acetonitrile/0.2% aq. TFA 50/50.

Fig. 1. *In vitro* envenomation experiment



For MALDI MSI protein analysis, the section was washed (70 and 90 % ethanol) and "seeded" by 1min immersion in 25 mg/ml sinapinic acid in 90/10 ethanol/0.2% aq. TFA. It was then spray coated with 25 mg/ml sinapinic acid in 60/40 acetonitrile/0.2% aq. TFA MALDI MSI analyses were performed on a MALDI TOF TOF Ultraflex III (Bruker Daltonics, Germany).

In vivo model

Materials- living honeybees, rats (Fig. 2)

Method- 7 stages procedure: A) the rat is anesthetized for the duration of the experiment; B) the honeybee is forced to sting his posterior leg; C) the rat is sacrificed and the leg dissected to obtain the muscle. Sample pre-paration procedeed from here as stages A-D of *in vitro* model experiment.

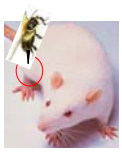


Fig. 2 *In vivo* envenomation experiment

In vivo results

Using the muscle of an anesthetized rat as envenomation site, a MALDI MSI time course experiment at 1 and 120 min p.e. was performed; data indicate a general trend of low intensity HBV ion signals. As an example, Fig. 7 shows detection at 1 min of Api m 4, Apamine and Api m 6 exhibiting a much more contained tissutal diffusion, and mainly around the sting, if compared to what observed in the pig ear. Biochemical response was also investigated. Two sul-

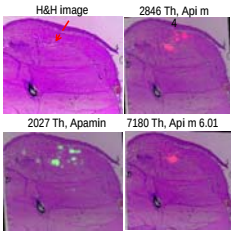


Fig. 7 *In vivo* MALDI MS HBV images at 1 min p.e.

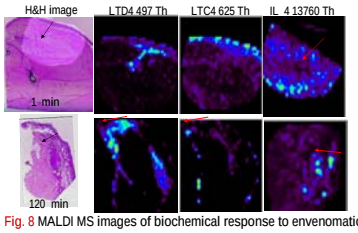
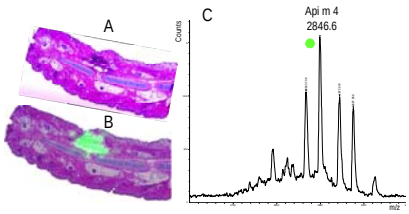


Fig. 8 MALDI MS images of biochemical response to envenomation

-phidoleukotrienes (LTs) LTC4 and LTD4 at 497.2 and 624.3 Th respectively were detected (Fig 8). LTs are important markers, responsive to an inflammation event. At 1 min, they were found nicely distributed where the sting was left and in its proximity. An ion signal at 13760 Th, compatible with the molecular weight of the Interleukin 4 (IL-4) was also found. At 120 min, distribution maps for LCT4, LTD4 and putative IL-4 change: LTD4 is spread and LTC4 is almost undetectable, whereas IL-4 is more localized in an area close to the sting. If confirmed by further analysis, this result would be in agreement with what reported by Mustafa et al [11] who observed presence of LTs within five minutes form the sting, whilst IL-4 was induced at later time points.

RESULTS

Fig. 3 MALDI MSI analysis after 5 min envenomation



MALDI MSI analysis showed a progressive decrease in the detection of the HBV peptides over time (Fig 3, ubiquitary phosphatidilcoline image shown to visualize the section). They are readily mapped at 1 min p.e. and appear to have spread after 30 min. At 60 min there seems to be an accumulation in the proximity of the cartilage possibly acting as a barrier to the longitudinal diffusion. At 120 min, HBV peptides localization maps are considerably reduced, although Api m 4 and MCD are detected also within the cartilage; a selective Apamine ion suppression by the cartilage is hypothesized. (Data processing with Biomap, Novartis, Switzerland)

In vitro results

Pepditic range

An explorative analysis at 5 min post envenomation (p.e.) revealed an irritated area of the tissue (Fig 3A, H&H image) which in fact corresponds to the area interested by the presence of HBV. This is shown by the superimposition of MALDI MS image of Api m 4 (m/z 2846.6, mass spectrum shown in Fig 3C) on the histological one (Fig 3B). (Data processing with Flex Imaging 2.0 and Flex Analysis 3.0)

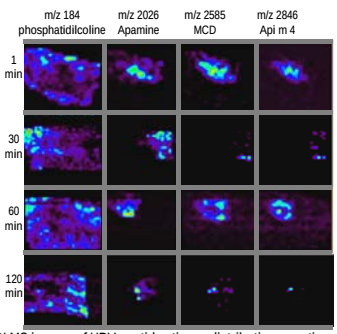
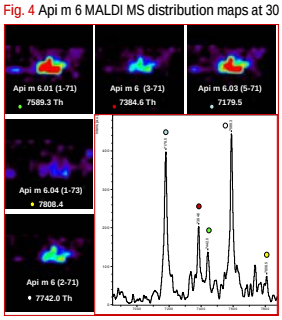


Fig. 3 MALDI MS images of HBV peptides tissue distribution over time

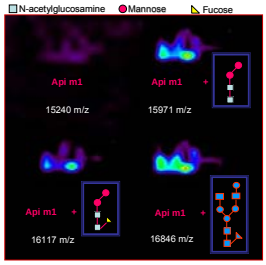
Proteic range

Fig. 4 Api m 6 MALDI MS distribution maps at 30 min p.e.



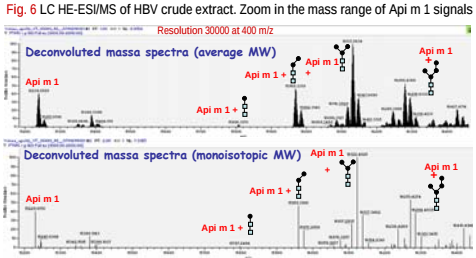
Due to gene misassembly, Api m 6 comprises of 4 isoforms: 1-71 (Api 6.01), 1-73 (Api m 6.04), 5-71 (Api m 6.03) and 5-73 (Api m 6.02) which are reported to be all allergenic and equimolar [20]. MALDI MSI analysis at 30 min p.e. (Fig. 4), reveals only three of the isoforms (Api m 6.01, 6.03 and 6.04) and only Api m 6.01 and 6.03 have similar density maps. Given the quasi-total structural overlapping, equimolarity becomes questionable. Noticeably other two isoforms, never reported were observed (2-71, 3-71) which are also likely to be allergenic.

Fig. 5 Api m 1 MALDI MS HBV images at 30 min p.e.



Api m 1 (15240 Th) was found to co-localize with Api m 6 (Fig. 5). Other three ion signals at 15971, 16116 and 16846 Th were as well observed which are compatible with the presence of Api m 1 isoforms exhibiting different glycation levels (Fig. 5, structures in the frames). The correct m/z assignment was aided by the LC HR-ESI/MS analysis of HBV extract (Fig. 6) on an LTQ-Orbitrap mass spectrometer (ThermoFisher, USA) coupled to an Ultimate 3000 HPLC system (Dionex, USA).

Fig. 6 LC HE-ESI/MS of HBV crude extract. Zoom in the mass range of Api m 1 signals



Conclusions

The ability of MALDI MSI to provide molecular maps of HBV in envenomed tissues over time is demonstrated. Remarkably, the opportunity of detecting HBV time distributions maps and organism response simultaneously, suggests the possibility of using MALDI MSI in the pre-clinical trial stage development of more efficient Venom Immunotherapy, to be one day even designed down to single individuals.

References:

1. Kettner, A. et al., *J. Allergy Clin. Immunol.* 2001, 107, 914-920; 2. Mustafa, et al., *Clin. Exp. Immunol.* 2007, 151, 94–100.