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Shotgun proteome analysis of honeybee venom using targeted enrichment strategies

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ABSTRACT

The aim of this study was to explore the honeybee venom proteome applying a shotgun proteomics approach using different enrichment strategies (combinatorial peptide ligand libraries and solid phase extraction). The studies were conducted using nano-LC/MALDI-TOF/TOF-MS system. The MS analysis of peptide profiles (in the range of 900–4500 Da) and virtual gel-image of proteins from Lab-on-Chip assay (in the range of 10–250 kDa) confirm that use of targeted enrichment strategies increase detection of honeybee venom components. The gel-free shotgun strategy and sophisticated instrumentation led to a significant increase of the sensitivity and higher number of identified peptides in honeybee venom samples, comparing with the current literature. Moreover, 11 of 12 known honeybee venom allergens were acknowledged and 4 new, so far uncharacterized proteins were identified. In addition, similarity searches were performed in order to investigate biological relations and homology between newly identified proteins sequences from *Apis mellifera* and other *Hymenoptera*.

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1. Introduction

Stinging by *Hymenoptera* represents one of the main causes of anaphylaxis both in adults and in children (Matysiak et al., 2011a; Silva et al., 2012). In Central Europe most post-stinging anaphylactic reactions are caused by honey bees (*Apis mellifera*), and less by other *Hymenoptera*, such as wasps (*Vespula vulgaris*, *Vespula germanica*), hornets (*Vespa crabro*) and bumble bees (*Bombus* spp.). The frequency of post-stinging allergic reaction and its intensity may vary depending the intra-individual characteristics of both the person and of the stinging insect (Przybilla and Rueff, 2010). Consequently the qualitative and quantitative differences in composition of the venom may affect the severity of allergic reaction after the sting. At present 12 allergens are identified in honeybee venom and are listed by the Allergen Nomenclature Sub-Committee of the International Union of Immunology Societies (IUIS) (Cichocka-Jarosz et al., 2011). According to the literature the most important bee venom allergens are: phospholipase A_2 (Api m1), hyaluronidase (Api m2), acid phosphatase (Api m3) and melittin (Api m4) (Bilo et al., 2005; Peiren et al., 2005). It is known that in 97% of patients with bee venom-specific IgE antibodies, IgE antibodies to Api m1 are detected (Dotimas and Hider, 1987; Muller et al., 2009). In addition, IgE antibodies to Api m2 (hyaluronidase), Api m3 (acid phosphatase) and Api m4 (melittin) are present in 51%, 60% and 31% of patients, respectively (Kemeny et al., 1983). Hence, whereas the most abundant allergens are the primary targets for venom immunotherapy, minor protein components may represent new opportunities of exploration into the organism response to this toxin. Due to the fact that the *A. mellifera* genome is fully sequenced, we

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can take advantage of searching the complete genomic DNA sequence and getting matches from unidentified open reading frames.

The current literature presents many strategies for proteomic characterization of complex biological matrices (Freour et al., 2013; Moore et al., 2013; Rees et al., 2013; Wang et al., 2013; Yu et al., 2013). Due to the complexity of this material, some laboratories employ bottom-up strategies, where two-dimensional SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is used to separate protein mixture. Subsequently after in-gel digestion, the resulting peptide mixture is analyzed by tandem mass spectrometry techniques (Wilkins et al., 2007). Nonetheless, the results obtained from the bottom-up strategy often have consistency issues (low reproducibility, inter-sample agreement). SDS-PAGE is both sample and time-consuming, and becomes very tedious particularly when the runs are repeated several times. Advancement of mass spectrometry fragmentation methods allowed the development of a less complicated top-down strategy, which involves the analysis of purified proteins. This technique is preferably used for analysis of post-translational modifications of single proteins or isoforms of proteins. The limitations of bottom-up and top-down strategies include: complex process of sample preparation and relatively high costs of analysis. Accordingly, most of the recent proteomic studies are based on the shotgun strategy, which requires proteolytic in-solution digestion of the whole sample and skips the laborious and low reproducible 2-D PAGE steps. In view of the large number of the peptides obtained, optimization of the LC method (including selection of proper column and gradient program) in this strategy is required.

In complex biological matrices, such as bee venom, the dynamic range of the proteins is very high (Kokot et al., 2011). Thereby a major challenge is to concentrate the analyte in order to identify proteins present at lower levels. To overcome this problem and allow access to the "hidden proteome", several multidimensional prefractionation methods and tools have been developed for removing the most abundant proteins. These methods include: organic solvent extraction (Chertov et al., 2004), ultrafiltration (Tirumalai et al., 2003; Zheng et al., 2006), solid phase extraction (Hu et al., 2009; Koomen et al., 2005), extraction by mesoporous silica particles (Tian et al., 2007), extraction using magnetic beads (Baumann et al., 2005), chromatography, electrophoresis (Marshall et al., 2004), immunoaffinity fractionation (Brand et al., 2006; Gong et al., 2006; Pieper et al., 2003) and fractionation using hydrogel nanoparticles (Rainczuk et al., 2010). Recently, especially methods using immunodepletion (only restricted to physiological fluids such as: serum, plasma, cerebrospinal fluid etc.) and the combinatorial peptide ligand library strategy (CPLL or ProteoMiner, Bio-Rad) have been employed (Krief et al., 2012; Whiteaker et al., 2007). The CPLL is helpful for revealing lower abundance proteins, undetectable by classical analytical methods (Boschetti and Righetti, 2008) and can be applied to the broad range of biological samples, including not only physiological fluids (Castagna et al., 2005; Sennels et al., 2007) but also extracts of plants (Boschetti et al., 2009; Boschetti and Righetti, 2014), food (D'Ambrosio et al., 2008; Farinazzo et al., 2009) or animal toxins (Calvete et al., 2009; Fasoli et al., 2010). This strategy is based on the interaction of complex protein matrices with a large, highly diverse library of hexapeptides bound to chromatographic supports. Due to the fact that bead capacity limits binding capacity of the proteins, the signal of high-abundance proteins is reduced while low-abundance proteins bring their signal increasing detection. Therefore, this technique is particularly useful for the removal of proteins with the highest concentrations, which can mask the other components.

In addition to the problem with compressing the dynamic range of protein concentration in complex biological samples, the second problem in the proteomic analysis is the contamination of the analyzed sample by salts, detergents and many other impurities (Gobom et al., 1999). The widely used solid phase extraction (SPE) with easy to handle pipette tips allows for desalting, concentration and purification of the sample, results in a significant improvement in the identification of the proteins and peptides analyzed by mass spectrometry techniques (Palmblad and Vogel, 2005). Commercially available pipette tips such as ZipTip (Millipore, Bedford, MS, USA) or NuTip (Glygen, Columbia, MD, USA) are packed with conventional particles, while MonoTip (GL Sciences, Tokyo, Japan) pipette tips are packed with functionalized monolithic silica. The OMIX tips (Agilent, Palo Alto, CA, USA) used in this study, are classified as a monolith-like silica gel product. However, there are divergent references in available literature about the effectiveness of extractive pretreatments of SPE techniques with use pipette tips in mass spectrometry analyses (Hennion, 1999; Huck and Bonn, 2000; Kumazawa et al., 2010; Rossi and Zhang, 2000).

The aim of this study was to examine the shotgun proteomics strategy for the characterization of honeybee venom samples, as well as the evaluation of suitable system for purification and concentration of the natural biological sample.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (ACN), DL-dithiothreitol (DTT), ammonium phosphate monobasic, iodoacetamide, heptafluorobutyric acid, trifluoroacetic acid (TFA), ultrapure water and ammonium bicarbonate were supplied by Sigma–Aldrich (St. Louis, MO, USA). 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and α-cyano-4-hydroxycinnamic acid (HCCA) were obtained from Bruker (Bremen, Germany). Ethanol and isopropanol was supplied by J. T. Baker (Center Valley, PA, USA). All reagents used were of analytical grade or better.

2.2. Honeybee venom samples

Samples of honeybee venom were collected from an apiary of the Department of Inorganic and Analytical Chemistry, Poznan University of Medical Sciences by stimulating the bees with electric current pulses. Venom collecting frames were placed in the upper body of the hive

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